The molecular regulation of resistin expression in cultured vascular smooth muscle cells under hypoxia

Huei-Fong Hung[a], Bao-Wei Wang[a], Hang Chang[b] and Kou-Gi Shyu[a,c]

Objectives **Resistin has a potential role in atherosclerosis because resistin produces proinflammatory effects in the vascular wall. However, the molecular mechanism of resistin increase in atherosclerosis remains unclear. Hypoxia plays an important role in vascular remodeling and directly affects vascular smooth muscle cells functions. We sought to investigate the molecular regulation of resistin expression under hypoxia in cultured vascular smooth muscle cells.**

Methods **Vascular smooth muscle cells from thoracic aorta of adult Wistar rats were cultured and subjected to hypoxia at 2.5% oxygen in a hypoxic chamber. Western blot, real-time PCR, reactive oxygen species assay, and promoter activity were measured.**

Results **Hypoxia significantly increased the resistin protein (3.5-fold, $P<0.001$) and mRNA (4.8-fold, $P<0.001$) expression as compared with the control cells. The specific extracellular signal-regulated kinase (ERK) inhibitor PD98059, antioxidant *N*-acetylcysteine, and ERK siRNA attenuated the induction of resistin protein by hypoxia. It increased the phosphorylated ERK protein expression (3.2-fold, $P<0.001$), whereas pretreatment with PD98059 and *N*-acetylcysteine significantly blocked the increase of phosphorylated ERK by hypoxia. It also increased the reactive oxygen species production (9.3-fold, $P<0.001$), and pretreatment with *N*-acetylcysteine significantly blocked the induction of reactive oxygen species by hypoxia. Hypoxia increased resistin promoter activity (5.1-fold, $P<0.001$), and the activity was abolished when nuclear factor of activating T cells in the promoter area was mutated. Pretreatment with PD98059 and *N*-acetylcysteine significantly attenuated the resistin promoter activity induced by hypoxia.**

Conclusion **Hypoxia increases the resistin expression in cultured rat vascular smooth muscle cells under hypoxia. The hypoxia-induced resistin is mediated through reactive oxygen species, ERK mitogen-activated protein (MAP) kinase and nuclear factor of activating T cells pathway.**
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Keywords: hypoxia, resistin, signal pathway, vascular smooth muscle cell

Abbreviations: EMSA, electrophoretic mobility shift assay; NAC, N-acetylcysteine; NFATc, nuclear factor of activating T cells; PCR, polymerase chain reaction; PDGF, platelet-derived growth factor; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; VSMCs, vascular smooth muscle cells

[a]Division of Cardiology, [b]Department of Emergency Medicine, Shin Kong Wu Ho-Su Memorial Hospital and [c]Graduate Institute of Clinical Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan

Correspondence to Kou-Gi Shyu, Division of Cardiology, Shin Kong Wu Ho-Su Memorial Hospital, 95 Wen-Chang Rd, Taipei 111, Taiwan
Tel: +886 2 28332211; fax: +886 2 28365775; e-mail: shyukg@ms12.hinetnet

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Introduction

Obesity and atherosclerosis are major public health problems in developed countries and are increasingly viewed as inflammatory states [1,2]. For clinical applications, biomarkers that integrate metabolic and inflammatory signals are powerful candidates for defining risk of atherosclerotic cardiovascular disease [3]. Rodent resistin is derived almost exclusively from fat tissue, and adipose expression and serum levels are elevated in models of obesity and insulin resistance [4–6]. Resistin was shown to have potent proinflammatory properties [7]. It promotes endothelial cell activation [8] and causes endothelial dysfunction of porcine coronary arteries [9]. Recently, resistin was found to have a potential role in atherosclerosis because resistin increased proinflammatory cytokine expression in vascular endothelial cells [10], and it promoted vascular smooth muscle cell (VSMC) proliferation [11]. In the atheroma, resistin may contribute to atherogenesis by virtue of its effects on vascular endothelial cells and smooth muscle cells [12]. Although resistin has been shown to increase in atherosclerosis, its molecular regulation mechanism remains unclear. Resistin may represent a novel link between metabolic signals, inflammation, and atherosclerosis [13].

Several lines of evidence indicate that hypoxia is a stimulus to VSMCs proliferation and migration, a process known as the vascular remodeling [14]. Blaschke *et al.* [15] have shown that hypoxia plays an important role in vascular remodeling and directly affects VSMCs functions. The effect of hypoxia on resistin expression in VSMCs has not been previously reported. Nuclear factor of activating T cells (NFAT) has been demonstrated to play a key role in various vasculopathies such as atherosclerosis and restenosis [16–19]. Hypoxia has been proved to stimulate the production of reactive oxygen

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species (ROS) in VSMCs [20]. Furthermore, ROS can activate NFAT [21,22]. We hypothesized that hypoxia may regulate resistin expression in VSMCs via ROS and NFAT. Therefore, in this study, we designed to investigate the resistin expression in VSMCs under the hypoxia model and tried to seek the possible molecular mechanisms and signal pathways mediating the expression of resistin under hypoxia condition.

Materials and methods

Vascular smooth muscle cell culture

Primary cultures of VSMCs were grown by the explant technique from the thoracic aorta of 200-g to 250-g male Wistar rats, as described previously [23,24]. Cells were cultured in medium 199 containing 20% fetal calf serum, 0.1 mmol/l nonessential amino acids, 1 mmol/l sodium pyruvate, 4 mmol/l L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C under 5% CO_2/95% air in a humidified incubator. When confluent, VSMC monolayers were passaged every 6–7 days after trypsinization and were used for experiment from the third to sixth passages. These third to sixth passage cells were incubated for an additional 2 days to render them quiescent before the initiation of each experiment. The study conforms to *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The study was reviewed and approved by the Institutional Animal Care and Use Committee of the Shin Kong Wu Ho-Su Memorial Hospital.

Hypoxic stimulation

A humidified temperature controlled incubator Proox model 110 (BioSpherix, Redfield, New York, USA) was used as a hypoxic chamber. For hypoxia conditions, the concentration of oxygen was reduced to 2.5% by replacement with N_2, keeping CO_2 constant at 5%, and incubated at 37°C for different times. Control was defined as 95% air and 5% CO_2. For the investigation of signal pathways, cells were pretreated with inhibitors for 30 min, and then exposed to hypoxia without changing medium. SP600125 (20 μmol/l; Calbiochem, San Diego, California, USA) is a potent, cell-permeable, selective, and reversible inhibitor of JNK. SB203580 (3 μmol/l; Calbiochem) is a highly specific, cell-permeable inhibitor of p38 kinase. PD98059 (50 μmol/l; Calbiochem) is a specific and potent inhibitor of extracellular signal-regulated kinase (ERK) kinase. *N*-Acetylcysteine (NAC, 500 μmol/l; Calbiochem) is a free radical scavenger.

Western blot analysis

Western blot was performed as previously described [25]. Rabbit antiresistin rat polyclonal antibody (Chemicon, Temecula, California, USA), polyclonal anti-ERK and monoclonal antiphospho ERK kinase antibodies (Cell Signaling, Beverly, Massachusetts, USA), polyclonal anti-$NFAT_c3$, and antiphospho $NFAT_c3$ antibodies (Santa Cruz Biotechnology, Santa Cruz, California, USA) were used.

Real-time reverse transcription-PCR

Total RNA from cultured VSMCs was extracted using the single-step acid guanidinium thiocyanate/phenol/chloroform extraction method [26]. Real-time reverse transcription-PCR was performed as described previously [24]. The rat resistin primers were 5′-ACTTCAGCTCCCTACTG-3′ and 5′-GTCTATGCTTCCGCACT-3′.

RNA interference

Cultured VSMCs were transfected with 800 ng ERK annealed siRNA (Dharmacon Inc., Lafayette, Colorado, USA) or resistin siRNA oligonucleotide (Invitrogen, Carlsbad, California, USA). ERK or resistin siRNA is a target-specific 20–25 nt siRNA designed to knockdown gene expression. ERK sense and antisense of siRNA sequences were 5′-GACCGGAUGUUAACCUUUAUU and 5′-PUAAAGGUUAACAUCCGGUCUU, respectively. Resistin sense and antisense of siRNA sequences were ACACAUUGUAUCCUCACGGACGUCCC and GGACGUCCGUGAGGATACAAUGUGU, respectively. As a negative control, a nontargeting siRNA (scrambled siRNA) purchased from Dharmacon was used. VSMCs were transfected with siRNA oligonucleotides using Effectene Transfection Reagent as suggested by the manufacturer (Qiagen Inc., Valencia, California, USA).

Reactive oxygen species assay

ROS production was measured using the cell permeant probe 2′-7′-dichlorodihydrofluorescin diacetate, which passively diffuses into cells in which intracellular esterases cleave the acetate groups to form the impermeable $DCFH_2$ that remains trapped within the cell [27]. After hypoxia treatment, cells were collected by trypsinization and resuspended in phosphate-buffered saline medium. ROS assay was performed according to the manufacture's instruction (Invitrogen, Eugene, Oregon, USA). Fluorescence microscopy was used to detect the green fluorescence.

Electrophoretic mobility shift assay

Nuclear protein concentrations from cultured VSMCs were determined by Biorad protein assay. Consensus and control oligonucleotides (Research Biolabs, Singapore) were labeled by polynucleotides kinase incorporation of [γ^{32}P]-dATP. Oligonucleotides sequences of $NFAT_c$ were consensus 5′-CGCCCAAAGAGGAAAATTTGTTTCATA-3′. The mutant oligonucleotides sequences were 5′-CGCCCAAAGCTTAAAATTTGTTTC-3′. Electrophoretic mobility shift assay (EMSA) was performed as previously described [26]. Controls were performed in each case with mutant oligonucleotides or cold oligonucleotides to compete with labeled sequences.

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Promoter activity assay

A −741 to +22 bp rat resistin promoter construct was generated as follows. Rat genomic DNA was amplified with forward primer (ACGCGTCTCAGCGGTAGAGCTCTTG) and reverse primer (AGATCTGGAGAAATGAAAGGTTCTTCATC). The amplified product was digested with *Mlu*I and *Bgl*II restriction enzymes and ligated into pGL3-basic luciferase plasmid vector (Promega Corp., Madison, Wisconsin, USA) digested with the same enzymes. The resistin promoter contains $NFAT_c$ conserved sites (AGG) at −365 to −363 bp and HIF-1α conserved sites (CGT) at −112 to −114 bp. For the mutant, the $NFAT_c$ and HIF-1α binding sites were mutated using the mutagenesis kit (Stratagene, La Jolla, California, USA). Site-specific mutations were confirmed by DNA sequencing. Plasmids were transfected into VSMCs using a low pressure-accelerated gene gun (Bioware Technologies, Taipei, Taiwan) essentially following the protocol from the manufacturer. In brief, 2 μg of plasmid DNA was suspended in 5 μl of PBS and was delivered to the cultured VSMCs at a helium pressure of 15 psi. The transfection efficiency using this method is 30%. Following hypoxia treatment for 1.5 h, cell extracts were prepared using Dual-Luciferase Reporter Assay System (Promega) and measured for dual luciferase activity by luminometer (Turner Designs Inc., Sunnyvale, California, USA).

Glucose uptake in cultured vascular smooth muscle cells

VSMCs were seeded on ViewPlate for 60 min (Packard Instrument Co., Meriden, Connecticut, USA) at a cell density of 5×10^3 cells/well in serum free medium with transferrin 5 μg/ml, insulin 5 μg/ml for overnight. Recombinant mouse resistin 20 μg/ml (R&D Systems, Minneapolis, Minnesota, USA), resistin siRNA, or NAC were added to the medium. Glucose uptake was performed by adding 0.1 mmol/l glucose and 500 nCi/ml D-[3-^{3}H]-glucose (Perkin Elmer, Boston, Massachusetts, USA) for various periods. Cells were washed with PBS twice. Nonspecific uptake was performed in the presence of 10 μmol/l cytochalasin B and subtracted from all of the measured value. MicroScint-20 50 μl was added and the plate was read with TopCount (Packard Instrument Co.).

Measurement of resistin concentration

Conditioned media from cultured VSMCs under hypoxia and those from control cells (normoxia) were collected for resistin measurement. The level of resistin was measured by a quantitative sandwich enzyme immunoassay technique (R&D Systems). The lower limit of detection of resistin was 5 pg/ml. Both the intraobserver and interobserver coefficient of variance were less than 10%.

Balloon injury of rat carotid artery

Adult Wistar rats were anesthetized with isoflurane (3%) and subject to balloon catheter injury of the right carotid artery. Briefly, a 2F Forgarty balloon catheter (Biosensors International Inc., Newport Beach, California, USA) was inserted through the right external carotid artery, inflated and passed three times along the length of the isolated segment (1.5–2 cm in length), then the catheter was removed. Resistin siRNA was injected to the segment and electric pulses using CUY21-EDIT Square Wave Electroporator (Nepa Gene Co., Ltd, Chiba, Japan) were administered with five pulses and five opposite polarity pulses at 250 V/cm, 50 ms duration, 75 ms interval using Parallel fixed platinum electrode (CUY610P2-1, 1 mm tip, 2 mm gap). The injected siRNA was incubated for 10 min. After incubation, unbound siRNA was aspirated. The carotid artery was then tied off and the neck was closed. The rats were sacrificed at 14 days after balloon injury. The carotid artery was harvested and fixed in 10% formaldehyde and sliced into 5 μm paraffin sections. Then immunohistochemical study was performed as previously described [28].

Statistical analysis

The data were expressed as mean ± SD. Statistical significance was performed with analysis of variance (GraphPad Software Inc., San Diego, California, USA). The Dunnett's test was used to compare multiple groups to a single control group. Tukey–Kramer comparison test was used for pairwise comparisons between multiple groups after the analysis of variance. A value of $P < 0.05$ was considered to denote statistical significance.

Results

Hypoxia increases resistin expression in cultured vascular smooth muscle cells

To test the effect of hypoxia on the resistin expression, different degrees of hypoxia were used. As shown in Fig. 1a and b, hypoxia at 2.5% oxygen for 2 h significantly induced resistin expression, whereas hypoxia at 5 and 10% oxygen had no effect on resistin expression. We then used 2.5% oxygen as hypoxia to the following experiments. As shown in Fig. 1c and d, the level of resistin induction by 2.5% oxygen was similar to that induced by growth factor such as platelet-derived growth factor (PDGF) at 5 ng/ml (PeproTech Inc., Rocky Hill, New Jersey, USA) and proinflammatory stimuli, such as TNF-α at 100 pg/ml (R&D systems). We further showed that hypoxia at 2.5% oxygen induced resistin protein expression maximally at 2 h after hypoxia treatment and maintained elevated for 8 h (Fig. 2a and b). The levels of resistin mRNA also significantly increased from 1.5 to 6 h after hypoxia treatment (Fig. 2c). The immunohistochemical staining demonstrated that resistin was present in the smooth muscle cells in the atheroma induced by balloon injury for 14 days as shown in Supplementary I. We used resistin siRNA by electric pulse to deliver the resistin siRNA to the intimal area. As shown in Supplementary Fig. SI, the neointimal area decreased and resistin labeling decreased by siRNA in the atheroma.

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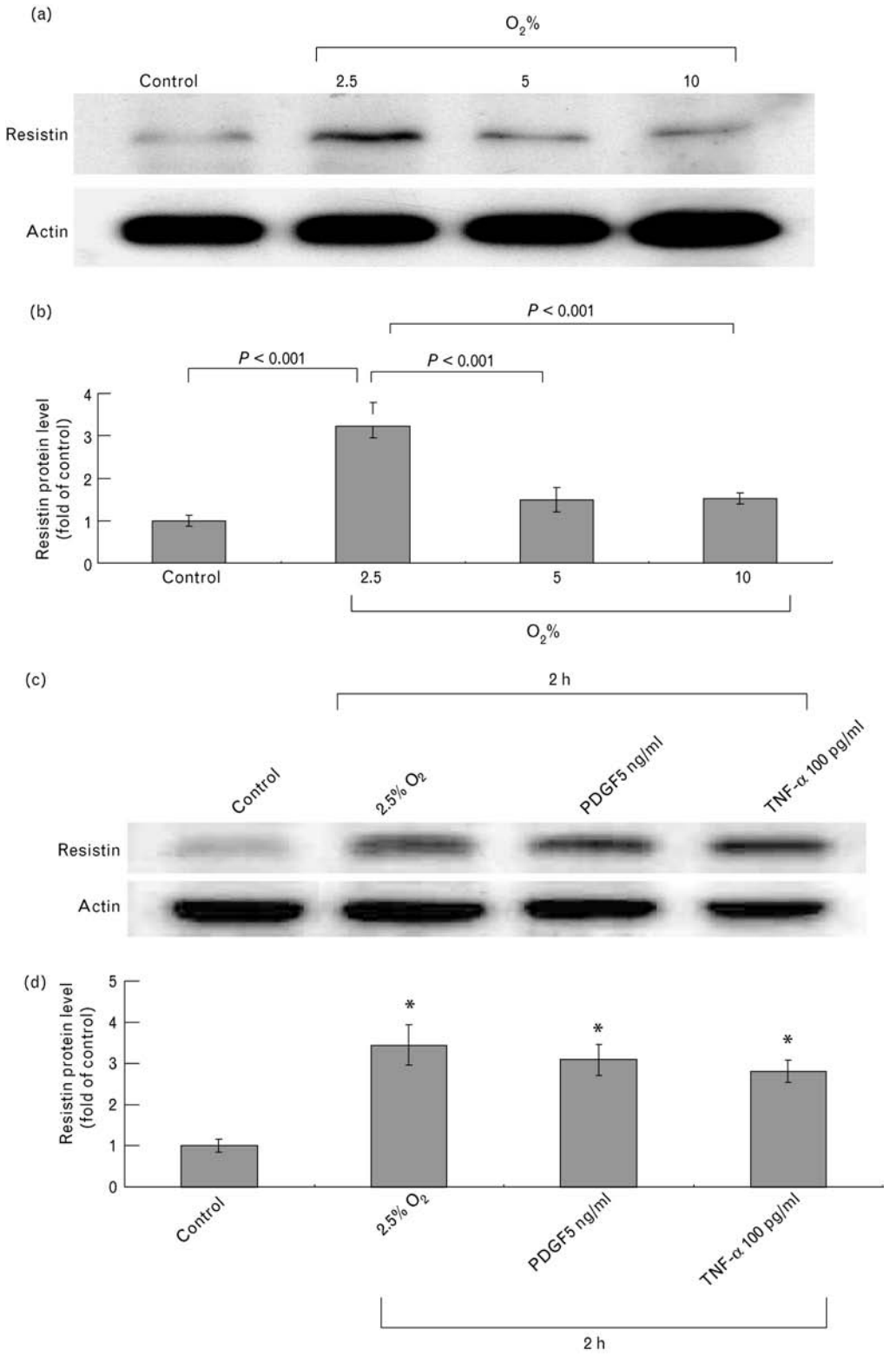
Fig. 1



Effect of hypoxia and proinflammatory cytokines on resistin expression in cultured vascular smooth muscle cells. (a and c) Representative western blots for resistin in VSMCs treated with different concentrations of oxygen, platelet-derived growth factor, or TNF-α for 2 h. (b and d) Quantitative analysis of resistin protein levels. The values from treated VSMCs have been normalized to values in control cells ($n = 4$ per group). $^*P < 0.001$ vs. control. The *P*-value for comparing control with 5 and 10% O_2 is 0.170. The *P*-value for comparing PDGF, TNF-α with 2.5% O_2 is 0.125. PDGF, platelet-derived growth factor; VSMC, vascular smooth muscle cell.

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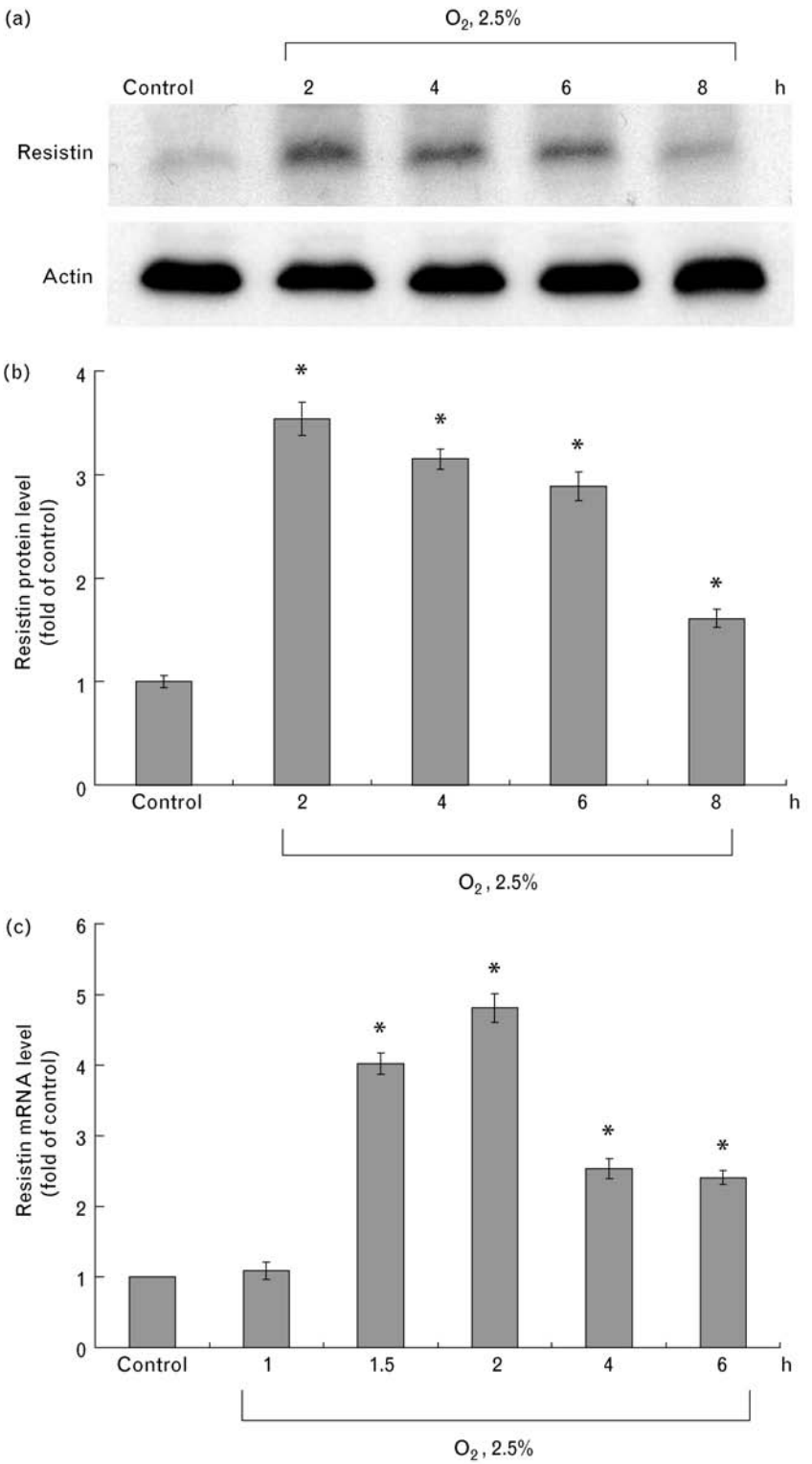
Fig. 2



Hypoxia increases resistin protein and mRNA expression in vascular smooth muscle cells. (a) Representative western blots for resistin in VSMCs subjected to 2.5% oxygen stimulation for various periods. (b) Quantitative analysis of resistin protein levels. The values from hypoxic VSMCs have been normalized to values in control cells ($n = 3$ per group). $^*P < 0.001$ vs. control. (c) Quantitative analysis of resistin mRNA levels. The mRNA levels were measured by real-time PCR. The values from hypoxic VSMCs have been normalized to matched actin measurement and then expressed as a ratio of normalized values to mRNA in control cells ($n = 3$ per group). $^*P < 0.01$ vs. control. VSMC, vascular smooth muscle cell.

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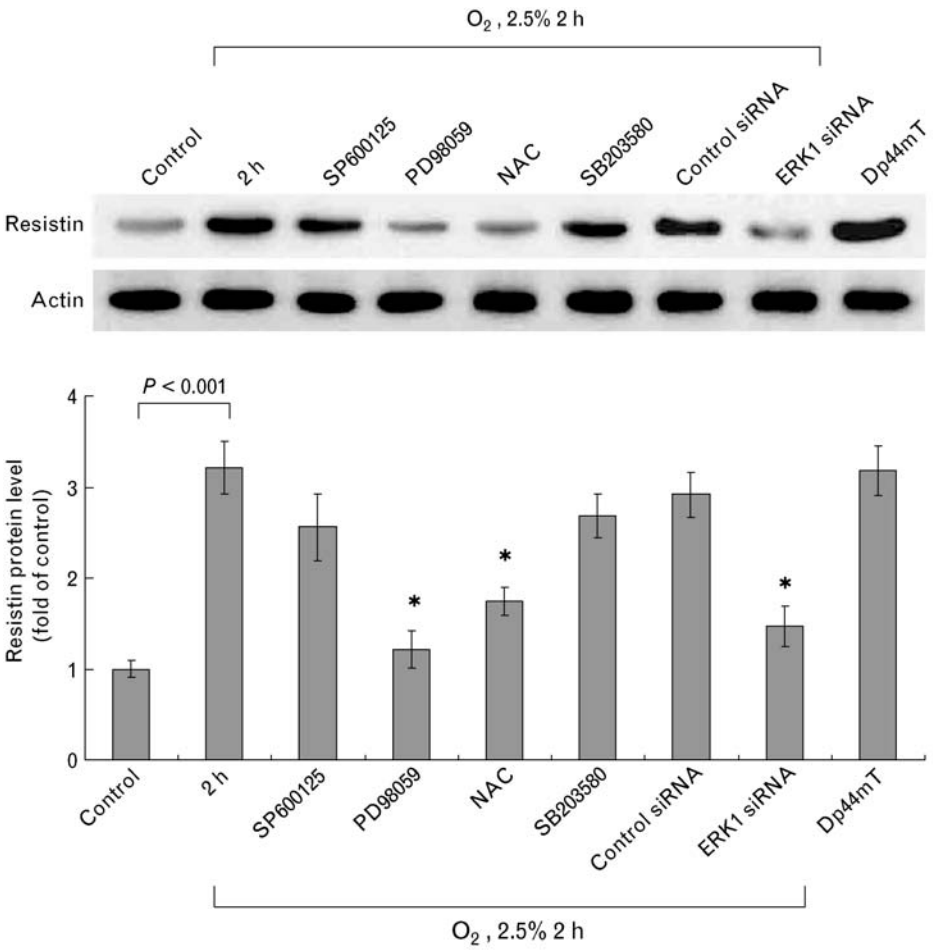
Fig. 3



Reactive oxygen species and extracellular signal-regulated and mitogen-activated protein kinases are important regulators that mediate hypoxia-induced resistin expression in vascular smooth muscle cells. Upper panel, representative western blots for resistin protein levels in VSMCs subjected to hypoxia stimulation for 2 h or control cells without hypoxia in the absence or presence of inhibitors, and siRNA. Dp44mT was added to VSMCs without hypoxia. Lower panel, quantitative analysis of resistin protein levels. The values from stimulated VSMCs have been normalized to values in control cells ($n = 4$ per group). $^*P < 0.001$ vs. 2 h. VSMC, vascular smooth muscle cell.

Macrophage was also found in the atheroma with anti-CD68 antibody staining.

Hypoxia-induced resistin protein expression in vascular smooth muscle cells is mediated by reactive oxygen species and extracellular signal-regulated kinase

To investigate the possible signaling pathways mediating the hypoxia-induced resistin expression, different inhibitors were used. As shown in Fig. 3, the western blots demonstrated that the hypoxia-induced increase of resistin protein was significantly attenuated after the addition of PD98059 or NAC 30 min before hypoxia treatment. The resistin protein induced by hypoxia was not affected by the addition of SP600125 and SB203580. Dimethyl sulfoxide as the vehicle for PD98059 did not affect resistin expression induced by hypoxia. Addition of 2,2′-dipyridyl-*N*,*N*-dimethylsemicarbazone (Dp44mT, 30 μmol/l; Calbiochem) alone without hypoxia treatment significantly increased resistin expression. ERK siRNA also completely blocked the resistin expression induced by hypoxia. The control siRNA did not affect the resistin expression induced by hypoxia.

As shown in Fig. 4, hypoxia for 1.5 and 2 h significantly increased the ROS production by using ROS assay with fluorescent microscope. Pretreatment with NAC significantly blocked the induction of ROS by hyperbaric oxygen. In the control group with normoxia treatment, very few VSMCs expressed green fluorescence.

As shown in Fig. 5a and b, phosphorylated ERK protein was induced by hypoxia in a time-dependent manner. The phosphorylated ERK protein was maximally induced at 1.5 h of hypoxia treatment and remained elevated until 4 h. The pattern of increase in phosphorylated ERK protein after hypoxia was slightly earlier than

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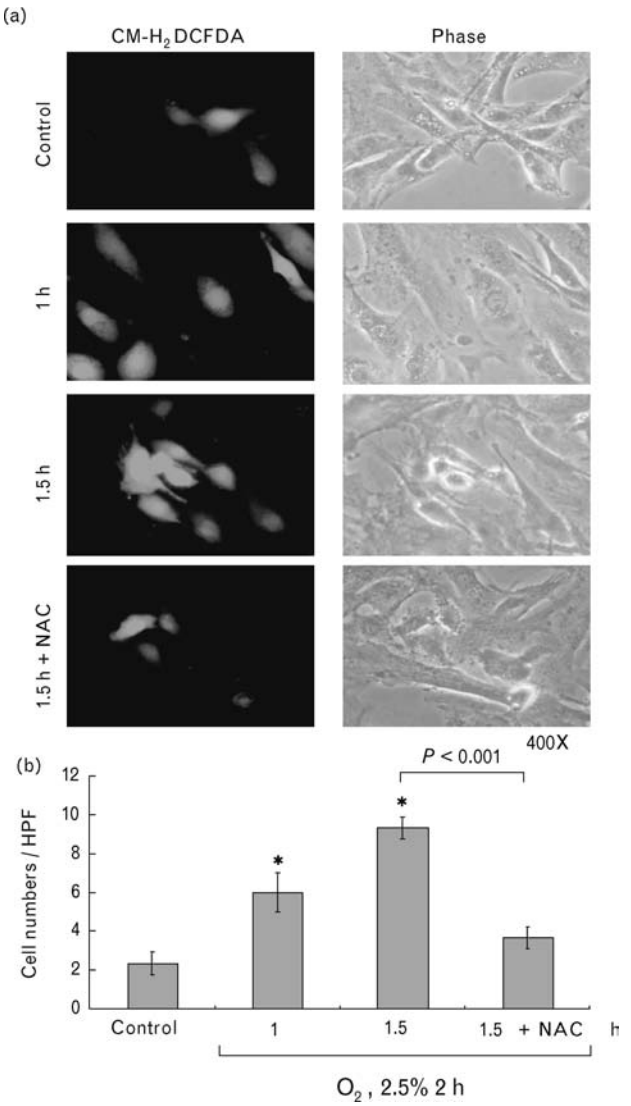
Fig. 4



Effect of hypoxia on reactive oxygen species formation in vascular smooth muscle cells. (a) Representative microscopic image for ROS assay with (left panel) or without green fluorescence (right panel) in VSMCs subjected to hypoxia stimulation for 2 h or control cells without hypoxia in the absence or presence of NAC. (b) Quantitative analysis of the positive fluorescent cells. Control group indicates normoxia group ($n = 4$ per group). $^*P < 0.001$ vs. control. NAC, *N*-acetylcysteine; ROS, reactive oxygen species; VSMC, vascular smooth muscle cell.

that of resistin protein after hypoxia as shown in Fig. 2. The phosphorylated ERK was abolished by pretreatment with PD98059 or NAC. ERK siRNA knocked down the ERK protein expression by 72% (from 3.2-fold to 0.89-fold). The phosphorylated $NFAT_c$ protein activated by hypoxia was similar to the pattern of phosphorylated ERK activated by hypoxia. The phosphorylated $NFAT_c$ induced by hypoxia was attenuated by pretreatment with PD98059, NAC, or ERK siRNA (Fig. 5c and d). These data indicate that ROS was generated before the ERK activation and ERK was activated before $NFAT_c$ activation, clarifying the sequence of ROS generation, ERK and $NFAT_c$ activation.

Hypoxia increases nuclear factor of activating T cells binding activity

Hypoxia treatment for cultured VSMCs for 1–4 h significantly increased the DNA-protein binding activity of NFAT (Fig. 6). An excess of unlabeled NFAT oligonucleotide competed with the probe for binding NFAT protein, whereas an oligonucleotide containing a 3-bp substitution in the NFAT-binding site did not compete for binding. Addition of PD98059 30 min before hypoxia abolished the DNA-protein binding activity induced by hypoxia. DNA-binding complexes induced by hypoxia could be supershifted by a monoclonal $NFAT_c$ antibody, indicating the presence of this protein in these complexes.

Hypoxia increases resistin promoter activity

The rat resistin promoter construct contains Stat-3, AP-1, $NFAT_c$, NF-κB, and HIF-1α binding sites. Hypoxia for 1.5 h significantly increased the resistin promoter activity by 5.2-fold as compared with control without hypoxia (Fig. 7). When the $NFAT_c$-binding sites were mutated, the increased promoter activity induced by hypoxia was abolished. Addition of PD98059 and NAC 30 min before hypoxia abolished the increased resistin promoter. The promoter activity of HIF-1α mutant was significantly enhanced after hypoxia treatment and addition of PD98059 and NAC did not change the promoter activity. The promoter activity of wild resistin promoter after hypoxia was significantly higher than that of HIF-1α mutant resistin promoter. This finding indicates that hypoxia regulates resistin in VSMCs at transcriptional level and that $NFAT_c$-binding sites in the resistin promoter is essential for the transcriptional regulation. Combing mutation of NFATC and HIF-1α binding sites did not further reduce the promoter activity as compared with mutation of NFATC-binding site alone. This finding indicates that the HIF-1α binding site is not the major part for the transcriptional regulation of resistin expression in the hypoxic model of VSMC.

Recombinant resistin reduces glucose uptake

Recombinant mouse resistin at 20 μg/ml significantly reduced glucose uptake at various periods of incubation as compared with control VSMCs without treatment (Fig. 8). The dose of recombinant mouse resistin used in the study was according to the study by Graveleau *et al.* [29]. The glucose uptake in hypoxic cells was similar to that in exogenous addition of resistin. Addition of resistin siRNA or NAC before recombinant resistin treatment reversed the glucose uptake to baseline levels. Addition of Dp44mT, a ROS generating agent, reduced the glucose uptake in cultured VSMCs similar to exogenous addition of resistin. Using serum free medium in the

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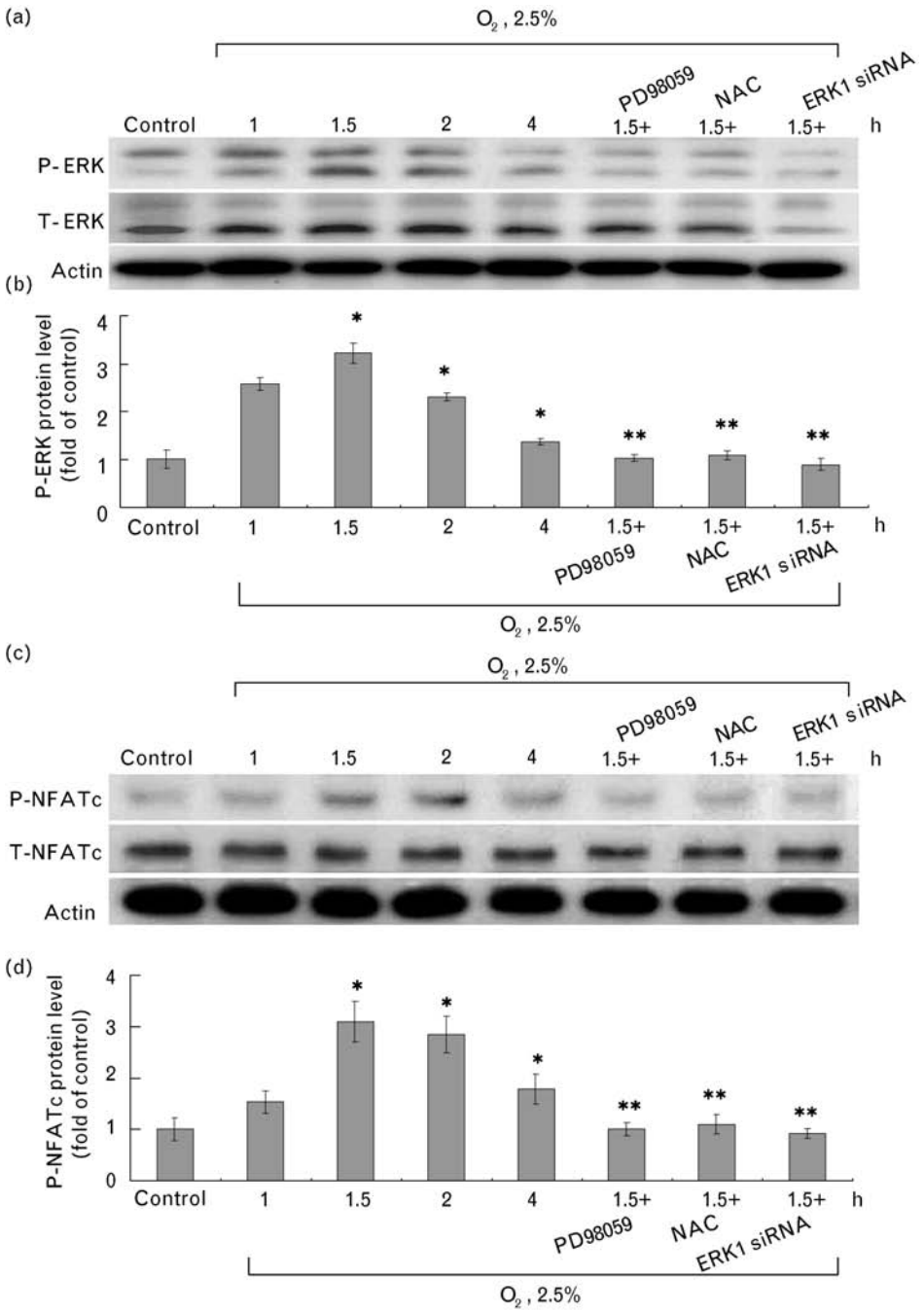
Fig. 5



Expression of extracellular signal-regulated and mitogen-activated protein kinases and nuclear factor of activating T cells in vascular smooth muscle cells. (a and c) Representative western blot for phosphorylated and total ERK MAP and $NFAT_c$ in VSMCs after treatment with hypoxia for various periods with or without inhibitor. (b and d) Quantitative analysis of phosphorylated protein levels. The values from hypoxic VSMCs have been normalized to matched actin and corresponding total protein measurement and then expressed as a ratio of normalized values to each phosphorylated protein in control cells ($n = 3$ per group). $^*P < 0.001$ vs. control. $^{**}P < 0.001$ vs. 1.5 h. ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; NAC, *N*-acetylcysteine; $NFAT_c$, nuclear factor of activating T cells; VSMC, vascular smooth muscle cell.

absence of insulin and resistin, the glucose uptake for VSMCs was 140 ± 10 cpm, whereas addition of resistin reduced the glucose uptake to 106 ± 8 cpm ($P < 0.05$, $n = 3$).

Hypoxia stimulates secretion of resistin from vascular smooth muscle cells

As shown in Fig. 9, hypoxia significantly began to increase the resistin secretion from VSMCs at 1 h after

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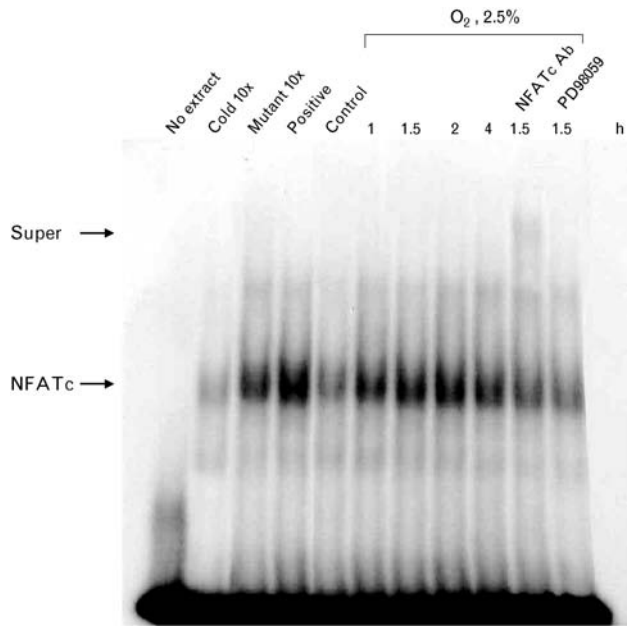
Fig. 6



Hypoxia increases nuclear factor of activating T cells binding activity. Representative electrophoretic mobility shift assay showing protein binding to the $NFAT_c$ oligonucleotide in nuclear extracts of vascular smooth muscle cells after hypoxia stimulation in the presence or absence of inhibitors. Arrow indicates the mobility of the complex. Similar results were found in another two independent experiments. Cold oligo means unlabeled $NFAT_c$ oligonucleotides. A significant supershifted complex (S) after incubation with $NFAT_c$ antibody was observed. $NFAT_c$, nuclear factor of activating T cells.

hypoxia treatment and remained elevated for 6 h. The mean concentration of resistin rose from 102 ± 10 pg/ml before hypoxia to 540 ± 20 pg/ml after hypoxia for 2 h ($P < 0.01$).

Discussion

In this study, we demonstrated several significant findings. First, hypoxia at 2.5%, but not 5 and 10% oxygen upregulates resistin expression in VSMCs; second, ROS, ERK kinase and NFAT transcription factor are involved in the signaling pathway of resistin induction; third, resistin impairs glucose uptake in cultured VSMCs, and finally, hypoxia-induced resistin secretion from VSMCs. Our data clearly indicate that moderate hypoxia plays a crucial role in the modulation of resistin expression in VSMCs. Our data also demonstrated that functional consequence of resistin upregulation by hypoxia resulted in reduction of glucose uptake. We have demonstrated that secreted resistin is already elevated at 1 h after hypoxia; however, resistin RNA levels are not increased yet. The resistin mRNA increased at 2 h after hypoxia. There are possibly two purely hypothetical explanations. First, the sercretion of stored resistin protein may be a possible explanation for the finding that the secreted resisitn protein level is elevated before the increase in mRNA level. Second, hypoxia may first protect resistin protein degradation and then enhance resistin protein synthesis by increased mRNA. To date, no resistin receptor has been reported yet. The resistin functions as mediator of insulin resistance. In this study, we analyzed the expression of resistin after PDGF and TNF-α and found a remarkable induction of resistin protein level even after stimulation with low level of PDGF and TNF-α, comparable to the level after 2.5% hypoxia. This finding indicates that effect on resistin expression in VSMC by hypoxia at 2.5% oxygen is similar to that by PDGF and TNF-α. Hypoxia at 2.5% has proinflammatory and growth factor effect on VSMCs.

The induction of resistin protein by hypoxia was largely mediated by ROS and ERK kinase pathway because the potent antioxidant, NAC and specific and potent inhibitors of an upstream ERK kinase, PD98059, inhibited the induction of resistin protein. Hypoxia increased ROS formation in VSMCs and NAC reduced the ROS formation induced by hypoxia. A ROS generating agent, Dp44mT, increased resistin protein expression similar to hypoxia. The signaling pathway of ERK was further confirmed by the finding that ERK siRNA inhibited the induction of resistin protein by hypoxia. Hypoxia increased phosphorylated ERK protein and NAC attenuated the induction of phosphorylated ERK protein. Our finding indicates that hypoxia generates ROS first and then ROS activates ERK protein.

Hypoxia is an important stimulus for the neointimal angiogenesis in the vessel wall because neoangiogenesis develops in response to inadequate perfusion through the thickened atherosclerotic plaques [30]. Hypoxia may cause plaque thrombosis during atherosclerosis because of poor perfusion caused by thickened atherosclerotic plaques. Sato *et al.* [20] have demonstrated that hypoxia stimulates the production of ROS in VSMCs. In this study, we clearly demonstrated that hypoxia generated ROS in VSMCs. NFAT can be activated by ROS in mouse embryo fibroblast [21] and human colon cancer cell [22]. NFAT, a Ca^{2+}-dependent transcription factor that regulates the expression of genes in both immune and nonimmune cells [31,32], has been linked to smooth muscle phenotypic maintenance [33,34]. In this study, we demonstrated that hypoxia stimulation of NFAT–DNA binding activity required at least phosphorylation of the ERK as ERK inhibitor abolished the NFAT-binding activity. $NFAT_c$ monoclonal antibody shifted the NFAT–DNA binding complex, indicating the specificity of the NFAT–DNA binding activity induced by hypoxia. We further demonstrated that hypoxia increased resistin promoter activity and the binding site of NFAT in the resistin promoter is essential for the transcriptional regulation. Taken together, our results indicate that hypoxia may increase the NFAT transcriptional activity in VSMCs. Recently, NFAT has been demonstrated to play a key

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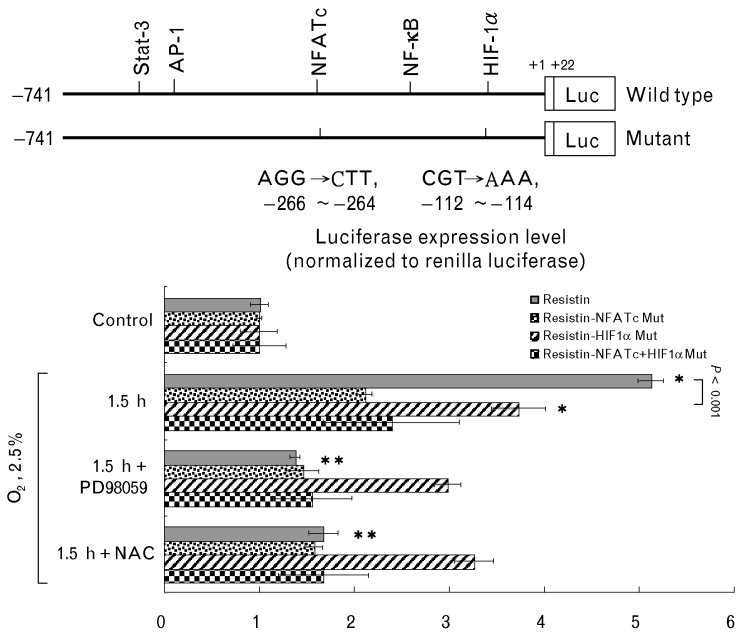
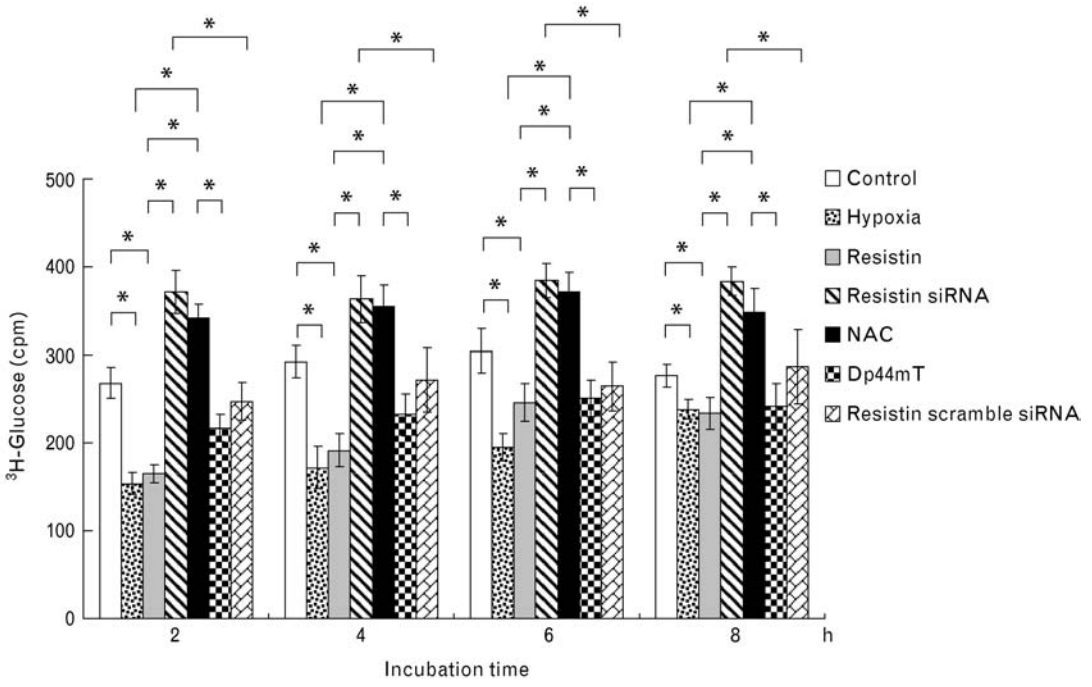
Fig. 7



Effect of hypoxia on resistin promoter activity in vascular smooth muscle cells. Upper panel, constructs of resistin promoter gene. Positive +1 demonstrates the initiation site for the resistin transcription. Lower panel, quantitative analysis of resistin promoter activity. VSMCs were transiently transfected with pResistin-Luc by gene gun. The luciferase activity in cell lysates was measured and was normalized with renilla activity ($n = 3$ per group). $*P < 0.001$ vs. control. $**P < 0.001$ vs. 1.5 h. NAC, *N*-acetylcysteine; VSMC, vascular smooth muscle cell.

Fig. 8



Effect of hypoxia and recombinant resistin on glucose uptake in vascular smooth muscle cells. Glucose uptake was measured in VSMCs treated for 90 min with 20 μg/ml recombinant mouse resistin with or without siRNA or NAC or Dp44mT. $*P < 0.001$. Data are from three independent experiments. NAC, *N*-acetylcysteine; VSMC, vascular smooth muscle cell.

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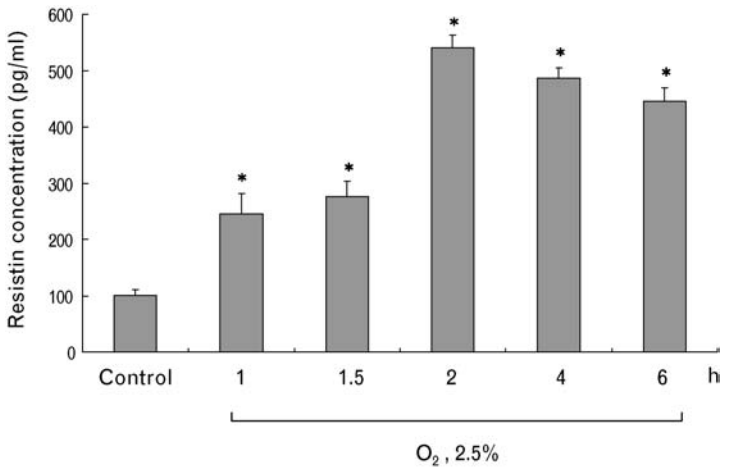
Fig. 9



Hypoxia increases release of resistin from vascular smooth muscle cells subjected to 2.5% oxygen for various periods ($n = 3$). $^*P < 0.001$ vs. control.

role in various vasculopathies such as atherosclerosis and restenosis [16–19]. $NFAT_c$ has been demonstrated to mediate hypoxia-induced pulmonary arterial remodeling [19] and blockade of NFAT can reduce SMC proliferation and restenosis [16–18]. The increased NFAT promoter activity by hypoxia may contribute to the vasculopathy induced by hypoxia.

Biomarkers that integrate metabolic and inflammatory signals are powerful candidates for defining risk of atherosclerotic cardiovascular disease [3]. Hyperresistinemia impairs glucose tolerance and induces hepatic insulin resistance in rodents [35], whereas mice deficient in resistin are protected from obesity-associated insulin resistance [36]. Our study using VSMC culture system demonstrated the impaired glucose transport by resistin. The glucose uptake in VSMCs was reduced by resistin upregulation. Impairment of glucose transport may explain the potential mechanism of resistin induction of insulin resistance. Recently, Yang *et al.* [37] have reported that NFAT contributes to glucose and insulin homeostasis and NFAT regulates resistin expression upon insulin stimulation. In the present study, we demonstrated that hypoxia increased resistin secretion from VSMCs, indicating that resistin plays an autocrine or paracrine function in VSMCs. We also demonstrated that the binding site of $NFAT_c$ in the resistin promoter is essential for the transcriptional regulation. We have demonstrated significant reduction in resistin promoter activity after mutation of HIF-1α binding site as compared with the wild-type promoter under hypoxic conditions. This may indicate that HIF-1α binding site in the resistin promoter is also involved in the regulation of resistin expression under hypoxia in VSMCs. However, combing mutation of NFATC and HIF-1α binding sites did not further reduce the promoter activity as compared with mutation of NFATC binding site alone. This finding indicates that the HIF-1α binding site is not the major part for the transcriptional regulation of resistin expression in the hypoxic model of VSMC. The link between resistin and NFAT may contribute to glucose homeostasis in VSMCs. The present study suggests resistin as a metabolic link between hypoxia, inflammation and atherosclerosis.

In summary, our study reports for the first time that hypoxia enhances resistin expression in cultured rat VSMCs. The hypoxia-induced resistin is mediated through ROS, ERK kinase and NFAT pathway. Glucose uptake in VSMCs is reduced by resistin upregulation. The resistin induced by hypoxia may contribute to the pathogenesis of atherosclerosis under hypoxia.

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