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Upregulation of IL-8, osteonectin, and myonectin mRNAs by intermittent hypoxia via OCT1- and NRF2-mediated mechanisms in skeletal muscle cells

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Abstract

Sleep apnoea 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. The induction of insulin resistance in skeletal muscle is a key phenomenon to develop diabetes. However, the mechanisms linking IH stress and insulin resistance remain elusive. We exposed human RD and mouse C2C12 muscle cells to normoxia or IH and measured their mRNA levels by real-time RT-PCR. We found that IH significantly increased the mRNA and protein levels of muscle-derived insulin resistance-factors (myokines) such as IL-8, osteonectin (ON), and myonectin (MN) in muscle cells. We further analysed the IH-induced expression mechanisms of *IL-8*, *ON*, and *MN* genes in muscle cells. Deletion analyses of the human myokine promoter(s) revealed that the regions −152 to −151 in *IL-8*, −105 to −99 in *ON*, and −3741 to −3738 in *MN* promoters were responsible for the activation by IH in RD cells. The promoters contain consensus transcription factor binding sequences for OCT1 in *IL-8* and *MN* promoters, and for NRF2 in *ON* promoter, respectively. The introduction of siRNA for OCT1 abolished the IH-induced expression(s) of *IL-8* and *MN* and siRNA for NRF2 abolished the IH-induced expression of *ON*.

KEYWORDS
intermittent hypoxia, myokine(s), NRF2, OCT1, sleep apnoea syndrome

1 | INTRODUCTION

Sleep apnoea syndrome (SAS) is a common disorder characterized by repetitive episodes of oxygen desaturation during sleep, by development of daytime sleepiness, and by deterioration of quality of life.[1,2] SAS is caused by the obstruction of the upper airway, and moderate to severe cases of SAS affect 10%–17% of men and 3%–9% of women aged between 30 and 70 years.[3] During sleep, the repeated upper airway obstruction in SAS patients can cause serious recurrent apnoea, and it exposes these patients to alternating low oxygen pressure and normal oxygen pressure levels, that is, intermittent hypoxia (IH).[4] SAS is associated with many systemic

complications, such as obesity, type 2 diabetes,[4–7] dyslipidaemia,[4,7] cardiovascular diseases (e.g., hypertension, coronary disease, heart failure, and stroke),[8] pulmonary hypertension,[5,9] neurocognitive deficits,[10,11] depression,[12] and impaired memory.[13]

Recently, several proteins called myokines, which are exclusively or predominantly secreted in muscle tissue, were established as directly affecting glucose and lipid metabolism.[14–16] For example, some myokines such as interleukin (IL)-6, IL-8, IL-15, and tumour necrosis factor-α (TNFα) were found to express highly in muscles of Type 2 diabetes patients[17] decreasing insulin sensitivity.[18] Moreover, newly diagnosed Type 2 diabetes and impaired glucose tolerance subjects had higher circulating erythroferrone (ERFE)/myonectin (MN) concentrations than normal subjects; also, plasma MN is correlated positively with waist/hip ratio, body fat percentage, triglyceride, fasting blood glucose, 2-hour blood glucose after glucose overload, fasting insulin, haemoglobin A1c, and with the homeostasis model assessment of insulin resistance.[19] However, the IH-induced changes in the levels of these myokines in myocytes remain elusive.

In the present study, using muscle cells and an in vitro IH system, which is a controlled gas delivery system that regulates the flow of nitrogen and oxygen to generate IH, we investigated the direct effect of IH, a hallmark of SAS, on the gene expression levels of *IL-6*, *IL-8*, *IL-15*, *TNFα*, *myostatin* (*MSTN*), *brain-derived neurotrophic factor* (*BDNF*), *IRISIN*, *Decorin* (*DCN*), *secreted protein acidic and cysteine rich* (*SPARC*)*/osteonectin* (*ON*), *ERFE/MN*, *glucose transporter type 4* (*GLUT4*), *mitogen-activated protein kinase 14* (*MAPK14*), *phosphatidylinositol 3-kinase regulatory subunit β* (*PI3KR2*), and *sirtuin 2* (*SIRT2*). A significant increase in the mRNA levels of *IL-8*, *ON*, and *MN* in two different muscle cells in response to IH treatment was detected. We also showed that the IH-induced upregulation of *IL-8* and *MN* requires octamer binding transcription factor 1 (OCT1) and that the IH-induced upregulation of *ON* requires nuclear factor erythroid 2-related factor 2 (NRF2) as transcriptional factors.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Mouse C2C12 skeletal myoblasts were purchased from Riken BioResource Research Center (Tsukuba, Japan). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (FUJIFILM Wako Pure Chemical Corporation) containing 10% (v/v) fetal calf serum (FCS), 100 units/ml penicillin G (FUJIMILM Wako), and 100 μg/ml streptomycin (FUJIFILM Wako). Once a suitable of cell proliferation was achieved (90% confluency), the medium was changed to the differentiation medium (DMEM containing 2% [v/v] horse serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin). Human rhabdomyosarcoma RD cells were purchased from the Japanese Collection of Research Bioresources Cell Bank (JCRB). The cells were grown in Eagle's Minimum Essential Medium (E-MEM) medium (FUJIFILM Wako) containing 10% (v/v) FCS, 100 units/ml penicillin G, and 100 μg/ml streptomycin. The cells were exposed to either normoxia (21% O_2, 5% CO_2, and balanced N_2) or intermittent hypoxia (IH: 64 cycles of 5 min of sustained hypoxia [SH: 1% O_2, 5% CO_2, and balanced N_2] and 10 min of 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, Wakenbtech CO., Ltd), as described.[4,20–24] These conditions are similar to those reported in studies involving patients with severe SAS, wherein 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,[4] which was nearly equivalent to the medium condition in the present study.

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

Total RNA was isolated from C2C12 and RD cells using an RNeasy Protect Cell Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized from the total RNA as template by using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as described.[4,20–25] Real-time PCR was performed using an SYBR® Fast qPCR kit (KAPA Biosystems) and a Thermal Cycler Dice Real Time System (Takara Bio). All PCR primers were synthesized by Nihon Gene Research Laboratories, Inc. (NGRL); the primer sequences for each primer set are described in Table 1. PCR was performed as follows: an initial step at 95°C for 3 min followed by 40 cycles of 95°C for 3 sec and 60°C for 20 sec for *β-actin*, *rat insulinoma gene (Rig)/RpS15*, *IL-6*, *CCL2*, *IL-8*, *IL-15*, *TNFα*, *MSTN*, *BDNF*, *IRISIN*, *DCN*, *SPARC/ON*, *ERFE/MN*, *NOX2*, *GLUT4*, *MAPK14*, *PI3KR2*, and *SIRT2*. The mRNA expression levels were normalized to the mRNA level of *Rig/RpS15* in mouse samples or of *β-actin* in human samples.

2.3 | Measurement of IL-8, ON, and MN in culture medium by ELISA

RD cells (1×10^5 cells/ml in E-MEM containing 10% (v/v) FCS, 100 units/ml penicillin G, and 100 μg/ml streptomycin) were seeded in 24-well plate. Cells were exposed to either normoxia or IH for 24 h; the culture medium was collected, and the concentrations of IL-8, SPARC (ON), and ERFE (MN) were measured by using an IL-8 ELISA Kit (Proteintech Group Inc), a Human/Pig Osteonectin EIA Kit (Takara Bio), and an Erythroferrone (ERFE) ELISA kit (CLOUD-CLONE Corp), respectively.

2.4 | RNA interference

Small interfering RNA (siRNA) directed against human *OCT1* and *NRF2* were synthesized by NGRL. The sense sequences of siRNA for human *OCT1* and *NRF2* were 5′-CCUCGGAAGAGAUCACUAUtt-3′ (corresponding to the 1295–1313 of NM_002697.4) and

TABLE 1 PCR primers for real-time RT-PCR.

Target mRNA	Primer sequence (Accession number: Position)
Human	
GLUT4	5′-CCCCCTCAGCAGCGAGTGA-3′ (NM_001042.3: 260–278)
	5′-GCACCGCCAGGACATTGTTG-3′ (NM_001042.3: 559–578)
MAPK14	5′-CGAGCGTTACCAGAACCTGT-3′ (NM_001315.3: 413–432)
	5′-GGAGAGCTTCTTCACTGCCA-3′ (NM_001315.3: 499–518)
PI3KR2	5′-ATGGCACCTTCCTAGTCCGAGA-3′ (NM_005027.4: 1601–1622)
	5′-CTCTGAGAAGCCATAGTGCCCA-3′ (NM_005027.4: 1707–1728)
SIRT2	5′-CAGACCCCTCTCACCCTCTG-3′ (NM_012237.4: 108–127)
	5′-GTCATAGAGGCCGGTGGATG-3′ (NM_012237.4: 393–412)
IL-6	5′-GGTACATCCTCGACGGCATC-3′ (NM_000600.5: 236–255)
	5′-GCCTCTTTGCTGCTTTCACAC-3′ (NM_000600.5: 294–314)
IL-8	5′-TAGCAAAATTGAGGCCAAGG-3′ (NM_000584.4: 683–702)
	5′-GGACTTGTGGATCCTGGCTA-3′ (NM_000584.4: 868–887)
IL-15	5′-ATGGATGGCTGCTGGAAAC-3′ (NM_000585.5: 313–331)
	5′-TGCACTGAAACAGCCCAAAA-3′ (NM_000585.5: 491–510)
TNFα	5′-CTTCTCCTTCCTGATCGTGG-3′ (NM_000594.4: 282–301)
	5′-TCTCAGCTCCACGCCATT-3′ (NM_000594.4: 529–537)
MSTN	5′-TGGTCATGATCTTGCTGTAACCTT-3′ (NM_005259.3: 832–855)
	5′-TGTCTGTTACCTTGACCTCTAAAAACG-3′ (NM_005259.3: 885–911)
BDNF	5′-CAGGGGCATAGACAAAAG-3′ (NM_170735.6: 1682–1699)
	5′-CTTCCCCTTTTAATGGTC-3′ (NM_170735.6: 1817–1834)
IRISIN	5′-AGGTGCTGATCATCGTCGT-3′ (NM_001171941.3: 454–472)
	5′-CCTCTGCAGTCCAGGGATT-3′ (NM_001171941.3: 679–697)
DCN	5′-CGCCTCATCTGAGGGAGCTT-3′ (NM_001920.5: 999–1018)
	5′-TACTGGACCGGGTTGCTGAA-3′ (NM_001920.5: 1184–1203)
SPARC/ON	5′-CACGGCAAGGTGTGCGAG-3′ (NM_003118.4: 299–316)
	5′-AGAAGTGGCAGGAAGAGTCGAA-3′ (NM_003118.4: 419–440)
ERFE/MN	5′-AGTCCCGGTGCCAGCGCAA-3′ (NM_001291832.2: 904–922)
	5′-CGCCCAGGAGGACAGCACTGAA-3′ (NM_001291832.2: 1077–1098)
β-Actin	5′-GCGAGAAGATGACCCAGA-3′ (NM_001101.5: 431–448)
	5′-CAGAGGCGTACAGGGATA-3′ (NM_001101.5: 503–520)
Mouse	
Il-6	5′-ACAACCACGGCCTTCCCTACTT-3′ (NM_031168.2: 139–160)
	5′-CAGGATTTCCCAGCGAACATGTG-3′ (NM_031168.2: 245–264)
Il-8	5′-CAGAAAGGAAGTGATAGCAGTCCCA-3′ (NM_011339.2: 211–235)
	5′-CAAAGTGTCTAGAGGTCTCCCGAA-3′ (NM_011339.2: 441–464)
Il-15	5′-ACATCCATCTCGTGCTACTTGT-3′ (NM_008357.2: 537–558)
	5′-GCCTCTGTTTTAGGGAGACCT-3′ (NM_008357.2: 629–649)
Tnfα	5′-CCTCCCTCTCATCAGTTCTA-3′ (NM_013693.3: 368–387)
	5′-ACTTGGTGGTTTGCTACGAC-3′ (NM_013693.3: 450–469)
Mstn	5′-ACTGGAATCCGATCTCTGAAACTT-3′ (NM_010834.3: 688–711)
	5′-GACCTCTTGGGTGTGTCTGTCAC-3′ (NM_010834.3: 898–920)
Bdnf	5′-ATTAGCGAGTGGGTCACAGC-3′ (NM_007540.4: 1097–1116)
	5′-TCAGTTGGCCTTTGGATACC-3′ (NM_007540.4: 1180–1199)
Irisin	5′-TGAAGTGGTCATTGGCTTTGC-3′ (NM_027402.4: 248–268)
	5′-GCGGGTGGTGGTGTTCAC-3′ (NM_027402.4: 318–335)
Dcn	5′-GCTGCGGAAATCCGACTTC-3′ (NM_001190451.2: 791–809)
	5′-TTGCCGCCCAGTTCTATGAC-3′ (NM_001190451.2: 831–850)
Sparc/On	5′-AAACATGGCAAGGTGTGTGA-3′ (NM_009242.5: 535–554)
	5′-AAGTGGCAGGAAGAGTCGAA-3′ (NM_009242.5: 658–677)

(Continues)

(Continues)

TABLE 1 (Continued)

Target mRNA	Primer sequence (Accession number: Position)
Erfe/Mn	5′-GGTGGATCGGCGTGCGTTG-3′ (NM_173395.2: 654–672)
	5′-TCCCGGGGTCGTGTTGGTC-3′ (NM_173395.2: 830–848)
Rig/RpS15	5′-ACGGCAAGACCTTCAACCAG-3′ (NM_009091.2: 343–362)
	5′-ATGGAGAACTCGCCCAGGTAG-3′ (NM_009091.2: 392–412)

5′-CCCAUUGAUGUUUCUGAUCUAtt-3′ (corresponding to the 1097–1117 of NM_006164.5), respectively. The Silencer® Select Human Scrambled siRNA was purchased from Ambion® and was used as a control. Transfection of siRNAs into RD cells was carried out using Lipofectamine® RNAiMAX Transfection Reagent (Life Technologies). Cells were transfected with 5 pmol each of siRNA in a 24-well culture dish as described.[20–25]

2.5 | Construction of reporter plasmid and luciferase assay

Reporter plasmids were prepared by inserting the promoter fragments of human *IL-8* (−1914 to +98, −521 to +98, −194 to +98, −178 to +98, −152 to +98, −151 to +98, −149 to +98, −147 to +98, −141 to +98, −133 to +98, −115 to +98, and −95 to +98), *ON* (−2702 to +145, −997 to +145, −712 to +145, −516 to +145, −105 to +145, −99 to +145, −92 to +145, −72 to +145, and −41 to +145), and *MN* (−3741 to −221, −3738 to −221, −3729 to −221, −3721 to −221, −3702 to −221, −3692 to −221, −3634 to −221, −3582 to −221, −3492 to −221, −3249 to −221, and −3013 to −221) upstream of a firefly luciferase reporter gene in the pGL4.17[*luc2*/Neo] vector (Promega). The reporter plasmids were transfected into human RD cells using Lipofectamine® 3000 (Invitrogen), as described,[20–25] and the cells were exposed to either 64 cycles/24 h of IH, mimicking the myocytes of SAS patients, or to normoxia for 24 h. The cells were harvested, and cell extracts were prepared in Extraction Buffer (0.1 M potassium phosphate, pH 7.8/0.2% Triton X-100; Life Technologies). To monitor transfection efficiency, we co-transfected pCMV-SPORT-βgal plasmid (Life Technologies) in all experiments at a 1:10 dilution. Luciferase activity was measured using a PicaGene Luciferase Assay System (Toyo-ink) and was normalized by the β-galactosidase activity as described previously.[20–25]

2.6 | Data analysis

Results are expressed as mean ± SE. Statistical significance was determined by Student's *t*-test using the GraphPad Prism software (GraphPad Software).

3 | RESULTS

3.1 | Gene expression levels of IL-8, ON, and MN in muscle cells were increased by IH

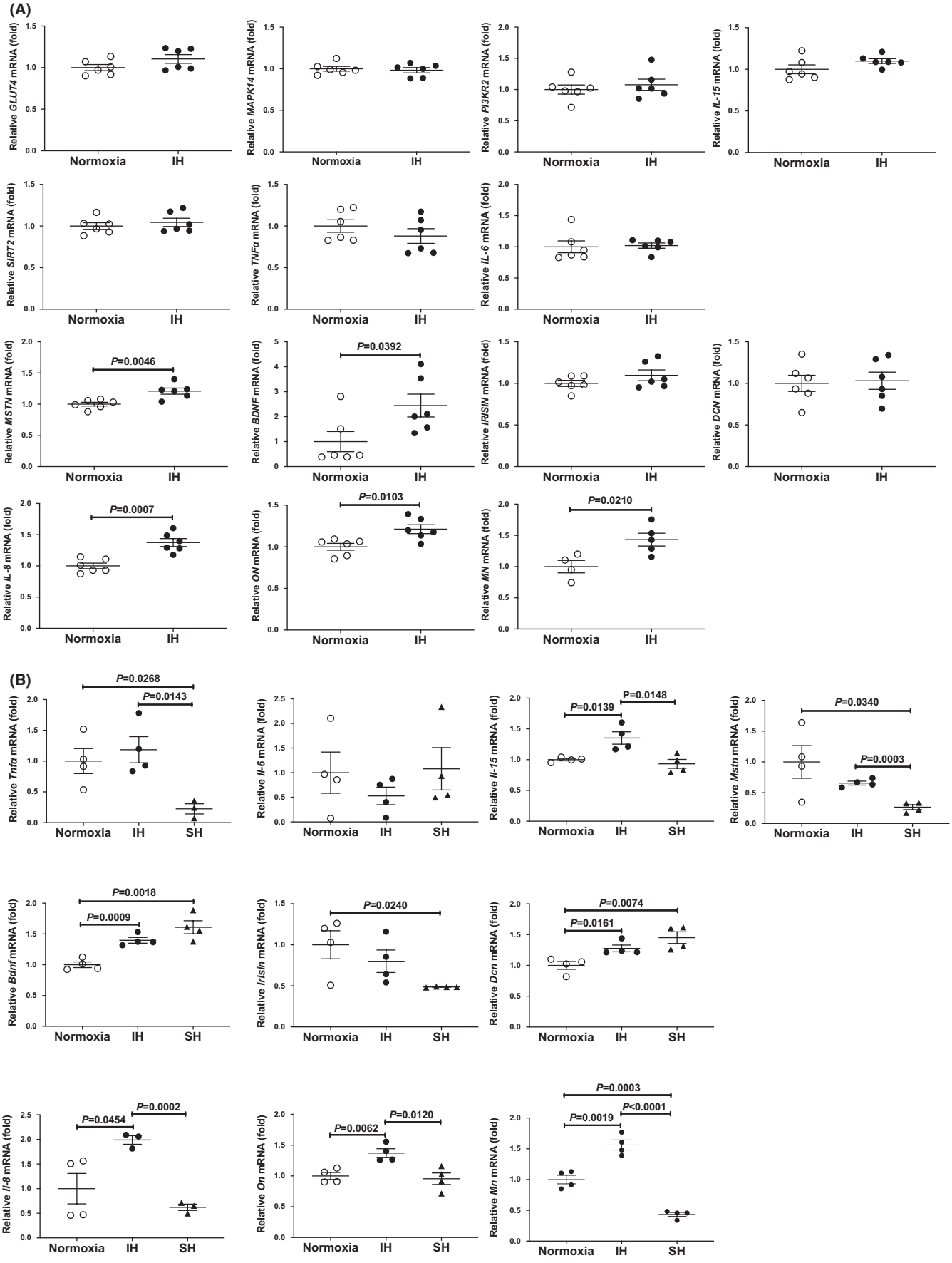
We exposed human RD muscle cells and differentiated mouse C2C12 muscle cells to normoxia or IH for 24 h. After the treatment, we measured the mRNA levels of *GLUT4*, *MAPK14*, *PI3KR2*, *SIRT2*, *TNFα*, *IL-6*, *IL-8*, *IL-15*, *MSTN*, *BDNF*, *IRISIN*, *DCN*, *SPARC/ON*, and *ERFE/MN* by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). As shown in Figure 1A, IH significantly increased the mRNA levels of *MSTN*, *BDNF*, *IL-8*, *ON*, and *MN* in the human RD muscle cells, whereas IH-specific elevations were not observed in *GLUT4*, *MAPK14*, *PI3KR2*, *SIRT2*, *TNFα*, *IL-6*, *IL-15*, *IRISIN*, and *DCN*. In mouse C2C12 cells that have differentiated into muscle cells, IH significantly increased the mRNA levels of *IL-8*, *On*, and *Mn*, but no IH-specific elevations in *Mstn* and *Bdnf* were observed (Figure 1B).

Furthermore, we measured the IL-8, ON (SPARC), and MN (ERFE) protein levels in the RD cell culture medium by using enzyme-linked immunosorbent assay (ELISA), and found that the levels of IL-8, ON, and MN were significantly increased by IH: IL-8 ($p < 0.0001$), ON ($p < 0.0001$), and MN ($p = 0.0079$) (Figure 2).

3.2 | Regions essential for the IH-induced IL-8, ON, and MN promoter activities are localized

In order to investigate the mechanism by which the mRNA levels of *IL-8*, *ON*, and *MN* were upregulated by IH, we prepared the reporter plasmids by inserting various lengths of *IL-8*, *ON*, and *MN* promoter fragments upstream of a firefly luciferase reporter gene in the pGL4.17 vector, transfected them into RD cells, and measured their luciferase activities after IH treatment. As shown in Figure 3A, a deletion down to position −152 of the *IL-8* promoter region resulted in the IH-induced upregulation of the reporter gene expression, but an additional deletion to nucleotide −151 attenuated the IH-induced promoter activity. Concerning *ON* promoter, the deletion down to

FIGURE 1 The mRNA levels of *IL-8*, *ON*, *MN*, *GLUT4*, *MAPK14*, *PI3KR2*, *SIRT2*, *TNFα*, *IL-6*, *IL-15*, *MSTN*, *BDNF*, *IRISIN*, and *DCN*. (A) Human RD and (B) differentiated mouse C2C12 cells treated with normoxia, IH, or sustained hypoxia (SH). The mRNA levels were measured by real-time RT-PCR and normalized by *β-actin* for human cells or by *ribosomal protein S15* (*RpS15*) for mouse cells as internal standard. Data are expressed as mean ± SE of the samples ($n = 4–6$). Statistical analyses were performed using Student's *t*-test. IH significantly increased the mRNA levels of *IL-8*, *ON*, and *MN* in both muscle (RD and C2C12) cells. *GLUT4*, *MAPK14*, *PI3KR2*, *SIRT2*, *TNFα*, *IL-6*, *IL-15*, *IRISIN*, and *DCN* mRNAs levels in IH-stimulated RD cells were 1.104, 0.9821, 1.007, 1.044, 0.8798, 1.020, 1.099, 1.031, and 1.031-folds against normoxia ($p = 0.1359$, 0.6857, 0.5248, 0.5073, 0.3248, 0.8539, 0.1328, 0.2225, and 0.8294, respectively). The mRNA levels of *Tnfα*, *Il-6*, *Mstn*, and *Irisin* in IH-stimulated differentiated C2C12 cells were 1.185, 0.5283, 0.6559, and 0.7994-folds ($p = 0.5533$, 0.3395, 0.6559, and 0.3952, respectively).


(A)
Relative GLUT4 mRNA (fold)
Relative MAPK14 mRNA (fold)
Relative PI3KR2 mRNA (fold)
Relative IL-15 mRNA (fold)
Relative SIRT2 mRNA (fold)
Relative TNFα mRNA (fold)
Relative IL-6 mRNA (fold)
Relative MSTN mRNA (fold)
P=0.0046
Relative BDNF mRNA (fold)
P=0.0392
Relative IRISIN mRNA (fold)
Relative DCN mRNA (fold)
Relative IL-8 mRNA (fold)
P=0.0007
Relative ON mRNA (fold)
P=0.0103
Relative MN mRNA (fold)
P=0.0210
Normoxia
IH
(B)
Relative Tnfα mRNA (fold)
P=0.0268
P=0.0143
Relative Il-6 mRNA (fold)
Relative Il-15 mRNA (fold)
P=0.0139
P=0.0148
Relative Mstn mRNA (fold)
P=0.0340
P=0.0003
Relative Bdnf mRNA (fold)
P=0.0018
P=0.0009
Relative Irisin mRNA (fold)
P=0.0240
Relative Dcn mRNA (fold)
P=0.0074
P=0.0161
Relative Il-8 mRNA (fold)
P=0.0454
P=0.0002
Relative On mRNA (fold)
P=0.0062
P=0.0120
Relative Mn mRNA (fold)
P=0.0003
P=0.0019
P<0.0001
Normoxia
IH
SH

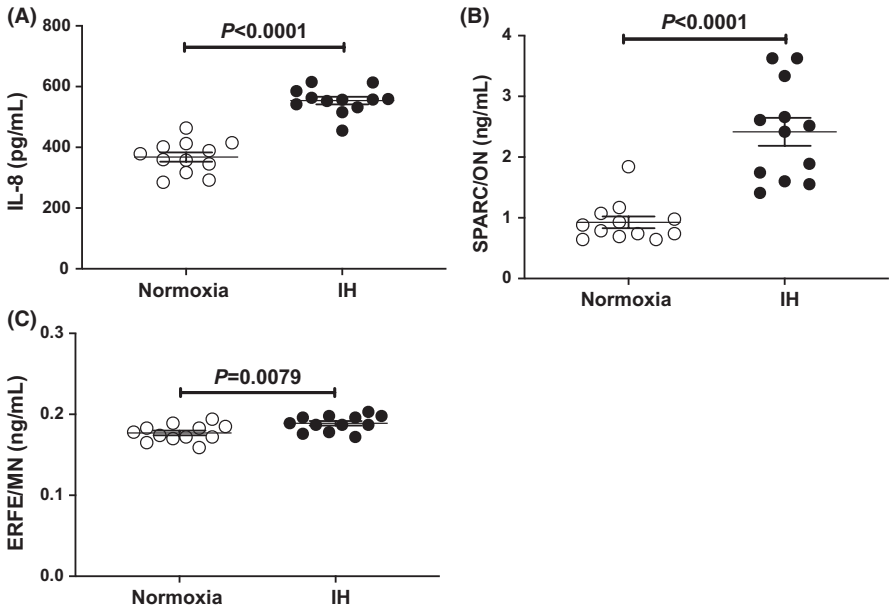


FIGURE 2 Concentrations of IL-8, ON, and MN in RD muscle cell culture medium. RD cells were treated with normoxia or IH for 24 h. The (A) IL-8, (B) ON, and (C) MN concentrations in the medium were measured by ELISA. Data are expressed as mean ± SE for each group ($n = 12$). Statistical analyses were performed using Student's *t*-test.

position −105 caused the IH-induced upregulation of the reporter gene expression, but an additional deletion to nucleotide −99 attenuated the IH-induced promoter activity (Figure 3B). Similarly, a deletion down to position −3741 of the *MN* promoter caused the IH-induced upregulation of the reporter gene expression, but an additional deletion to nucleotide −3738 attenuated the IH-induced promoter activity (Figure 3C). These results indicate that the IH-induced upregulation of *IL-8*, *ON*, and *MN* mRNAs is caused by the transcriptional activation of *IL-8*, *ON*, and *MN* genes and that the −152 to −151 promoter region of the *IL-8* gene, −105 to −99 region of the *ON* gene, and the −3741 to −3738 region of the *MN* gene are essential for the IH-induced promoter activity.

3.3 | OCT1 and NRF2 are key factors for the IH-induced upregulation of IL-8, ON, and MN mRNA expression levels

To further investigate the mechanism by which IH upregulates the *IL-8*, *ON*, and *MN* mRNA levels, we conducted a computer-aided search for sequences similar to known *cis*-acting elements containing the −152 to −151 promoter region of the *IL-8* gene, the −105 to −99 promoter region of the *ON* gene, and the −3741 to −3738 region of the *MN* gene using the TFBIND program (http://tfbind.hgc.jp). The result showed that the −152 ~ promoter region of *IL-8* and the −3741 ~ promoter region of *MN* contained the possible OCT1 binding sequences, whereas the −151 ~ promoter region of *IL-8* and the −3738 ~ promoter region of *MN* lost the OCT1 binding sequences. The −105 ~ promoter region of *ON* contains a possible NRF2 transcription factor binding sequence, whereas there is no NRF2 binding sequence in the −99 ~ promoter region.

To investigate whether OCT1 and/or NRF2 were essential for the IH-induced upregulation of *IL-8*, *ON*, and *MN* mRNA levels, we introduced small interfering RNA (siRNA) s against human *OCT1* and *NRF2* mRNAs into RD cells and analysed the IH-induced mRNA expression levels of *IL-8*, *ON*, and *MN* by real-time RT-PCR. As shown in Figures 4A–C, the IH-induced upregulation of *IL-8*, *ON*, and *MN* mRNAs, respectively, were abolished by the human *OCT1* and *NRF2* siRNAs. Furthermore, IH-induced upregulation of IL-8, ON, and MN in the culture medium were also abolished by the introduction of human *OCT1* and *NRF2* siRNAs (Figures 5A–C). Specifically, the results indicated that OCT1 is a key factor for the IH-induced upregulation of *IL-8* and *MN* mRNA expression levels and that NRF2 serves as an essential factor for the IH-induced upregulation of *ON* mRNA expression.

4 | DISCUSSION

In this study, we demonstrated that IH exposure induced the increase in the *IL-8*, *ON*, and *MN* mRNA levels in muscle cells. Furthermore, we elucidated the mechanisms by which IH upregulates the mRNA levels of the myokines *IL-8*, *ON*, and *MN*, and we revealed the OCT1- and NRF2-mediated transcriptional regulation in IH-stimulated myocytes.

The causal mechanisms mediating the association between IH and insulin resistance/glucose intolerance are not well established; however, augmented dysfunction/inflammation in muscle cells might be involved.[18,26–30] It is well known that macrophages, which infiltrate into muscle tissue, are increased in obese patients, resulting in the upregulation of pro-inflammatory cytokines, such as IL-8.[31] Some mechanisms linking IH stress and muscle tissue inflammation have been reported.[32,33] Recently, IH was shown to induce impairment of muscle tissue, leading to various changes in the secretion of inflammatory cytokines called myokines.[34] Myokines, which are bioactive mediators produced and released by myocytes, play important roles in many physiological and pathophysiological processes that contribute in the modulation

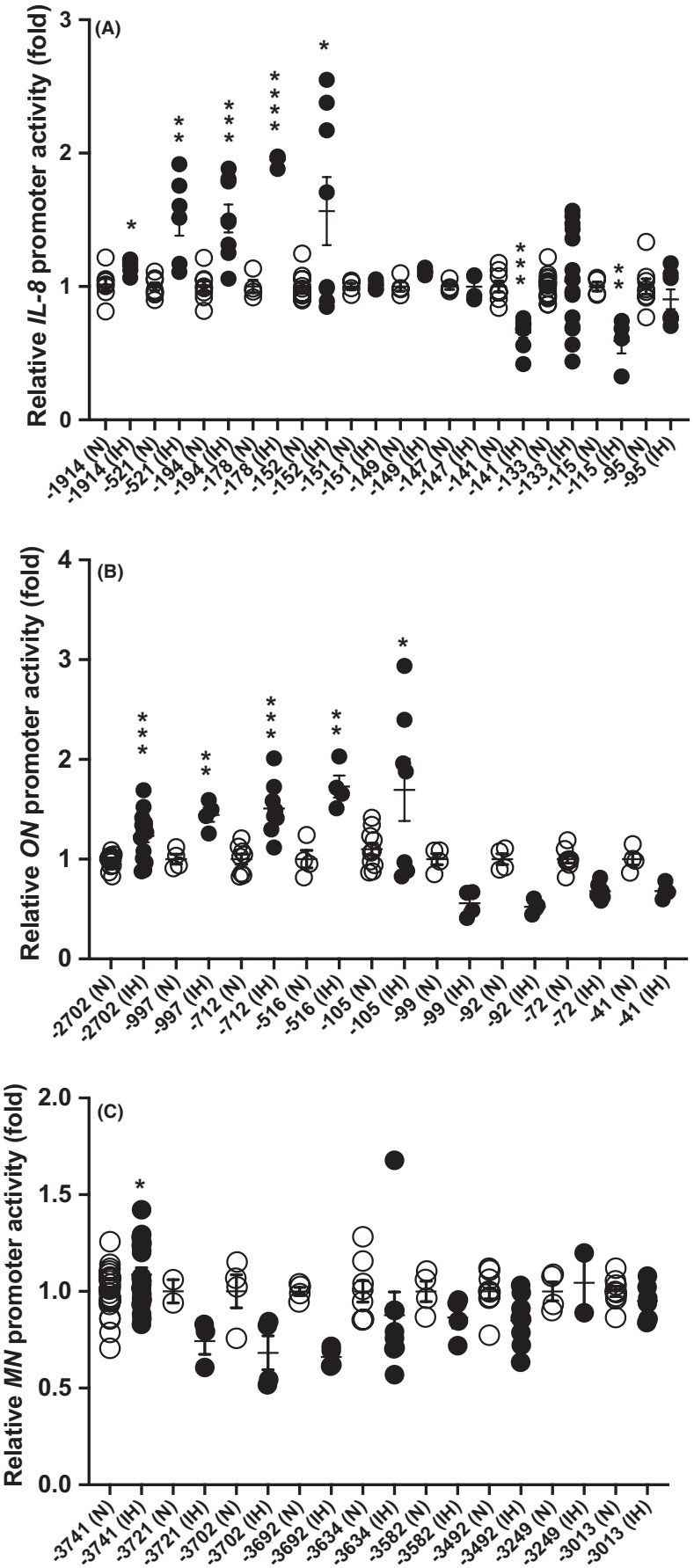


FIGURE 3 Localization of the essential region for IH-induced *IL-8*, *ON*, and *MN* promoter activities. RD cells were transfected with constructs containing various lengths of promoter fragments upstream of a firefly luciferase reporter gene in the pGL4.17 vector. After treatment with normoxia (N) or IH, the luciferase activity was measured. The diagram represents relative luciferase activities of (A) "-1914" in *IL-8*, (B) "-2702" in *ON*, and (C) "-3741" in *MN* in the normoxia group. All data are expressed as the mean ± SE for each group ($n = 4$–16). *: $p < 0.05$ vs Normoxia, **: $p < 0.01$ vs Normoxia, ***: $p < 0.001$ vs Normoxia, and ****: $< p < 0.0001$ vs Normoxia. Statistical analyses were performed using Student's *t*-test.

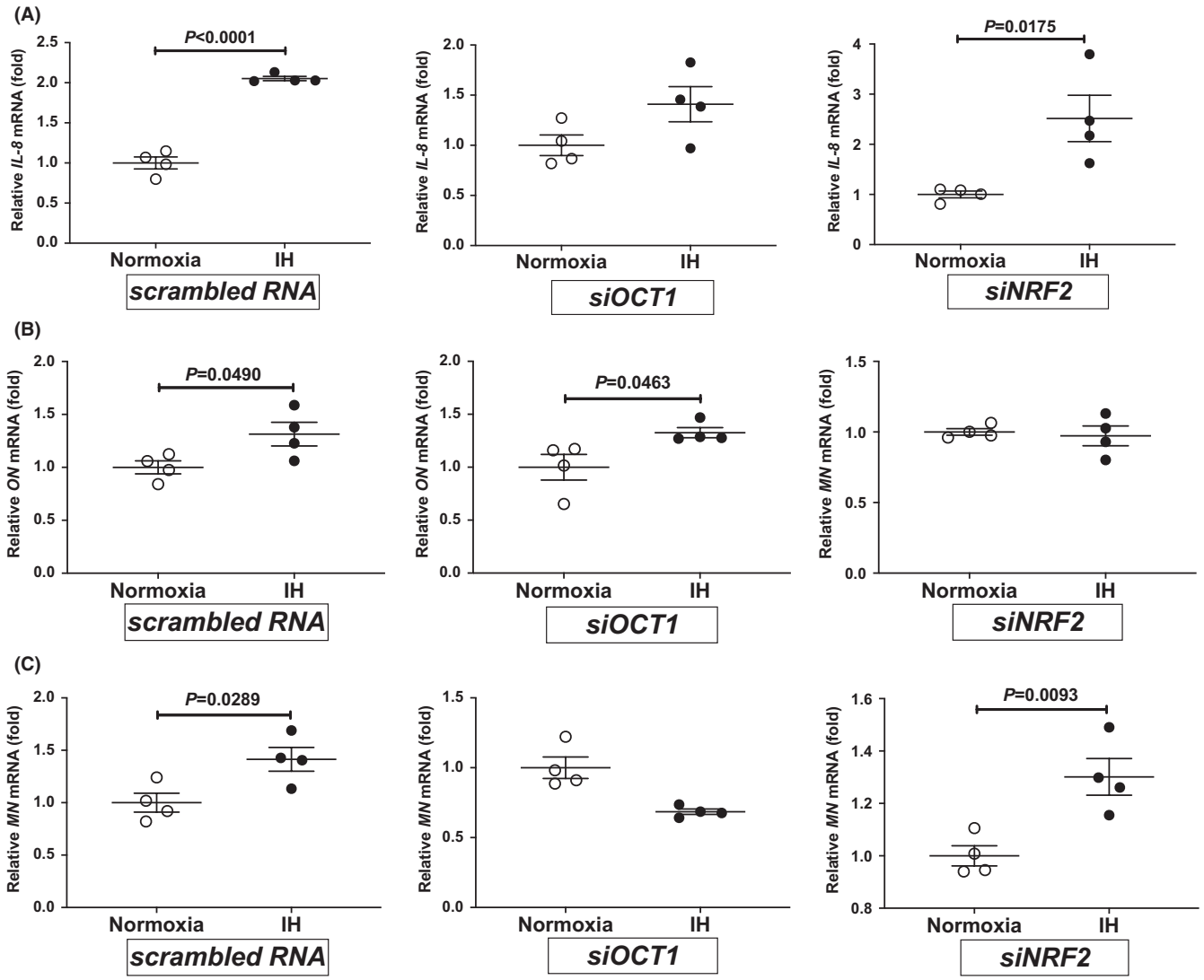


FIGURE 4 Inhibition of the IH-induced upregulation of the *IL-8*, *ON*, and *MN* mRNAs by transfection of OCT1 and NRF2 siRNAs into RD cells. After the introduction of OCT1 and NRF2 siRNAs, RD cells were treated with normoxia or IH for 24 h. The mRNA expression levels of (A) *IL-8*, (B) *ON*, and (C) *MN* were measured by real-time RT-PCR and normalized by β-Actin as internal standard. Data are expressed as the mean ± SE for each group ($n = 4$). Statistical analyses were performed using Student's *t*-test. IH-induced expressions of *IL-8* mRNA in siOCT1 introduction, of *ON* mRNA in siNRF2 introduction, and of *MN* mRNA in siOCT1 introduction were not upregulated (1.409, 0.972, and 0.6851-fold, respectively).

of homeostasis, lipid and/or glucose metabolism, and inflammation.[18,35,36] IL-8, also referred to as chemokine (C-X-C motif) ligand 8, is a key regulator of monocyte infiltration into skeletal muscle and is involved in muscle tissue inflammation and insulin resistance.[37–39] In this study, the mRNA levels of *IL-8* were significantly increased in the IH condition in mouse C2C12 and human RD muscle cells. ON, also referred to as SPARC, is an acidic extracellular matrix glycoprotein that plays a vital role in bone mineralization, cell-matrix interactions, and collagen binding.[40] ON plays a key role in obesity-related insulin resistance, and increased levels of ON contribute to impaired glucose homeostasis; however, the role of ON as a myokine in the IH condition has not yet been fully elucidated.[41,42] In this study, ON was produced/secreted by C2C12 and RD myocytes in the IH condition. In SAS patients, the circulating levels of IL-8 were reportedly elevated,[43] and the production of IL-8 in monocytes was upregulated.[44] ON has also been reported to be associated with insulin resistance, dyslipidaemia, and inflammation.[42] Overexpression of ON was observed in diet-induced obese rats, and ON caused insulin resistance in 3 T3-L1 mouse adipocytes.[45]

MN, also referred to as erythroferrone (ERFE) or C1q/TNF-related protein isoform 15 (CTRP15), is a pro-inflammatory myokine and reported to be associated with insulin resistance.[19,46] Recently, MN was proposed as a marker of insulin resistance, obesity, and diabetes[19]; however, the relationship between insulin resistance and MN expression remains controversial.[19,47] Our results demonstrated that MN was produced/secreted by RD myocytes in the IH condition (Figures 1 and 2). In addition, recent studies indicated that *MN*

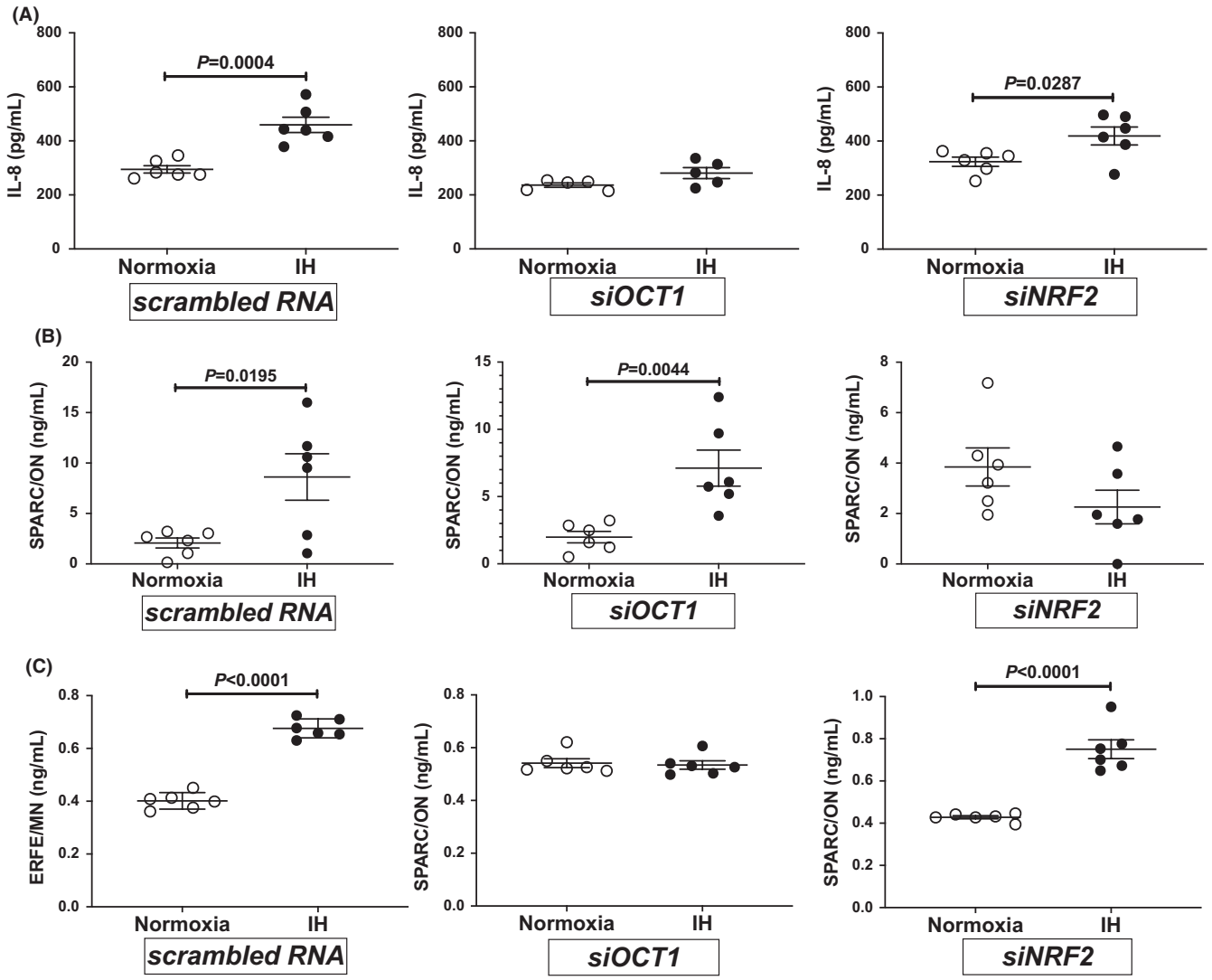


FIGURE 5 Concentrations of IL-8, ON, and MN in RD muscle cell culture medium. After introduction of OCT1 and NRF2 siRNA, RD cells were treated with normoxia or IH for 24 h. The (A) IL-8, (B) ON, and (C) MN concentrations in the medium were measured by ELISA. Data are expressed as mean ± SE for each group ($n = 5–6$). Statistical analyses were performed using Student's *t*-test. IH-induced expressions of IL-8 in siOCT1 introduction, ON in NRF2 introduction, and MN in siOCT1 introduction were not upregulated ($p = 0.0773$, 0.1465, and 0.7654, respectively).

gene knockout female mice had larger gonadal fat pads and had developed mild insulin resistance when fed with a high-fat diet, and *MN*-deficient male mice showed impaired lipid tolerance.[48] On the basis of our results and of the reported findings, *MN* may be upregulated in SAS patients and can lead these patients to develop insulin resistance/Type 2 diabetes and dyslipidaemia.[49] In addition to *IL-8* and *ON*, *MN* is possibly one of the myokines that promote insulin resistance in IH condition. There are some reports that upregulated myokines (IL-8, ON, and MN) in muscle cells under insulin-resistant conditions. Anti-diabetic 5′ AMP-activated protein kinase (AMPK) activator 5-aminoimidazole-4-carboxamide-1-beta-d-ribonucleoside (AICAR) reduced IL-6 and IL-8 in human skeletal muscle cells.[50] In insulin-resistant condition, ON was upregulated in skeletal muscle cells via microRNA-29a downregulation mechanism.[51] Plasma levels of MN are associated with insulin resistance in adult humans.[52] Therefore, IL-8, ON, and MN expressed in skeletal muscle cells could be involved in IH-induced diabetes and/or insulin resistance.

We investigated the mechanisms by which IH upregulates the mRNA levels of *IL-8*, *ON*, and *MN*, and we found that the promoter activities of these genes were increased by IH via OCT1 and NRF2. This finding suggests that the IH-induced upregulation of *IL-8*, *ON*, and *MN* mRNAs is regulated in the transcriptional step.

Interestingly, significant increases in the *IL-8*, *ON*, and *MN* gene expression levels induced by IH were observed. The subsequent promoter assays indicated that the IH-induced upregulation of *IL-8*, *ON*, and *MN* mRNAs was caused by the transcriptional activation of these genes. In addition, RNA interference experiments revealed that the transcriptional activation of *IL-8* and *MN* by IH required OCT1 and that the transcriptional activation of *ON* by IH needed NRF2. Furthermore, we demonstrated that both OCT1 and NRF2

are essential for the IH-induced upregulation of *IL-8*, *MN*, and *ON* mRNA expression levels. The gene expression levels of *IL-8*, *MN*, and *ON*, which are expressed in muscle cells, are remarkably upregulated by IH through the transcription factors OCT1 and NRF2. As a computer-aided search revealed that the −152 to −151 region of the *IL-8* promoter and the −3741 to −3738 region of the *MN* promoter, as well as the −105 to −99 region of the *ON* promoter, (both promoters are essential for the IH-induced *IL-8*, *MN*, and *ON* transcription) include the possible OCT1 and NRF2 transcription factor binding sequences; thus, we focused on OCT1 and NRF2 as important players in the IH-induced upregulation of *IL-8*, *MN*, and *ON* mRNAs in muscle cells.

OCT1, a POU class 2 homeobox1 (POU2F1), transcription factor, was among the first identified members of the POU transcription factor family.[53] The members of this family contain the POU domain, a 160-amino acid region necessary for DNA binding to the octameric sequence ATGCAAAT.[54] Oct1 was reported to function as a sensor for metabolic and stress signals in pancreatic islets.[55] Although OCT1 expression has not yet been reported in SAS patients or in cells in the IH condition, OCT1 could function as an intramuscular IH sensor to express IL-8 and MN.

NRF2, which was originally isolated as a homologue of the haematopoietic transcription factor NF-E2 p45,[56] is the key transcription factor regulating antioxidant response. NRF2 signalling is repressed by Kelch-like ECH-associated protein 1 (KEAP1)[57] at basal condition and is induced by oxidative stress. Most papers reporting on the relationship between NRF2 and SAS/IH suggested some protective functions of NRF2 in SAS/IH conditions.[58–61] Stress-induced upregulation of ON was reported in vascular smooth muscle cells.[62,63] It is quite reasonable that the KEAP1-NRF2 system functions as an intracellular sensor for oxygen and oxidative stress in IH condition. As a result, *ON* transcription could be activated by NRF2.

In this study, we observed no elevation of *GLUT4* mRNA in IH-treated human RD cells (Figure 1A). Siquws et al. exposed rats to intermittent hypoxia (IH) for 30 days and reported that there is no change in the expressions of Glut1 and Glut4 in soleus muscle but an increase in the translocation of Glut4 from vesicles to the plasma membrane.[64] In contrast, Wang et al. exposed rat to IH for 28 days and reported that the expressions of *Glut4* mRNA, total Glut4, and plasma membrane protein of Glut4 in skeletal muscle were decreased.[65] Thus, the effect of IH on Glut4 expression and localization seems to be controversial. *Bdnf* mRNA was elevated in IH and further elevated in SH in human RD and mouse C2C12 cells (Figure 1A,B). Although there is no report concerning Bdnf elevation in IH condition, Bdnf is reported to be a positive-acting myokine that is abundantly expressed in slow-twitch skeletal muscle fibres, and its beneficial effects in skeletal muscle are mediated through AMPK-PGC1α-mediated mitochondrial function and β-oxidation.[66–69] In SH condition, Nagahisa and Miyata reported that the muscle fibre area was decreased and mRNA expression of Bdnf was significantly increased in young soleus muscle.[70] Therefore, in stress condition (SH and/or IH), it is quite possible that muscle cells express Bdnf to maintain muscle function and cell numbers. Although the mechanism of hypoxia-induced Bdnf expression is interesting, it is not IH-specific phenomenon (Figure 1B) and therefore we did not further investigated in this paper.

In this study, we use in vitro IH model to investigate the change in gene expression and its molecular mechanism. How similar/different to/from SAS patients are problems in in vitro system. Although the O_2 concentrations of in vitro system is similar to IH patients and the in vitro system can examine direct effects of IH excluding effects of other organs and cells,[71] the results from in vitro system sometimes are different from that occur in SAS patients. Therefore, our results obtained in this study indicated a possible occurrence in SAS patients and clinical studies using SAS patients are required.

In conclusion, this study revealed that the gene expression levels of *IL-8*, *ON*, and *MN* in IH-treated myocytes were increased by OCT1, which acts on the −152 to −151 region of the *IL-8* promoter and on the −3741 to −3738 region of the *MN* promoter, and by NRF2, which acts on the −105 to −99 region of the *ON* promoter. Our results suggest that in SAS patients, the upregulation of *IL-8*, *ON*, and *MN* may induce a pro-inflammatory phenotype in muscle tissue, leading to the development of insulin resistance and decreased insulin sensitivity.

AUTHOR CONTRIBUTIONS

Shin Takasawa: Conceptualization (lead); funding acquisition (equal); investigation (lead); methodology (lead); project administration (lead); writing – original draft (lead). **Ryogo Shobatake:** Conceptualization (supporting); data curation (supporting); funding acquisition (equal); investigation (supporting); validation (supporting); writing – review and editing (supporting). **Asako Itaya-Hironaka:** Data curation (supporting); methodology (supporting); writing – review and editing (supporting). **Mai Makino:** Data curation (supporting); writing – review and editing (supporting). **Tomoko Uchiyama:** Data curation (supporting); writing – review and editing (supporting). **Sumiyo Sakuramoto-Tsuchida:** Data curation (supporting); writing – review and editing (supporting). **Yoshinori Takeda:** Data curation (supporting); writing – review and editing (supporting). **Hiroyo Ota:** Data curation (supporting); funding acquisition (equal); writing – review and editing (supporting). **Akiyo Yamauchi:** Data curation (supporting); writing – review and editing (supporting).

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CONFLICT OF INTEREST

The authors confirm that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data are available on request from the authors.

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