



# Hyperbaric oxygen activates discoidin domain receptor 2 via tumour necrosis factor- $\alpha$ and the p38 MAPK pathway to increase vascular smooth muscle cell migration through matrix metalloproteinase 2

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## A B S T R A C T

DDR2 (discoidin domain receptor 2) regulates collagen turnover mediated by SMCs (smooth muscle cells) in atherosclerosis. HBO (hyperbaric oxygen) has been used in medical practice; however, the molecular mechanism of the beneficial effects of HBO is poorly understood. Furthermore, the effect of HBO on DDR2 has not been reported previously. In the present study, we investigated the cellular and molecular mechanisms of DDR2 regulation by HBO in VSMCs (vascular SMCs). Cells were exposed to 2.5 ATA (atmosphere absolute) of oxygen in a hyperbaric chamber. DDR2 protein (3.63-fold) and mRNA (2.34-fold) expression were significantly increased after exposure to 2.5 ATA HBO for 1 h. Addition of SB203580 and p38 MAPK (mitogen-activated protein kinase) siRNA (small interfering RNA) 30 min before HBO inhibited the induction of DDR2 protein. HBO also significantly increased DNA–protein binding activity of Myc/Max. Addition of SB203580 and an anti-TNF- $\alpha$  (tumour necrosis factor- $\alpha$ ) monoclonal antibody 30 min before HBO abolished the DNA–protein binding activity induced by HBO. HBO significantly increased the secretion of TNF- $\alpha$  from cultured VSMCs. Exogenous addition of TNF- $\alpha$  significantly increased DDR2 protein expression, whereas anti-TNF- $\alpha$  and anti-(TNF- $\alpha$  receptor) antibodies blocked the induction of DDR2 protein expression. HBO significantly increased VSMC migration and proliferation, whereas DDR2 siRNA inhibited the migration induced by HBO. HBO increased activated MMP2 (matrix metalloproteinase 2) protein expression, and DDR2 siRNA abolished the induction of activated MMP2 expression induced by HBO. In conclusion, HBO activates DDR2 expression in cultured rat VSMCs. HBO-induced DDR2 is mediated by TNF- $\alpha$  and at least in part through the p38 MAPK and Myc pathways.

**Key words:** angiogenesis, discoidin domain receptor 2 (DDR2), hyperbaric oxygen, matrix metalloproteinase (MMP), p38 mitogen-activated protein kinase (p38 MAPK), smooth muscle cell.

**Abbreviations:** Ang2, angiotensin II; ATA, atmosphere absolute; DDR, discoidin domain receptor; EMSA, electrophoretic mobility-shift assay; HBO, hyperbaric oxygen; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; RNAi, RNA interference; siRNA, small interfering RNA; SMC, smooth muscle cell; TNF- $\alpha$ , tumour necrosis factor- $\alpha$ ; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling; VEGF, vascular endothelial growth factor; VSMC, vascular SMC.

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## INTRODUCTION

HBO (hyperbaric oxygen), a safe and non-invasive modality, is increasingly being used in a number of areas of medical practice and has been used primarily in the treatment of carbon monoxide poisoning, air embolism and enhancement of wound healing [1–3]. HBO has been shown to induce VEGF (vascular endothelial growth factor) expression in HUVECs (human umbilical vein endothelial cells) [4] and to increase NO levels in perivascular tissues via the stimulation of NOS (NO synthase) [5]. The beneficial effects of HBO in treating ischaemia-related wounds may be mediated by stimulating collagen synthesis [6], cell proliferation [7] and promoting angiogenesis [8]. However, the mechanism of HBO-induced vessel formation is still not well understood.

DDR1 (discoidin domain receptor 1) and DDR2 have emerged as non-integrin-type receptors for collagen [9]. DDR1 is mainly expressed in epithelial cells, whereas DDR2 is found in mesenchymal cells [10]. DDR1 and DDR2 play potential roles in the regulation of collagen turnover mediated by VSMCs [vascular SMCs (smooth muscle cells)] in obstructive diseases of blood vessels [11]. Both DDRs are highly expressed by SMCs within the fibrous cap [11]. The activation of DDR2 by collagen results in increased production of MMP1 (matrix metalloproteinase 1) [12] and MMP2 [13]. Prolonged stimulation of DDR2 has been associated with the up-regulation of MMP1 expression [14]. These findings suggest that DDRs play an important role in regulating collagen matrix degradation and re-organization. DDR2 is regulated by mechanical stretch in VSMCs [15], and DDR2 increases VSMC migration and proliferation, indicating the potential role for mature vessel formation in the process of neovascularization. In addition, HBO has potential effects on angiogenesis [7]. However, the effect of HBO on DDR2 has not been reported previously. We hypothesized that HBO may induce DDR2 expression in VSMCs. Therefore in the present study we investigated the cellular and molecular mechanisms of DDR2 regulation by HBO in VSMCs. The induction of DDR2 in VSMCs by HBO may elucidate the mechanisms responsible for the therapeutic effect of HBO.

## MATERIALS AND METHODS

### VSMC culture

Primary cultures of VSMCs were grown using an explant technique from the thoracic aorta of 200–250 g male Sprague–Dawley rats, as described previously [15,16]. Cells were cultured in Medium 199 containing 20% (v/v) FCS (fetal calf serum), 0.1 mmol/l non-essential amino acids, 1 mmol/l sodium pyruvate, 4 mmol/l L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C under 5% CO<sub>2</sub>/95% air in a humidified incubator.

When confluent, VSMC monolayers were passaged every 6–7 days after trypsinization and were used in the experiments from passages 3–6. These cells were incubated for a further 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.

### HBO treatment

For HBO treatment, cells were exposed to 2.5 ATA (atmosphere absolute) of oxygen (98% O<sub>2</sub> plus 2% CO<sub>2</sub>) in a hyperbaric chamber for 2 h at 37°C. The oxygen tension was chosen based on human treatment protocols [17]. For the inhibition of signalling pathways, cells were pretreated with inhibitors (SP600125, SB203580 and PD98059) for 30 min, and then exposed to HBO without changing the medium. SP600125 (20 µmol/l; Calbiochem) is a potent cell-permeant selective and reversible inhibitor of JNK (c-Jun N-terminal kinase), SB203580 (3 µmol/l; Calbiochem) is a highly specific cell-permeant inhibitor of p38 MAPK (mitogen-activated protein kinase), and PD98059 (50 µmol/l; Calbiochem) is a specific and potent inhibitor of MEK [MAPK/ERK (extracellular-signal-regulated kinase) kinase].

### Immunoprecipitation and Western blot analysis

The cultured VSMCs were homogenized in a reporter lysis buffer (Promega) and centrifuged at 10 600 g for 20 min at 4°C. Protein content of the supernatant was determined using a protein assay (Bio-Rad Laboratories) with BSA as the standard. The lysate was then incubated with a polyclonal anti-DDR2 antibody for 2 h at 4°C, followed by precipitation on Protein A-agarose beads. The immunoprecipitated proteins were washed three times with lysis buffer before SDS/PAGE. For detection of the phosphorylation of DDR2, cell lysates were immunoprecipitated using the anti-DDR2 antibody, followed by Western blotting with an anti-phosphotyrosine antibody. Western blotting was performed as described previously [18]. Briefly, equal amounts of protein (15 µg) were mixed with sample buffer, boiled for 10 min, separated by SDS/PAGE under denaturing conditions and electroblotted on to nitrocellulose membranes. The blots were incubated overnight in TBS (Tris-buffered saline) containing 5% (v/v) skimmed milk to block non-specific binding of the antibodies. Proteins of interest were revealed with specific antibodies [mouse anti-phosphotyrosine (BD Bioscience), goat polyclonal anti-DDR1 and anti-DDR2 (Santa Cruz Biotechnology), polyclonal anti-p38 MAPK and monoclonal anti-(phospho-p38 MAPK)

antibodies (Cell Signaling) at 1:1000 dilutions] for 1 h at room temperature (22°C), followed by incubation with HRP (horseradish peroxidase)-conjugated polyclonal anti-(rabbit IgG) or anti-(goat IgG) antibodies (1:5000 dilution) for 1 h at room temperature. Antibody binding was then detected using ECL<sup>®</sup> (Amersham Biosciences). Equal protein loading of the samples was verified by staining with an anti-actin monoclonal antibody. All Western blots were quantified by densitometry.

### Northern blot analysis

Total RNA was isolated from VSMCs using the single-step acid guanidinium thiocyanate/phenol/chloroform extraction method. Reverse transcription was performed as described previously [19]. The cDNA produced by reverse transcription was used to generate a DDR2 cDNA probe by PCR. The following PCR primer sequences for DDR2 were chosen: forward, 5'-GGCGGAACGA-AAGTGCT-3'; and reverse, 5'-ACCGTGACAAAC-CGGG-3'. Total RNA (20 µg) was fractionated in formaldehyde/agarose gels, transferred on to a Hybond-N<sup>+</sup> nylon membrane and hybridized with [ $\alpha$ -<sup>32</sup>P]dCTP-labelled cDNA probes generated from mouse DDR2 cDNA. The membrane was pre-hybridized at 65°C for 1 h, and hybridized with radioactively labelled probes at 65°C for 3 h in Rapid-hyb buffer (Amersham Biosciences). A post-hybridization wash was performed with a final stringency of 0.2× standard saline citrate containing 0.1% SDS at 65°C. Quantitative analysis was performed with a PhosphorImager.

### RNAi (RNA interference)

Rat VSMCs were transfected with 800 ng of p38 MAPK-annealed siRNA (small interfering RNA) or DDR2 siRNA (Santa Cruz Biotechnology). The p38 MAPK and DDR2 siRNAs are target-specific 20–25 nt siRNAs designed to knockdown gene expression. DDR2 sense and antisense of siRNA sequences were 5'-GAUG-AUAGCAACACUCGGAUU-3' and 5'-PUCCGAG-UGUUGCUAUCUUCU-3' respectively. As a negative control, a non-targeting siRNA (scrambled siRNA) from Dharmacon was used. VSMCs were transfected with siRNA oligonucleotides using Effectene transfection reagent (Qiagen), according to the manufacturer's instructions. After incubation at 37°C for 24 h, cells were treated with HBO for 2 h and subjected to analysis. No off-target effect was found with DDR2 or p38 MAPK siRNA. No significant similarities to any genes other than DDR2 were found using BLAST against the rat reference sequence database. We have demonstrated previously that DDR2 siRNA does not affect the DDR1 protein expression [20]. Other proteins, such as MMP14, were also not affected by DDR2 siRNA (see Supplementary Figure S1 at <http://www.ClinSci.org/cs/116/cs1160575add.htm>). This indicates the specificity of the DDR2 siRNA to knockdown DDR2 gene expression.

### EMSA (electrophoretic mobility-shift assay)

EMSA were carried out to detect the formation of Myc/Max–DNA complexes. Nuclear protein concentrations from VSMCs were determined using a protein assay (Bio-Rad Laboratories). Consensus and control oligonucleotides (Santa Cruz Biotechnology) were labelled by polynucleotide kinase incorporation of [ $\gamma$ -<sup>32</sup>P]ATP. Oligonucleotide sequence for the Myc/Max consensus was 5'-GGAAGCAGACCACGTGGTCTGCTTCC-3'. The Myc/Max mutant oligonucleotide sequence was 5'-GGAAGCAGACCACGGAGTCTGCTTCC-3'. The EMSA was performed as described previously [19]. Controls were performed in each case with mutant oligonucleotides or unlabelled oligonucleotides to compete with labelled sequences.

### Promoter activity assay

A –490 to +66 bp rat DDR2 promoter construct was generated. Rat genomic DNA was amplified with forward primer, 5'-GACAGAAGGGAAGTGCATC-TTTAAG-3', and reverse primer, 5'-GATTCAAAGTGCCTCCGGCCGCTT-3'. The amplified product was digested with M1uI and Bg1II restriction enzymes and ligated into pGL3-basic luciferase plasmid vector (Promega) digested with the same enzymes. The DDR2 promoter contains Myc/Max conserved sites (ACGTG) at –258 to –254 bp. For the mutant, the Myc/Max-binding sites were mutated using a mutagenesis kit (Stratagene). Site-specific mutations were confirmed by DNA sequencing. Plasmids were transfected into VSMCs using a low-pressure-accelerated gene gun (Bioware Technologies), essentially according to the manufacturer's instructions. Briefly, 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 lbf/in<sup>2</sup> (1 lbf/in<sup>2</sup> ≈ 6.9 kPa). The transfection efficiency using this method was 25%. Following 0.5 h of HBO treatment, cell extracts were prepared using the dual luciferase reporter assay system (Promega) and were measured for dual luciferase activity by a luminometer (Turner Designs).

### Zymography

ECM (extracellular matrix)-degrading activity was detected by zymography. The protein was extracted from cultured VSMCs and equal amounts of sample protein were subjected to SDS/PAGE on gelatin-containing acrylamide gels [7.5% (w/v) polyacrylamide and 2 mg/ml gelatin] under non-reducing conditions. The zymogram was performed as described previously [21].

### Measurement of TNF- $\alpha$ (tumour necrosis factor- $\alpha$ ) concentration

Conditioned medium from VSMCs subjected to HBO and those from control cells were collected for TNF- $\alpha$

measurement using a quantitative sandwich ELISA (R&D Systems). The lowest limit of detection of the TNF- $\alpha$  ELISA kit was 52 pg/ml.

### Migration assay

The migration activity of VSMCs was determined using the growth-factor-reduced Matrigel invasion system (Becton Dickinson), according to the manufacturer's instructions, as described previously [21]. A total of  $5 \times 10^4$  VSMCs were seeded on top of the ECMatrix gel (Chemicon International) prepared as described previously [22]. Cells were then incubated at 37°C for 2–6 h without or with HBO. Three different phase-contrast microscopic high-power fields per well were photographed. The positively stained migrating VSMCs were counted by an observer blinded to the treatment protocol.

### Proliferation assay

The proliferation of VSMCs was determined using [ $^3$ H]thymidine incorporation. VSMCs were seeded on to ViewPlate (Packard Instrument) at a density of  $5 \times 10^3$  cells/well in serum-free medium. Thymidine uptake was studied by addition of 500 nCi/ml [ $^3$ H]thymidine (PerkinElmer) for 1–6 h without or with HBO. Cells were washed twice with PBS. Non-specific uptake was studied in the presence of 10  $\mu$ mol/l cytochalasin B and was subtracted from the measured value. MicroScint-20 (50  $\mu$ l) was added, and the plate was read with TopCount (Packard Instrument).

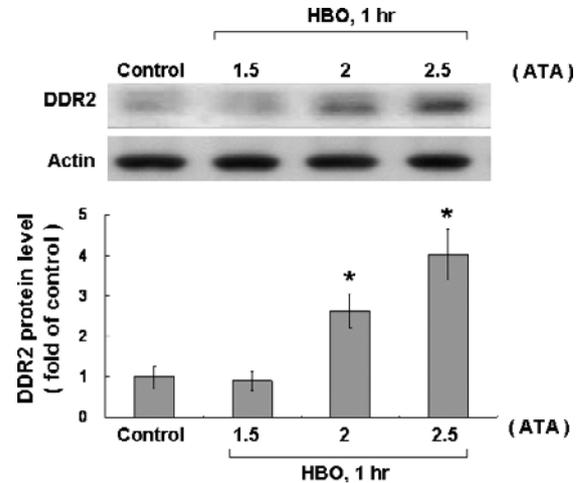
### Statistical analysis

Results expressed as means  $\pm$  S.D. Statistical significance was performed by ANOVA (GraphPad Software). A Tukey–Kramer comparison test was used for pairwise comparisons between multiple groups after the ANOVA. A value of  $P < 0.05$  was considered to denote statistical significance.

## RESULTS

### HBO transiently increases DDR2 expression

To investigate the effect of HBO on the expression of DDR2 protein, different degrees of ATA were used. As shown in Figure 1, DDR2 protein was significantly induced by HBO at 2 and 2.5 ATA for 1 h, but not at 1.5 ATA. As 2.5 ATA provided most powerful induction of DDR2 protein, subsequent experiments used 2.5 ATA as the hyperbaric stimulation. In time-course experiments, the levels of DDR2 protein determined by Western blot analysis increased significantly at 1 and 2 h after HBO treatment (Figures 2A and 2B) compared with controls without treatment. The level of DDR2 protein tended to return to baseline levels after 4 and 6 h of HBO treatment. DDR1 protein expression was



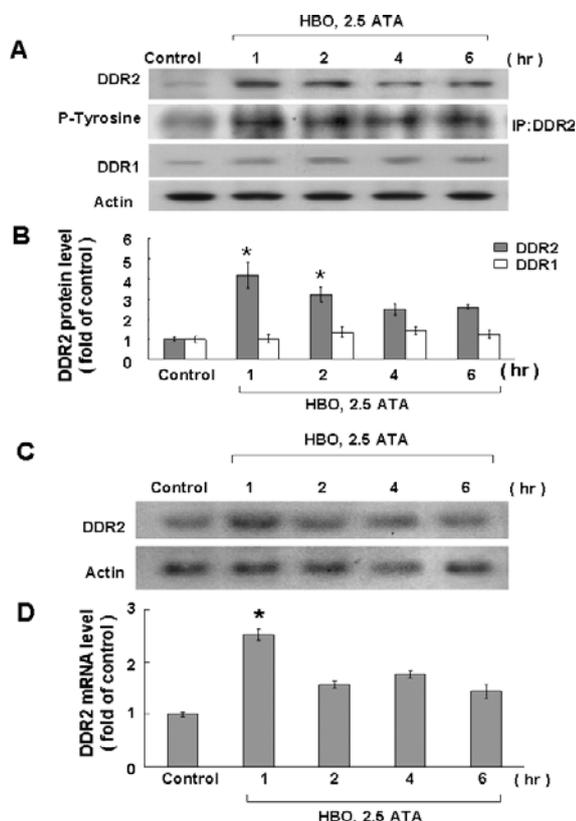
**Figure 1** Effect of HBO on DDR2 protein expression

Upper panel, a representative Western blot of DDR2 protein expression in VSMCs treated with different degrees of HBO for 1 h. Lower panel, quantification of DDR2 protein levels ( $n = 4$  per group). \* $P < 0.001$  compared with control.

not significantly altered by HBO treatment. Western blot analysis with an anti-phosphotyrosine antibody demonstrated that DDR2 was phosphorylated after HBO (Figure 2A). DDR2 mRNA increased significantly at 1 h after HBO treatment and returned to baseline levels from 2 to 6 h after HBO treatment (Figures 2C and 2D). These findings indicate that DDR2 in VSMCs is activated and functional after HBO treatment.

### HBO-induced DDR2 protein expression in VSMCs is mediated by MAPK

As shown in Figure 3, the Western blot analysis demonstrated that the HBO-induced increase in DDR2 protein was significantly reduced after the addition of PD98059, SB203580 and SP600125 30 min before HBO treatment. These findings implicated the MAPK pathways in mediating the induction of DDR2 protein expression by HBO in VSMCs. As the p38 MAPK inhibitor SB203580 decreased DDR2 protein expression most significantly, we focused on the role of the p38 MAPK pathway in DDR2 protein expression induced by HBO. DMSO, the vehicle for SB203580, had no effect on the increase in DDR2 by HBO (Figures 3A and 2B). p38 MAPK siRNA significantly attenuated DDR2 protein expression induced by HBO ( $P < 0.001$ ; Figures 3C and 3D) and also knocked-down p38 MAPK protein expression (results not shown). The scrambled siRNA did not affect DDR2 expression induced by HBO (Figures 3C and 3D). HBO at 2.5 ATA also significantly increased the phosphorylation of p38 MAPK (Figure 4), and this effect of HBO was significantly attenuated by SB203580. p38 MAPK siRNA significantly attenuated the phosphorylation of p38 MAPK induced by HBO (Figure 4).

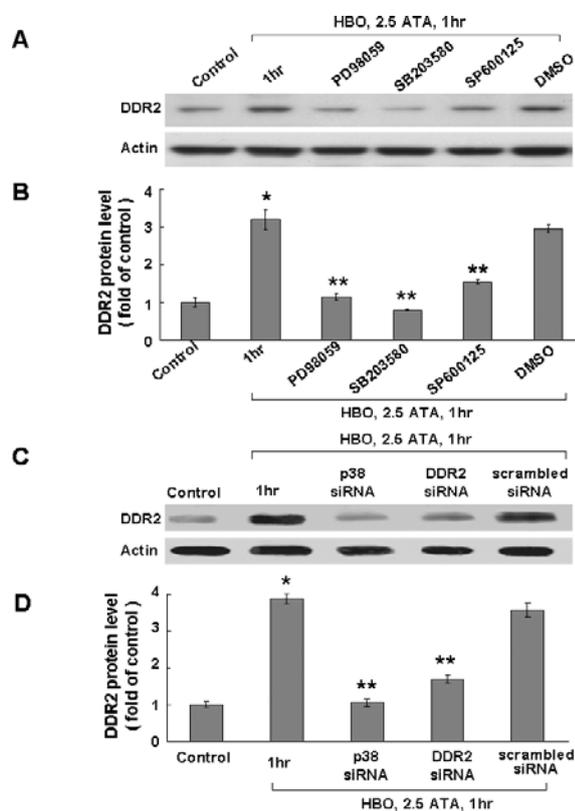


**Figure 2 HBO increases DDR2, but not DDR1, expression**

(A) Representative Western blots of DDR2 and DDR1 protein expression in VSMCs treated with HBO for various time periods. IP, immunoprecipitation; P-Tyrosine, phosphotyrosine. (B) Quantification of DDR2 and DDR1 protein levels. The values from treated VSMCs were normalized to matched actin measurements and then expressed as a ratio of normalized values to protein in the control cells ( $n = 4$  per group). Parallel aliquots of the same immunoprecipitate were probed for actin, confirming equal amounts of total protein in all samples. (C) Representative Northern blot of DDR2 in VSMCs treated with HBO for various time periods. (D) Quantification of DDR2 mRNA levels. The values from treated VSMCs were normalized to matched actin measurements and then expressed as a ratio of normalized values to mRNA in the control cells ( $n = 4$  per group). \* $P < 0.001$  compared with control.

### HBO-induced DDR2 protein expression in VSMCs is mediated by TNF- $\alpha$

As shown in Figure 5(A), TNF- $\alpha$  secretion from VSMCs increases significantly at 0.5 h after HBO stimulation at 2.5 ATA and expression remained elevated for 1.5 h before returning to baseline levels after 2 and 6 h. HBO-induced DDR2 protein expression was significantly attenuated by the addition of an anti-TNF- $\alpha$  antibody (5  $\mu\text{g}/\text{ml}$ ) or anti-(TNF- $\alpha$  receptor) antibody (5  $\mu\text{g}/\text{ml}$ ) (Figures 5B and 5C). Addition of control IgG did not abolish the induction of DDR2 protein by HBO. Exogenous addition of TNF- $\alpha$  at 20  $\text{pg}/\text{ml}$  also induced DDR2 protein expression (Figures 5B and 5C). These results indicate that TNF- $\alpha$  mediates the induction of DDR2 protein expression by HBO.

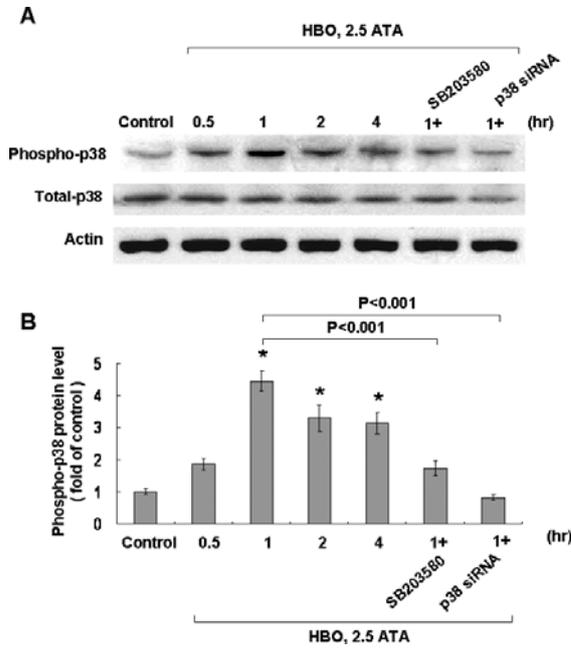


**Figure 3 HBO-induced DDR2 expression in VSMCs is via MAPK**

(A and C) Representative Western blots of DDR2 protein levels in VSMCs subjected to HBO stimulation for 1 h in the absence or presence of inhibitors (A) and siRNA (C). (B and D) Quantification of DDR2 protein levels. Values from stimulated VSMCs were normalized to those in control cells ( $n = 4$  per group). \* $P < 0.001$  compared with control; \*\* $P < 0.001$  compared with HBO alone (1 hr).

### HBO increases Myc/Max binding activity and DDR2 promoter activity

Treatment of HBO for 0.5–1.5 h significantly increased the DNA–protein binding activity of Myc/Max (Figure 6A). An excess of unlabelled Myc/Max oligonucleotide competed with the probe for binding Myc/Max protein, whereas an oligonucleotide containing a 2 bp substitution in the Myc/Max-binding site did not. Addition of SB203580 and the anti-TNF- $\alpha$  antibody 30 min before HBO stimulation abolished the DNA–protein binding activity induced by HBO. To study whether DDR2 expression induced by HBO was regulated at the transcriptional level, we cloned the promoter region of the rat DDR2 gene (–490 to +66), and constructed a luciferase reporter plasmid (pGL3-Luc). The DDR2 promoter construct contains SRE (sterol-regulatory element)-, Ets-, Myc/Max-, GATA- and MEF2 (myocyte enhancer factor 2)-binding sites. As shown in Figures 6(B) and 6(C), transient transfection experiments in VSMCs using this reporter gene revealed



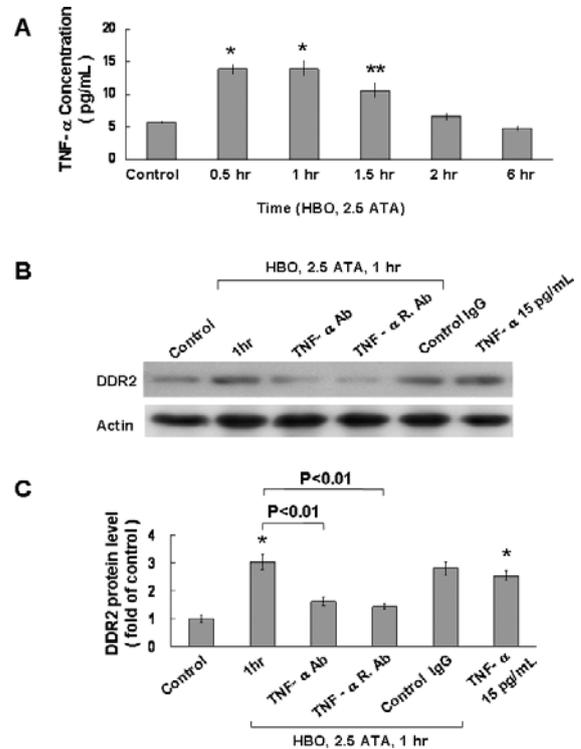
**Figure 4** HBO induces the phosphorylation of p38 MAPK in VSMCs

(A) Representative Western blot of phosphorylated and total p38 MAPK in VSMCs after treatment with HBO at 2.5 ATA for 0.5–4 h without or with SB203580 or siRNA. (B) Quantification of phosphorylated protein levels. The values from treated VSMCs were normalized to matched actin and corresponding total protein measurements and then expressed as a ratio of normalized values to each phosphorylated protein in control cells ( $n = 4$  per group). \* $P < 0.001$  compared with control.

that HBO for 0.5 h significantly induced DDR2 promoter activation, suggesting that DDR2 expression is induced at the transcriptional level by HBO. When the Myc/Max-binding sites were mutated, this increased promoter activity induced by HBO was abolished. Moreover, addition of SB203580 and the anti-TNF- $\alpha$  antibody caused an inhibition of transcription. The increased promoter activity induced by exogenous addition of TNF- $\alpha$  was similar to that induced by HBO at 2.5 ATA. These results suggest that the Myc/Max-binding site in the DDR2 promoter is essential for the transcriptional regulation by HBO and that HBO regulates the DDR2 promoter via TNF- $\alpha$  and p38 MAPK pathways.

### HBO increases MMP2 activity

As shown in Figure 7(A), zymography demonstrated that activated MMP2 significantly increased after treatment of HBO at 2.5 ATA for 4 h. DDR2 siRNA, the anti-TNF- $\alpha$  antibody and the p38 MAPK inhibitor SB203580 all significantly attenuated the increase in MMP2 activity induced by HBO. Scrambled siRNA had no effect on activated MMP2 activity.



**Figure 5** TNF- $\alpha$  mediates the induction of DDR2 by HBO

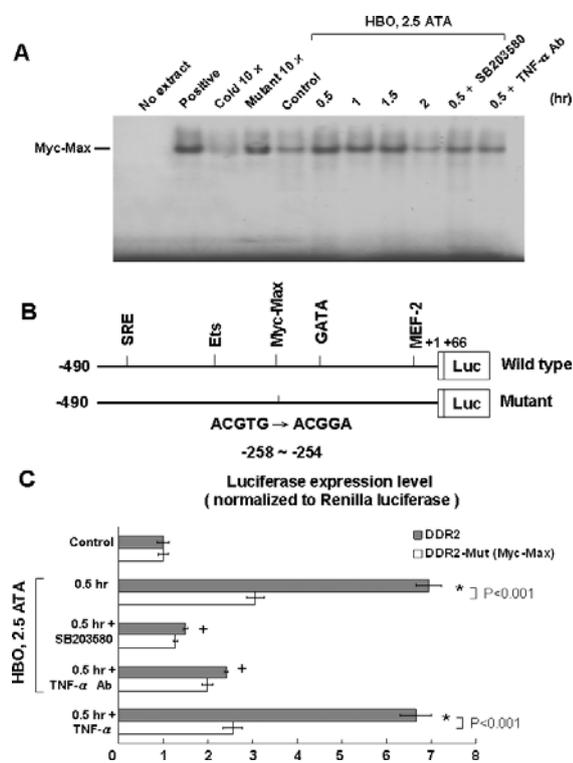
(A) HBO increased TNF- $\alpha$  secretion from VSMCs after HBO treatment. The secreted TNF- $\alpha$  was measured by ELISA. (B) Representative Western blots of DDR2 protein levels in VSMCs subjected to HBO stimulation for 1 h in the absence or presence of TNF- $\alpha$ , the anti-TNF- $\alpha$  antibody, the anti-TNF- $\alpha$  receptor (TNF- $\alpha$  R) antibody and control IgG. (C) Quantification of DDR2 protein levels. The values from stimulated VSMCs were normalized to values in control cells ( $n = 4$  per group). \* $P < 0.001$  compared with control.

### HBO increases VSMC migration and proliferation

To test the effect of HBO on the function of VSMCs, the migration activity of VSMCs was examined. As shown in Figures 7(B) and 7(C), HBO at 2.5 ATA for 2–6 h significantly increased VSMC migration activity compared with control cells under normoxia. Addition of DDR2 siRNA significantly abolished the migration activity of VSMCs induced by HBO, whereas control siRNA did not affect migration activity. HBO for 1–6 h also significantly increased the proliferation of cultured VSMCs (Figure 7D). To investigate the effect of HBO on apoptosis of cultured VSMCs, a TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) assay was performed. The TUNEL assay showed that HBO did not increase VSMC apoptosis (results not shown).

### DISCUSSION

In the present study, we have demonstrated several significant findings: (i) HBO induces transient DDR2



**Figure 6 HBO increases Myc/Max binding activity and DDR2 promoter activity**

(A) A representative EMSA showing protein binding to the Myc/Max oligonucleotide in nuclear extracts of VSMCs after HBO treatment in the absence or presence of inhibitors and the anti-TNF- $\alpha$  antibody. Similar results were found in another two independent experiments. Cold oligo, unlabelled Myc/Max oligonucleotides. (B) Constructs of the promoter of the DDR2 gene. +1 represents the initiation site for the DDR2 transcription. Mutant, mutation of the Myc/Max-binding sites in the DDR2 promoter as indicated. (C) Quantification of DDR2 promoter activity. VSMCs were transiently transfected with pDDR2-Luc using a gene gun. The luciferase activity in cell lysates was measured and was normalized with *Renilla* activity ( $n=3$  per group). \* $P < 0.001$  compared with control; + $P < 0.001$  compared with 0.5 h.

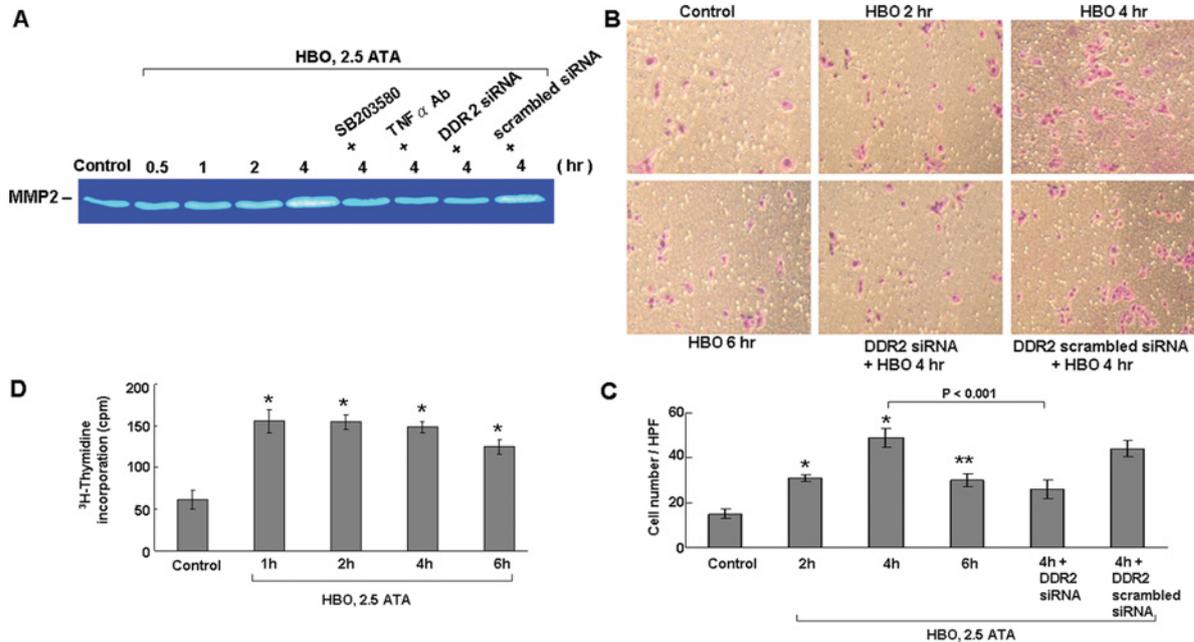
expression in cultured VSMCs in a time- and load-dependent manner; (ii) TNF- $\alpha$  acts as an autocrine factor in mediating HBO-induced DDR2 protein expression in VSMCs; (iii) the p38 MAPK pathway and Myc/Max transcription factors are involved in the signalling pathways leading to DDR2 induction by HBO; (iv) HBO increases MMP2 activity; and (v) HBO increases VSMC migration and proliferation. As VSMCs are required for mature vessel formation, the findings that HBO induces DDR2 expression in VSMCs and HBO increases VSMC migration and proliferation, but does not affect apoptosis, may strengthen further the effect of HBO on angiogenesis.

We have demonstrated previously that activation of the Myc/Max transcriptional complex significantly induces DDR2 expression in VSMCs after cyclic mechanical stretch [15]. The Myc oncoprotein is a transcription factor that can both activate and repress genes [23]. In

the present study, we have demonstrated that DDR2 expression was induced at the transcriptional level by HBO. TNF- $\alpha$ , similar to HBO, could also activate DDR2 promoter activity. TNF- $\alpha$  and p38 MAPK pathways mediated the increased transcriptional activity of Myc/Max in the HBO model because a TNF- $\alpha$  neutralizing antibody and p38 MAPK inhibitor caused an inhibition of Myc/Max transcription. TNF- $\alpha$ , a potent pro-inflammatory cytokine, has been shown to modulate a wide spectrum of responses, including the activation of many genes [24]. Furthermore, our present results indicate that the Myc/Max-binding site in the DDR2 promoter is essential for the transcriptional regulation by HBO because a mutant Myc/Max-binding site in the DDR2 promoter abolished the transcriptional activity induced by HBO.

Molecular oxygen plays a central role in the reparative process and is one of the critical nutrients in wound healing [25]. Collagen synthesis, matrix deposition, angiogenesis and epithelialization all require molecular oxygen during the reparative process. Degradation of the basement membrane by MMP2 and MMP9 may be critical for wound remodelling and angiogenesis. MMPs are involved in normal physiological processes, such as embryogenesis and tissue remodelling, and may play an important role in tumour metastasis and angiogenesis [26,27]. MMP2 promotes matrix protein degradation in vascular disease remodelling [28] and facilitates VSMC migration [29]. MMP2 production by endothelial cells or surrounding cells may be vital to the formation of new functional blood vessels. Olaso et al. [30] have demonstrated that DDR2 mediated fibroblast migration and proliferation, and established a role for DDR2 in critical events during wound repair. In the present study, we have demonstrated that HBO induced DDR2 expression in VSMCs. Taken together, the induction of DDR2 by HBO in the *in vivo* condition may augment wound repair further because fibroblasts and SMCs co-activated MMP2. HBO has been shown to increase VEGF and Ang2 (angiopoietin 2) expression in endothelial cells [31,32]. VEGF and Ang2 are also critical for angiogenesis. Angiogenic protein induction, fibroblast proliferation and SMC migration induced by HBO may have a synergistic effect on wound healing. DDR2 siRNA inhibited activated MMP2 expression induced by HBO, and DDR2 siRNA also inhibited the migration of VSMCs induced by HBO. These findings may indicate that HBO-induced SMC migration is mediated by DDR2 through activation of MMP2.

RNA has become the tool of choice for gene function studies once the gene-silencing effect of RNAi in mammalian cells has been confirmed [33]; however, off-target effects of siRNA are a major biological restriction [33]. In the present study, we have used BLAST, a set of similarity search programs designed to find regions of local similarity between sequences, against the Rat RefSeq database and found no significant similarities to any genes other



**Figure 7** HBO increases MMP2 activity, migration activity and proliferation of VSMCs

(A) A representative zymogram of activated MMP2 in VSMCs treated with HBO for various time periods in the absence or presence of siRNA and inhibitors. Similar results were observed in another two independent experiments. (B) VSMCs migrating through the filter were stained. (C) Migration of VSMCs was quantified by staining and counting the number of cells that migrated to the bottom of the filter in five fields under a  $\times 400$  high-power field (HPF) ( $n = 4$  per group). (D) Quantification of VSMC proliferation determined using a [ $^3$ H]thymidine incorporation assay. \* $P < 0.001$  and \*\* $P < 0.01$  compared with control.

than DDR2. In addition, the scrambled DDR2 siRNA did not have any effect on DDR2 expression. Furthermore, DDR2 siRNA did not affect DDR1 protein expression or major migratory proteins such as MMP14. This indicates the specificity of the DDR2 siRNA to specifically knock-down DDR2 gene expression. Therefore the off-target effects of our designed DDR2 siRNA are unlikely.

In summary, our present study reports for the first time that HBO enhances DDR2 expression in cultured rat VSMCs. HBO-induced DDR2 is mediated by TNF- $\alpha$  and at least, in part, through the p38 MAPK and Myc pathways. HBO-induced VSMC migration is mediated by DDR2 through activation of MMP2. The induction of DDR2 in VSMCs by HBO may elucidate the potential mechanism responsible for the clinical use of HBO.

## FUNDING

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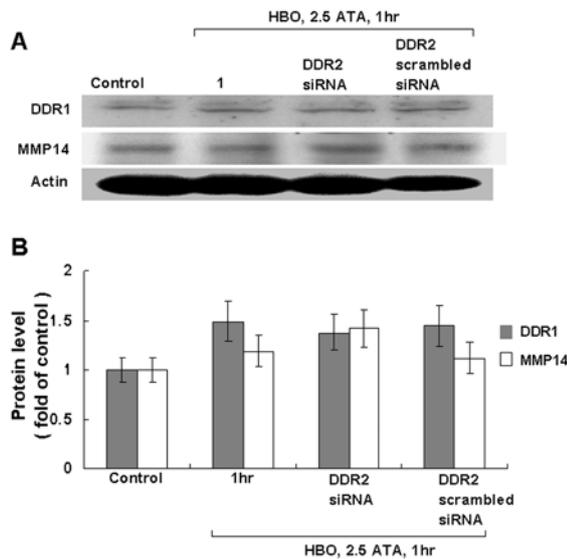
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# Hyperbaric oxygen activates discoidin domain receptor 2 via tumour necrosis factor- $\alpha$ and the p38 MAPK pathway to increase vascular smooth muscle cell migration through matrix metalloproteinase 2

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**Figure S1** DDR2 siRNA has no off-target effects

(A) Representative Western blot of DDR1 and MMP14 in VSMCs treated with DDR2 siRNA and scrambled DDR2 siRNA for 1 h. (B) Quantification of DDR1 and MMP14 protein levels. No significant statistical difference was observed between DDR2 siRNA- and scrambled DDR2 siRNA-treated groups ( $n = 3$ ).

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