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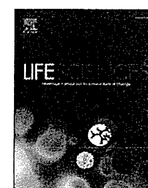

Pancreatic β cell proliferation by intermittent hypoxia via up-regulation of *Reg* family genes and *HGF* gene

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ABSTRACT

Aims: Although accumulating evidence suggests the associations between sleep apnea syndrome (SAS) and type 2 diabetes, the direct effect of intermittent hypoxia (IH) on pancreatic β cell proliferation remains a missing piece of the puzzle.
Main methods: Rat RINm5F β cells, hamster HIT-T15 β cells, and human 1.1B4 β cells were exposed to normoxia (21% O_2, 5% CO_2, and balance N_2), to sustained hypoxia (SH: 1% O_2, 5% CO_2, and balance N_2), or to intermittent hypoxia (IH: 64 cycles of 5 min SH and 10 min normoxia) for 24 h. After the treatment, cellular proliferation and apoptosis were measured by WST-8 assay and TUNEL method, respectively. The expression of regenerating gene (*Reg*) family, *interleukin* (*IL*)*-6*, and *hepatocyte growth factor* (*HGF*) was determined by real-time RT-PCR.
Key findings: The cellular proliferation of HIT-T15, RINm5F and 1.1B4 cells by IH was significantly increased, whereas apoptosis of these cells was unchanged. Real-time RT-PCR revealed that the mRNA levels of *Reg* family genes, *IL-6*, a typical *Reg* family gene inducer, and *HGF*, an inhibitor of high-concentration of Reg protein-induced apoptosis, were increased in IH-treated cells. In addition, siRNAs against rat *Reg* family genes except for *PAP I/Reg 2* attenuated IH-induced β cell proliferation.
Significance: IH stress stimulates pancreatic β cell to induce *IL-6* gene expression. By the IL-6 stimulation, β cells over-express *Reg* family genes as well as *HGF* gene. Reg family proteins stimulate β cell proliferation and HGF inhibits apoptosis of β cells. As a result, β cell numbers are increased by IH.
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Introduction

Sleep apnea syndrome (SAS) is characterized by recurrent episodes of oxygen desaturation during sleep, the development of daytime sleepiness, and deterioration in the quality of life. SAS results in long-term exposure to intermittent hypoxia (IH), which represents a form of oxidative stress leading to the increased generation of reactive oxygen species during reoxygenation, similar to that seen in ischemia-reperfusion injury (Lavie, 2003; Zhan et al., 2005). Recently, SAS comes to be considered as an inflammatory systemic disease. Accumulating evidence suggests that recurrent intermittent hypoxia/reoxygenation in SAS may lead to a state of oxidative stress (Schulz et al., 2002; Lavie, 2003; Ryan et al., 2005), and activation of inflammatory transcription factors (Lavie, 2003; Ryan et al., 2005). We reported that SAS-induced hypoxic stress activates the production of inflammatory mediators such as tumor necrosis factor-α, matrix metalloproteinase-9, and monocyte chemoattractant protein-1 in human monocytes, contributing to the development of atherosclerosis (Tamaki et al., 2009). Obesity is recognized as a major health care problem and the high prevalence of the association with the metabolic syndrome, the commonly used term for the cluster of obesity, insulin resistance, hypertension, and dyslipidemia. Recently, it has been suggested that SAS may contribute to the development of metabolic syndrome (Coughlin et al., 2004; Tasali and Ip, 2008). Obesity is a predominant risk factor for SAS (Young et al., 2002), insulin resistance, hyperglycemia, and type 2 diabetes (Mokdad et al., 2001). Multiple epidemiological studies have provided evidence implicating the presence of SAS as a risk factor of insulin resistance and type 2 diabetes (Tasali et al., 2008). Punjabi et al. reported that type 2 diabetes is associated with SAS independently of age, sex, and body habitus (Punjabi et al., 2004). Muraki et al. prospectively showed the association between nocturnal intermittent hypoxia and the risk of developing type 2 diabetes among community-dwelling Japanese participants (Muraki et al., 2010). One of the postulated mechanisms for the metabolic alterations associated with SAS is that the intermittent hypoxia (IH) that characterizes this condition during sleep leads to substantial alterations in both pancreatic beta cell function

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and organ glucose homeostasis. The progression to type 2 diabetes depends on both the impairment of glucose-induced insulin secretion from pancreatic β cells and the presence of insulin resistance. On the other hand, hyperglycemia is known to increase the rate of β cell replication (Bonner-Weir et al., 1989; Steil et al., 2001), which can provide an enlarged source of insulin to combat the insulin resistance. Yokoe et al. (Yokoe et al., 2008) and Xu et al. (Xu et al., 2009) reported that IH causes β cell replication without hyperglycemia in mice model, suggesting a possible mechanism that IH directly stimulates β cell replication. However, the impact of β cell replication remains elusive. In this study we investigated direct effects of IH on β cell replication and the changes of gene expression by IH.

Materials and methods

Cell culture

Hamster insulinoma HIT-T15 cells (ATCC number: CRL-1777) were purchased from American Type Culture Collection (Manassas, VA, USA) and were grown in RPMI1640 medium (Sigma, St. Louis, MO, USA) containing 5.5 mM glucose, 10% (v/v) fetal calf serum (FCS), 100 units/ml penicillin G (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and 100 μg/ml streptomycin (Wako) as described (Ota et al., 2012). RINm5F cells, a rat insulinoma-derived β cell line, were grown in RPMI1640 medium (Sigma) containing 10% (v/v) FCS, 100 units/ml penicillin G (Wako), and 100 μg/ml streptomycin (Wako) as described (Akiyama et al., 2001; Shervani et al., 2004; Takasawa et al., 2006; Ota et al., 2012). Rat islets of Langerhans were isolated from Wistar male rats (250–350 g) (Kiwa Laboratory Animals Co., Ltd., Kimino, Japan) as described previously (Takasawa et al., 1993, 1998, 2006, 2010; Ota et al., 2012) with approval of the local Ethics Committee and cultured in RPMI1640 medium supplemented with 10% (v/v) FCS, 100 units/ml penicillin G (Wako), and 100 μg/ml streptomycin (Wako) as described (Ota et al., 2012). 1.1B4 cells, an insulin-releasing human pancreatic β cell line, were purchased from European Collection of Cell Culture (Salisbury, UK) and were maintained as described (McCluskey et al., 2011). Cells were either exposed to normoxia (21% O_2, 5% CO_2, and balance N_2), to sustained hypoxia (SH: 1% O_2, 5% CO_2, and balance N_2), or to intermittent hypoxia (IH: 64 cycles of 5 min SH 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, 9200E SP, Wakenyaku Co., Ltd, Kyoto, Japan) as described (Ota et al., 2012). This condition is almost similar with that reported in patients with severe degree of SAS: In severe degree of SAS, patients are repeatedly exposed to severe hypoxemia followed by mild hypoxemia or normoxic condition, i.e., IH. We previously reported that the magnitude of IH expressed by SpO_2 was fluctuated between 75–98% and 50–80% in SAS (Niijima et al., 1999), which was almost equivalent to the medium condition in the present study.

Measurement of viable cell numbers by tetrazolium salt cleavage

RINm5F cells, HIT-T15 cells, and 1.1B4 cells (2.5×10^4 cells/100 μl in 96-well plate) were incubated 37 °C over night and the medium was replaced with RPMI1640 + 1% FCS. After a 24-h treatment of normoxia, IH, or SH, the viable cell numbers were determined by a Cell Counting kit-8 (Dojindo Laboratories, Mashikimachi, Japan) according to the manufacturer's instructions. Briefly, WST-8 (2-(2-methoxy-4-nitrophenyl) -3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt) solution was added to cells in 96-well plates, and the cells were incubated at 37 °C for 60–90 min. The optical density of each well was read at 450 nm (reference wave length at 650 nm) using a Sunrise™ microplate reader (Tecan, Männedorf, Swizerland) as described (Ota et al., 2012).

Measurement of apoptosis

RINm5F cells and HIT-T15 cells (2.5×10^4 cells/100 μl in 96-well plate) were incubated 37 °C over night and the medium was replaced with RPMI1640 + 1% FCS. After a 24-h treatment of normoxia or IH, apoptosis was detected by the TUNEL method using an apoptosis screening kit (Wako) as described (Kobayashi et al., 2000; Ota et al., 2012). For measurement of Reg I protein (high concentration)-induced apoptosis, RINm5F cells were cultured in RPMI1640 with 1% FCS in the presence of 1000 nM rat Reg I protein (Watanabe et al., 1994; Kobayashi et al., 2000; Akiyama et al., 2001; Shervani et al., 2004; Takasawa et al., 2006) with/without recombinant human hepatocyte growth factor (HGF) (Genzyme, Cambridge, MA, USA) for 24 h, and apoptosis was measured as described above.

Real-time RT-PCR

Total RNA was isolated using a RNA protect cell mini kit (Qiagen, Hilden, Germany) from RINm5F and 1.1B4 cells, and cDNA was synthesized from total RNA as template using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) as described (Ota et al., 2012). Real-time PCR was performed using SYBR® Fast qPCR Kit (KAPA Biosystems, Woburn, MA, USA). All the PCR primers were synthesized by NGRL (Sendai, Japan), and the primer sequences for each primer set are described in Table 1. The mRNA expression levels were normalized to the mRNA level of hypoxanthine phosphoribosyltransferase (*HPRT*) in rat or β-actin in human.

RNA interference (RNAi)

RNAi was performed using *Silencer®* Select predesigned small interfering RNAs (siRNAs) (Life Technologies, Carlsbad, CA, USA) directed against rat *Reg* family genes (*Reg I, PAP I/Reg2, PAP II/Reg III, PAP III*, and *Reg IV*) (Okamoto and Takasawa, 2002). The sense sequences of siRNAs for rat *Reg I, PAP I/Reg2, PAP II/Reg III, PAP III*, and *Reg IV* were 5′-GAAAUGGAGAGAUAACAGUtt-3′, 5′-CCCUGUUUCAGAUACCACAtt-3′, 5′-UGUCAACCGUGGUAACUGUtt-3′, 5′-GAAUAAUUGUAUCUCAGAAtt-3′ and 5′-GGGUACUUCCGGAAGCUAAtt-3′, respectively. The *Silencer®* Select GAPDH siRNA (Hs, Mm, Rn) was used as a control. Transfection of siRNAs to RINm5F cells was carried out using siPORT™ *NeoFx*™ Transfection Agent (Life Technologies). Cells were transfected with 0.5 pmol each of siRNA in a 96-well culture dish (7.5×10^4 cells/ml).

Data analysis

Results are expressed as mean ± SE. Statistical significance was determined by Student's *t* test using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

Results

β-cell proliferation was increased by IH

To evaluate direct effects of IH on β cell proliferation, RINm5F, HIT-T15, and 1.1B4 β cells were exposed to normoxia, IH, or SH for 24 h. After the treatment, the cell viability was determined by WST-8 assay. The viable cell numbers of rat RINm5F, hamster HIT-T15, and human 1.1B4 β cells were significantly increased by IH ($P = 0.0013$, $P = 0.0064$, and $P = 0.0113$, respectively; Fig. 1), whereas the cell numbers were decreased in RINm5F and HIT-T15 cells ($P = 0.004$ and $P = 0.0001$, respectively) and were not changed in 1.1B4 cells by SH. To determine whether IH decreases apoptosis, we measured apoptosis of RINm5F and HIT-T15 cells by TUNEL method. As shown in Fig. 2, there was no statistically significant difference in apoptosis between normoxia- and IH-treated cells. In addition, overexpression of cell proliferation regulators such as cyclin-dependent kinase 4 (CDK4) and

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

Primer (position)
Rat *HPRT*
Forward 5′-CTCATGGACTGATTATGGACAGGAC-3′ (179–203 of NM_012583.2)
Reverse 5′-GCAGGTCAGCAAAGAACTTATAGCC-3′ (277–301 of NM_012583.2)
Rat *CDK4*
Forward 5′-TTTGATCTCATTGGATTGCC-3′ (864–883 of NM_053593)
Reverse 5′-AGGTCAGCATTTCCAGCAG-3′ (999–1017 of NM_053593)
Rat *E2F*
Forward 5′-TTCTTGGAGCTGCTGAGCC-3′ (562–580 of NM_001100778)
Reverse 5′-TGGTGATGTCATAGATGCG-3′ (643–661 of NM_001100778)
Rat *Reg I*
Forward 5′-GGACACTGGGTATCCTAACAATTCC -3′ (424–448 of M18962)
Reverse 5′-CTCTCCATTTCTTGTATCCTGAGTTTG -3′ (477–503 of M18962)
Rat *Reg IV*
Forward 5′-CTGCTGAGCTGGGTAGCTGGCCC-3′ (31–53 of AB164049)
Reverse 5′-TTTATCCTTGGGGTTCATCTCAG-3′ (386–408 of AB164049)
Rat *PAP I/Reg II*
Forward 5′-AAAATACCCTCTGCACGCATTAG-3′ (153–171 of NM_053289)
Reverse 5′-GGGCATAGCAGTAGGAGCCATA-3′ (198–219 of NM_053289)
Rat *PAP II/Reg III*
Forward 5′-CCAGAAGGCAGTGCCCTCTA-3′ (240–259 of L10229)
Reverse 5′-GCAGTAAGAACGATAAGCCTTGGA-3′ (283–306 of L10229)
Rat *PAP III*
Forward 5′-TGTGCCCACTTCACGTATCAG-3′ (121–140 of L_20869)
Reverse 5′-GTTGTTGATCTTCCCATTGGG-3′ (162–184 of L_20869)
Rat *IL-6*
Forward 5′-AAGTCGGAGGCTTAATTACATATGTTC-3′ (213–239 of NM_012589)
Reverse 5′-TGCCATTGCACAACTCTTTTCT-3′ (260–281 of NM_012589)
Rat *IL-6 receptor*
Forward 5′-ACCTGTATGGTCAAAGACGTTC-3′ (784–805 of NM_017020)
Reverse 5′-GTCTTCAACAGAGACCTGTGTG-3′ (1000–1021 of NM_017020)
Rat *gp130*
Forward 5′-CCGTCAGTGCAAGTGTTCTCA-3′ (NM_001008725: 2260–2280)
Reverse 5′-CACTATCCACCAGCTGCAGGT- 3′ (NM_001008725: 2330–2350)
Rat *HGF*
Forward 5′-GGCTGAAAAGATTGGATCAGGAC-3′ (2131–2153 of NM_017017)
Reverse 5′-ATCCACGACCAGGAACAATG-3′ (2221–2240 of NM_017017)
Human *REG Iα*
Forward 5′-AGGAGAGTGGCACTGATGACTT-3′ (369–390 of NM_002909)
Reverse 5′-TAGGAGACCAGGGACCCACTG-3′ (445–465 of NM_002909)
Human *REG Iβ*
Forward 5′-GCTGATCTCCTCCCTGATGTTC-3′ (108–129 of NM_006507)
Reverse 5′-GGCAGCTGATTCGGGGATTA-3′ (170–190 of NM_006507)
Human *REG III*
Forward 5′-GAATATTCTCCCCAAACTG-3′ (695–713 of AB161037)
Reverse 5′-GAGAAAAGCCTGAAATGAAG-3′ (765–784 of AB161037)
Human *HIP/PAP*
Forward 5′-AGAGAATATTCGCTTAATTCC-3′ (645–665 of NM_138937)
Reverse 5′-AATGAAGAGACTGAAATGACA-3′ (716–736 of NM_138937)
Human *REG IV*
Forward 5′-ATCCTGGTCTGGCAAGTC-3′ (470–487 of AY007243)
Reverse 5′-CGTTGCTGCTCCAAGTTA-3′ (538–555 of AY007243)
Human *IL-6*
Forward 5′-GGTACATCCTCGACGGCATC-3′ (289–308 of NM_000600)
Reverse 5′-GCCTCTTTGCTGCTTTCACAC-3′ (347–367 of NM_000600)
Human *IL-6 receptor*
Forward 5′-TGAGCTCAGATATCGGGCTGAAC-3′ (1196–1218 of X12830)
Reverse 5′-CGTCGTGGATGACACAGTGATG-3′ (1260–1281 of X12830)
Human *gp130*
Forward 5′-AGGACCAAAGATGCCTCAACT-3′ (1097–1117 of NM_002184)
Reverse 5′-TTGGACAGTGAATGAAGATCG-3′ (1154–1174 of NM_002184)
Human *HGF*
Forward 5′-GGCTGAAAAGATTGGATCAGGAC-3′ (2145–2167 of NM_000601)
Reverse 5′-ATCCACGACCAGGAACAATG-3′ (2235–2254 of NM_000601)
Human *E2F*
Forward 5′-ACCCTGCAGAGCAGATGGTT-3′ (907–926 of NM_005225)
Reverse 5′-TTTGCTCTTAAGGGAGATCTGAA-3′ (985–1007 of NM_005225)
Human *β-actin*
Forward 5′-GCGAGAAGATGACCCAGA-3′ (420–437 of NM_001101)
Reverse 5′-CAGAGGCGTACAGGGATA-3′ (492–509 of NM_001101)

E2F has been recognized (Cohn et al., 2010; Lu et al., 2013). We therefore measured *CDK4* and *E2F* mRNAs in rat isolated islets and *E2F* mRNA in 1.1B4 human β cells. Real-time RT-PCR revealed that *CDK4* and *E2F* mRNAs were up-regulated in IH-stimulated rat islets and that *E2F* mRNA was significantly increased in IH-stimulated 1.1B4 β cells (Fig. 3). These results indicate that IH directly increases β cell proliferation.

Reg family gene expression was increased by IH

Regenerating gene (*Reg*) family genes, which encode autocrine/paracrine β cell growth factors, were reported to be involved in β cell proliferation. We analyzed the mRNA levels of *Reg* family genes, by real-time RT-PCR. As shown in Fig. 4, the mRNA levels of *Reg I*, *PAP II/Reg III*, *PAP III*, and *Reg IV* were significantly increased in rat RINm5F β cells by IH stimulation. The mRNA level of *REG Iα* was significantly increased in human 1.1B4 β cells by IH ($P = 0.0052$; Fig. 5).

In order to evaluate independent Reg family members, siRNAs for *Reg* mRNAs were introduced into RINm5F β cells and IH-induced cell proliferation was measured. As shown in Fig. 6, the introduction of siRNAs for *Reg I*, *PAP II/Reg III*, *PAP III*, and *Reg IV* suppressed the IH-induced β cell proliferation, whereas siRNA for *PAP I/Reg2* did not change the cell numbers.

IL-6 gene expression was increased by IH

As *Reg* family genes were activated by IL-6 (Akiyama et al., 2001; Broekaert et al., 2002; Gurr et al., 2002; Kiji et al., 2005; Luo et al., 2013), we tested whether IL-6 also increases human *REG Iα* mRNA. As shown in Fig. 7A, IL-6 significantly increased human *REG Iα* mRNA in 1.1B4 human β cells. We then determined *IL-6* mRNA expression in RINm5F cells and 1.1B4 β cells exposed to IH. As shown in Fig. 7B and C, the mRNA levels of *IL-6* in RINm5F and 1.1B4 cells were increased by IH ($P = 0.0093$ and $P = 0.0302$, respectively), suggesting that IH stimulation up-regulates *Reg* family mRNAs through IL-6 expression in pancreatic β cells. As IL-6 has been also reported to down-regulate its signaling molecules such as IL-6 receptor and gp130, we measured mRNA levels of *IL-6 receptor* and *gp130* in RINm5F cells, rat islets, and 1.1B4 cells exposed to normoxia, IH, or SH. As shown in Fig. 8, any of IH-treated cells (RINm5F cells, rat islets, and 1.1B4 cells) did not show down-regulation of *IL-6 receptor/gp130* mRNAs, suggesting that IL-6 induced by IH up-regulates *Reg* family mRNAs to stimulate β cell proliferation.

HGF gene expression was increased by IH

Although Reg protein is well known as a pancreatic β cell growth factor, it was also reported that a high concentration of Reg protein induced β cell apoptosis (Kobayashi et al., 2000). These findings lead us to an idea that IH-stimulated β cells express some anti-apoptotic factors against a high concentration of Reg protein. It was reported that HGF protected rat RINm5F β cells against free fatty acid-induced apoptosis (Santangelo et al., 2007). Therefore, we tested possible anti-apoptotic action of HGF against a high concentration of Reg protein (1000 nM) and found that HGF (2.5 and 25 ng/ml) protected RINm5F β cells against apoptosis induced by a high concentration of Reg I protein (Fig. 9A). We next examined whether HGF expression is induced by IH or not. As shown in Fig. 9B and C, *HGF* mRNA was significantly induced by IH in RINm5F and 1.1B4 β cells. This simultaneous gene expression of *HGF* with *Reg* family genes may work as a safety valve for pancreatic β cells to minimize apoptotic effects of Reg family proteins.

Discussion

Recently, there has been great interest in the interaction between SAS and metabolic dysfunction. SAS is commonly found in patients with type 2 diabetes. Recent research demonstrates the likelihood of a relationship between the two conditions independent of obesity. The Sleep Heart Health Study (Punjabi et al., 2004) showed a significant association between oxygen desaturation during sleep and elevated

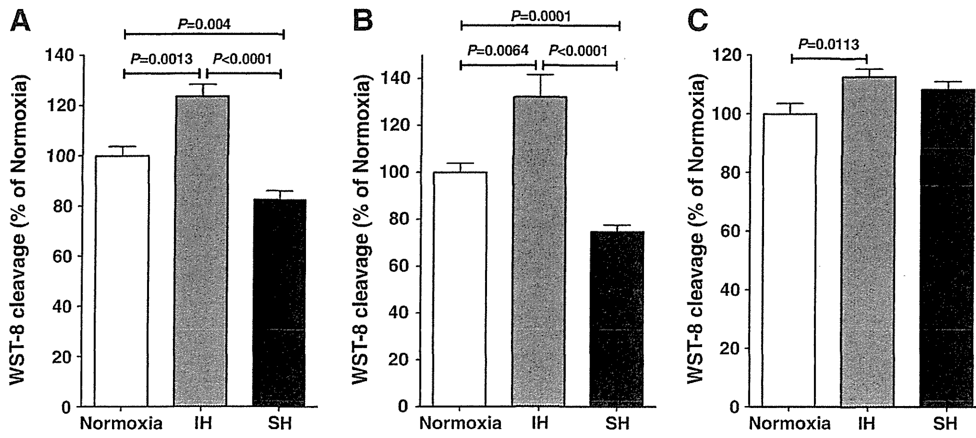

Fig. 1. Effects of hypoxia on cellular proliferation of RINm5F cells (A), HIT-T15 cells (B), and 1.1B4 cells (C). RINm5F, HIT-T15, and 1.1B4 cells were exposed with normoxia, IH, or SH and cellular proliferation was measured by WST-8 assay. Data are expressed as means ± SEM for each group ($n = 6$–8).

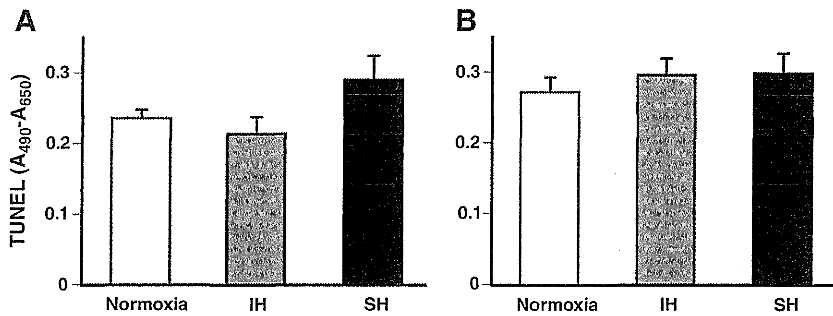

Fig. 2. Apoptosis of RINm5F (A) and HIT-T15 (B) insulinoma cells treated by normoxia, or IH, or SH. RINm5F cells and HIT-T15 cells were incubated in RPMI1640 medium supplemented with 1% FCS for 24 h in normoxia, IH or SH. Apoptosis was detected by the TUNEL method. Data are expressed as means ± SEM for each group ($n = 8$).

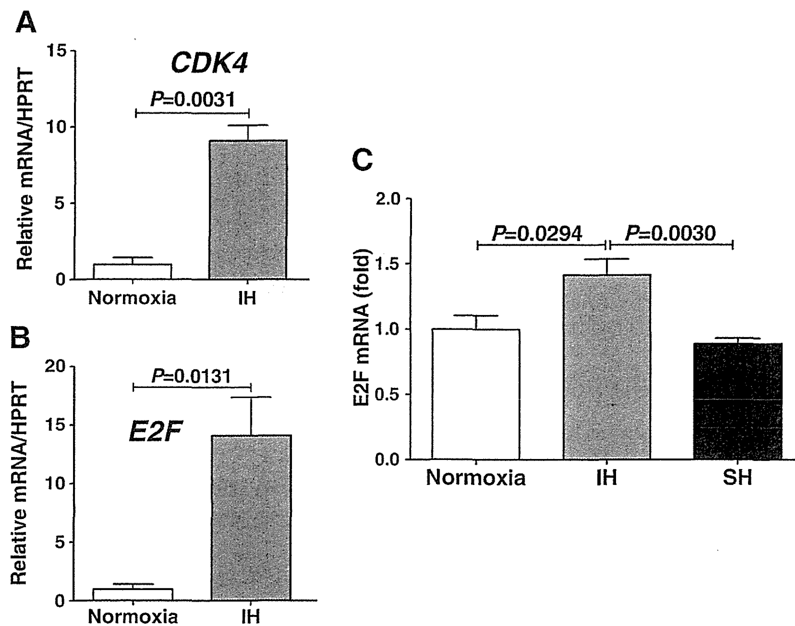

Fig. 3. The mRNA levels of rat *CDK4* (A) and *E2F* (B) in rat islets, and human *E2F* (C) in human 1.1B4β cells. Cells were treated by normoxia, IH or SH for 24 h. The levels of rat *CDK4* and *E2F* mRNAs were measured by real-time RT-PCR using *HPRT* as an endogenous control. Data are expressed as means ± SEM for each group ($n = 4$). The levels of human *E2F* mRNA were measured by real-time RT-PCR using *β-actin* as an endogenous control. Data are expressed as means ± SEM for each group ($n = 4$).

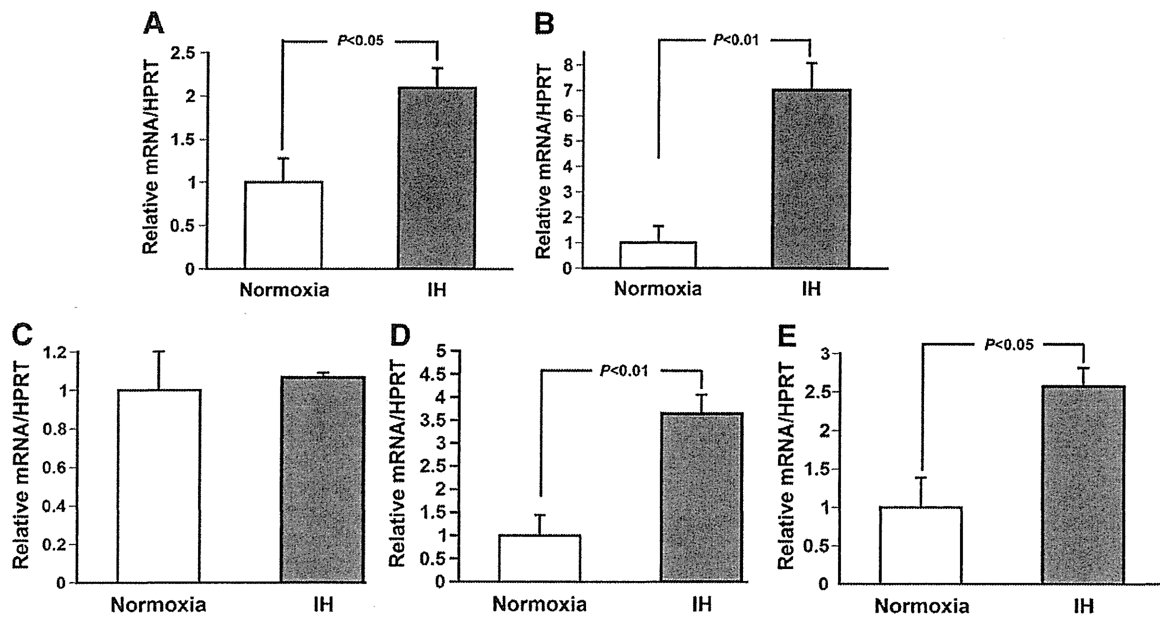

Fig. 4. The mRNA levels of *Reg I* (A), *Reg IV* (B), *PAP I/Reg 2* (C), *PAP II/Reg III* (D), and *PAP III* (E) in RINm5F cells treated by normoxia or IH for 24 h. The levels of *Reg* family mRNAs were measured by real-time RT-PCR using *HPRT* as an endogenous control. Data are expressed as means ± SEM for each group ($n = 4$).

fasting glucose tolerance test (OGTT). The Wisconsin Sleep Study ($n = 1387$) showed a significant cross-sectional association between SAS and type 2 diabetes for all degrees of SAS, which was preserved for those with moderate–severe SAS after adjustment for obesity (OR = 2.3) (Reichmuth et al., 2005). In addition to the development of glucose intolerance and insulin resistance, the progression to type 2 diabetes is dependent on the impairment of glucose-induced insulin secretion from pancreatic β cells and the compensatory replication of pancreatic β cells to combat the presence of insulin resistance. However, the direct effects of SAS/IH on β cell have been obscured. In the present study we examined the relationship between IH and experimentally induced replication and apoptosis of pancreatic β cells and their mechanisms. Here we use pancreatic β cell lines and rat isolated islets and expose to IH, mimicking β cells of SAS patients to show that 24 h-exposure to IH can cause a comparable degree of β cell replication as hyperglycemia.

We evaluated cellular proliferation by WST-8 assay, and found that the HIT-T15 cell proliferation was significantly increased by IH ($p < 0.01$) and decreased by SH ($p < 0.01$) compared to that by normoxia. The cellular proliferation of rat insulinoma RINm5F cells was also increased by IH. Yokoe et al. and Xu et al. showed that intermittent hypoxia causes β cell replication in mice (Yokoe et al., 2008; Xu et al., 2009), but its mechanism and direct effect in β cell by IH are

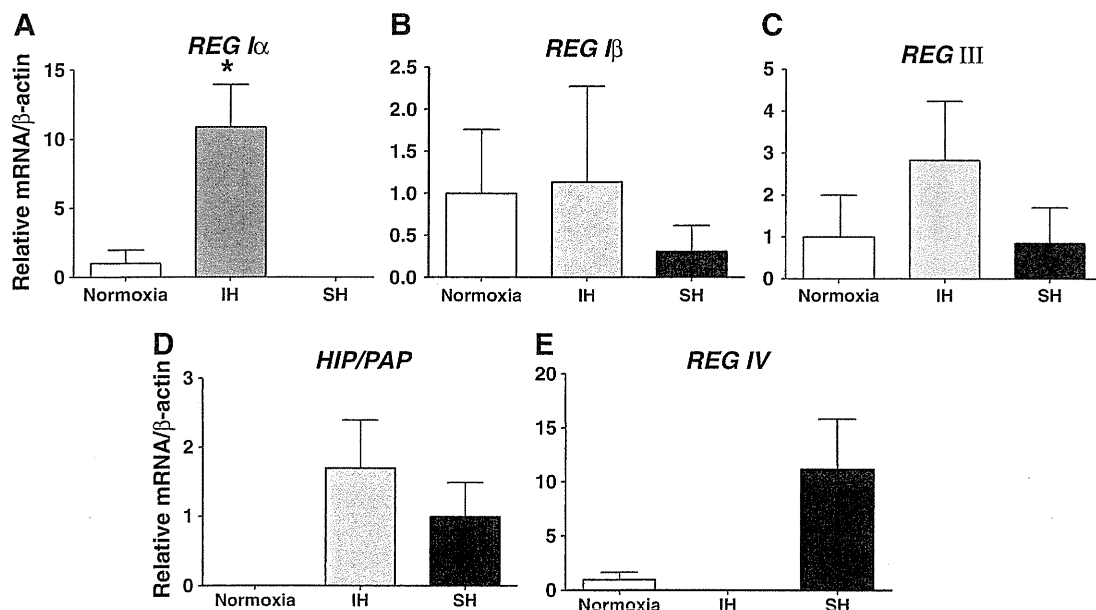

Fig. 5. The mRNA levels of *REG Iα* (A), *REG Iβ* (B), *REG III* (C), *HIP/PAP* (D), and *REG IV* (E) in 1.1B4 human β-cells treated by normoxia, IH or SH for 24 h. The levels of *REG* family mRNAs were measured by real-time RT-PCR using *β-actin* as an endogenous control. Data are expressed as means ± SEM for each group ($n = 4$). *: $P < 0.01$.

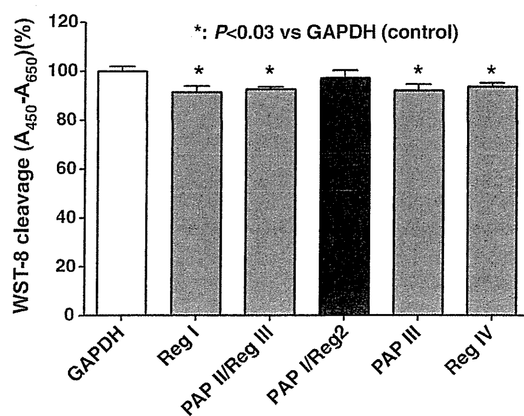

Fig. 6. Effects of *Reg* family gene knockdown on cell proliferation. To determine which *Reg* family gene is involved in the IH-induced β-cell proliferation, siRNAs for *Reg* family genes were introduced into RINm5F cells. After siRNA introduction, RINm5F cells were exposed with IH and cellular proliferation was determined by WST-8 cleavage. Efficacy of siRNAs for *Reg I*, *PAP II/Reg III*, *PAP I/Reg 2*, *PAP III*, and *Reg IV* determined by real-time RT-PCR was 73.9 ± 13.00%, 90.39 ± 13.65%, 83.1 ± 10.44%, 63.79 ± 16.20%, and 55.46 ± 12.69%, respectively. Data are expressed as means ± SEM for each group ($n = 7$). *: $P < 0.03$.

elusive. We measured the mRNA levels of *Reg I*, *PAP II/Reg III*, *PAP III*, and *Reg IV*, autocrine β cell growth factors, and found that they were significantly increased by IH. Reg protein induces β cell replication via the Reg receptor and ameliorates experimental diabetes (Watanabe et al., 1994; Okamoto and Takasawa, 2002; Unno et al., 2002; Cui et al., 2009). This result suggests that IH stimulates β cell proliferation via *Reg* gene expression. As IL-6 induces expression of *Reg* family genes (Akiyama et al., 2001; Okamoto and Takasawa, 2002; Broekaert et al., 2002; Kiji et al., 2005) and the elevation of blood IL-6 in SAS patient was reported (Tam et al., 2007), we measured the mRNA level of *IL-6* and found that the expression of *IL-6* mRNA was significantly increased by IH ($p < 0.01$). As IH stimulus induces NFκB activation (Supplementary Figure) and *IL-6* gene promoter is reported to be activated via NFκB (Keller et al., 1996; Ye and Johnson, 2001), it is quite reasonable that IL-6 is upregulated by IH stimulus. In contrast, IL-6 has been also reported to down-regulate its signaling molecules such as IL-6 receptor and gp130 (Schoester et al., 1994; Wang et al., 1998). Thus, we measured mRNA levels of *IL-6 receptor* and *gp130* in RINm5F cells, rat islets, and 1.1B4 cells exposed to normoxia, IH, or SH and found that IL-6 receptor and gp130 expression were essentially unchanged by IH (Fig. 8). These results indicate that IH increases IL-6 production in pancreatic β cells and induces *Reg* gene expression, resulting in β cell proliferation.

In contrast to the proliferative effect of Reg family proteins to pancreatic β cells, high concentrations of rat Reg I protein (300–1000 nmol/l) were reported to induce β cell apoptosis in Reg receptor overexpressing β cells (Kobayashi et al., 2000), and transgenic mice overexpressing mouse *Reg I* gene under the control of rat glucagon promoter showed β cell apoptosis and diabetes (Yamaoka et al., 2000). These findings indicate that IH-stimulated β cells express some anti-apoptotic factors against a high concentration of Reg protein(s). HGF was reported to protect rat RINm5F β cells against free fatty acid-induced apoptosis (Santangelo et al., 2007). Therefore, we tested possible anti-apoptotic action of HGF against a high concentration of Reg I protein and found that HGF (2.5 and 25 ng/ml) protected RINm5F β cells against apoptosis induced by a high concentration Reg I protein (Fig. 9A). We also found that IH induced both *IL-6* and *HGF* mRNAs (Figs. 7, 9B, and C). Thus, we assumed that the Reg-Reg receptor system could regulate both the proliferation and apoptosis of pancreatic β cells to maintain the insulin-producing cell mass by controlling the concentrations of Reg protein(s) (Okamoto and Takasawa, 2002). In this study, we have demonstrated that expression of *HGF* gene as well as *Reg* family gene was induced in β cells in response to IH exposure and that HGF attenuated the high concentration of Reg I-induced apoptosis. HGF is well known as a mesenchyme-derived multifunctional protein that plays a critical role in cell survival, proliferation, migration, and differentiation (Stella and Comoglio, 1999). Earlier studies demonstrated that HGF receptor, a receptor tyrosine kinase encoded by *c-met* proto-oncogene, was expressed in various cells of epithelial origin including pancreatic islet β cells (Calvo et al., 1996; Otonkoski et al., 1996). HGF is also shown to promote pancreatic β cell differentiation, proliferation, and regeneration (Otonkoski et al., 1996; Zhang et al., 2001) and to increase the expression of *REG* mRNA in human fetal pancreatic cells Otonkoski et al., 1994). Transgenic mice overexpressing *HGF* in pancreatic β cells resisted the diabetogenic effects of β cell toxin streptozotocin (García-Ocaña et al., 2000; García-Ocaña et al., 2001) and administrations of *HGF* expression vectors attenuated β cell destruction and hyperglycemia in animal models of type 1 diabetes (Dai et al., 2003; Park et al., 2003). Intraperitoneal injection of HGF protein also exhibited a favorable effect for amelioration of hyperglycemia in diabetic mice receiving a marginal mass of islet grafts (Nakano et al., 2000). HGF was reported to show anti-inflammatory effects via inhibiting NFκB activation (Bendinelli et al., 2010). These observations led us to speculate that HGF might play a critical role in promoting insulin-producing pancreatic β cell survival after injurious stimuli. When islet β cells receive injurious

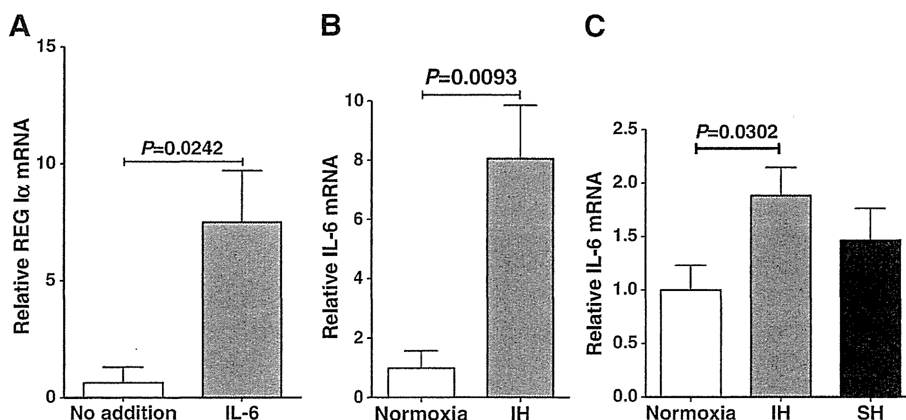

Fig. 7. Induction of *REG Iα* mRNA in human 1.1B4 β cells by IL-6 (A), and the mRNA levels of *IL-6* in RINm5F cells (B) and 1.1B4 cells (C) treated by normoxia, IH, or SH for 24 h. Human 1.1B4 β cells were treated with 20 ng/ml human IL-6 for 24 h. The level of *REG Iα* mRNA and the levels of *IL-6* mRNAs were measured by real-time RT-PCR using *β-actin* as an endogenous control. Data are expressed as means ± SEM for each group ($n = 4$).

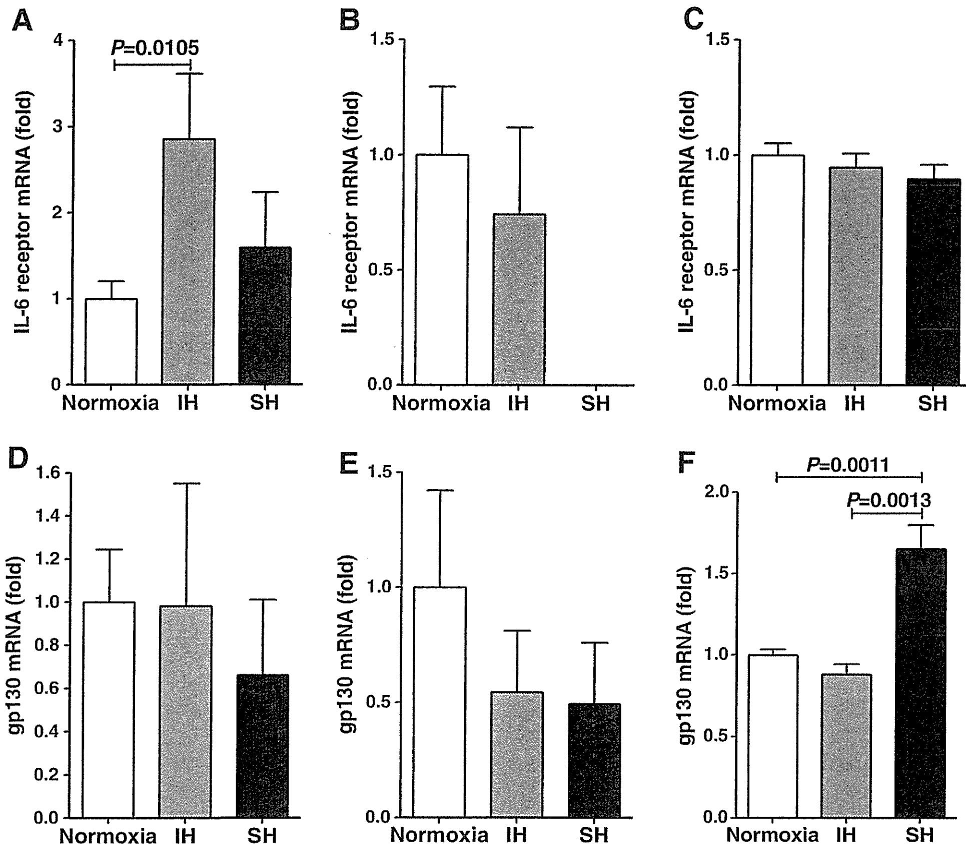

Fig. 8. The mRNA levels of *IL-6 receptor* (A-C) and *gp130* (D-F) in normoxia-, IH-, or SH-treated cells. RINm5F cells (A and D), rat islets (B and E), and 1.1B4 cells (C and F) were exposed to normoxia, IH, or SH for 24 h. The levels of *IL-6 receptor* and *gp130* mRNAs were measured by real-time RT-PCR using *HPRT* (for RINm5F cells and rat islets) and *β-actin* (for 1.1B4 cells) as an endogenous control. Data are expressed as means ± SEM for each group ($n = 4$–8).

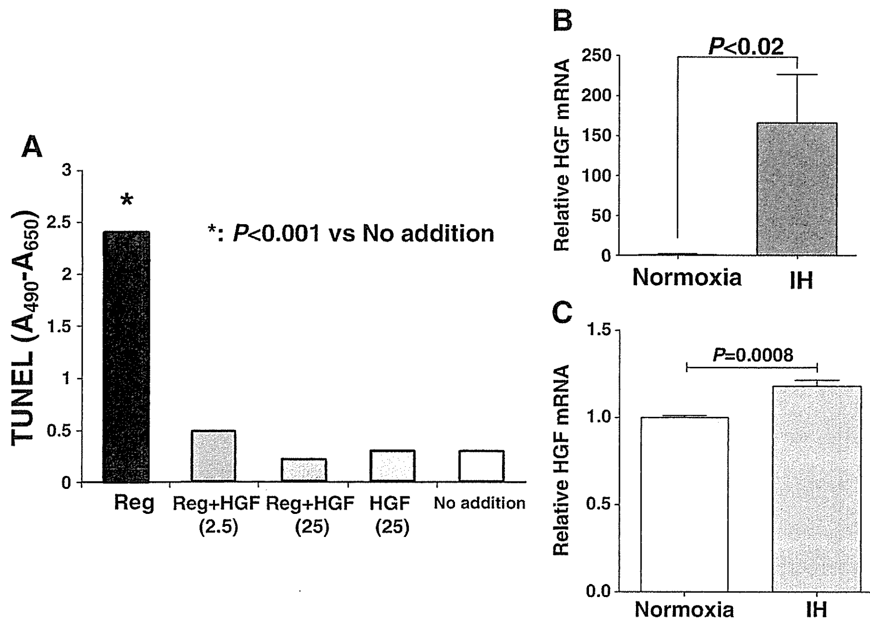

Fig. 9. Attenuation of Reg protein-induced apoptosis in RINm5Fβ cells by HGF (A), and the mRNA levels of *HGF* in RINm5F cells (B) and 1.1B4 cells (C) treated by normoxia or IH for 24 h. Cells were incubated with rat Reg (1000 nmol/l) and HGF (HGF (2.5); 2.5 ng/ml, HGF (25); 25 ng/ml). Apoptosis of RINm5F cells was quantitated by the TUNEL method. Statistical analysis was performed using Student's t test. Values are mean ± SEM of 7 independent experiments. Asterisk indicates significant difference from the value with no addition at $P < 0.001$. The mRNA levels of *HGF* were measured by real-time RT-PCR using *HPRT* (for RINm5F) or *β-actin* (for 1.1.B4) as an endogenous control. Data are expressed as means ± SEM for each group ($n = 4$).

stimuli such as inflammation or IH, *Reg* gene transcription initiates via IL-6 (Akiyama et al., 2001) and islets could recover their insulin-producing cell mass by proliferation/regeneration of β cells by Reg protein. Promoters for *HGF* gene as well as for *Reg* gene were activated by IH stimulus in β cells. This means β cells produce both Reg and HGF under inflammatory situations such as in 90% pancreatectomy, autoimmune models of diabetes, and IH (Terazono et al., 1988; Kulseng et al., 1998; Anastasi et al., 1999; Park et al., 2003). Thus, HGF could enhance the Reg-induced proliferation/regeneration of islet β cells efficiently by inhibiting the high concentration Reg-induced apoptosis. In fact, Gharib et al. recently reported that IH increased plasma HGF level in mouse model (Gharib et al., 2010). It is quite possible that the IH-induced up-regulation of *Reg* and *HGF* genes in pancreatic β cells (Figs. 4, 5 and 9) could lead to proliferation of pancreatic β cells efficiently. Accumulating evidence suggests that IH causes decreased insulin sensitivity (Lesser et al., 2012). IH also increased β cell mass by up-regulation of Reg and HGF to overcome the insulin resistance, causing hyperinsulinemia to make patients more obese to worsen their SAS anatomically.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.lfs.2013.09.001.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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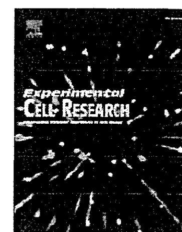

Research Article

Intermittent hypoxia induces the proliferation of rat vascular smooth muscle cell with the increases in epidermal growth factor family and erbB2 receptor

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ABSTRACT

Obstructive sleep apnea is characterized by intermittent hypoxia (IH), and associated with cardiovascular diseases, such as stroke and heart failure. These cardiovascular diseases have a relation to atherosclerosis marked by the proliferation of vascular smooth muscle cells (VSMCs). In this study, we investigated the influence of IH on cultured rat aortic smooth muscle cell (RASMC). The proliferation of RASMC was significantly increased by IH without changing the level of apoptosis. In order to see what induces RASMC proliferation, we investigated the influence of normoxia (N)-, IH- and sustained hypoxia (SH)-treated cell conditioned media on RASMC proliferation. IH-treated cell conditioned medium significantly increased RASMC proliferation compared with N-treated cell conditioned medium, but SH-treated cell conditioned medium did not. We next investigated the epidermal growth factor (EGF) family as autocrine growth factors. Among the EGF family, we found significant increases in mRNAs for epiregulin (ER), amphiregulin (AR) and neuregulin-1 (NRG1) in IH-treated cells and mature ER in IH-treated cell conditioned medium. We next investigated the changes in erbB family receptors that are receptors for ER, AR and NRG1, and found that erbB2 receptor mRNA and protein expressions were increased by IH, but not by SH. Phosphorylation of erbB2 receptor at Tyr-1248 that mediates intracellular signaling for several physiological effects including cell proliferation was increased by IH, but not by SH. In addition, inhibitor for erbB2 receptor suppressed IH-induced cell proliferation. These results provide the first demonstration that IH induces VSMC proliferation,

Abbreviations: AGE, advanced glycation end product; AR, amphiregulin; CDK4, cyclin-dependent kinase 4; CPAP, continuous positive airway pressure; ER, epiregulin; ErbB2 inhibitor II, 4-(3-phenoxyphenyl)-5-cyano-2H-1,2,3-triazole; HIF-1, hypoxia inducible factor-1; IH, intermittent hypoxia; NRG1, neuregulin-1; OSA, obstructive sleep apnea; POD, peroxidase; PVDF, polyvinylidene difluoride; RASMC, rat aortic smooth muscle cell; ROS, reactive oxygen species; SH, sustained hypoxia; TBS-T, tris-buffered saline containing 0.1% Tween-20; TEMPOL, 1-oxyl-2,2,6,6-tetramethyl-4-hydroxypiperidine; VSMC, vascular smooth muscle cell; WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt

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and suggest that EGF family, such as ER, AR and NRG1, and erbB2 receptor could be involved in the IH-induced VSMC proliferation.

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Introduction

Obstructive sleep apnea (OSA) is a highly prevalent disorder [1], and characterized by repetitive episodes of intermittent hypoxia (IH), i.e. transient oxygen desaturation and resaturation. It is reported that OSA is an independent risk factor for systemic hypertension [2] and consequent cardiovascular diseases, including coronary artery disease, congestive cardiac failure, and stroke [3,4]. Continuous positive airway pressure (CPAP) therapy reduces IH episodes and is clinically used as a treatment for OSA; it is reported that CPAP therapy improves several parameters for atherosclerosis [5,6] and reduces cardiovascular morbidity and mortality [7,8].

Atherosclerosis is characterized by the accumulation of lipids and fibrous elements in large arteries. In the progression of atherosclerosis, excessive proliferation and accumulation of vascular smooth muscle cells (VSMCs) are observed in fibrous lesions [9]. Therefore, we inferred that IH induces the VSMC proliferation, which is characterized in atherosclerosis, to lead cardiovascular diseases.

In this study, we investigate an influence of IH on VSMC proliferation using cultured rat aortic smooth muscle cells (RASMCs).

Materials and methods

Cell culture

Treatment of animals was based on the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1985) and approved by the ethical committee of Animal Laboratory of Nara Medical University. Thoracic aortae of male Sprague-Dawley rats (weighing 250–300 g) were excised and immediately immersed in DMEM containing penicillin (100 U/ml) and streptomycin (100 μg/ml). The isolated specimens of the aorta were cut open, and VSMCs were allowed to grow with the culture medium. As described previously [10], after 24 h culture in DMEM containing 1% fetal bovine serum or serum-free DMEM, early subcultured cells (from passage 2–5) were used for the experiments. Purity of the VSMCs was estimated to be >90% on the basis of cell morphology and expression of myosin, as described previously [10]. Cell viability was determined to be >98% by the exclusion of 0.2% Trypan Blue. Cells were maintained at atmospheric oxygen concentrations (21% O_2, 5% CO_2; 37 °C) (normoxia; N). In order to expose sustained hypoxia (SH), cells were maintained in a hypoxia chamber (1% O_2, 5% CO_2; balance N_2 and water vapor). For IH, cells were exposed to cycled changes of hypoxic (5 min) and normoxic (10 min) conditions as described previously [11]. Cells exposed to N, SH or IH were cultured for 24 h under the conditions described above. To address direct involvement of erbB2 receptor in IH-induced cell proliferation, cells were treated with ErbB2 inhibitor II (4-(3-phenoxyphenyl)-5-cyano-2H-1,2,3-triazole, Calbiochem, San Diego, CA) before cells were exposed to N, IH or SH.

Cell counting assay (WST-8 assay)

Cell counting assay was performed essentially according to the manufacturer's protocol as described [12,13]. In brief, after exposing to N, IH or SH for 24 h, WST-8 reagent [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] from Cell Counting Kit-8 (Dojindo, Mashikimachi, Japan) was added to each cell medium (finally 10% (v/v)). Then, cells were incubated at 37 °C for another 0.5 ~ 1.5 h. The formation of formazan was determined photometrically at 450 nm (reference wavelength at 650 nm) with a Sunrise™ microplate reader (Tecan, Männedorf, Switzerland).

Apoptosis assay

Apoptosis was detected by the TUNEL method using an Apoptosis Screening Kit (Wako Pure Chemicals, Osaka, Japan) as described [12]. After exposing to N, IH or SH for 24 h, cells were labeled with dUTP by terminal deoxynucleotidyl transferase. After then, peroxidase (POD)-conjugated anti-dUTP antibody was added and reacted with chromogen. The absorbance of each well was measured at 490 nm (reference wavelength at 650 nm).

Collection of conditioned media

RASMCs were cultured with serum-free DMEM for 24 h before exposing to N, IH or SH. After exposure, conditioned media were collected to examine conditioned media-induced RASMC proliferation.

Real-time RT-PCR assay

Total RNA was extracted from RASMCs using RNAprotect Cell Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol as described [11]. After quantifying the isolated RNA using spectrophotometer, 1 μg aliquots were reverse transcribed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). PCR primers were synthesized by Nihon Gene Research Laboratories (Sendai, Japan), and the primer sequences used to amplify cyclin-dependent kinase 4 (CDK4), EGF family and erbB family receptor mRNAs are shown in Table 1. Real-time PCR was carried out using a SYBR® qPCR kit (KAPA Biosystems, Woburn, MA) in a Thermal Cycler Dice (Takara, Otsu, Japan). The mRNA expression levels were normalized to that of β-actin.

Immunoblot analysis

After exposing RASMCs to N, IH or SH, conditioned media and cells were collected for dot and western blotting, respectively.

Table 1 – Primers used for RT-PCR.

Target gene	Primer sequence (position)
CDK4	5′-TTTGATCTCATTGGATTGCC-3′ (NM_053593: 864-883)
	5′-AGGTCAGCATTTCCAGCAG-3′ (NM_053593: 999-1017)
EGF	5′-CCCGTGTTCTTCTGAGTTCC-3′ (NM_012842: 840-859)
	5′-TGTAACCGTGGCTTCCTTCT-3′ (NM_012842: 1054-1073)
ER	5′-CAAAGTGTAGCTCTGACATG-3′ (NM_021689: 311-330)
	5′-CTGTACCATCTGCAGAAATA-3′ (NM_021689: 529-548)
AR	5′-TTTCGCTGGCGCTCTCA-3′ (NM_017123: 192-208)
	5′-TTCCAACCCAGCTGCATAATG-3′ (NM_017123: 239-259)
Heparin-binding	5′-CTCTTTCTGGCCGCAGTGTT-3′ (NM_012945: 62-81)
EGF-like growth factor	5′-GCCCATGACACCTCTGTCCA-3′ (NM_012945: 448-467)
Betacellulin	5′-GCTTCGTGATGGACGAACAAAC-3′ (NM_022256: 296-317)
	5′-AGCAGACCACCAGGATCTGC-3′ (NM_022256: 399-418)
TGFα	5′-ATGGTCCCCGCGGCCGGACAG-3′ (NM_012671: 145-165)
	5′-GGCCTGCTTCTTCTGGCTGGCA-3′ (NM_012671: 417-438)
Neuregulin-1	5′-GAAGGCGCAAACACTTCTTC-3′ (NM_031588: 825-844)
	5′-TTGGCAACGATCACCAGTAA-3′ (NM_031588: 991-1010)
Neuregulin-2	5′GAGACAGCCAAGTCCTACTG-3′ (NM_001136151: 1319-1338)
	5′-CCCTCGATGTAGTAGCAGAC-3′ (NM_001136151: 1352-1371)
Neuregulin-3	5′-AGGACCTGGCGTATTGTCTC-3′ (XM_002728367.1: 924-943)
	5′-ACTCCTTGGTAGCCTTCCTT-3′ (XM_002728367.1: 1004-1023)
Neuregulin-4	5′-ATGCCAACAGATCACGAGCAGCCC-3′ (NM_001191109: 177-200)
	5′-ACAGGTTACTTTTGCTTTGGATG-3′ (NM_001191109: 338-360)
erbB1 receptor	5′-AAATGCTCTCTACGAAAACACC-3′ (NM_031507: 480-501)
	5′-CAGTTCTCCTCTCCTCTTCC-3′ (NM_031507: 754-773)
erbB2 receptor	5′-CAGTGTGTCAACTGCAGTCA-3′ (NM_017003: 1696-1715)
	5′-CAGGAGTGGGTGCAGTTGAT-3′ (NM_017003: 1999-2018)
erbB3 receptor	5′-CTGTTTAGGCCAAGCAGAGG-3′ (NM_017218: 2022-2041)
	5′-GACTTTGTTTGCCTTCTCGC-3′ (NM_017218: 2210-2229)
erbB4 receptor	5′-GCTAGAGACCCTCAAAGATACC-3′ (NM_021687: 2965-2986)
	5′-GCATGGGCATTCCTTGTTGTGT-3′ (NM_021687: 3250-3271)

TGF, transforming growth factor.

For dot blotting, conditioned media were spotted onto a polyvinylidene difluoride (PVDF) membrane (0.2 μm pore size; Bio-Rad, Hercules, CA) using dot-blot apparatus (DP-48 plate; Advantec Toyo, Ltd., Tokyo, Japan). For western blotting, after the cells were lysed, cellular proteins were denatured and subjected to SDS-polyacrylamide gel electrophoresis. The proteins were transferred onto PVDF membranes (0.45 μm pore size), according to the method as described previously [14]. The membranes for each blot were blocked by 5% skim milk (Nacalai Tesque, Kyoto, Japan) in tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 1 h at room temperature. The blots were incubated overnight at 4 °C with primary antibody against erbB2, phospho-erbB2, epiregulin (ER), amphiregulin (AR) or neuregulin-1 (NRG1) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and then, with secondary antibody conjugated with POD (Santa Cruz Biotechnology, Inc.) at room temperature for 1 h. Immunoreactive bands were visualized using enhanced chemiluminescence (Amersham, Buckinghamshire, UK) and quantified by densitometry.

Statistical analysis

All the experiments were performed in triplicate or more, and the values obtained are described as means ± SEM. After performing a 2-way analysis of variance (ANOVA) to determine the significance among groups, we used a modified *t* test with Fisher's post-hoc test for intergroup comparison. *P* value of <0.05 was considered to be statistically significant.

Results

IH induces RASMC proliferation without apoptosis

Several clinical studies had reported that cardiovascular diseases are associated with patients with OSA [3,4]. It is also reported the correlation of OSA with atherosclerosis [5,6]. These reports let us speculate that IH induces the proliferation of VSMC, which is characterized in atherosclerosis, to induce cardiovascular diseases. Therefore, we investigated the effect of IH on RASMC proliferation. As shown in Fig. 1A, the numbers of RASMC were increased by IH compared with N, whereas SH did not increase the RASMC numbers. In addition, CDK4 mRNA, which correlates well with cell proliferation [15], was increased by IH, but not by SH (Fig. 1B), indicating that IH induced RASMC proliferation. On the other hand, IH did not decrease apoptosis of RASMCs as shown in Fig. 1C. These results indicate that the IH-induced increase in RASMC numbers was caused mainly from the increase in RASMC proliferation but not by the decrease in apoptosis.

IH-treated cell conditioned medium induces RASMC proliferation

In order to investigate whether some autocrine growth factors have a positive effect on IH-induced RASMC proliferation, we examined the influence of conditioned media on RASMC

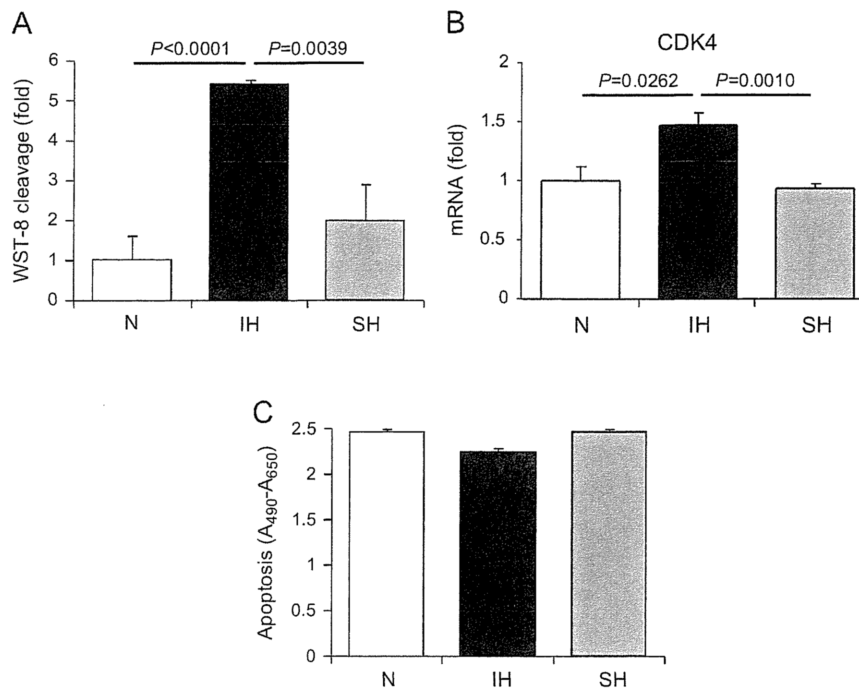

Fig. 1 – Exposure of RASMCs to IH induced the cell proliferation without apoptosis. RASMCs were cultured for 24 h with 1% FBS containing medium and exposed to N, IH or SH. (A) RASMC proliferation was measured as described under Materials and methods. Each value was normalized by arbitrarily setting the absorbance of WST-8 cleavage of the cells, exposed to N, to 1.0. This is representative of six independent experiments. (B) Total RNAs were extracted and real-time RT-PCR was performed using the specific primers for CDK4 mRNAs as described under Materials and methods. Each value was normalized by arbitrarily setting the value of β-actin of the cells, exposed to N, to 1.0. This is representative of six independent experiments. (C) RASMC apoptosis was measured as described under Materials and methods. This is representative of twelve independent experiments. Each point represents the mean ± SEM.

proliferation. As shown in Fig. 2, IH-treated cell conditioned medium induced RASMC proliferation compared with N- and SH-treated cell conditioned media, which is consistent with the result that IH induced RASMC proliferation (Fig. 1A and B).

IH increases mRNAs for ER, AR and NRG1 and mature ER in the conditioned medium

We next investigated the changes in mRNAs for EGF family as autocrine growth factors. As shown in Fig. 3A, ER, AR and NRG1 mRNAs were increased by IH but not by SH. In contrast, the IH-specific increases in the mRNA levels of other members of EGF family such as EGF, heparin-binding growth factor, betacellulin, TGFα, NRG2, NRG3, and NRG4 were not observed (data not shown). As membrane-anchored precursors of EGF family are enzymatically processed to release mature soluble forms that act as paracrine/autocrine growth factors [16], we measured ER in conditioned medium. The level of ER in the IH-treated cell conditioned medium was significantly increased compared with N- and SH-treated cell conditioned media (Fig. 3B).

IH increases erbB2 receptor mRNA and protein

We next investigated the changes in mRNAs for erbB receptors for EGF family. Among erbB receptor family, we found that the level of erbB2 receptor mRNA in RASMC was significantly increased by

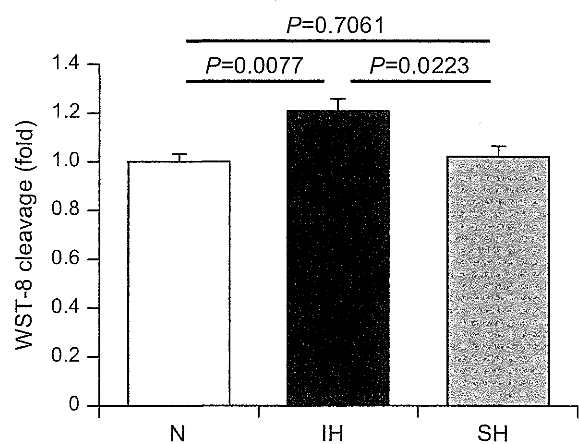

Fig. 2 – IH-treated cell conditioned medium induced the proliferation of RASMCs. RASMCs were cultured for 24 h with serum-free medium and exposed to N, IH or SH. Conditioned media of N-, IH- and SH-treated cells were collected and added to other cultured RASMCs respectively. After cell culture for 28 h, RASMC proliferation was measured by WST-8 assay as described under Materials and methods. Each value was normalized by arbitrarily setting the absorbance of WST-8 cleavage of the cells, exposed to N, to 1.0. This is representative of five independent experiments. Each point represents the mean ± SEM.

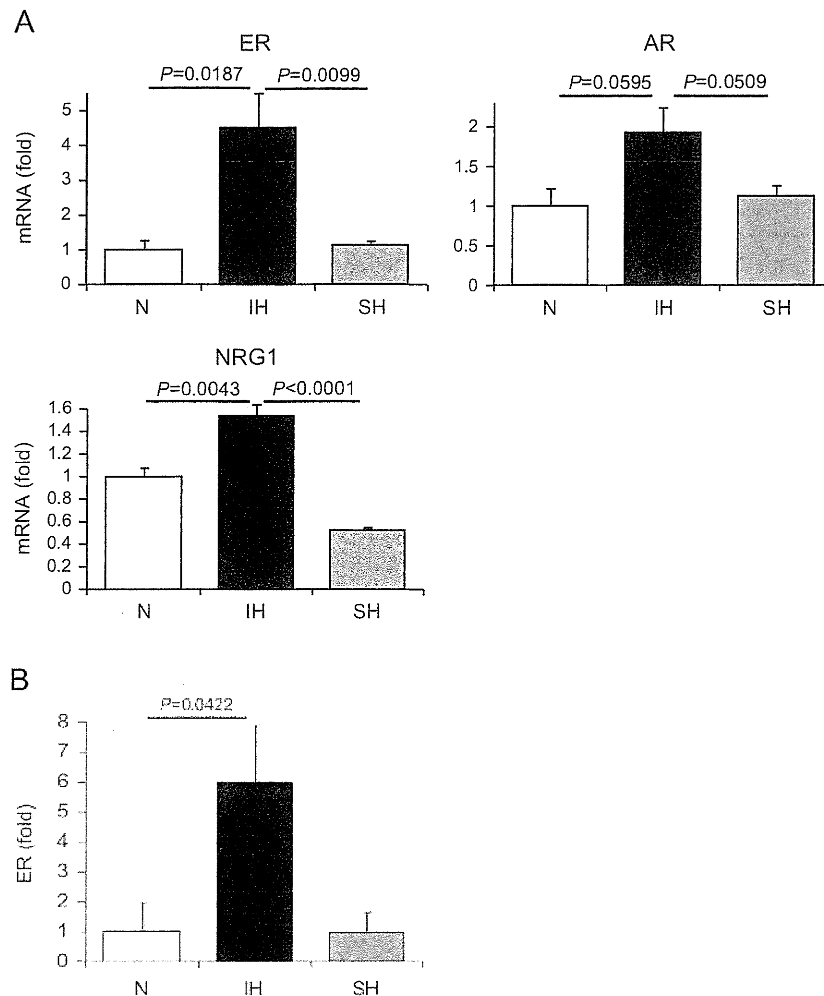

Fig. 3 – IH exposure induced up-regulation of ER, AR and NRG1 mRNAs in RASMCs and mature ER in conditioned medium. (A) After the exposure of RASMCs to N, IH or SH for 24 h, total RNAs were extracted and real-time RT-PCR was performed using the specific primers for indicated mRNAs as described under Materials and methods. Each value was normalized by arbitrarily setting the value of β-actin of the cells, exposed to N, to 1.0. This is representative of four to five independent experiments. (B) After the exposure of RASMCs to N, IH or SH for 24 h, the conditioned medium of N-, IH- and SH-treated cells were collected and filtrated by size-limited filtration. Immunoblot was performed by an ER specific antibody as described under Materials and methods. Densitometric analysis of each value was normalized by arbitrarily setting the densitometric value of the cells, exposed to N, to 1.0. This is representative of six independent experiments. Each point represents the mean±SEM.

IH but not by SH (Fig. 4A). We next measured the expression of erbB2 receptor protein. As shown in Fig. 4B, expression of erbB2 receptor protein was increased by IH, but not by SH, consistent with the increase in erbB2 mRNA expression.

IH induces the phosphorylation of erbB2 receptor

It is established that tyrosine phosphorylation at specific residues on EGF receptor family mediates downstream signals for several physiological effects including cell proliferation [17–20]. We examined the phosphorylation status of specific Tyr-1248 residue of erbB2 with a commercially available anti-phospho-erbB2 antibody in N-, IH-, and SH-treated RASMCs. As shown in Fig. 5, the level of phosphorylation at Tyr-1248 of erbB2 receptor was significantly increased by IH.

Inhibitor for erbB2 receptor inhibits IH-induced cell proliferation

We next investigated the direct involvement of erbB2 receptor in IH-induced cell proliferation using ErbB2 inhibitor II, a specific inhibitor for erbB2 receptor. As shown in Fig. 6, IH-induced cell proliferation was attenuated by ErbB2 inhibitor II in a dose-dependent manner.

Discussion

The major finding of our study provides the first demonstration that IH induces the proliferation of VSMCs. Our other findings provide the possibility that the IH-induced increases in EGF family, such as

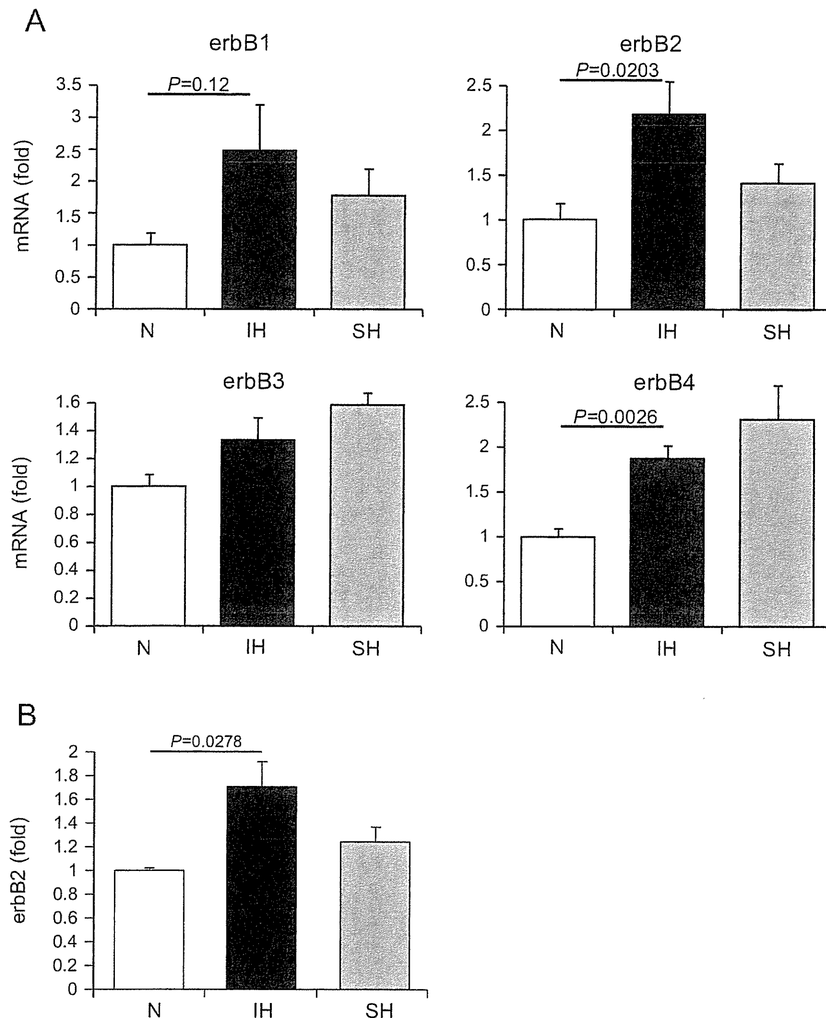

Fig. 4 – IH exposure increases erbB2 receptor mRNA and protein in RASMCs. (A) After the exposure of RASMCs to N, IH or SH for 24 h, total RNAs were extracted and real-time RT-PCR was performed using the specific primers for each erbB family receptor mRNA as described under Materials and methods. Each value was normalized by arbitrarily setting the value of β-actin of the cells, exposed to N, to 1.0. This is representative of four to five independent experiments. (B) After the exposure of RASMC to N, IH or SH for 24 h, RASMCs were collected and lysed. Cellular proteins of RASMCs were denatured and subjected to SDS-polyacrylamide gel electrophoresis. Immunoblot was performed as described under Materials and methods. Densitometric analysis of each value was normalized by arbitrarily setting the densitometric value of the cells, exposed to N, to 1.0. This is representative of three independent experiments. Each point represents the mean±SEM.

ER, AR and NRG1 are involved in the IH-induced proliferation of VSMCs. Data to support the involvement of EGF family in the IH-induced VSMC proliferation include the following findings: 1) IH-treated cell conditioned medium induced RASMC proliferation. 2) The levels of EGF family mRNAs, such as ER, AR and NRG1, and mature ER in cell conditioned medium were increased by IH, but not by SH. 3) erbB2 receptor mRNA and erbB2 receptor protein were increased by IH, but not by SH. 4) Phosphorylation of erbB2 receptor at Tyr-1248, which mediates intracellular signaling for several physiological effects including cell proliferation, was increased in the IH-treated RASMCs compared with those in N- and SH-treated cells. 5) IH-induced cell proliferation was attenuated by a specific inhibitor for erbB2 receptor.

The initiation step of atherosclerosis process is disruption of endothelial barrier by an inflammatory response of endothelial cells to several stresses. After the subendothelial accumulation of foam-cells, fibrous plaques which are characterized by the accumulation of lipid-rich necrotic debris and VSMCs are developed [9]. Therefore, the proliferation and the migration of VSMCs as well as inflammatory responses of endothelial cells are important steps for the progression of atherosclerosis. It was previously reported that repetitive episodes of hypoxia, especially reoxygenation induces generation of reactive oxygen species (ROS) [21] and that increased levels of ROS have been detected in patients with OSA [22–24]. ROS triggers vascular inflammation with characteristic increases in endothelial cell barrier disruption, and is thought to induce/accelerate atherosclerosis [25,26]. OSA patients are probably exposed to the condition of partially disruption of endothelial barrier in the vasculature caused by inflammatory factors such as ROS. We have recently reported that

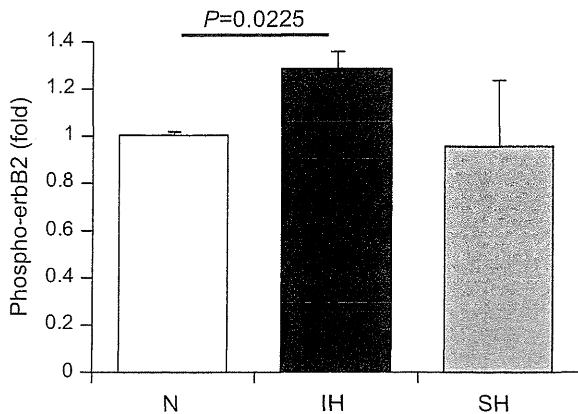

Fig. 5 – IH exposure induced the phosphorylation of erbB2 receptor in RASMCs. After the exposure of RASMC to N, IH or SH for 24 h, RASMCs were collected and lysed. Cellular proteins of RASMCs were denatured and subjected to SDS-polyacrylamide gel electrophoresis. Immunoblot was performed as described under Materials and methods. Densitometric analysis of each value was normalized by arbitrarily setting the densitometric value of the cells, exposed to N, to 1.0. This is representative of three independent experiments. Each point represents the mean±SEM.

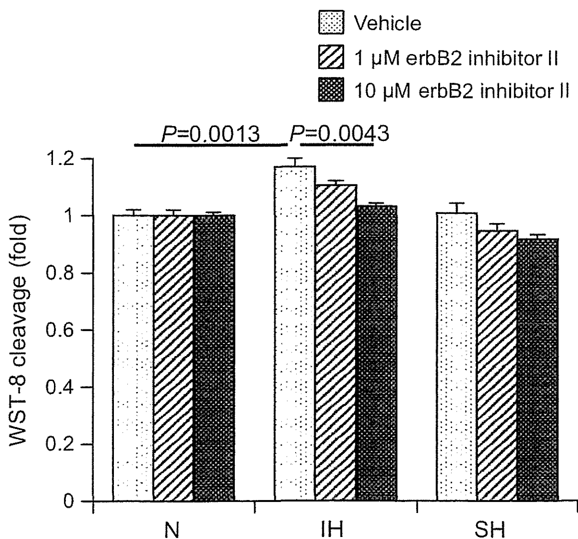

Fig. 6 – Inhibition of erbB2 receptor suppressed IH-induced RASMC proliferation. RASMCs were cultured for 24 h with serum-free medium and treated with vehicle or indicated concentration of ErbB2 inhibitor II. After exposing to N, IH or SH, RASMC proliferation was measured as described under Materials and methods. Each value was normalized by arbitrarily setting the absorbance of WST-8 cleavage of the cells, exposed to N, to 1.0. This is representative of six independent experiments. Each point represents the mean±SEM.

24 h IH attenuated glucose-induced insulin secretion from pancreatic β cells via down-regulation of CD38 [11]. It is well known that impaired glucose-induced insulin secretion is one of major factors of diabetes and that atherosclerosis is a major complication of diabetes. Therefore, IH can cause/accelerate vascular dysfunction/atherosclerosis not only by IH itself but also by IH-induced diabetes.

In the present study, we obtained the following findings concerned with EGF family; the increases in several EGF family mRNAs for ER, AR and NRG1, and mature soluble form of ER in the cell conditioned medium by IH (Fig. 3A and B), the up-regulation of erbB2 receptor mRNA and erbB2 receptor protein by IH (Fig. 4A and B), the cell proliferation with IH-treated cell conditioned medium (Fig. 2), the increase in phosphorylation of erbB2 receptor at Tyr-1248 in the IH-treated cells (Fig. 5), inhibitory effect of ErbB2 inhibitor II for IH-induced cell proliferation (Fig. 6), all of which are observed specifically in the IH-treated cells but not in SH-treated cells. Taken our findings together, it is inferred that the IH-induced VSMC proliferation is, in part, derived from the activation of erbB2 receptor through increasing of EGF family and erbB2 receptor, as shown in Fig. 7. As EGF family and erbB2 receptor have important roles in mitogenesis and differentiation of smooth muscle cells [27–29] and erbB2 has been identified immunocytochemically on aortic intimal smooth muscle cells within human atherosclerotic plaque [30], our results support the idea of Dreux et al. [31] that EGF family and EGF receptor family including erbB2 have important roles in development and progression of atherosclerosis.

It was previously proposed the involvement of ROS in response to IH [21]. However, Ryan et al. suggested that ROS signaling might not be involved in IH using endothelial cells [32]. In addition, we found that TEMPOL (1-oxyl-2,2,6,6-tetramethyl-4-hydroxypiperidine), a superoxide dismutase mimetic, did not suppress the IH-induced RASMC proliferation (data not shown). This discrepancy might be dependent on cell types and methodological pitfalls. On the other hand, Ryan et al. also suggested that IH induces NF-κB activation through p38 MAP kinase, without stabilizing hypoxia inducible factor-1 (HIF-1) [32,33]. It is consistent with our result of NF-κB activation by IH using reporter gene assay (data not shown). HIF-1 has an important role as a

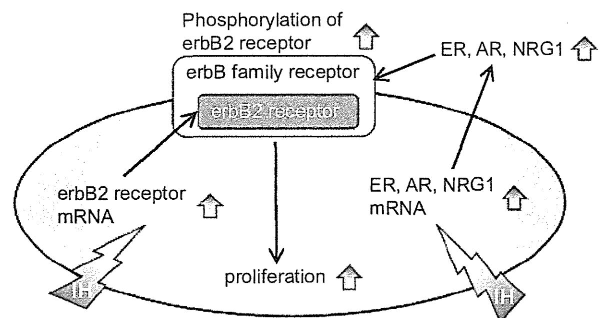

Fig. 7 – A possible mechanism of IH-induced RASMC proliferation. IH stimulation induces several EGF family mRNAs encoding ER, AR and NGR1 precursors. These precursors were processed by proteolytic cleavage, resulting in the release of soluble, biologically active growth factors. On the other hand, erbB2 receptor is also up-regulated by IH. IH-induced EGF family growth factors bind to erbB family receptor and lead to heterodimerization with erbB2 receptor. Activation/phosphorylation of erbB2 receptor induces proliferation of VSMCs via several intracellular signal transduction cascades.

transcription factor in hypoxia, which is destabilized by oxygen [34]. Therefore, we inferred that IH-related intracellular mechanisms are different from those in SH. Therefore, the initial signaling mechanisms in response to IH remain to be determined. These are subjects of a future investigation.

Conclusions

In this study, we suggested that IH induced VSMC proliferation, and that IH-induced changes of EGF family and erbB2 receptor might have important roles in IH-induced VSMC proliferation as shown in Fig. 7. Our findings might be useful to clarify the mechanisms of OSA-induced cardiovascular diseases and develop the treatment for it.

Acknowledgments

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ORIGINAL ARTICLE
Pulmonary Circulation

Oral Administration of a Novel Long-Acting Prostacyclin Agonist With Thromboxane Synthase Inhibitory Activity for Pulmonary Arterial Hypertension

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Background: Continuous administration of prostacyclin has improved the survival of patients with pulmonary arterial hypertension (PAH). However, this treatment has some problems, including its short duration of activity and difficult delivery. Therefore, we developed ONO-1301, an orally active, long-acting prostacyclin agonist with thromboxane synthase inhibitory activity.

Methods and Results: We investigated whether oral administration of ONO-1301 can both prevent and reverse monocrotaline (MCT)-induced PAH in rats. Rats were randomly assigned to receive repeated oral administration of ONO-1301 twice daily beginning either 1 or 8 days after subcutaneous injection of MCT. A control group received oral saline, and a sham group received a subcutaneous injection of saline instead of MCT. MCT-treated controls developed significant pulmonary hypertension. Treatment with ONO-1301 from day 1 or 8 significantly attenuated the increases in right ventricular systolic pressure and the increase in medial wall thickness of pulmonary arterioles. Kaplan-Meier survival curves demonstrated that the effect of ONO-1301 was equivalent to that of an endothelin receptor antagonist and a phosphodiesterase-5 inhibitor. A single oral dose of ONO-1301 increased plasma cAMP levels for up to 6h. Treatment with ONO-1301 significantly decreased urinary 11-dehydro-thromboxane B_2 and increased the plasma hepatocyte growth factor concentration.

Conclusions: Oral administration of ONO-1301 ameliorated PAH in rats, an effect that may occur through cAMP and hepatocyte growth factor. (*Circ J* 2013; **77:** 2127–2133)

Key Words: cAMP; Hepatocyte growth factor; Monocrotaline; Pulmonary arterial hypertension; Thromboxane synthase

Pulmonary arterial hypertension (PAH) is a rare but life-threatening disease characterized by progressive pulmonary hypertension that leads to right ventricular failure and death.[1,2] Continuous intravenous infusion of prostacyclin has become recognized as a therapeutic breakthrough[3–5] and the dramatic success of long-term intravenous prostacyclin has led to the development of prostacyclin analogs.[6–9] Nevertheless, treatment with prostacyclin or its analogs has some problems in the clinical setting, viz., short-term activity and difficulty of drug delivery.

Editorial p1994

ONO-1301 has long-lasting prostacyclin activity in association with thromboxane synthase inhibitory effects.[10,11] We have reported that repeated subcutaneous injections of this compound ameliorated PAH induced by monocrotaline (MCT) in rats.[10,11] However, whether oral administration has the same beneficial effects on PAH remains unknown, so in the present study we investigated the effects of oral ONO-1301 on pulmonary hemodynamics and survival in MCT rats.

Thus, the aims of this study were as follows: (1) to investi-

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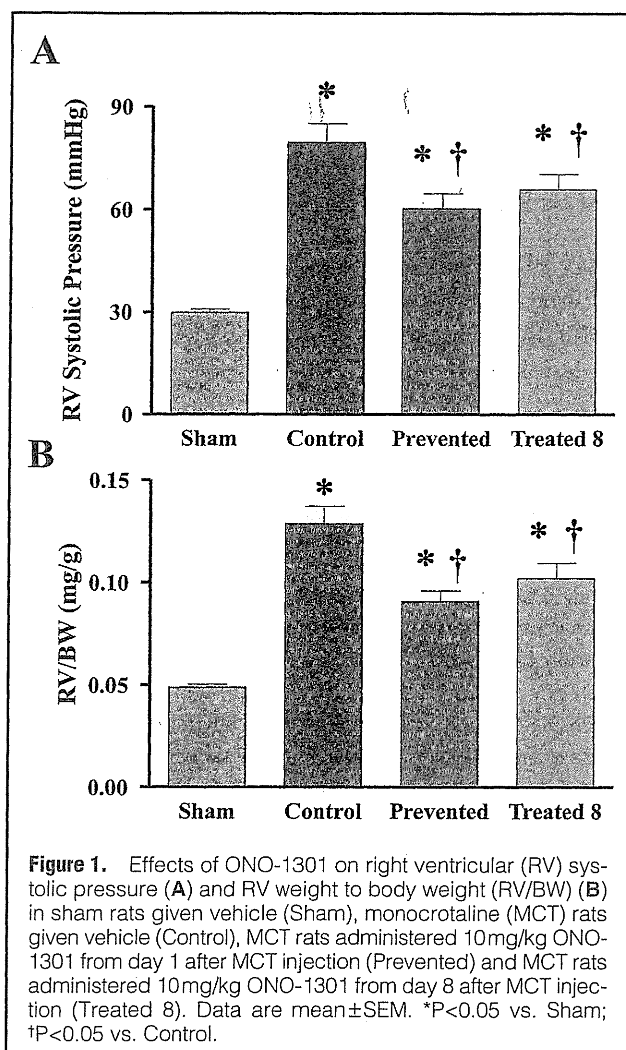

Figure 1. Effects of ONO-1301 on right ventricular (RV) systolic pressure (**A**) and RV weight to body weight (RV/BW) (**B**) in sham rats given vehicle (Sham), monocrotaline (MCT) rats given vehicle (Control), MCT rats administered 10mg/kg ONO-1301 from day 1 after MCT injection (Prevented) and MCT rats administered 10mg/kg ONO-1301 from day 8 after MCT injection (Treated 8). Data are mean±SEM. *P<0.05 vs. Sham; †P<0.05 vs. Control.

gate whether oral administration of ONO-1301 attenuates PAH induced by MCT in rats, (2) to compare the effect of this compound with that of conventional treatments, and (3) to assess the underlying mechanisms of its therapeutic effect.

Methods

Animal Model

In this study to evaluate the effects of oral administration of ONO-1301 on MCT-induced PAH, we used 60 5-week-old male Wistar rats weighing 100–120g; 45 were chosen randomly to receive a subcutaneous injection of 60mg/kg MCT on day 1 of the trial, while the remaining 15 received a subcutaneous injection of 0.9% saline. The MCT-injected rats were then assigned to one of 3 treatment protocols: oral treatment with ONO-1301 from day 1 (prevented group, n=15), oral treatment with ONO-1301 from day 8 (treated 8 group, n=15), and oral 0.9% saline (control group, n=15). Saline-injected rats received 0.9% saline orally from day 1 (sham group, n=15). In addition, 65 rats were studied to evaluate plasma ONO-1301 (n=20), and 11-dehydro-thromboxane B_2 (TXB_2) level (n=45) concentration s. Furthermore, the effect of ONO-1301 on survival of MCT rats was also evaluated.

In Vivo Experimental Protocol

Following anesthesia by intraperitoneal injection of 30mg/kg pentobarbital, rats were given a subcutaneous injection of either 60mg/kg MCT or 0.9% saline. Subsequently, 10mg/kg ONO-1301 was administered twice daily by oral gavage from either the 1st or 8th day after MCT injection. It has been previously shown that 10mg/kg is the maximum dose that does not cause significant hypotension. Hemodynamic measurements and histologic analyses were performed on day 25; this time point was based on survival curve analyses. Hemodynamic measurements were performed with the rats anesthetized by continuous inhalation of 1% isoflurane. A polyethylene catheter (model PE-50; BD Biosciences, San Jose, CA, USA) was inserted into the right carotid artery to measure heart rate and mean arterial pressure. A second catheter was inserted through the right jugular vein into the right ventricle (RV) for the measurement of pressure. Heart rate, mean arterial pressure, and systolic RV pressure were calculated from 20 consecutive heart beats in each rat. Finally, cardiac arrest was induced by blood collection through the catheter. Blood was immediately transferred into a chilled glass tube containing disodium ethylenediaminetetraacetic acid (EDTA; 1mg/ml) and aprotinin (500U/ml) and centrifuged. The ventricles and lungs were excised and weighed. The ratio of RV weight to body weight (RV/BW) was calculated as an index of ventricular hypertrophy, as previously reported.[12] All protocols were performed in accordance with the guidelines of the Animal Care and Ethics Committee of the National Cardiovascular Center Research Institute (Osaka, Japan).

Morphometric Analysis of Pulmonary Arterioles

Paraffin sections of 4μm thickness were obtained from the lower region of the right lung and stained with hematoxylin-eosin. Analysis of the medial wall thickness of the pulmonary arterioles was performed as described previously.[13] In brief, the external diameter and medial wall thickness were measured in 20 muscular arteries (25–100μm external diameter) per lung section. For each artery, the medial wall thickness was expressed as follows: %wall thickness = ([medial thickness × 2] / external diameter) × 100.

Comparison With Conventional Drugs

To evaluate the effect of oral administration of ONO-1301 on survival in MCT rats, the following survival analyses were performed: whether an oral dose of ONO-1301 improved the survival rate in MCT rats as compared with vehicle (Control), an endothelin receptor antagonist (bosentan) or a phosphodiesterase-5 inhibitor (sildenafil).

Survival was evaluated from the date of MCT injection to the death of the rat or 6 weeks after injection.

Assay of Plasma ONO-1301 and cAMP Levels

To investigate whether prostacyclin activity induced by a single, oral dose of ONO-1301 is comparatively equivalent with that with a subcutaneous injection, we measured the plasma level of ONO-1301. Rats were assigned to receive a single, oral or subcutaneous dose of 10mg/kg ONO-1301 (n=10 in each group), and blood was drawn at 0, 1, 2, 4, 8, 12, and 24h. Blood was immediately transferred to a chilled glass tube containing 1mg/ml disodium EDTA and centrifuged. The plasma ONO-1301 level was measured by liquid chromatography tandem mass spectrometry assay.

We investigated whether a single, oral dose of ONO-1301 induced long-lasting prostacyclin activity in MCT rats. In this study, 20 rats were assigned to orally receive the dose of ONO-