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Up-regulation of *selenoprotein P* and *HIP/PAP* mRNAs in hepatocytes by intermittent hypoxia via down-regulation of miR-203

Tomoko Uchiyama[a,b], Hiroyo Ota[a,c], Asako Itaya-Hironaka[a], Ryogo Shobatake[a], Akiyo Yamauchi[a], Sumiyo Sakuramoto-Tsuchida[a], Mai Makino[a], Hiroshi Kimura[c], Maiko Takeda[b], Chiho Ohbayashi[b], Shin Takasawa[a,*]

[a] *Department of Biochemistry, Nara Medical University, Kashihara 634-8521, Japan*
[b] *Department of Diagnostic Pathology, Nara Medical University, Kashihara 634-8522, Japan*
[c] *Second Department of Internal Medicine, Nara Medical University, 840 Shijo-cho, Kashihara 634-8522, Japan*

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ABSTRACT

Sleep apnea syndrome is characterized by recurrent episodes of oxygen desaturation and reoxygenation (intermittent hypoxia [IH]) and is a risk factor for insulin resistance/type 2 diabetes. However, the mechanisms linking IH stress and insulin resistance remain elusive. We exposed human hepatocytes (JHH5, JHH7, and HepG2) to experimental IH or normoxia for 24 h, measured mRNA levels by real-time reverse transcription polymerase chain reaction (RT-PCR), and found that IH significantly increased the mRNA levels of *selenoprotein P* (*SELENOP*) — a hepatokine — and *hepatocarcinoma-intestine-pancreas/pancreatitis-associated protein* (*HIP/PAP*) — one of *REG* (Regenerating gene) family. We next investigated promoter activities of both genes and discovered that they were not increased by IH. On the other hand, a target mRNA search of micro RNA (miRNA) revealed that both mRNAs have a potential target sequence for miR-203. The miR-203 level of IH-treated cells was significantly lower than that of normoxia-treated cells. Thus, we introduced miR-203 inhibitor and a non-specific control RNA (miR-203 inhibitor NC) into HepG2 cells and measured the mRNA levels of *SELENOP* and *HIP/PAP*. The IH-induced expression of *SELENOP* and *HIP/PAP* was abolished by the introduction of miR-203 inhibitor but not by miR-203 inhibitor NC. These results demonstrate that IH stress up-regulates the levels of *SELENOP* in human hepatocytes to accelerate insulin resistance and up-regulates the levels of *HIP/PAP* mRNAs to proliferate such hepatocytes, via the miR-203 mediated mechanism.

1. Introduction

Sleep apnea syndrome (SAS) is characterized by the narrowing or collapse of the upper airway during sleep that leads to a cessation of airflow. Apnea and hypopnea are often accompanied by a drop in oxygen saturation. Accumulating evidence suggests that recurrent episodes of oxygen desaturation and reoxygenation (intermittent hypoxia [IH]), which are typical features of SAS, contribute to the development of β cell dysfunction and impaired glucose tolerance [1].

Epidemiological and clinical evidence postulates that SAS may be a causal factor of type 2 diabetes. The increasing severity of SAS is associated with worsening insulin resistance [2,3]. The recent report by Priou et al. suggested that an increase in the severity of SAS may worsen glucose control in patients with asymptomatic, untreated, or early stages of type 2 diabetes [4]. Nocturnal IH is associated with an increased risk of type 2 diabetes among community-dwelling Japanese people independent of its traditional risk factors such as age, sex, and body habitus [5]. However, the mechanisms by which IH induces insulin resistance in SAS patients are not well established.

Recently, several proteins that are exclusively or predominantly secreted from the liver, called hepatokines, were established as directly affecting glucose and lipid metabolism [6,7]. For example, fibroblast growth factor 21 (FGF21) has recently emerged as a novel hormone, leading to beneficial effects on glucose metabolism and lipid

Abbreviations: AHSG, α2 HS-glycoprotein; ANGPTL6, angiopoietin-related growth factor; DROSHA, ribonuclease type III; DICER, endoribonuclease Dicer; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; FGF21, fibroblast growth factor 21; HIP/PAP, hepatocarcinoma-intestine-pancreas/pancreatitis-associated protein; IH, intermittent hypoxia; LECT2, leukocyte cell-derived chemotaxin 2; MCPIP1, monocyte chemotactic protein-induced protein 1; miRNA, micro RNA; Reg, regenerating gene; Rig, rat insulinoma gene; RpS15, ribosomal protein S15; SAS, sleep apnea syndrome; *SELENOP*, selenoprotein P; SHBG, sex hormone-binding globulin; siRNA, small interfering RNA; TP63, transformation-related protein 63; WST-8, 2-(2-methoxy-4-nitrophenyl) – 3-(4-nitrophenyl) – 5-(2,4-disulfophenyl) – 2H-tetrazolium monosodium salt

* Corresponding author.
E-mail address: shintksw@naramed-u.ac.jp (S. Takasawa).

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homeostasis [8], while selenoprotein P is correlated positively with insulin resistance and could be a therapeutic target for type 2 diabetes [9]. The changes of these hepatokine expressions in hepatocytes by IH remain elusive.

The regenerating gene (*Reg*) was identified in regenerating islets [10,11] and a *Reg* gene product — Reg protein — acts as a growth factor and promotes cell proliferation and regeneration [11–13]. In humans, five functional *Reg* family genes (*REG Iα*, *REG Iβ*, *REG III*, *hepatocarcinoma-intestine-pancreas/pancreatitis-associated protein* [*HIP/PAP*] and *REG IV*) have been isolated. For several cells, *Reg* family proteins have been suggested to be involved in cellular proliferation [11]. We have reported that IH stress stimulates pancreatic β cell proliferation via up-regulation of *Reg* family mRNAs and may cause hyperinsulinemia, which makes patients more obese [1,14]. However, the direct effects of IH on hepatocyte proliferation and the IH-induced changes in *Reg* family gene expression in hepatocytes remain unknown.

In the present study, we investigated the changes of gene expression in hepatokines and *Reg* family genes, as well as their regulation mechanisms in response to IH stress in hepatocytes.

2. Materials and methods

2.1. Cell culture

Rat H4IIE hepatocytes were grown in DMEM medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 10% (v/v) fetal calf serum (FCS), 100 units/mL penicillin G (Wako) and 100 μg/mL streptomycin (Wako) as described [15]. Human hepatocarcinoma JHH5 cells and JHH7 cells were purchased from Japanese Collection of Research Bioresources (Sennan, Japan) and human hepatocarcinoma HepG2 cells were purchased from RIKEN BRC CELL BANK (Tsukuba, Japan). JHH5 and JHH7 cells were grown in William's E medium (Sigma, St. Louis, MO) containing 10% (v/v) FCS, 100 units/mL penicillin G (Wako), and 100 μg/mL streptomycin (Wako), and HepG2 cells were grown in DMEM medium containing 10% (v/v) FCS, 100 units/mL penicillin G, and 100 μg/mL streptomycin as described [16]. Cells were exposed to either normoxia (21% O_2, 5% CO_2, and balance N_2) or intermittent hypoxia (IH: 64 cycles of 5 min sustained hypoxia [1% O_2, 5% CO_2, and balanced N_2] and 10 min normoxia) using a custom-designed, computer-controlled incubation chamber attached to an external O_2-CO_2-N_2 computer-driven controller (O_2 programmable control, 9200EX, Wakenyaku CO., Ltd, Kyoto, Japan), as described [1,16]. These conditions are similar to the conditions reported in patients with severe degree of SAS — in severe cases of SAS, patients are repeatedly exposed to severe hypoxemia followed by mild hypoxemia or normoxia (i.e., IH). We previously reported that the magnitude of IH expressed by SpO_2 fluctuated between 75–98% and 50–80% in SAS [1,17], which was almost equivalent to the medium condition in the present study.

2.2. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated using a RNA protect cell mini kit (Qiagen, Hilden, Germany) from JHH5, JHH7, HepG2, and H4IIE cells, and cDNA was synthesized from total RNA as template using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) as described [14,16,18–20]. Real-time polymerase chain reaction (PCR) was performed using SYBR® Fast qPCR kit (KAPA Biosystems, Boston, MA) and a Thermal Cycler Dice Real Time System (Takara, Kusatsu, Japan). All the PCR primers were synthesized by Nihon Gene Research Laboratories, Inc. (NGRL; Sendai, Japan), and the primer sequences for each primer set are described in Table 1. PCR was performed with an initial step of 3 min at 95 °C followed by 40 cycles of 3 s at 95 °C and 20 s at 60 °C for *β-actin*, *rat insulinoma gene (Rig)/ribosomal protein S15* (*RpS15*), *REG III*, and *HIP/PAP*, 40 cycles of 3 s at 95 °C and 20 s at 64 °C for *REG Iα, REG Iβ*, and *REG IV,* 45 cycles of 3 s at 95 °C and 20 s at 60 °C for *α2 HS-glycoprotein* (*AHSG*), *angiopoietin-related growth factor 6* (*ANGPTL6*), *FGF21*, *leukocyte cell-derived chemotaxin 2* (*LECT2*), *LIPASIN*, *sex hormone-binding globulin* (*SHBG*), *Selenoprotein P* (*SELENOP*), *ribonuclease type III* (*DROSHA*), *endoribonuclease Dicer* (*DICER*), *monocyte chemotactic protein-induced protein 1* (*MCPIP1*), *transformation-related protein p63* (*TP63*), and *miR-203*. The mRNA expression levels were normalized to the mRNA level of *Rig/RpS15* in rat samples or *β-actin* in human samples, and the *miR-203* level was normalized to the *U6* RNA level.

Table 1
Primers used for real-time RT-PCR.

Target mRNA/miR	Primer sequence
Rat *Selenop* (NM_001082911)	5′-GACAGTGGTTGCTCTTCTTCAA-3′
	5′-TCGCAGGTCTTCCAATCTG-3′
Rat *Rig/RpS15* (NM_017151)	5′-ACGGCAAGACCTTCAACCAG-3′
	5′-ATGGAGAACTCGCCCAGGTAG-3′
Human *SELENOP* (NM_001093726)	5′-TCATCAAGGAATCTCTTCTCG-3′
	5′-CAAGACGGCCACATCTATCA-3′
Human *ANGPTL6* (NM_031917)	5′-CTGTGGTTCCGGTCCGTCTT-3′
	5′-GCTGCTCACACCATACTGACACT-3′
Human *SHBG* (NM_001040)	5′-TCAATCTCCGAGACATTCCC-3′
	5′-TGGTGTCCCAAGAGCAAG-3′
Human *LIPASIN* (NM_018687)	5′-GGCCGCACAATAGAACTCC-3′
	5′-CAGCGTGAGCCTTTAAGACC-3′
Human *LECT2* (NM_002302)	5′-GTGTTCGAATATCTGGAAGAGGT-3′
	5′-AAGGGCAATAGAGTTCCAAGT-3′
Human *FGF21* (NM_019113)	5′-ACCTGGAGATCAGGGAGGAT-3′
	5′-AGTGGAGCGATCCATACAGG-3′
Human *AHSG* (NM_001622)	5′-CCCCGGAAAACACGCACA-3′
	5′-GTGCCAAACCTCCTCATCTCT-3′
Human *REG Iα* (NM_002909)	5′-AGGAGAGTGGCACTGATGACTT-3′
	5′-TAGGAGACCAGGGACCCACTG-3′
Human *REG Iβ* (NM_006507)	5′-GCTGATCTCCTCCCTGATGTTC-3′
	5′-GGCAGCTGATTCGGGGATTA-3′
Human *REG III* (AB161037)	5′-GAATATTCTCCCCAAACTG-3′
	5′-GAGAAAAGCCTGAAATGAAG-3′
Human *HIP/PAP* (NM_138937)	5′-AGAGAATATTCGCTTAATTCC-3′
	5′-AATGAAGAGACTGAAATGACA-3′
Human *REG IV* (AY007243)	5′-ATCCTGGTCTGGCAAGTC-3′
	5′-CGTTGCTGCTCCAAGTTA-3′
Human *SOCS3* (NM_003955)	5′-CCACTCTTCAGCATCTCTGT-3′
	5′-ATCGTACTGGTCCAGGAACT-3′
Human *TP63* (NM_003722)	5′-TGTATCCGCATGCAGGACT-3′
	5′-CTGTGTTATAGGGACTGGTGGAC-3′
Human *DICER* (NM_177438)	5′-GAGCTGTCCTATCAGATCAGGG-3′
	5′-ACTTGTTGAGCAACCTGGTTT-3′
Human *DROSHA* (NM_013235)	5′-GGCCCGAGAGCCTTTTATAG-3′
	5′-TGCACACGTCTAACTCTTCCAC-3′
Human *MCPIP1* (NM_025079)	5′-TGCCTATCACAGACCAGCAC-3′
	5′-CTCACCTTCGCGAAGTAGCTC-3′
Human *β-actin* (NM_001101)	5′-GCGAGAAGATGACCCAGA-3′
	5′-CAGAGGCGTACAGGGATA-3′
Human *miR-203* (NR_029620)	5′-GCCGGTGAAATGTTTAGGAC-3′
	5′-GTGCAGGGTCCGAGGT-3′
Human *U6* (NR_004394)	5′-CTCGCTTCGGCAGCACA-3′
	5′-AACGCTTCACGAATTTGCGT-3′

2.3. Measurement of Sepp1 in culture medium by enzyme-linked immunosorbent assay (ELISA)

Cells were exposed either normoxia or IH for 24 h, culture medium was collected, and the concentration of Sepp1 was measured by using a Human Selenoprotein P (SELENOP) ELISA kit (Cusabio, Wuhan, China) for human cells and Rat Selenoprotein P (Selenop) ELISA kit (Cusabio) according to the instructions of supplier.

2.4. Measurement of viable cell numbers by tetrazolium salt cleavage

HepG2 cells (2.5×10^4 cells/100 μL in 96-well plate) were incubated at 37 °C over night and the medium was replaced with DMEM +10% FCS just before normoxia/IH exposure. After a 24-h treatment of normoxia or IH, the viable cell numbers were determined by a Cell