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Hypoxia induces discoidin domain receptor-2 expression via the p38 pathway in vascular smooth muscle cells to increase their migration

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ABSTRACT

Discoidin domain receptor-2 (DDR2) is a receptor tyrosine kinase that binds to the extracellular matrix. We investigated the role of hypoxia in DDR2 expression in vascular smooth muscle cells (VSMCs) and the underlying mechanism. Subjecting VSMCs to hypoxia (2.5% O_2) induced DDR2 expression; treatments with a specific inhibitor (SB203580) of p38 mitogen-activated protein kinase (MAPK) or p38-specific small interference RNA (siRNA) abolished this hypoxia-induced DDR2 expression. Gel shifting assays showed that hypoxia increased the Myc–Max–DNA binding activity in the promoter region of DDR2; inhibition of p38 MAPK activation by SB203580 and p38-specific siRNA blocked hypoxia-induced DDR2 promoter activity. Hypoxia also induced matrix metalloproteinase-2 (MMP-2) activity in VSMCs and increased their migration. These VSMC responses to hypoxia were inhibited by DDR2- and p38-specific siRNAs. Our results suggested that hypoxia induces DDR2 expression in VSMCs at the transcriptional level, which is mediated by the p38 MAPK pathway and contributes to VSMC migration.

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Communication between cells and their environment is mediated by specific cell surface receptors that transduce external signals to the inside of cells. An important class of signaling receptors are receptor tyrosine kinases (RTKs), which play crucial roles in many fundamental cellular processes [1]. Two members of this family, discoidin domain receptors 1 and 2 (DDR1 and 2) are unusual RTKs in that their ligands are extracellular matrix (ECM) rather than growth factor-like peptides [2,3]. DDRs bind to several collagens and stimulate production of matrix metalloproteinase (MMP) [3,4], which can digest the ECM proteins and promote cell migration. DDR1 is mainly expressed in epithelial cells bound to collagen types I through V and VIII, whereas DDR2 is found in vascular and mesenchymal cells and responds to the fibrillar collagen types I and III [5]. It has been shown that DDR2 interacts with Src following its activation by collagen type I [6]. Thus, DDR2 requires Src activity to exhibit maximal levels of tyrosine-phosphorylation [6].

The ECM is a dynamic structure that not only provides a scaffold for mechanical support and organization of tissues, but also regulates critical events in normal development and pathological conditions. The neointimal is composed of vascular smooth muscle cells (VSMCs), which synthesize abundant ECM. Obstructive vascular diseases are characterized by degradation and synthesis of new ECM components; for example, interactions between VSMCs and ECM proteins are important in cell migration during atherosclerosis and restenosis. Evidence from *in vitro* and *in vivo* studies using DDR1 and DDR2-null mice suggests that DDR can regulate cell proliferation and matrix metalloproteinase (MMP)-mediated ECM remodeling [4,7–9]. DDR1 is expressed on migrating VSMCs after balloon catheter injury of the rat carotid artery, and intimal thickening after arterial injury is reduced in DDR1-knockout mice [4]. The activation of DDR2 by collagen results in increased production of MMP-1 [2] and MMP-2 [8]. Prolonged stimulation of DDR2 is also associated with the up-regulation of MMP-1 expression [3]. Previous studies have shown that DDR2 associated with the MMP-2-dependent mechanism plays an important role in migration and proliferation of hepatic stellate cells [8] and fibroblasts [9]. Recent studies have shown that DDR1 and DDR2 play potential roles in regulating VSMC-mediated collagen turnover in obstructive vascular diseases [10]. These findings suggest an important role for DDRs in regulating collagen matrix degradation and cellular reorganization in the vascular system.

Hypoxia elicits a variety of functional responses in VSMCs, including cell migration. In order to maintain vascular homeostasis, VSMCs cope with hypoxia by regulating the expression of a number of genes that are mediated by a variety of signaling cascades [11]. Hypoxia enhances endothelial cell migration in an

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MMP-2-dependent manner [12]. However, the mechanisms by which hypoxia regulates VSMC migration remain unclear. Moreover, little is known about the role of DDR2 in modulating hypoxia-induced VSMC migration. The aim of this study is to elucidate the role of DDR2 in hypoxia-induced VSMC migration and isolate the mechanism by which hypoxia induces DDR2 expression. Understanding the oxygen-sensitive adaptive pathways in VSMCs may help develop therapeutic strategies to treat various hypoxia-related vascular diseases.

Materials and methods

VSMC cultures. VSMCs were grown by explanting the thoracic aorta of 200–250-g male Sprague–Dawley rats, as described [13]. Cells were cultured in medium 199 containing 20% FCS, 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, VSMCs were passaged after trypsinization and cultured in a Flexcell I flexible membrane dish, in medium 199 containing 0.5% FCS. The cells were incubated for another 2 days to render them quiescent before the hypoxia experiment. VSMCs between passages 3–6 were used.

Hypoxia apparatus. Hypoxic conditions (2.5% O_2) were achieved by adding medium pre-equilibrated with nitrogen gas to cells prior to incubating them in a Plexiglas chamber purged with water-saturated nitrogen gas to a pO_2 = 20 mmHg by an oxygen controller (PROOX model 110, BioSpherix, Ltd., Redfield, NY) as previously described [14].

Western blot analysis. Western blot was performed as previously described [13]. Goat monoclonal anti-DDR2 antibody (Santa Cruz Biothchnology) was used.

Real-time RT-PCR. Total RNA was isolated from VSMCs using the single-step acid guanidinium thiocyanate/phenol/chloroform extraction method. Real-time RT-PCR was performed as described [13]. The DDR2 primers used were as follows: sense, 5′-GGCGGAACGAAAGTGCT-3′; antisense, 5′-ACCGTGACAAA CCGGG-3′.

Electrophoretic mobility shift assay (EMSA). Nuclear proteins from VSMCs were collected, and the EMSA was performed as described [13]. Consensus and control oligonucleotides (Santa Cruz Biotechnology) were labeled by polynucleotide kinase incorporation of [γ^{32}-P]-dATP. Consensus oligonucleotide sequences for Myc–Max, the responsive element in the promoter region of DDR2, were 5′-GGAAGCAGACCACGTGGTCTGCTTCC-3′. The Myc–Max mutant oligonucleotide sequences were 5′-GGAAGCAGACCACGGAGTCTGCT TCC-3′. In parallel experiments, controls were performed in each case with either mutant oligonucleotides or unlabeled oligonucleotides to compete with the labeled sequences.

siRNA transfection. VSMCs were transfected with DDR2- or p38-specific siRNA (800 ng each) (Dharmacon) for 48 h before the hypoxia experiment. DDR2-specific siRNA is a target-specific 21 nt siRNA as designed by a computer program provided by Dharmacon. The sequences of sense and antisense DDR2-specific siRNA were 5′-GAUGAUAGCAACACUCGGAUU-3′ and 5′-PUCCGAGUGUUGCUAUCAUCUU-3′, respectively. The p38-specific and control siRNA sequences were used as previously described [13].

Promoter activity assay. A −490 to +66 bp rat DDR2 promoter construct was generated. In brief, rat genomic DNA was amplified with sense (5′-GACAGAAGGGAACTGCATCTTTAAG-3′) and antisense (5′-GATTCAAACTGTCCTCCGGCCGCTT-3′) primers. The amplified product was digested with M1uI and Bg1II restriction enzymes and ligated into a pGL3-basic luciferase plasmid vector (Promega, Madison, WI) that was digested with the same enzymes. The DDR2 promoter contains Myc–Max conserved sites (ACGTG) at −258 to −254 bp. To construct a DDR2 mutant, the Myc–Max binding sites were mutated using the mutagenesis kit (Stratagene, La Jolla, CA). Plasmids were transfected into VSMCs using a low pressure-accelerated gene gun (Bioware Technologies, Taipei, Taiwan). Two micrograms of plasmid DNA was suspended in 5 μl of PBS and delivered to the VSMCs. The transfection efficiency using this method is 25%. Cell extracts were prepared using the Dual-Luciferase Reporter Assay System (Promega) and measured by luminometer (Turner Designs, Sunnyvale, CA).

Zymography. ECM-degrading activity was detected by zymography. Proteins were extracted from cultured VSMCs, and equal amounts of sample protein were subjected to SDS–PAGE on gelatin-containing acrylamide gels (7.5% polyacrylamide and 2 mg/mL gelatin) under nonreducing conditions. Zymogram was performed as described [15].

Migration assay. The migration activity of VSMCs was determined using the growth factor-reduced Matrigel invasion system (Becton Dickinson) following the manufacturer's protocol. The migration assay was performed as previously described [13].

Statistical analysis. Results were expressed as mean ± SEM. Statistical significance was evaluated using analysis of variance (GraphPad Software Inc., San Diego, CA, USA). The Dunnett's test was used to compare multiple groups to a single control group. Turkey–Kramer comparison was used for pairwise comparisons between multiple groups after the ANOVA. A value of $P < 0.05$ was considered significant.

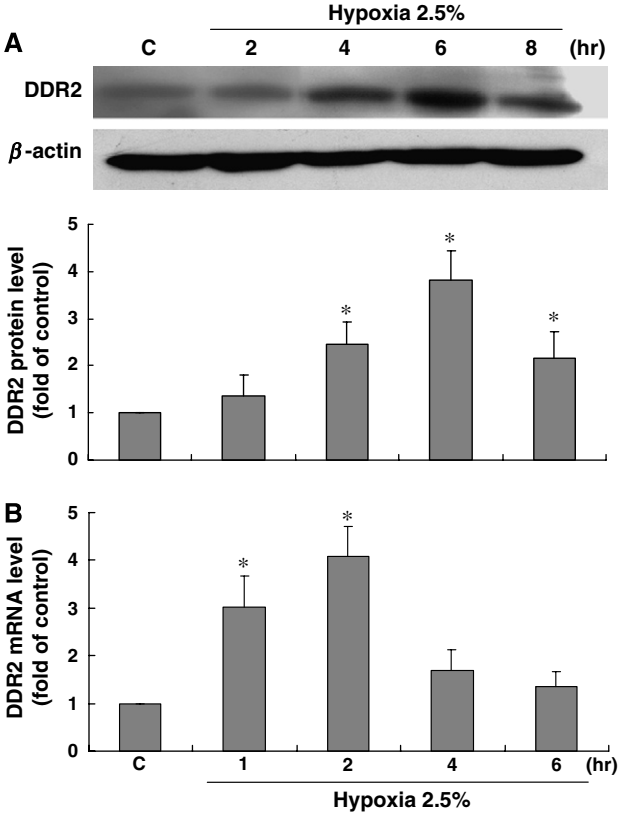

Fig. 1. Hypoxia induces DDR2 mRNA and protein expressions in VSMCs. VSMCs were kept as controls (C) or subjected to hypoxia (2.5% O_2) for the indicated times, and their protein (A) and mRNA (B) expressions were determined by western blot and quantitative RT-PCR analyses, respectively, as described in Materials and methods. Data are shown as mean ± SEM from 3 independent experiments. $^{*}P < 0.05$ vs. controls.

Results

Hypoxia induces DDR2 expression in VSMCs

VSMCs were incubated in the hypoxic chamber (2.5% O_2) for different periods of time, and DDR2 protein expression was examined by Western blot analysis. DDR2 protein expression in VSMCs was induced by hypoxia within 4 h, reached a maximal level at 6 h, and then declined but remained elevated after exposure to hypoxia for 8 h (Fig. 1A). RT-PCR analysis showed that hypoxia-induced mRNA expression of DDR2 in VSMCs as early as 1 h after exposure (Fig. 1B). This increase in DDR2 mRNA expression reached a maximal level after exposure to hypoxia for 2 h and then decreased to the basal level within 6 h. These results suggest that hypoxia induces transient increases in DDR2 expression in VSMCs.

Hypoxia-induced DDR2 expression in VSMCs is predominantly mediated by the p38 mitogen-activated protein kinase (MAPK) pathway

MAPK signaling pathways regulate gene expression and cellular function in response to hypoxia. To determine whether MAPK signaling pathways are involved in the hypoxia-induced DDR2 expression in VSMCs, VSMCs were pre-treated with specific inhibitors of ERK (PD98059), p38 MAPK (SB203580), and JNK (SP600125) and then subjected to hypoxia at 2.5% O_2 for 6 h. Treatment with PD98059 and SP600125 resulted in partial inhibition of hypoxia-induced DDR2 expression in VSMCs (Fig. 2). In contrast, treatment with SB203580, a specific inhibitor of p38 MAPK, abolished the hypoxia-induced DDR2 expression in VSMCs. Involvement of the p38 MAPK signaling pathway in hypoxia-induced DDR2 expression in VSMCs was substantiated by treating VSMCs with p38-specific siRNA, which also abolished the hypoxia-induced DDR2 expression (compared to control siRNA). These results suggest that the p38 MAPK signaling pathway plays a predominant role in modulating DDR2 expression in VSMCs in response to hypoxic conditions.

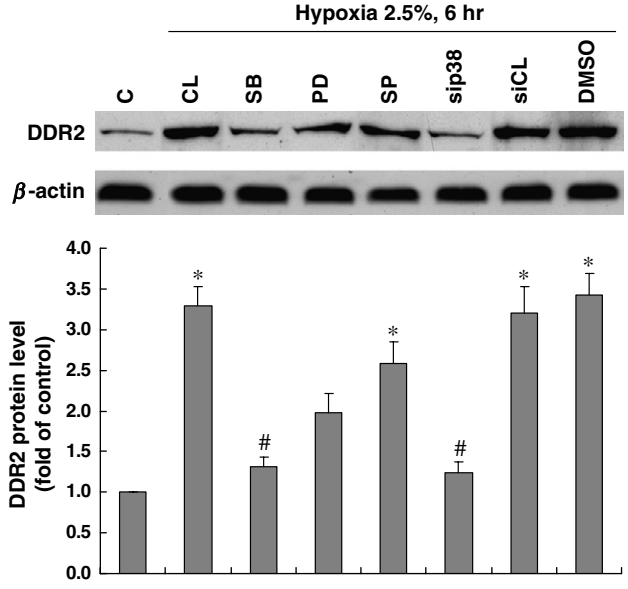

Fig. 2. Hypoxia-induced DDR2 expression is mediated by the p38 MAPK pathway. VSMCs were kept as controls (C) or subjected to hypoxia for 6 h (CL). In parallel experiments, the cells were either pre-treated with SB203580 (SB; 3 μM), PD98058 (PD; 50 μM), or SP600125 (SP; 20 μM) for 30 min or transfected with control (siCL) or p38-specific siRNA (sip38; 40 nM) for 24 h. Data are shown as mean ± SEM from 3 independent experiments. $^{*}P<0.05$ vs. controls. $^{\#}P<0.05$ vs. hypoxic cells without pre-treatment.

Hypoxia induces increases in Myc–Max–DNA binding activity in the nucleus of VSMCs

Because the promoter regions of the DDR2 gene contain the Myc–Max binding domain that is responsible for expression in response to various stimuli, we investigated whether Myc–Max–DNA binding activity in the VSMC nuclei was influenced by hypoxia. Nuclear protein extracts from VSMCs subjected to hypoxia for 0.5–4 h were prepared for EMSA. The EMSA results obtained from incubating VSMC nuclear protein extracts with oligonucleotides corresponding to the Myc–Max binding sequences showed that treatment with hypoxia for 0.5 and 1 h caused an increase in binding activity (Fig. 3A). Pre-treatment of VSMCs with p38-specific siRNA (compared to control siRNA) or SB203580 significantly inhibited this hypoxia-induced promoter activity. The formation of the Myc–Max–DNA complex required the presence of a wild-type Myc–Max binding site, as demonstrated by a lack of competition by providing 10-fold excess of mutant oligonucleotide, whereas a

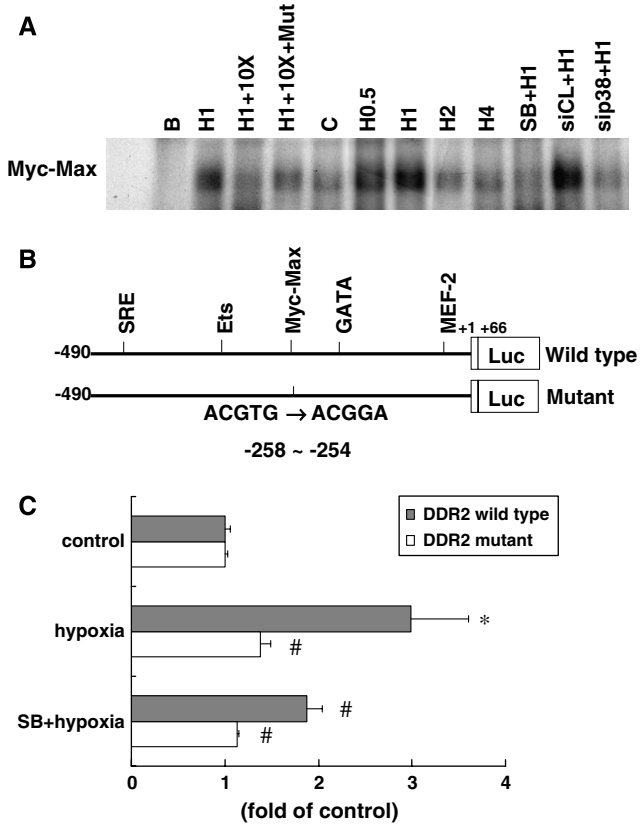

Fig. 3. Hypoxia-induced DDR2 expression in VSMCs is a transcriptional event. (A) VSMCs were kept as controls (C) or exposed to hypoxia for 0.5 h (H0.5), 1 h (H1), 2 h (H2), or 4 h (H4). EMSA was performed using total nuclear extracts and ^{32}P-labeled oligonucleotides containing wild-type or mutant (Mut) DDR2 Myc–Max binding sites. The specificity of the retarded complexes (Myc–Max) was assessed by pre-incubating the nuclear extracts with 10-fold excess unlabeled oligonucleotides (wild-type or mutant) as a competitor. In parallel experiments, the cells were either pre-treated with SB203580 (SB; 3 μM) for 0.5 h or transfected with control (siCL) or p38-specific siRNA (sip38; 40 nM) for 24 h. B: Blank. (B and C) VSMCs were transfected with plasmids containing wild-type or mutant DDR2 promoter regions (B) and then were kept as controls or subjected to hypoxia for l h. Their promoter activities were determined using the luciferase assay (C). In some experiments, the cells were pre-treated with SB203580 (SB; 3 μM) for 30 min. Data are shown as mean ± SEM from 3 independent experiments. $^{*}P<0.05$ vs. controls. $^{\#}P<0.05$ vs. hypoxic cells transfected with wild-type constructs without pre-treatment.

10-fold concentration of unlabeled wild-type oligonucleotide effectively competed with ^{32}P-labeled oligonucleotide for Myc–Max binding. These results suggest that hypoxia plays a role in modulating DDR2 expression via the modulation of Myc–Max binding activity at the transcriptional level.

Hypoxia induces DDR2 promoter activity in VSMCs

To further determine whether the hypoxic modulation of DDR2 expression is a transcriptional event, VSMCs were transfected with promoter constructs containing the promoter regions of DDR2 (−490 bp) and the reporter gene luciferase (Fig. 3B) and then exposed to hypoxic condition for 1 h. VSMCs subjected to hypoxia at 2.5% O_2 for 1 h significantly increased DDR2 promoter activities by approximately 3-fold compared with control cells in normoxia (Fig. 3C). In contrast, hypoxic exposure of VSMCs transfected with promoter constructs containing DDR2 promoter regions in which the Myc–Max binding sites (between −258 and −254 bp) were mutated did not induce promoter activity. These results suggest that the Myc–Max binding elements are responsible for the hypoxic induction of DDR2 promoter activity. The addition of SB203580 inhibited hypoxia-induced DDR2 promoter activity, suggesting that the p38 MAPK pathway is involved in hypoxia-induced DDR2 promoter activity in VSMCs.

DDR2 is involved in hypoxia-induced migration of VSMCs

To investigate whether DDR2 modulates VSMC migration in response to hypoxia, we further investigated the effect of hypoxia on MMP-2 activity in VSMCs and the involvement of DDR2 in VSMC migration. Zymographic assays demonstrated that MMP-2 activity was induced in VSMCs subjected to hypoxia at 2.5% O_2 for 12 h (Fig. 4A). Pre-treatment of VSMCs with DDR2- and p38-specific siRNAs (compared to control siRNA) and SB203580 abolished hypoxia-induced MMP-2 activity. These results suggest that DDR2 and p38 MAPK are involved in MMP-2 activation in VSMCs in response to hypoxia. We further explored the role of DDR2 in VSMC migration in response to hypoxia. Matrigel

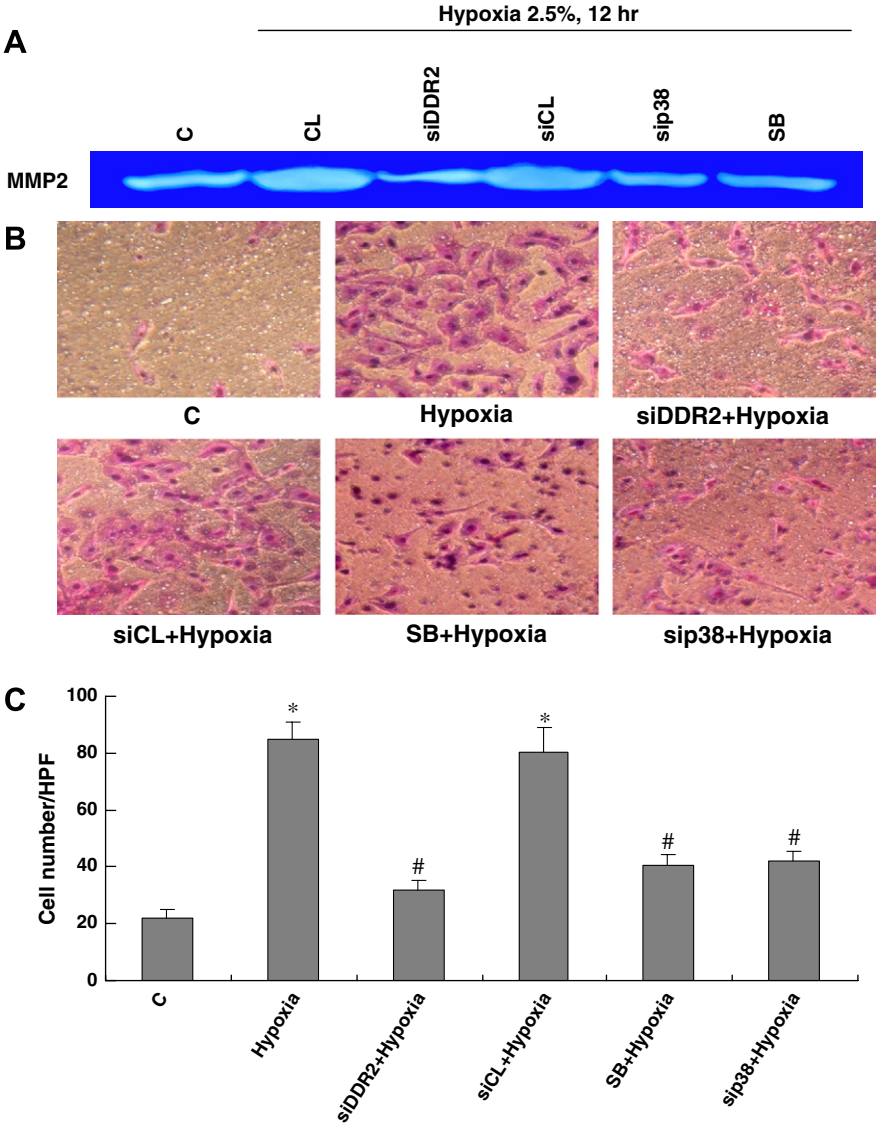

Fig. 4. Hypoxia induces MMP-2 activity in VSMCs and increases their migration through the p38/DDR2 signaling pathway. VSMCs were kept as controls (C) and exposed to hypoxia (CL) for 12 h, and their MMP-2 activity and migratory ability were determined by zymography (A) and Matrigel migration assays (B), respectively, as described in Materials and methods. In parallel experiments, the cells were either pre-treated with SB203580 (SB; 3 μM) for 30 min or transfected with control (siCL), p38- (sip38) or DDR2-specific siRNA (siDDR2; 40 nM) for 48 h. Data in (B) are shown as mean ± SEM from 3 independent experiments. The stained migrated VSMCs were counted in 4 fields under a 400× high-power field (HPF). $^{*}P < 0.05$ vs. control. $^{\#}P < 0.05$ vs. hypoxic cells without pre-treatment.

assays showed that VSMCs migrated significantly through the filter membrane under hypoxic conditions for 12 h (Fig. 4B). This hypoxia-induced increase in SMC migration was inhibited by transfecting the VSMCs with DDR2- and p38-specific siRNAs (compared to control siRNA), as well as pre-treating with SB203580. These results suggest that hypoxia increases VSMC migration, and this migration is mediated by the DDR2 and p38 MAPK signaling pathways.

Discussion

The main findings of our present study are summarized as follows. First, hypoxia induces DDR2 mRNA and protein expression in VSMCs. Second, the hypoxia-induced DDR2 expression in VSMCs is predominantly mediated by the p38 MAPK pathway. Third, hypoxia induces increased binding activity of Myc–Max–DNA, an important responsive element in the promoter region of DDR2, in the VSMC nuclei. In addition, mutation of the Myc–Max binding sequences in the promoter regions of the DDR2 gene significantly attenuated hypoxia-induced DDR2 promoter activity. These results suggest that Myc–Max transcription activity is responsible for the expression of DDR2 in response to hypoxia. Fourth, hypoxia induces MMP-2 activity in VSMCs. This hypoxia-induced MMP-2 activity is mediated by the DDR2 signaling pathway. Finally, VSMCs subjected to hypoxic conditions exhibit increased migratory ability that is mediated by the DDR2 and p38 MAPK signaling pathways. Because ECM is abundant in the atherosclerotic plaque, our present results suggest that acute hypoxia plays a pivotal role in regulating MMP-2 activity via DDR2 in VSMCs and, hence, modulates their migration, which may contribute to atherosclerosis.

DDR2 is associated with increased MMP-2 activity in hepatic stellate cells [8] and skin fibroblasts [9]. Overexpression of DDR2 in SMCs has been shown to lead to a down-regulation of collagen production and an up-regulation of MMP activity [10]. Recent studies have also demonstrated that hypoxia can up-regulate MMP-2 activity [16,17]. However, it is not clear whether hypoxia can regulate MMP-2 activity in VSMCs via a DDR2-dependent pathway. In the present study, we explored the role of DDR2 in regulating MMP-2 activity in hypoxic VSMCs. Our results showed for the first time that hypoxia increases DDR2 expression in VSMCs, with concurrent up-regulation of MMP-2 activity. Pre-treatment of VSMCs with DDR2-specific siRNA significantly inhibited the hypoxia-induced MMP-2 activity. Our results suggest that MMP-2 is a downstream target of the DDR2 signaling pathway in hypoxic VSMCs.

MAPKs comprise a family of serine/threonine kinases that function as pivotal mediators of signal transduction pathways. Three isoforms, p38, ERK, and JNK, play major roles in the regulation of cellular responses to hypoxia [14,18,19]. In the present study, pre-treatment of VSMCs with either a specific inhibitor of p38 MAPK (SB203580) or p38-specific siRNA abolished the hypoxia-induced DDR2 expression in VSMCs. In contrast, a specific inhibitor of ERK (PD98059) and JNK (SP600125) had only partially inhibitory effects on hypoxia-induced DDR2 expression. Our findings suggest that p38 MAPK plays a predominant role in the hypoxia-induced DDR2 expression in VSMCs.

The c-Myc transcription factor is a member of the "max network," which consists of a family of basic helix–loop–helix leucine zipper (B-HLH-LZ) proteins that heterodimerize with Max [20]. As a regulator of gene transcription, Myc has multiple mechanisms through which it activates and represses target genes [21]. The promoter region of the DDR2 gene contains the Myc–Max binding elements that are responsible for DDR2 expression in cells in response to various stimuli. We found that hypoxia can increase Myc–Max–DNA binding. This hypoxia-induced Myc–Max binding activity is mediated by the p38 MAPK pathway, inasmuch as treatment of VSMCs with p38-specific siRNA and SB203580 significantly inhibited hypoxia-induced Myc–Max binding activity. When VSMCs were transfected with the constructs that contain the DDR2 promoter region with mutated Myc–Max binding elements, hypoxia-induced DDR2 promoter activity was inhibited. These findings suggest that the Myc–Max binding elements in the DDR2 promoter regions are responsible for its expression in VSMCs in response to hypoxia.

Our present study has elucidated the role of DDR2 in VSMC migration under hypoxic conditions. Pre-treating VSMCs with DDR2-specific siRNA inhibited hypoxia-induced VSMC migration. In addition, the p38 MAPK specific inhibitor SB203580 and p38-specific siRNA also had inhibitory effects on hypoxia-induced VSMC migration. DDR2-specific siRNA attenuates neointimal formation after carotid injury [22]. DDR2 also plays pivotal roles in regulating VSMC-mediated collagen turnover in atherosclerosis. Our results confirm the role of DDR2 in hypoxia-induced VSMC migration.

In summary, our data have elucidated both the role of hypoxia in DDR2 expression and VSMCs and the contributions of DDR2 to VSMC migration in response to hypoxic conditions. Hypoxia-induced VSMC migration may contribute to the pathophysiological effects of hypoxia on the vasculature and, thus, may play a role in the development of atherosclerosis. Our findings suggest that DDR2 may be an important therapeutic target in the treatment of atherosclerosis.

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