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Indazole-Cl inhibits hypoxia-induced cyclooxygenase-2 expression in vascular smooth muscle cells

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Abstract

Atherosclerosis is the most common root cause of arterial disease, such as coronary artery disease and carotid artery disease. Hypoxia is associated with the formation of macrophages and increased inflammation and is known to be present in lesions of atherosclerotic. Vascular smooth muscle cells (VSMCs) are one of the major components of blood vessels, and hypoxic conditions affect VSMC inflammation, proliferation and migration, which contribute to vascular stenosis and play a major role in the atherosclerotic process. Estrogen receptor (ER)- β is thought to play an important role in preventing the inflammatory response in VSMCs. In this report, we studied the anti-inflammatory effect of indazole (In-Cl), an ER β -specific agonist, under conditions of hypoxia. Expression of cyclooxygenase-2 reduced by hypoxia was inhibited by In-Cl treatment in VSMCs, and this effect was antagonized by an anti-estrogen compound. Additionally, the production of reactive oxygen species induced under conditions of hypoxia was reduced by treatment with In-Cl. Increased cell migration and invasion by hypoxia were also dramatically decreased following treatment with In-Cl. The increase in cell proliferation following treatment with platelet-derived growth factor was attenuated by In-Cl in VSMCs. RNA sequencing analysis was performed to identify changes in inflammation-related genes following In-Cl treatment in the hypoxic state. Our results suggest that ER β is a potential therapeutic target for the suppression of hypoxia-induced inflammation in VSMCs.

Key Words

- hypoxia
- ER beta
- COX-2
- VSMC

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Introduction

Oxygen homeostasis in blood vessels has been linked to atherosclerosis (Sluimer *et al.* 2008). Hypoxia is a major regulator of physiological processes and diseases of chronic inflammation, including angiogenesis, glycolysis and erythropoiesis (Guillemin & Krasnow 1997). In the cardiovascular system, for example, the blood diffusion

capacity decreases, and the arterial walls thicken in atherosclerotic lesions under conditions of hypoxia, and hypoxia plays an important role in the progression of atherosclerosis (Nakano *et al.* 2005, Sluimer *et al.* 2008). VSMCs are one of the main components of vessels. The proliferation and migration of VSMCs contribute to

vascular stenosis and play a major role in atherosclerosis, and hypoxia has been reported to induce VSMCs proliferation and migration (Osada-Oka *et al.* 2008, Zhang *et al.* 2009). The mechanism by which hypoxic conditions regulate VSMC growth includes direct cell cycle-specific effects and indirect effects through the regulation of VSMC mitogen production (Kourembanas *et al.* 1998).

Hypoxia upregulates the expression of macrophage migration inhibitory factor in VSMCs through the hypoxia-inducible factor (HIF)-1- α -dependent pathway (Fu *et al.* 2010). Hypoxia and cellular injury induce cyclooxygenase (COX)-2 in many cell types (Chida & Voelkel 1996, Schmedtje *et al.* 1997). COX-2 is a significant mediator of the vascular response to injury; COX-2 is increased in response to lipopolysaccharides and ischemia-reperfusion (Davidge 2001). Upregulation of COX-2 is important in vascular smooth muscle hyperreactivity (Guo *et al.* 2005). Moreover, the promotion of COX-2 expression, along with increased levels of prostanoids, mainly prostaglandin E₂ (PGE₂), are hallmarks of inflammation in many tissues (Dubois *et al.* 1998, Vane *et al.* 1998). In hypoxia, both nuclear factor kappa B (NF- κ B) and HIF-1 are important in transducing inflammatory signals, and these transcription factors appear to regulate COX-2 expression and PGE₂ secretion both directly and indirectly (Lukiw *et al.* 2003). These processes result in smooth muscle cell hypertrophy (Orton *et al.* 1992), hyperplasia and migration. Reducing COX-2 expression in VSMCs under conditions of hypoxia is expected to help prevent or mitigate vascular injury.

Estrogen has been widely used as an atheroprotective agent. According to some reports, premenopausal women have a significantly lower risk of cardiovascular disease than postmenopausal women of the same age (Kannel & Wilson 1995, Mendelsohn & Karas 1999, Reckelhoff 2001), and women with ovarian dysfunction have a higher risk of cardiovascular disease (Punnonen *et al.* 1997). Additionally, menopausal women undergoing estrogen replacement have been reported to have a reduced risk of coronary heart disease (Stampfer *et al.* 1991, Grady *et al.* 1992, Grodstein *et al.* 1996). The biological function of estrogen is mediated primarily by nuclear estrogen receptors (ERs), which function as ligand-activated transcription factors in the regulation of gene expression. Two ER isoforms exist: ER α and ER β (Mangelsdorf *et al.* 1995, Kuiper *et al.* 1996). Whereas the physiological effects of ER α have been studied extensively, the effects of ER β are less well defined (Harris 2007). Estrogen exhibits protective effects via the reduction of inflammatory mediators, such as cytokines, and has been shown to suppress inflammation in experimental models

of carrageenan-induced pleurisy in the lungs (Cuzzocrea *et al.* 2000, 2001) as well as adjuvant-induced arthritis (Badger *et al.* 1999). However, estrogen also stimulates edema (Tchernitchin & Galand 1983), increases prostatitis (Naslund *et al.* 1988), promotes an influx of macrophages and increases vascular permeability in the uterus (De & Wood 1990, Kaushic *et al.* 1998). On the other hand, ER β ligands are more effective in attenuating the inflammatory response in neural cells and may therefore be promising therapeutic candidates for neural anti-inflammatory therapies (Lewis *et al.* 2008).

Previous studies have shown that estrogen suppressed NF- κ B-dependent inflammation by cytokines in incubated cerebral endothelial cells (Galea *et al.* 2002). However, to the best of our knowledge, no study has shown the direct effects of COX-2 regulation by ER β -specific ligands under conditions of hypoxia. We hypothesized that, in the hypoxic state, ER β -specific ligands reduce the expression of inflammatory mediators in VSMCs and aimed to investigate the vasoprotective effects of the ER β -specific agonist indazole (In)-CI through the regulation of inflammatory responses under hypoxia using COX-2 as an indicator of vascular inflammation.

Materials and methods

Materials

Celecoxib, 17 β -estradiol (E2) and In-CI were purchased from Sigma. Diarylproprionitrile (DPN) and ICI 182, 780 (ICI) were purchased from Tocris. Platelet-derived growth factor (PDGF)-BB (S20-BB/CF) was purchased from R&D System.

Cell culture and hypoxic conditions

The thoracic aortas from 3-month-old Sprague-Dawley rats (160–180g) were removed, and VSMCs were enzymatically isolated as previously described (Yoon *et al.* 2012). This study protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Sungkyunkwan University School of Medicine (Permission No. H-A9-003). VSMCs were cultured with phenol red-free Dulbecco's modified Eagle medium: Nutrient Mixture F-12 (DMEM/F12) (WelGENE, Daegu, South Korea) containing 10% fetal bovine serum (FBS) (WelGENE, Daegu, South Korea) and 1% penicillin/streptomycin (GIBCO Invitrogen). Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂. All treatments were done in media containing 5% charcoal-dextran stripped (CD)-FBS. For the hypoxic state, cells were placed in a

forma anaerobic system (Thermo Fisher Scientific) at 37°C containing 5% CO₂/1% O₂/94% N₂ atmosphere.

Transfection and luciferase assays

For reporter assays, VSMCs were transiently transfected with 3x(NF-κB)tk-luciferase and COX-2-luciferase reporter plasmids (Lim *et al.* 2014) using the polyethylenimine method (Polysciences, Warrington, PA, USA). Approximately 16h after transfection, the cell medium was changed and treated with In-Cl 10μM for 24h. Luciferase activity was measured with an AutoLumat LB9507 luminometer (EG & G Berthold, Bad Wildbad, Germany) using the luciferase assay system (Promega). The activities were expressed as relative light units.

Quantitative real-time PCR

Total RNA was extracted and the first-strand cDNA was synthesized according to a previously reported method (Park & Lee 2014). Quantitative real-time PCR was performed using the Power SYBR® Green PCR Master Mix (Applied Biosystems). The primers used were GAPDH sense primer, 5'-AGTTCAACGGCACAGTCAAG-3'; GAPDH anti-sense primer, 5'-TACTCAGCACCAGCATCACC-3'; COX-2 sense primer, 5'-GCAGGAAGTCTTTGGTCTGG-3'; COX-2 anti-sense primer, 5'-CTCGTCATCCCACTCAGGAT-3'. Real-time PCR was conducted using a StepOnePlus Real-Time PCR System (Life Technologies). The amplification data were analyzed using the 2.3 version of StepOne optical system software and calculated by the $\Delta\Delta C_T$ method.

Western blot analysis

Protein isolation and western blot analysis were performed according to previously reported methods (Park & Lee 2014). Anti-COX-2 (sc-1745, Santa Cruz Biotechnology) was diluted to 1:1000, and blots were incubated overnight. Anti-β-actin (A5441, Sigma) was diluted to 1:5000, and blots were incubated. Then, blots were incubated with the secondary antibody diluted 1: 5000. Immunoreactive bands were detected using enhanced chemiluminescence blotting detection reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Reactive oxygen species (ROS) measurement by flow cytometry

VSMCs were pretreated with In-Cl 0.1μM for 6h and cultured with or without PDGF-BB (20ng/mL).

Measurement of intracellular ROS levels were according to previously reported methods (Song *et al.* 2017). The data were analyzed by FlowJo v.10 (FlowJo LLC, Ashland, OR, USA).

Cell invasion and migration assays

Cells were treated with In-Cl 0.1μM and/or 1μM ICI. After 24h or 48h of incubation under normoxic or hypoxic conditions, migration and invasion assays were performed according to previously reported methods (Park & Lee 2014).

Cell proliferation assay

VSMC proliferation was determined with a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma) assay. In brief, cells were plated in 96-well culture media and incubated for 48h. Later, cells were pretreated with or without In-Cl and treated with PDGF-BB for 24, 48 and 72 h. At the indicated time points, cells were incubated with MTT (0.5 mg/mL) at 37°C. Then the medium was discarded and DMSO was added for 15 min. The optical density was measured for each well at 590 nm, with a reference wavelength of 620 nm using the Microplate Autoreader (Bio-Tek Instruments Inc.).

mRNA library preparation and sequencing

RNA purity and mRNA sequencing libraries were determined according to previously reported methods (Kim *et al.* 2018). And then the flow cell loaded on HISEQ 2500 sequencing system (Illumina), performed sequencing with 2 × 100 bp read length.

Transcriptome data analysis

QuantSeq 3' mRNA-Seq reads were aligned using Bowtie2 (Langmead & Salzberg 2012). Differentially expressed genes were determined based on counts from unique and multiple alignments using coverage in Bedtools (Quinlan & Hall 2010). The RT (Read Count) data were processed based on quantile normalization method using EdgeR within R using Bioconductor. Gene classification was based on searches performed by Medline databases (<http://www.ncbi.nlm.nih.gov/>) and DAVID (<http://david.abcc.ncifcrf.gov/>). High-throughput sequencing data have been deposited in the Gene Expression Omnibus (GEO) database under accession number GSE113940.

The functional annotation of differentially transcribed genes was analyzed using the DAVID web-server v.6.8.

Gene Ontology (GO) terms for biological processes included in the DAVID knowledgebase were considered. We used a P value <0.05 as a cut-off. GO terms were submitted to REVIGO, a web server that takes long lists of GO terms and summarizes them in categories and clusters of differentially expressed genes by removing redundant entries.

Statistical analysis

Data shown in this study are expressed as means $\pm$ s.d., and statistical analysis for single comparison was performed using the Student's t test. The criterion for statistical significance was $P < 0.05$.

Results

Inhibition of hypoxia-induced COX-2 expression by ER β agonist

In-Cl is an ER β agonist with a phenyl-2H-indazole core that was synthesized by the Katzenellenbogen group from a series of nonsteroidal compounds (De Angelis *et al.* 2005). COX-2 is induced by hypoxic conditions and has been implicated in angiogenesis, inflammation and cell cycle progression (Greenhough *et al.* 2009). ER β has been shown to exhibit anti-inflammatory effects in inflammatory diseases (Harris *et al.* 2003, Cristofaro *et al.* 2006, Follettie *et al.* 2006). Recent studies have shown that In-Cl suppresses inflammation in a model of endometriosis (Madak-Erdogan *et al.* 2016). Therefore, we determined the effects of In-Cl on regulation of COX-2 under conditions of hypoxia in VSMCs. To assess the role of ER β in the hypoxic induction of COX-2 in VSMCs, we pre-incubated cells with In-Cl and/or the ER antagonist ICI for 1 h and subjected the cells to hypoxia.

We confirmed the inhibitory effect of 100 nM In-Cl on the induction of COX-2 in hypoxia, which was blocked following treatment with 1 μ M ICI (Fig. 1A).

To further elucidate the molecular mechanism underpinning the inhibition of hypoxia-induced COX-2 by In-Cl, VSMCs treated with In-Cl were exposed to hypoxic conditions. The hypoxia-dependent increase in COX-2 mRNA expression was significantly reduced by In-Cl (Fig. 1B). To determine whether transcription of the COX-2 gene was regulated by In-Cl, the effect of In-Cl on COX-2 promoter activity was examined using a COX-2 reporter gene. Under hypoxic conditions, COX-2 promoter activity was effectively suppressed by In-Cl, suggesting that In-Cl inhibits hypoxia-induced COX-2 expression (Fig. 1C). The production of proinflammatory cytokines and proteins is regulated primarily by NF- κ B (Li & Verma 2002), and the promoter region of the COX-2 gene includes a binding site for NF- κ B (Crofford *et al.* 1997). Next, the effect of In-Cl on NF- κ B-mediated gene transcription was investigated using an NF- κ B reporter gene. The results indicated that transcriptional activity of NF- κ B induced by hypoxia was reduced following treatment with In-Cl (Fig. 1D).

Attenuation of ROS by In-Cl under conditions of hypoxia

Increased levels of ROS are important in the development of vascular pathologies and atherosclerosis, and hypoxia increases the accumulation of ROS via inflammation (Singh & Jialal 2006, Tafani *et al.* 2016). We assessed whether In-Cl decreased ROS accumulation under hypoxia in VSMCs. VSMCs were pretreated with In-Cl for 6 h and then incubated under conditions of hypoxia for 24 h to generate ROS. ROS levels were attenuated by In-Cl

Figure 1

Indazole (In-Cl) inhibits hypoxia-induced cyclooxygenase (COX)-2 transcriptional activity in vascular smooth muscle cells (VSMCs). (A) VSMCs were pretreated with In-Cl (0.1 μ M) and/or ICI (1 μ M) for 1 h prior to treatment under hypoxic conditions for 24 h and analysis by western blotting. (B) VSMCs were pretreated with In-Cl (0.1 μ M) for 1 h before treatment under hypoxic conditions for 16 h and analysis by real-time PCR. (C) VSMCs were transfected with a COX-2-Luc reporter and treated with In-Cl (10 μ M) for 24 h. (D) VSMCs were transfected with an NF- κ B-Luc reporter. Cells were treated with In-Cl (10 μ M) and incubated for 24 h. All experiments were repeated at least three times.

(Fig. 2), indicating that In-Cl can reduce the levels of ROS in cultured VSMCs.

Prevention of hypoxia-induced cellular migration and invasion by In-Cl

Vascular lesions are formed by migration and invasion of VSMCs and contribute to the progression of hyperplasia diseases (Park *et al.* 2015). To demonstrate the biological relevance of the decreased COX-2 expression caused by In-Cl in VSMCs, we confirmed the effect of In-Cl on VSMC migration and invasion under conditions of hypoxia. The effect of In-Cl on hypoxia-induced VSMC migration was determined by transwell assay. Hypoxic stimulation for 24 h induced VSMC migration potential compared with the control group, and the stimulatory effect of hypoxia

on VSMC migration was effectively inhibited by In-Cl. However, the effect of In-Cl was not blocked by the ER antagonist ICI (Fig. 3A), likely because migration was not a direct cause of ER activation.

Celecoxib, a selective inhibitor of COX-2, reduces gastrointestinal bleeding compared with typical nonsteroidal anti-inflammatory agents (NSAIDs; e.g., acetylsalicylic acid and indomethacin) (Goldenberg 1999). Figure 3B shows representative images of VSMCs treated with 5 μ M celecoxib. The migratory potential of VSMCs in the hypoxic state was significantly inhibited by treatment with celecoxib and In-Cl, suggesting that the decrease in cell migration is related to the suppression in COX-2 expression. The effect of In-Cl on the invasion potential of VSMCs induced by hypoxia was investigated using a Matrigel invasion assay. VSMC invasiveness was induced under hypoxia compared with normoxia (Fig. 3C). In the hypoxic state, VSMC invasiveness was significantly reduced by In-Cl, indicating that In-Cl induces anti-invasion and anti-migratory properties in VSMCs under hypoxic conditions.

Effects of In-Cl on the proliferation of PDGF-BB-stimulated VSMCs

VSMC growth and proliferation are key events in inflammation and atherogenesis, which can cause thickening of the arteries. VSMC proliferation was induced using PDGF-BB, a growth factor for VSMCs that is produced under conditions of hypoxia (Lu *et al.* 2015). The effect of In-Cl on VSMC proliferation was confirmed by MTT assay (Fig. 4A). Compared with that in the control group, proliferation was increased by 1.7-fold in the PDGF-BB (20 ng/mL) 48-h-treated group and by 1.4-fold in the 72-h-treated group. Increased VSMC proliferation following treatment with PDGF-BB tended to be reduced by treatment with In-Cl, suggesting that In-Cl attenuates VSMC proliferation.

To further confirm the regulation of VSMC proliferation by In-Cl, we constructed a heat map consisting of genes related to cell proliferation from RNA sequencing data (Fig. 4B). Downregulated and upregulated genes are shown in blue series and red series, respectively. Overall, proliferation-related genes downregulated in the hypoxic state were upregulated when treated with ER β agonists, whereas proliferation-related genes upregulated in the hypoxic state were downregulated when treated with ER β agonists. PDGF is a factor that alters the phenotype of VSMCs (Owens *et al.* 2004, Zimna & Kurpisz 2015); the phenotype conversion of VSMCs plays a major role

Figure 2

In-Cl decreases reactive oxygen species (ROS) levels under conditions of hypoxia. VSMCs were incubated under conditions of hypoxia following pre-treatment with In-Cl (0.1 μ M) for 3 h. ROS levels were measured by flow cytometry. VSMCs were treated with 1 μ M DCF-DA for 15 min. All experiments were repeated at least three times.

Figure 3

In-Cl inhibits hypoxia-induced cellular migration and invasion of VSMCs. (A) VSMCs were treated with 1 μ M In-Cl and/or 1 μ M ICI. Transwell migration assays were conducted under conditions of hypoxia or normoxia. (B) VSMCs were treated with 5 μ M celecoxib and/or 0.1 μ M In-Cl and incubated under hypoxia for 24 h. Cell migration was determined using a transwell migration assay. (C) VSMCs were untreated or treated with In-Cl (0.1 μ M), incubated for 48 h under hypoxic conditions, and invasion was assessed using Matrigel-coated transwell chambers. Translocated cells are visible on the lower surface of the filter. Migrating and invading cells were counted and are shown in the graph. All experiments were repeated at least two times.

in atherosclerosis. In particular, the PDGFB gene was increased by 1.9-fold in the hypoxic state and decreased by 0.78-fold in the hypoxic state following treatment with In-Cl. These results demonstrate that In-Cl treatment of VSMCs alters the expression of proliferation-related genes in hypoxic states, confirming our results that increased cell proliferation by PDGF-BB is reduced by In-Cl.

Differentially expressed genes (DEGs) under conditions of hypoxia

Because our results suggested that COX-2 is reduced by ER β agonists in VSMCs, we performed RNA sequencing analysis to identify the transcripts that were differentially regulated in VSMCs following treatment with the ER β agonists In-Cl and DPN, as well as the ER agonist E2. The Venn diagram using the standard two-fold change in expression as the threshold criterion shows that 578, 158 and 198 genes were differentially expressed in the hypoxic,

DPN-treated and In-Cl-treated states, respectively (Fig. 5A). We confirmed that 50 and 53 genes following DPN and In-Cl treatment, respectively, exhibited different levels of expression under the hypoxic condition compared with normoxia. On the other hand, we identified 18 upregulated and 10 downregulated genes with the same regulatory pattern in VSMCs treated with DPN or In-Cl under hypoxic conditions. GO was analyzed by selecting genes with a more than 1.5-fold difference in the hypoxic and hypoxic groups treated with reagents (In-Cl or E2). DEGs based on GO and grouped by REVIGO could be characterized in clusters according to biological processes (Fig. 5B). With the In-Cl treatment in hypoxia, the clusters included peptidyl-tyrosine dephosphorylation, immune system process, cytokine-mediated signaling pathway, cell-matrix adhesion, negative regulation of cell proliferation and positive regulation of interleukin-6 production. With the E2 treatment in hypoxia, the clusters included peptidyl-tyrosine dephosphorylation,

Figure 4
In-CI regulates proliferation of VSMCs. (A) VSMCs were plated on a 96-well plate. Cells were pretreated with In-CI (0.1 μ M) for 6 h and cultured with or without platelet-derived growth factor (PDGF)-BB (20 ng/mL). Cell proliferation was measured using the MTT assay. All experiments were repeated at least three times. (B) Heat map analysis of known cell proliferation-related genes regulated by ER β agonists (In-CI and DPN) in VSMCs. The blue bands indicate reduced gene expression; the red bands indicate increased gene expression.

metabolism, immune response, negative regulation of JAK-STAT cascade, cytokine-mediated signaling pathway and positive regulation of circadian rhythm. The expression patterns of 87 DEGs related to inflammation under hypoxia compared with hypoxia+ER agonist treatment are presented in Fig. 5C. RNA sequencing data confirmed our data showing that expression of prostaglandin H synthase 2 (also known as COX-2) was increased under hypoxia; this effect was counteracted by DPN and In-CI but strengthened by E2. Interleukin (IL)-17b, which has been reported to stimulate the release of tumor necrosis factor α and IL-1B, was present at higher levels in the hypoxic state; this effect was reversed by treatment with DPN, In-CI and E2. The expression patterns of the ER β -specific agonists DPN and In-CI were more similar to one another than to those of the ER agonist E2. These results showed that the ER β -specific agonists affect the expression of genes related to inflammation under hypoxic conditions.

Discussion

The results presented in this study show how the activation of ER β by In-CI contributes to the reduction of inflammatory response in VSMCs. COX-2 expression in VSMCs was increased in the hypoxic state, which increased proliferation, inflammation and migration. COX-2 contributes to vascular diseases, such as atherosclerosis. Recent studies have suggested that, in both brain and coronary VSMCs, dihydrotestosterone decreases cytokine- or hypoxia-induced COX-2 protein expression via an ER β -dependent mechanism (Osterlund *et al.* 2010, Zuloaga & Gonzales 2011). Using COX-2 protein expression as a marker of vascular inflammation, we examined the effects of In-CI on COX-2 expression in VSMCs. Our results showed that In-CI decreased hypoxia-induced COX-2 expression in VSMCs and that this was mediated by ER β .

Hypoxia stimulates excessive growth of VSMCs, which contributes to vascular remodeling (Tan *et al.* 2017). Vascular remodeling is a common feature of cardiovascular diseases, such as atherosclerosis and pulmonary arterial hypertension (Jeffery & Wanstall 2001, Stenmark *et al.* 2006, Hanze *et al.* 2007). Long-term hypoxia enhances the induction of COX-2 in human pulmonary artery cells (Bradbury *et al.* 2002). COX-2 is associated with proliferation, inflammation and tumor growth. These studies have indicated the importance of decreasing COX-2 expression to prevent

A

B

C

Figure 5

Hierarchical clustering and Venn diagram of differentially expressed genes (DEGs) following treatment with ER ligands under hypoxic conditions. (A) Venn diagram showing the overlap between DEGs following treatment with ER β ligand under hypoxia. (B) Enriched GO terms among the transcripts significantly regulated by In-CI or E2 in hypoxia. REVIGO uses multi-dimensional scaling of the dimensionality of a matrix of GO terms with pairwise semantic similarities. This results in semantically similar GO terms remaining close together in the plot. (C) Hierarchical clustering of DEGs using RNA sequencing data derived from VSMCs treated with ER ligands (In-CI, DPN and E2) in a hypoxic state.

hypoxia-induced cell migration and invasion. Celecoxib is among the new generation of currently approved NSAIDs that characteristically inhibit COX-2 activity. Recent studies have suggested that celecoxib downregulates HIF-1 α , PI3K and p-Akt expression in a dose-dependent manner (Sui *et al.* 2014). Our data demonstrate that hypoxia-induced cell migration is reduced by the COX-2 inhibitor celecoxib and the ER β agonist In-CI. These results suggest that In-CI has significant effects on the migration and invasion induced by COX-2 under conditions of hypoxia. It should be noted that ICI, which is an ER antagonist and breast cancer drug, did not prevent the inhibition of cell migration by In-CI, supporting the validity of ICI

as an anticancer agent. Lau and To (2016) showed that ICI interacts with ER β , and this complex translocates into the nucleus to activate the hsa-miR765 promoter via an ER β recognition site. Upon stimulation, the increase in hsa-miR765 expression suppresses HMGA1 protein expression, induces G2 cell cycle arrest and interferes with cell migration and invasion.

ROS are upregulated by hypoxia; their levels are increased in human atherosclerotic lesions, and their induction increases atherosclerosis and vascular disease. Previous studies have suggested that IL-19, an anti-inflammatory cytokine, stimulates heme oxygenase-1 and reduces ROS levels in VSMCs (Gabunia *et al.* 2012). Our

study showed that In-CI blocked the increased oxidative burden due to hypoxia. In-CI attenuated the promotion of cell migration following COX-2-induced ROS production under conditions of hypoxia.

COX-2 is increased by inflammatory stimuli and other factors important for cardiovascular diseases, such as endothelin (ET)-1 or angiotensin (Ang)-II (Ohnaka *et al.* 2000, Álvarez *et al.* 2007, Montezano *et al.* 2007, Martín *et al.* 2012) and is increased in pathological conditions (Félétou *et al.* 2011, Hernanz *et al.* 2014, Ozen & Norel 2017). ET-1 and Ang-II have been shown to cause vascular stiffness (Aroor *et al.* 2013, Muniyappa & Sowers 2013). Increased cytokine secretion and COX-2 expression and activity led to greater vascular contractile responses in the aorta (García-Redondo *et al.* 2018). The contractile state of VSMCs determines resistance vessel diameter, which is critical in the regulation of blood pressure and tissue blood flow. One of the fundamental manifestation of hypertension is increased stiffness of the aorta (Zhou *et al.* 2017a). Previous studies have demonstrated that intrinsic VSMC stiffening was significantly increased in the aortic stiffness of hypertension and aging (Zhou *et al.* 2017b). VSMC stiffness was enhanced after 24h of hypoxia treatment, but no further change was detected following treatment with In-CI (data not shown). Further studies investigating how to decrease the VSMC stiffness increased by hypoxia will be valuable in the development of novel treatment strategies.

Transcriptome characterization can help elucidate the functional complexity of the genome as well as the mechanisms underlying cellular activities, such as growth, development and immune responses. We observed that inflammation-related gene expression patterns were altered under conditions of hypoxia in the presence of ER β ligands. Hierarchical clustering showed that samples were more closely grouped based on treatment with the ER β -specific ligands DPN and In-CI than with ER ligands (E2) in the hypoxic state. The results of this study suggest that ER β agonists exhibit potential therapeutic value as a promising target for suppressing inflammation.

This study indicates that hypoxia-induced COX-2, an inflammatory marker, is reduced following treatment with the ER β agonist In-CI. Because other environmental factors may be associated with inflammation, the combined effects of multiple exposure factors as well as inflammatory factors, such as COX-2 and ROS, should be investigated further. The findings of the present study suggest that ER β may be targeted to suppress inflammation related to vascular diseases in a hypoxic environment.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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