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HuR mediates the synergistic effects of angiotensin II and interleukin 1 β on vascular COX-2 expression and cell migration

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Abstract

BACKGROUND AND PURPOSE. Angiotensin II (AngII) and interleukin 1 β (IL-1 β) are involved in cardiovascular diseases through induction of inflammatory pathways. HuR is an ARE-binding protein that contributes to the mRNA stabilization of many genes. This study investigated the contribution of HuR upon COX-2 expression induced by AngII and IL-1 β and its consequences on vascular smooth muscle cell (VSMC) migration and remodeling.

EXPERIMENTAL APPROACH. Rat and human VSMC stimulated with AngII (0.1 μ M) and/or IL-1 β (10 ng·mL⁻¹) and mice infused with AngII or subjected to carotid artery ligation were used. mRNA and protein levels were assayed by qPCR, western blot, immunohistochemistry, and immunofluorescence. Cell migration was measured by wound healing and transwell assays.

KEY RESULTS. In VSMC, AngII potentiated COX-2 and tenascin-C expressions and cell migration induced by IL-1 β . The effect of AngII on IL-1 β -induced COX-2 expression was accompanied by increased COX-2 3'UTR reporter activity and mRNA stability occurring through cytoplasmic HuR translocation and COX-2 mRNA binding. These effects were blocked by ERK1/2 and HuR inhibitors. VSMC migration was reduced by blockade of ERK1/2, HuR, COX-2, TXAS, TP and EP receptors. HuR, COX-2, mPGES-1 and TXAS expressions were increased in AngII-infused mouse aortas and in carotid-ligated arteries. AngII-induced tenascin-C expression and vascular remodeling were abolished by celecoxib and by mPGES-1 deletion.

CONCLUSIONS AND IMPLICATIONS. The synergistic induction of COX-2 by AngII and IL-1 β in VSMC involves HuR through an ERK1/2-dependent mechanism. The HuR/COX-2 axis participates in cell migration and vascular damage. HuR might be a novel target to modulate vascular remodeling.

Keywords: Cell migration, cytokines, inflammatory mediators, smooth muscle cells, vascular remodeling.

List of non-standard abbreviations: AngII, angiotensin II; ARE, adenylylate- and uridylylate - rich element; ECM, extracellular matrix; IL-1 β , interleukin 1 β ; PG, prostaglandin; PGS, prostaglandin synthases; PGIS, prostacyclin synthase; TN-C, tenascin-C; TXAS, thromboxane A₂ synthase; VSMC, vascular smooth muscle cells; 3'UTR, 3' untranslated region

Introduction

The constitutive and the inducible cyclooxygenases, COX-1 and COX-2, respectively, are expressed in the vasculature where they convert arachidonic acid into an endoperoxide intermediate, prostaglandin (PG) H_2 , which is immediately metabolized by different prostaglandin synthases (PGS) into specific prostanoids. COX-2 is the dominant source of PGs in inflammatory vascular diseases, such as atherosclerosis (Cipollone and Fazia, 2006), balloon-injured arteries (Yang *et al.*, 2004) and hypertension (Martínez-Revelles *et al.*, 2013). Angiotensin II (AngII) is a key player in vascular damage through its significant pro-inflammatory actions including the production of cytokines (Marchesi *et al.*, 2008) such as interleukin 1β (IL- 1β). Both AngII and cytokines induce COX-2 expression at vascular level (Ohnaka *et al.*, 2000; Galán *et al.*, 2011; Martín *et al.*, 2012).

COX-2 expression in response to pro-inflammatory stimuli is initiated through transcriptional induction (Harper and Tyson-Capper, 2008). As a means to control COX-2 expression, post-transcriptional mechanisms are in place that targets the mRNA for rapid degradation. A key feature present in the COX-2 3' untranslated region (3'UTR) that is implicated in controlling mRNA degradation is the adenylate- and uridylylate (AU)-rich element or ARE (Dixon *et al.*, 2000). HuR protein (Hu antigen R; ELAVL1) is an ARE RNA-binding protein that is primarily nuclear localized and contributes to the mRNA stabilization of many genes when exported to the cytoplasm (Abdelmohsen and Gorospe, 2010). Thus, HuR is implicated in a wide variety of physiological and pathological processes such as cell growth, differentiation and inflammation (Abdelmohsen and Gorospe, 2010). Our prior work has identified COX-2 mRNA as a HuR target in intestinal cancer cells and in myeloid leukocytes (Dixon *et al.*, 2001; Young *et al.*, 2012; Dixon *et al.*, 2006). Similarly, in human mesangial cells, AngII increases COX-2 mRNA stability through HuR (Doller *et al.*, 2008). However, the ability of HuR to influence COX-2 expression in vascular smooth muscle cells (VSMC) is not yet explored.

PGS downstream of COX-2 participate in pathological vascular remodeling (Wang *et al.*, 2011; Zhang *et al.*, 2013; Sparks *et al.*, 2013). The main COX-derived prostanoids responsible for this effect are PGE₂, derived from inducible microsomal PGE₂ synthase-1 (mPGES-1), and TXA₂ for their effects on promoting cell proliferation and migration along with PGI₂ having an opposing effect (Nie *et al.*, 2000; Bos *et al.*, 2004; Wang *et al.*, 2011; Zhang *et al.*, 2013). The influence of PGE₂ on cell migration appears mediated, in part, by the inducible extracellular matrix (ECM) glycoprotein, tenascin-C (TN-C) (Wang *et al.*, 2011). However, whether HuR is involved with these effects of COX-2-derived prostanoids upon cell migration and vascular damage is currently unknown.

This study investigated: 1) the participation of HuR in AngII and IL- 1β -induced COX-2 expression in VSMC and 2) the contribution of HuR to the effects of COX-2-derived prostanoids on cell migration and vascular remodeling. We demonstrate that the combination AngII+IL 1β induces HuR-dependent stabilization of COX-2 mRNA leading to increased prostanoid-mediated cell migration. This effect could contribute to exacerbate the vascular remodeling triggered by AngII on inflamed vascular beds.

Methods

All experimental procedures were approved by the Ethical Committees of Research of the Universidad Autónoma de Madrid and University of Kansas Medical Center and by the Reviewer Institutional Committee on Human Research of the Hospital de la Santa Creu i Sant Pau, conforms to the Declaration of Helsinki. These studies were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and with guidelines for ethical care of experimental animals of the European Community, the current Spanish and European laws (RD 223/88 MAPA and 609/86), and the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines.

Cell culture

Primary cultures of VSMC were obtained from isolated Sprague Dawley rat aortas and grown in DMEM-F12 medium supplemented with 10% fetal bovine serum (FBS) containing 100 U·mL⁻¹ of penicillin, 100 µg·mL⁻¹ of streptomycin as previously reported (Aguado *et al.*, 2013). Human VSMC were obtained from coronary arteries of hearts removed in transplant operations by using a modification of the explant technique (Orriols *et al.*, 2014). Human VSMC were grown in M199 supplemented with 20% FBS, 1% human serum, 2 mM of L-glutamine, 100 U·mL⁻¹ of penicillin and 100 µg·mL⁻¹ of streptomycin. Cells were serum-starved in serum free media for 24 h prior to stimulation. All products were purchased to Sigma-Aldrich (St. Louis, MO, USA).

Cells were stimulated with vehicle (control), AngII (0.1 µM; Sigma-Aldrich), IL-1β (10 ng·mL⁻¹; Sigma-Aldrich), AngII+IL-1β, 16,16-Dimethyl PGE₂ (Sigma-Aldrich) or U46619 (Cayman Chemical, Ann Arbor, MI, USA) at the times and concentrations indicated in the Results section. The effects of the following inhibitors were analyzed by its addition 30 min (or 4 h in the case of MS-444) before and throughout stimulation: L798106, SC19220, actinomycin D (Sigma-Aldrich); U0126, SP600125, LY294002, SB203580, N-(6-Aminoheptyl)-5-chloro-1-naphthalenesulfonamide (W7), N-(4-aminobutyl)-1-naphthalenesulfonamide (W12), KN93 and PD98059 (Calbiochem, Darmstadt, Germany); tyrphostin AG 1478 (AlomoneLabs, Jerusalem, Israel); chelerythrine (Tocris, Biogen Científica, Spain); SQ29548 (ICN Iberica, Barcelona, Spain); furegrelate (Cayman Chemical); celecoxib (generously provided by Pfizer Inc. Groton, CT, USA), ozagrel (generously provided by Kissei Pharmaceutical CO, Matsumoto, Japan); MS-444 (generously provided by Novartis, Basel, Switzerland). Compounds used for stimuli and ozagrel were dissolved in distilled water; remaining compounds were dissolved in DMSO. None of these inhibitors affected basal expression of COX-2, mPGES-1, TXAS, PGIS or TN-C (data not shown). MS-444 was originally characterized as myosin light chain kinase inhibitor in the µM-range (Aotani and Saitoh, 1995), however the concentrations used in these experiments did not affect myosin light chain phosphorylation induced by AngII+IL-1β or cell viability (data not shown).

RNA analysis

Cells were harvested in TRI Reagent (Sigma-Aldrich) or TRIzol (Life Technologies Inc., Gaithersburg, Maryland, USA) according to the manufacturer's recommendations to obtain total RNA and reverse transcribed using High Capacity cDNA Archive Kit (Life Technologies) with random hexamers. Quantitative PCR (qPCR) was performed in a 7500 Fast ABI System (Life Technologies). qPCR for human COX-2, mPGES-1, PGIS, TXAS and

18S rRNA, along with rat and mouse TXAS were performed using Taqman Gene Expression Assays (COX-2: Hs00153133_m1; mPGES-1: Hs00610420_m1; PGIS: Hs00168766_m1; TXAS: Hs01022706_m1; 18S rRNA: Hs99999901_s1 in humans; TXAS: Rn00562160_m1 in rats, TXAS: Mm00495553_m1 in mice, Life Technologies). qPCR for COX-2, mPGES-1, HuR, TN-C and β_2 -microglobulin in rats and mice and PGIS in rats were performed using SYBR green PCR master mix (iTaQ FAST SYBRGreen Supermix with ROX, Bio-Rad, USA). Primer sequence information is listed on Supporting Table 1. PCR cycles proceeded as described (Martínez-Revelles *et al.*, 2013). Data analyses used the $2^{-\Delta\Delta C_t}$ method, where β_2 -microglobulin or 18S rRNA served as the internal control.

Determination of COX-2 mRNA stability in stimulated cells was initiated by adding 5 $\mu\text{g}\cdot\text{mL}^{-1}$ actinomycin D (Sigma-Aldrich) to the medium at specified times. Total RNA was isolated and COX-2 and β_2 -microglobulin mRNA expression levels were measured by qPCR as indicated above.

Western blot analysis

Whole-cell lysates were harvested in RIPA buffer containing: 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM MgCl_2 , 1 mM EDTA, 1% Nonidet-P40 (NP-40), 0.5% deoxycholate Na, 1% sodium dodecyl sulfate (SDS), a protease inhibitor cocktail (Roche Applied Science, Barcelona, Spain) and a mix of phosphatase inhibitors (1 mM orthovanadate, 20 mM β -glycerophosphate, 10 mM NaF from Sigma-Aldrich). For cellular fractionation, cells were lysed in hypotonic lysis buffer (10 mM HEPES pH 8, 3 mM MgCl_2 , 40 mM KCl, 0.2% NP-40, 10% glycerol, 0.1 mM DTT with protease and phosphatase inhibitors) for 15 min at 4 °C. Samples were centrifuged 2 min at 10,000 rpm to separate cytoplasmic supernatant from nuclei. Nuclei were washed four times in hypotonic buffer and lysed in RIPA buffer. Samples were then centrifuged 10 min at 10,000 rpm to obtain the nuclear fraction. Protein content was determined with Lowry (Bio-Rad) or BCA protein assay reagent (Pierce, Rockford, IL, USA), using bovine serum albumin (BSA, Sigma-Aldrich or Pierce) as standard. Lysates (20–40 μg) were separated by 10% SDS-PAGE, transferred to PVDF or nitrocellulose membranes (Amersham, GE Healthcare, Buckinghamshire, UK) and probed with antibodies against HuR (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Nucleoporin p62 (1:5,000; BD Bioscience, San Jose, CA, USA), COX-2 and mPGES-1 (1:200; Cayman Chemical), PGIS (1:1,000; Cayman Chemical), MLC, p-MLC, p-ERK1/2, ERK1/2, p-p38 and p38 MAPK, p-JNK, JNK, p-Akt and Akt (1:1,000; Cell Signaling, Boston, MA, USA). Blots were stripped and then probed with antibodies against β -actin (1:50,000; Sigma-Aldrich) or tubulin (1:10,000; Sigma-Aldrich). Detection was accomplished using horseradish peroxidase-coupled anti-rabbit (1:2,000; Bio-Rad) or anti-mouse (1:5,000; Stressgen, Victoria, Canada) IgG antibodies for 1 h at room temperature. Signal was detected using the Luminata Forte (Millipore Corporation, Billerica, MA, USA) detection system. Immunoblot signals were quantified using NIH ImageJ using β -actin or tubulin expressions as loading controls.

Ribonucleoprotein complex immunoprecipitation

Ribonucleoprotein complex immunoprecipitation was performed as described (Lal *et al.*, 2004). Briefly, cells were lysed in polysome lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM MgCl_2 , 1 mM DTT, 0.5% NP-40, 1 mM PMSF, protease inhibitors, 100 U RNase inhibitor). 200 μg of cytoplasmic lysate was incubated with anti-HuR antibody or control IgG precoated to protein A/G PLUS agarose (Santa Cruz Biotechnology) overnight at 4 °C in NT2 buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM MgCl_2 , 0.05% NP-40).

adding 0.1 mM DTT, 40 U RNase inhibitor (Promega, Mannheim, Germany), 5 mM EDTA, 100 $\mu\text{g}\cdot\text{mL}^{-1}$ tRNA (Life Technologies). Immunoprecipitates were collected by centrifugation and washed 4 times with NT2 buffer. Total RNA was isolated from immunoprecipitates using 1 ml TRIzol reagent and then used for cDNA synthesis and qPCR as described above.

DNA and siRNA transfections

Luciferase reporter constructs containing 3.9 kb of human COX-2 promoter (pGL3-COX-2P) or the COX-2 3'UTR (from 3074 to 3236) (pGL3P-UTR2) were kindly provided Dr. W. Eberhardt (Klinikum der Johann Wolfgang Goethe-Universitat Frankfurt am Main, Germany). Cells were seeded in 12-well culture plates at a density of 6×10^4 cells per well and transiently transfected with 0.5 μg luciferase reporter gene construct per well using the Lipofectamine LTX Plus reagent (Life Technologies). After 6 h, media was changed and cells were transferred to depletion medium overnight. After stimulation, cells were harvested in reporter lysis buffer and luciferase activities were measured using a luminometer (Orion I, Berthold detection systems) according to the manufacturer's instructions. Relative luciferase activities were normalized to total protein.

In other set of experiments cells seeded in 6-well plates or transwells at a density of 1.2×10^5 or 3×10^4 cells per well, respectively, were transfected with a predesigned siRNA against HuR (Life Technologies) or a negative control siRNA (Qiagen-Izasa, Barcelona, Spain) using the Lipofectamine LTX Plus reagent (Life Technologies) according to the manufacturer's protocol. Cells were transfected for 48 h with 20 nM siRNA and silencing efficiency was determined by western blot.

Migration assays

VSMC migration was examined using a wound healing assay. VSMC monolayers were wounded using a sterile 10 μL pipette tip. Phase contrast images were taken immediately after wounding and at 24 h post-stimulation using a Nikon microscope (Tokyo, Japan) connected to a video camera (Sony Corporation, Tokyo, Japan). To measure migration, wound area was quantified using Adobe Photoshop and expressed as percentage of wound closure.

Cell migration was also determined using a 6.5 mm Transwell chamber with an 8 μm pore size (Corning Costar Inc., Corning, New York, NY, USA). 3×10^4 cells were seeded in the upper compartment of each chamber and after 16 h serum deprivation, inhibitors were added in the upper chamber and the stimuli were added in the bottom chamber. After 5 h, cells of the upper membrane surface were removed by a cotton swab and washed with PBS. Migration values were determined by counting three fields per chamber after fixing the membrane in 4% paraformaldehyde and staining with Hoechst 33342.

Animal models

The following animal models were used: 1) C57BL6 male mice with permanent ligation of the left common carotid artery for 3 weeks, as we described previously (Rodríguez-Calvo *et al.*, 2013). 2) C57BL6 male mice infused with saline, with AngII ($1.44\text{ mg}\cdot\text{Kg}^{-1}\cdot\text{day}^{-1}$, 2 weeks, subcutaneously by osmotic minipumps, Alza Corp., Cupertino, CA, USA) or with AngII plus celecoxib ($25\text{ mg}\cdot\text{Kg}^{-1}\cdot\text{day}^{-1}$ i.p. started 24 h before AngII-infusion), as described (Martínez-Revelles *et al.*, 2013). 3) mPGES-1-wild-type (WT) and mPGES-1-deficient (mPGES-1^{-/-}) mice infused with saline or with AngII. WT and mPGES-1^{-/-} mice were

maintained in a DBA/1 genetic background and were generated by corresponding heterozygous breeders derived from mPGES-1^{+/-} embryos that were a kind gift from Pfizer.

Immunofluorescence microscopy

Cells or frozen transverse aortic sections (14 μ m) were rinsed with PBS, fixed with 2% or 4% paraformaldehyde (Sigma-Aldrich), respectively, permeabilized with 0.2% Triton-PBS, blocked with 3% IgG-free BSA in PBS and incubated overnight at 4°C with anti-HuR monoclonal primary antibody diluted 1:200 in blocking solution. Detection was performed using fluorescein isothiocyanate-conjugated secondary IgG (1:200; Jackson ImmunoResearch, West Grove, PA, USA) diluted in blocking buffer and incubated for 1 h at room temperature, after which the cells were counterstained with Hoechst dye 33342 (Life Technologies). Slides were examined using an Evos XL Cell Imaging System or a Leica TCS SP2 confocal microscope with a 40X objective. Images were processed using Adobe Photoshop CS3 software (Adobe Systems Inc., San Jose, CA, USA).

Histological analysis

Histological analysis was performed as described (Avendaño *et al.*, 2014). Briefly, cross sections from fixed aortas were stained with hematoxylin-eosin. Morphometric determinations of the lumen and vessel areas were performed by using Metamorph image analysis software (Universal Imaging, Molecular Devices Corp., Downingtown, PA, USA).

Immunohistochemistry

Immunohistochemistry was performed as described (Orriols *et al.*, 2014). Briefly, mouse paraffin-fixed carotids were prepared in cross sections with a microtome (Jung RM 2055, Leica). After deparaffinization, rehydration and hydrogen peroxide treatment, sections were blocked and incubated with anti-HuR (1:100), anti-COX-2 (1:100), anti-mPGES-1 (1:100), and anti-TXAS (1:50) antibodies. After that, sections were incubated with a biotinylated secondary antibody (Vector Laboratories). Color was developed using 3,3'-diaminobenzidine and sections were counterstained with haematoxylin. Negative controls in which the primary antibody was omitted were included to test for non-specific binding.

Measurement of Prostaglandin E₂ production

The levels of PGE₂ metabolites were determined in plasma of mice using an enzyme immunoassay commercial kit (Cayman Chemical). The plasma was kept at -70°C until analysis and processed following the manufacturer's instructions.

Data analysis and statistical procedures

All values are expressed as mean \pm SEM of the number animals used in each experiment or independent cell culture-based experiments. Statistical analysis was done by Student's *t* test, Mann-Whitney test or by one-way ANOVA followed by a Bonferroni test. Values were considered to be significant when *P* < 0.05.

Results

AngII and IL-1 β synergistically induce COX-2 expression in VSMC through ERK1/2 pathway.

In basal conditions, rat VSMC express low-to-undetectable COX-2 mRNA and protein levels. After AngII stimulation, COX-2 mRNA and protein levels expression increased reaching a maximum at 1 h, being at 24 h as the basal expression. The exposure of cells to IL-1 β increased COX-2 mRNA and protein levels reaching a plateau at 2 h and 8 h, respectively. However, co-stimulation of rat VSMC with AngII+IL-1 β led to a synergistic increase in COX-2 mRNA and protein. This exacerbated induction of COX-2 was observed to occur as early as 1 h after stimulation and persisted out to 24 h (Figure 1A,B). Consistently, Figure 1C,D show that after 24 h stimulation of rat VSMC, IL-1 β , increased COX-2 mRNA and protein levels whereas AngII only slightly increased mRNA levels. However, at this time, AngII strongly enhanced both COX-2 mRNA and protein levels induced by IL-1 β . Similar results were obtained in human VSMC after 24 h stimulation (Supporting Figure 1A,B) indicating this effect is conserved and adds to this finding an additional relevance.

AngII+IL-1 β promoted rapid phosphorylation of ERK1/2, JNK, p38 MAPK and Akt as soon as 5 min after treatment (Figure 1E). Interestingly, only the ERK1/2 inhibitor U0126 (10 μ M) was able to decrease COX-2 mRNA expression observed with AngII+IL-1 β treatment, whereas the JNK (SP600125, 20 μ M), p38 MAPK (SB203580, 10 μ M) and PI3K (LY294002, 10 μ M) inhibitors did not (Figure 1F). These results suggest that ERK1/2 is the main pathway implicated in AngII+IL-1 β -induced COX-2 expression.

HuR is involved in the COX-2 mRNA stabilization induced by AngII in VSMC.

COX-2 expression is regulated by both transcriptional and post-transcriptional mechanisms (Harper and Tyson-Capper, 2008). To determine whether the effect of AngII on IL-1 β -induced COX-2 expression was due to changes in transcription, COX-2 transcriptional activity was measured in rat VSMC transfected with a reporter plasmid containing the human COX-2 promoter fused to luciferase cDNA. IL-1 β increased COX-2 promoter activity and the combination of AngII+IL-1 β resulted in a similar transcriptional activity (Figure 2A). These results suggested that post-transcriptional regulatory mechanisms to be the primary effector of the synergistic induction of COX-2 expression induced by AngII+IL-1 β . To test this hypothesis, we performed mRNA stability assays. Figure 2B shows that AngII stabilized COX-2 mRNA in IL-1 β -triggered VSMC. We also performed transfections of VSMC using a luciferase reporter plasmid containing a 150 bp 3'UTR from COX-2 mRNA, which included the binding site for HuR. Figure 2C shows that both stimuli were needed to increase COX-2 3'UTR luciferase activity.

In unstressed cells, HuR is predominantly localized to the nucleus and can be exported to the cytoplasm to stabilize different mRNAs including COX-2 mRNA (Dixon et al., 2001; Young et al., 2012). As shown in Figure 2D, treatment of VSMC with AngII+IL-1 β increased HuR cytoplasmic localization and this effect was abolished by ERK1/2 inhibition (+PD98059, 20 μ M). As expected, treatment of AngII+IL-1 β stimulated VSMC with MS-444 (8 μ M), a low-molecular weight inhibitor of HuR that prevents cytoplasmic trafficking of HuR by interfering with its homodimerization (Meisner and Filipowicz, 2011), yielded similar effects. Consistent with these observations on HuR trafficking, another ERK1/2 inhibitor (U0126, 10 μ M) and MS-444 also abolished the increased stability of COX-2 mRNA observed in AngII+IL-1 β -stimulated VSMC (Figure 2B). Moreover, MS-444 or HuR knockdown by siRNA reduced COX-2 mRNA levels induced by AngII+IL-1 β (Figure 2E). We then determined if AngII+IL-1 β signaling promotes increased cytoplasmic HuR binding to COX-2

mRNA by ribonucleoprotein immunoprecipitation using an antibody against HuR or control IgG. We observed an enrichment in COX-2 mRNA in anti-HuR precipitates from cells treated with AngII+IL-1 β , but not in control cells or in anti-IgG precipitates (Figure 2F). GAPDH mRNA co-precipitation was used as control to assess the nonspecific background (data not shown).

All together, these findings suggest that AngII+IL-1 β induce an ERK1/2-dependent activation of HuR which is responsible for increased COX-2 mRNA stability and COX-2 levels in VSMC.

Effect of AngII and IL-1 β on mPGES-1, PGIS and TXAS expression in rat and human VSMC.

To assess whether prostanoid synthases were also regulated by AngII, IL-1 β or the combination of both, we measured mPGES-1, PGIS and TXAS mRNA and protein levels.

IL-1 β treatment led to a time-dependent increase in mPGES-1 mRNA levels, whereas AngII treatment decreased mPGES-1 mRNA (Supporting Figure 2A). Accordingly, AngII+IL-1 β increased mPGES-1 mRNA levels less than IL-1 β alone. Similar results were observed when mPGES-1 mRNA and protein levels were only examined at 24 h stimulation (Figure 3A,B). In contrast to mPGES-1 expression, PGIS mRNA and protein levels were significantly induced by AngII and not affected by IL-1 β (Figure 3C, D and Supporting Figure 2B); the effects of AngII+IL-1 β on PGIS expression were similar to those produced by AngII alone. TXAS mRNA levels were increased in a time-dependent manner by IL-1 β , AngII, and AngII+IL-1 β (Figure 3E and Supporting Figure 2C). Similar results were observed at the protein level (Figure 3F). Human VSMC showed a pattern similar to rat VSMC in the changes observed in mPGES-1, PGIS and TXAS mRNA levels when cells were exposed during 24 h to AngII and/or IL-1 β , except PGIS that was induced by IL-1 β in human cells, and human mPGES-1 protein expression was similarly regulated by these stimuli (Supporting Figure 1C-F).

The increased mPGES-1 mRNA levels observed after 24 h stimulation with AngII+IL-1 β were blocked with ERK1/2 (U0126), JNK (SP600125), p38 MAPK (SB203580) and PI3K (LY294002) inhibitors (Supporting Figure 3A). However, PGIS mRNA levels were diminished only by the ERK1/2 inhibitor (Supporting Figure 3B). TXAS mRNA levels were reduced by ERK1/2, p38 MAPK and PI3K inhibitors (Supporting Figure 3C). These results indicate that different signaling pathways are involved in the regulation of mPGES-1, PGIS and TXAS expressions.

COX-2-derived prostanoids mediate the synergistic effect of AngII and IL-1 β -induced cell migration.

Wound healing and transwell assays were used to study cell migration in rat VSMC. Both AngII and IL-1 β induced VSMC migration that was synergistically increased in the presence of both stimuli (Figure 4A). The addition of inhibitors against ERK1/2 (U0126, 10 μ M), COX-2 (celecoxib, 10 μ M), TXAS (furegrelate, 10 μ M and ozagrel, 10 μ M), TP receptor (SQ29548, 3 μ M), EP1 receptor (SC19220, 10 μ M), EP3 receptor (L798106, 1 μ M), HuR (MS-444) and HuR siRNA reduced the ability of AngII+IL-1 β to promote VSMC cell migration (Figure 4B,C). These results indicate that PGE₂ and TXA₂ derived from COX-2, along with ERK1/2 and HuR activities are involved in cell migration induced by AngII+IL-1 β . In agreement, 16,16-Dimethyl PGE₂ (0.1-10 μ M) or the TP agonist U46619 (0.01-1 μ M) increased VSMC migration (Figure 5A-C), which was abolished by respective receptor

antagonists SQ29548, SC19220 and L798106 (Figures 5D,E).

To understand the signaling by which TXA₂ and PGE₂ mediate cell migration, VSMC were pretreated with inhibitors targeting various kinase pathways: ERK1/2 (U0126), JNK (SP600125), p38 MAPK (SB203580), PI3K (LY294002), PKC (chelerythrine, 20 μM), calmodulin (CaM) (W7, 10 μM), pCaMKII (KN93, 20 μM) and epidermal growth factor receptor (EGFR) (AG1478, 10 μM). Wound healing assays were then performed in the presence of TP agonist U46619 (0.1 μM) and 16,16-Dimethyl PGE₂ (1 μM). As a negative control, the less potent CaM inhibitor W12 (10 μM) was used. As shown in Figures 5D and E, only U0126, SP600125, W7, KN93 and AG1478 inhibited 16,16-Dimethyl PGE₂- and U46619-induced cell migration indicating the participation of ERK1/2, JNK, CaM, PKC and EGFR in such effects.

TN-C is expressed predominantly in pathological conditions and it is involved in cell migration (Wang *et al.*, 2011; Yu *et al.*, 2013). In addition, PGE₂ induces its expression in the mouse model of femoral artery wire injury (Wang *et al.*, 2011). We observed that TN-C mRNA levels were increased by AngII or IL-1β and this effect was potentiated in the presence of both stimuli (Figure 6A). The effect of AngII+IL-1β on TN-C mRNA levels was abolished by celecoxib, U0126, furegrelate, SC19220 and L798106 (Figure 6B).

All together our results demonstrate that PGE₂ and TXA₂ derived from COX-2 pathway participate of the effects induced by AngII and IL-1β on cell migration by mechanisms involving ERK1/2, HuR and TN-C.

Implication of COX-2-derived prostanoids in vascular remodeling.

To study the potential involvement of the results obtained in cultured VSMC, we used two models of vascular remodeling characterized by thickening of the vascular wall influenced by VSMC migration and inflammation. Increased expression of COX-2, mPGES-1, TXAS and HuR was observed in ligated carotid arteries compared to non-ligated controls (Figure 7A). Similar results were obtained in aorta from AngII-infused mice (Figure 7B). **In agreement, plasma PGE₂ levels were increased in the carotid ligation and AngII-infused mouse models (Supporting Figure 4).** Moreover, AngII-infused mice showed increased aortic TN-C expression that was reduced by pharmacological COX-2 blockade with celecoxib and by mPGES-1 deletion (Figure 7C). Aorta from AngII-infused mice also showed increased media:lumen ratio and cross sectional area (markers of hypertrophic remodeling) that was reduced by celecoxib treatment and mPGES-1 deletion (Figure 7D,E). These results suggest the role of COX-2- and mPGES-1-derived TXA₂ and PGE₂ in TN-C expression and vascular **hypertrophic** remodeling.

Discussion and Conclusions

In this study we present evidence that the combination of two inflammatory stimuli, AngII and IL-1 β , synergistically induce COX-2 expression and cell migration in VSMC via a HuR-mediated increase of mRNA stability and this mechanism would be implicated in the vascular remodeling associated **with** cardiovascular diseases.

AngII and cytokines have been implicated in several cardiovascular pathologies through their inflammatory actions in the vascular wall (Marchesi *et al.*, 2008). COX-2 is an early response gene overexpressed in pathological conditions associated with inflammatory processes, such as cancer (Dixon *et al.*, 2001; Young *et al.*, 2012), atherosclerosis (Cipollone and Fazia, 2006) and hypertension (Martínez-Revelles *et al.*, 2013). In this study, we found that AngII and IL-1 β synergistically increased COX-2 protein levels in VSMC, as we previously described in vascular fibroblasts (Galán *et al.*, 2011). More importantly, the effects of AngII+IL-1 β on COX-2 expression in VSMC had a functional consequence since the combination of both stimuli synergistically induced VSMC migration by a mechanism dependent on COX-2-derived prostanoids. The participation of COX-2-derived prostanoids on cell migration has been previously observed in VSMC stimulated with AngII (Zhang *et al.*, 2013).

COX-2 expression is tightly regulated by both transcriptional and post-transcriptional mechanisms (Dixon *et al.*, 2000; Harper and Tyson-Capper, 2008; Young *et al.*, 2012). Pham *et al.* (2008) described that the synergistic up-regulation of COX-2 induced by AngII and epidermal growth factor in intestinal epithelial cells was due to an increase of its transcriptional rate and its mRNA stability. Our results in VSMC demonstrate that the effect of AngII on IL-1 β -induced COX-2 expression was mainly due to a mRNA stabilization mechanism, as previously described in adventitial fibroblasts (Galán *et al.*, 2011). RNA stability is regulated by different mRNA binding proteins such as HuR, which is most noted to play a role in cancer through stabilization of various target genes such as COX-2 (Dixon *et al.*, 2001; Abdelmohsen and Gorospe, 2010). HuR is mainly located in the nucleus and shuttles between the nucleus and the cytoplasm. The presence of cytoplasmic HuR is noted to contribute in promoting cell growth, proliferation, angiogenesis and survival (Abdelmohsen and Gorospe, 2010), and in this capacity, HuR stabilizes ARE-containing mRNAs such as cyclins, tumor necrosis factor- α , vascular endothelial growth factor, and COX-2 (Abdelmohsen and Gorospe, 2010). This is evident in colorectal and other tumor types, where HuR is overexpressed and present within the cytoplasm leading to ARE-mRNA stabilization and increased of COX-2 expression (Dixon *et al.*, 2001; Young *et al.*, 2012). Post-translational modifications of HuR such as serine and threonine phosphorylation by several kinases can modify HuR subcellular location (Meisner and Filipowicz, 2011). Specifically, ERK1/2 can participate in HuR phosphorylation, modifying its activity in a lung cancer cell line (Yang *et al.*, 2004) or HuR cytoplasmic location in hepatic stellate cells (Woodhoo *et al.*, 2012). Herein, we demonstrate for the first time in VSMC that ERK1/2-mediated HuR activation is involved in the synergistic effect of AngII and IL-1 β on COX-2 expression and cell migration. This is supported by the following findings: 1) AngII+IL-1 β interfered with rapid decay of COX-2 mRNA resulting in increased 3'UTR reporter activity in VSMC. This effect was accompanied with HuR translocation to the cytoplasm and its binding to COX-2 mRNA; 2) HuR silencing and/or treatment with the HuR inhibitor MS-444 reduced COX-2 mRNA stability, AngII+IL-1 β -induced COX-2 mRNA levels and cell migration; 3) ERK1/2 phosphorylation was increased by AngII+IL-1 β and ERK1/2 inhibition reduced COX-2 levels, COX-2 mRNA stability, HuR cytoplasmic translocation and cell migration. All together, these results suggest that HuR cytoplasmic trafficking could be a central node implicated in vascular remodeling associated **with** cardiovascular pathologies by modulating

COX-2 expression and cell migration. In agreement, we observed increased vascular expression of HuR in AngII-infused mice and in the carotid ligation mouse model, as described in different vascular injuries such as intimal hyperplasia, arterialized saphenous vein or atherosclerotic plaque (Pullmann *et al.*, 2005).

During the last few years COX-2 downstream prostanoids involved in vascular remodeling has gained growing attention (Wang *et al.*, 2011; Zhang *et al.*, 2013; Sparks *et al.*, 2013). Our results demonstrate that COX-2-derived PGE₂ and TXA₂ are involved in cell migration induced by AngII+IL-1 β . Thus, we found that not only COX-2 but also mPGES-1 and TXAS expressions were increased in VSMC treated with AngII+IL-1 β . In addition, cell migration induced by AngII+IL-1 β was blocked by pharmacological inhibitors of COX-2 and the TXA₂ and PGE₂ pathways. Finally, a PGE₂ analogue and a TP agonist mimicked AngII+IL-1 β effects on cell migration, effects that were blocked by EP1 and EP3 and TP blockade, respectively. In agreement, PGE₂ and TXA₂ induce cell migration in VSMC (through EP3 receptor) or in endothelial cells (through TP receptor), respectively (Nie *et al.*, 2000; Zhang *et al.*, 2013). The effects of prostanoids on cell migration might be mediated by TN-C, an ECM protein which participates in cell proliferation and migration (Wang *et al.*, 2011; Yu *et al.*, 2013) and that it is regulated by PGI₂ or PGE₂ (Wang *et al.*, 2011). Our results demonstrate that not only EP1 and EP3 receptors but also TXA₂ participate in the AngII+IL-1 β -mediated TN-C induction. More importantly, the effects of AngII+IL-1 β on COX-2, mPGES-1, TXAS and TN-C expression were recapitulated in two animal models of vascular remodeling associated with inflammation, and increased cell migration/proliferation (the carotid artery ligation model and the AngII infusion model). **Accordingly, heightened plasma PGE₂ levels were observed in both models of vascular damage.** Moreover, the **hypertrophic** vascular remodeling and the increased TN-C expression induced by AngII were reversed by celecoxib treatment and genetic mPGES-1 deletion, demonstrating that COX-2/mPGES-1/TXAS-derived prostanoids are key mediators of vascular damage/remodeling. Supporting these findings, other authors have demonstrated a role for COX-2, PGE₂ and TXA₂ in animal models of restenosis or hypertension (Wang *et al.*, 2011; Zhang *et al.*, 2013; Sparks *et al.*, 2013). **The effect of celecoxib treatment and mPGES-1 deletion in vascular remodeling might depend on changes in blood pressure. However, while celecoxib prevented the increase in blood pressure induced by AngII (Martinez-Revelles *et al.*, 2013) mPGES-1 deletion did not (Facemire *et al.*, 2010). The effect of AngII on the hypertrophic vascular remodeling may depend on the generation of pro-inflammatory cytokines, as observed with increased levels of IL-1 β in hearts of AngII-infused rats (Dange *et al.*, 2014). In addition, we cannot exclude that a synergistic effect of AngII and IL-1 β is also occurring in vivo and thus modulate vascular remodeling.**

An intriguing finding that deserves further consideration is the fact that COX-2-selective nonsteroidal anti-inflammatory drugs seem to be associated with increased cardiovascular risk mainly through the reduced biosynthesis of endothelial COX-2-dependent PGI₂ leading to an increase in thrombotic events (reviewed in Patrignani and Patrono, 2014). Care is needed in drawing direct comparisons between results obtained from animals- and cell-based studies, similar to the ones presented here, to clinical trials. Various factors such as the well-controlled nature of animal models, the duration or type of the pathology studied, and the particular inhibitor or gene regulatory pathway assayed in these studies are factors that might explain differences between experimental and clinical studies. Our results are part of a proof of concept that builds on information available of effects of prostanoids on vascular structure. Whether these effects have a translational consequence remains to be identified.

Different intracellular signaling pathways are responsible for cell migration. Among the most extensively studied are ERK1/2 and JNK. Both are able to phosphorylate different proteins

leading to cytoskeleton reorganization and cell migration (Huang *et al.*, 2004). CaM and pCaMKII are also important mediators of VSMC migration (Scott *et al.*, 2012). CaM/pCaMKII is activated by Gq proteins which are associated to EP1, EP3 and TP receptors (Bos *et al.*, 2004; Yamaoka *et al.*, 2009). The participation of EGFR in VSMC migration has been suggested in response to AngII (Mugabe *et al.*, 2010). Interestingly EGFR can be activated directly by TN-C (Huang *et al.*, 2004) or transactivated by EP and TP receptors (Han *et al.*, 2006; Uchiyama *et al.*, 2009). Herein, we demonstrate that ERK1/2, JNK, CaM and pCaMKII and EGFR are key mediators responsible for PGE₂ and TXA₂-dependent VSMC migration.

In summary, we demonstrate in VSMC the synergistic effect of AngII and IL-1 β upon COX-2 expression occurs through post-transcriptional regulation mediated by ERK1/2-dependent effects on the activity and localization of HuR. COX-2-derived TXA₂ and PGE₂ acting on TP and EP1 and EP3 respectively, induce TN-C expression and promote cell migration (Figure 8). Increased COX-2, mPGES-1, TXAS, TN-C and HuR, and PGE₂ levels were observed in animal models of vascular damage. Furthermore, blockade of COX-2 and mPGES-1 protects the vessel against TN-C expression and vascular remodeling. In conclusion, this study suggests that HuR and COX-2/mPGES-1/TXAS pathway could be pharmacological targets for vascular remodeling in cardiovascular diseases.

Acknowledgments

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Conflict of interest

None declared

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FIGURE LEGENDS

Figure 1. AngII potentiates IL-1 β -induced COX-2 expression in VSMC through ERK1/2. Time course of COX-2 mRNA (A) and protein (B) expression in rat VSMC. Representative western blots of five independent experiments are shown. Effects of AngII, IL-1 β and AngII+IL-1 β (24 h) on COX-2 mRNA (C) and protein expression (D) in rat VSMC. (E) Time course of ERK1/2, JNK, p38 and Akt activation by AngII+IL-1 β . (F) Effect of inhibitors of ERK1/2 (U0126, U), JNK (SP600125, SP), p38 MAPK (SB203580, SB) and PI3K (LY294002, LY) on COX-2 mRNA levels induced by AngII+IL-1 β . Data are expressed as mean \pm SEM. * p <0.05, ** P <0.01, *** P <0.001 vs Control; † P <0.05, †† P <0.01 vs Ang II and IL-1 β ; ††† P <0.001 vs AngII+IL-1 β . n=4-9.

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Figure 2. The synergistic effect of AngII and IL-1 β on COX-2 expression in VSMC is due to an increase of COX-2 mRNA stability mediated by HuR. (A) Luciferase activity in VSMC transfected with a luciferase reporter construct containing the COX-2 promoter and incubated with vehicle (control), IL-1 β or AngII+IL-1 β for 4 h. (B) Effect of AngII+IL-1 β on COX-2 mRNA stability (24 h after stimulation) and reversion by ERK1/2 (U0126) and HuR (MS-444) inhibitors. Actinomycin D, a transcriptional inhibitor, was added for the indicated times, after which COX-2 mRNA levels were measured by qPCR. (C) Luciferase activity in VSMC transfected with a luciferase reporter construct containing COX-2 3'-UTR and incubated with vehicle (control), IL-1 β or AngII+IL-1 β for 4 h. (D) Effect of PD98059 (ERK1/2 inhibitor) and MS-444 on HuR subcellular localization assayed by HuR immunofluorescence and western blotting of cellular fractions from VSMC stimulated with AngII+IL-1 β for 24 h. (E) Effects of MS-444 and HuR siRNA on COX-2 mRNA levels in VSMC stimulated with AngII+IL-1 β for 24 h. Representative blot of six independent experiments of HuR expression in cells transfected with control siRNA or HuR siRNA is included. (F) Ribonucleoprotein immunoprecipitation of HuR or control IgG was done to isolate mRNA bound by HuR in VSMC stimulated with AngII+IL-1 β for 24 h. Data are expressed as mean \pm SEM. * P <0.05, ** P <0.01, *** P <0.001 vs Control; $^{+++}$ P <0.001 vs IL-1 β ; $^{++}$ P <0.01, $^{+++}$ P <0.001 vs AngII+IL-1 β . n=4-8.

Figure 3. AngII and IL-1 β regulate differentially mPGES-1, PGIS and TXAS expressions. Effect of AngII, IL-1 β or AngII+IL-1 β (24 h) on mPGES-1, PGIS and TXAS mRNA levels (A, C, E) and protein expression (B, D, F) in rat VSMC. Data are expressed as mean \pm SEM. * P <0.05, ** P <0.01 vs Control; † P <0.05 vs AngII and IL-1 β . n=5-8.

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Figure 4. AngII+IL-1 β potentiates cell migration through COX-2-derived PGE₂ and TXA₂. (A) Wound healing (left) and transwell (right) migration assays performed in rat VSMC unstimulated (Control) and stimulated with AngII, IL-1 β or AngII+IL-1 β . (B) Wound healing (left) and transwell (right) assays of rat VSMC stimulated with AngII+IL-1 β with and without inhibitors of ERK1/2 (U0126, U), COX-2 (celecoxib, Cele), HuR (MS-444, MS), TXAS (furegrelate, F; ozagrel, Oza), TP receptor (SQ29548, SQ), EP1 receptor (SC19220, SC) or EP3 receptor (L798106, L7). (C) Transwell assays of rat VSMC stimulated with AngII+IL-1 β with HuR siRNA or control siRNA. Data are expressed as mean \pm SEM. ** P <0.01, *** P <0.001 vs Control; † P <0.05, †† P <0.01 vs AngII and IL-1 β . ‡ P <0.05, ‡‡ P <0.01, ‡‡‡ P <0.001 vs AngII+IL-1 β . n=3-15.

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Figure 5. PGE₂ and TXA₂ induce cell migration. (A,B) Wound healing assays performed in rat VSMC unstimulated and stimulated with different concentrations of U46619 or PGE₂. (C) Transwell migration assay of VSMC unstimulated and stimulated with U46619 or PGE₂. (D,E) Wound healing assays performed in rat VSMC unstimulated and stimulated with U46619 or PGE₂ with or without inhibitors of TP receptor (SQ29548, SQ), EP1 receptor (SC19220, SC), EP3 receptor (L798106, L7), ERK1/2 (U0126, U), JNK (SP600125, SP), p38 MAPK (SB203580, SB), PI3K (LY294002, LY), PKC (chelerythrine, Ch), CaM (W7, W12), pCaMKII (KN93, KN) or EGFR (AG1478, AG). Data are expressed as mean \pm SEM. * P <0.05, ** P <0.01, *** P <0.001 vs Control; † P <0.05, †† P <0.01, ††† P <0.001 vs AngII+IL-1 β . n=3-6.

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Figure 6. AngII+IL-1 β potentiates Tenacin-C mRNA levels in VSMC through COX-2-derived PGE₂ and TXA₂. (A) Effect of AngII, IL-1 β or AngII+IL-1 β (24 h) on Tenacin-C (TN-C) mRNA levels in rat VSMC. (B) Effect of inhibitors of COX-2 (celecoxib, Cele), ERK1/2 (U0126, U), EP1 receptor (SC19220, SC), EP3 receptor (L798106, L7) or TXAS (furegrelate, F) on TN-C mRNA levels in rat VSMC stimulated with AngII+IL-1 β (24 h). Data are expressed as mean \pm SEM. ** P <0.01 vs Control; † P <0.05 vs AngII or IL-1 β ; ‡‡ P <0.01, ‡‡‡ P <0.001 vs AngII+IL-1 β . n=6.

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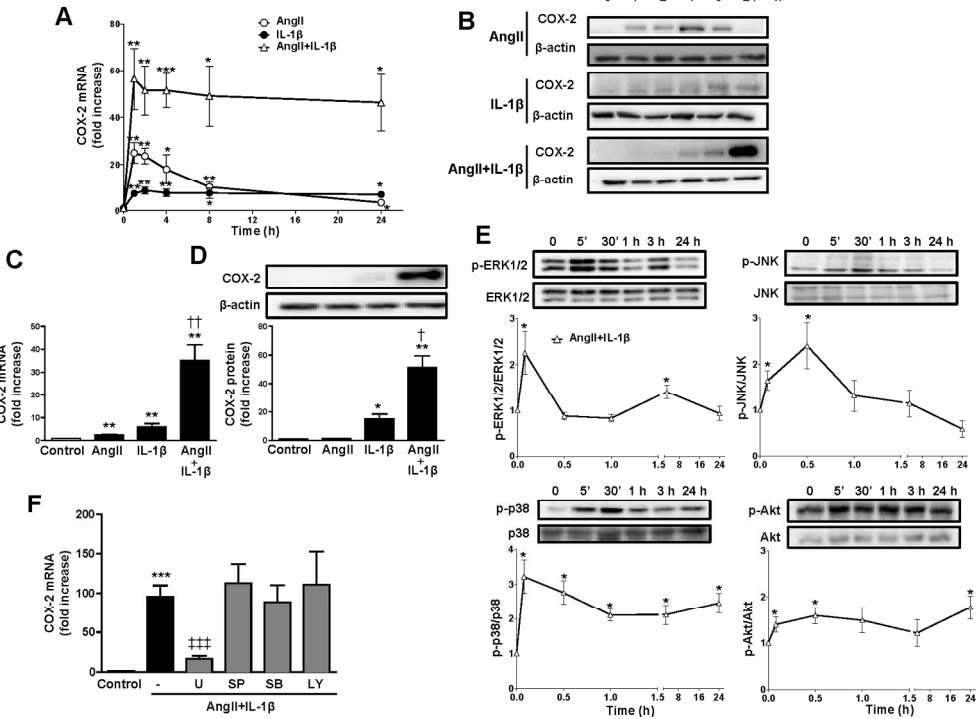
Figure 7. COX-2-derived PGE₂ participates in vascular remodeling. (A) Representative immunohistochemical staining of three independent experiments of COX-2, mPGES-1, TXAS and HuR expression in non-ligated and ligated carotid arteries. (B) COX-2, mPGES-1, TXAS and HuR mRNA levels and HuR immunofluorescence in aorta from untreated or AngII-treated mice. (C) TN-C mRNA levels in aorta from mice untreated or treated with AngII or AngII plus celecoxib and mPGES-1^{+/+} (wild type, WT) and mPGES-1^{-/-} mice infused or not with AngII. (D,E) Representative photographs of hematoxylin-eosin aortic sections, media/lumen ratio and cross-sectional-area (CSA) from untreated or treated with AngII or AngII plus celecoxib-treated and from WT and mPGES-1^{-/-} mice infused or not with AngII. Data are expressed as mean \pm SEM. * P <0.05, ** P <0.01 vs Untreated; [†] P <0.05, ^{††} P <0.01 vs AngII. n=3-7.

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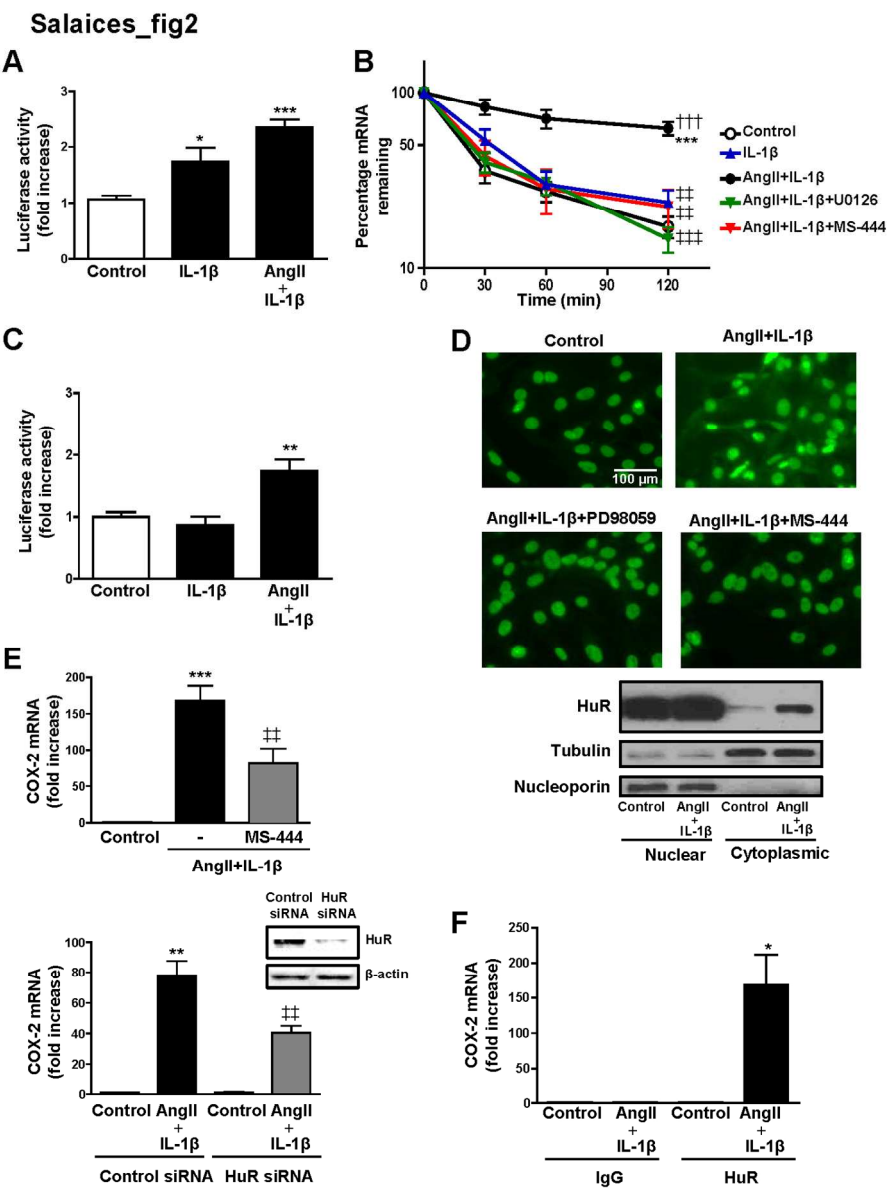
Figure 8. Scheme showing the role of HuR in AngII and IL-1 β effects on vascular COX-2 expression and cell migration. In VSMC AngII and IL-1 β synergistically induces COX-2 expression through ERK1/2 pathway. ERK1/2 activation leads to HuR binding to COX-2 3'UTR mRNA and exportation to the cytoplasm where HuR increases COX-2 mRNA stability. COX-2 is translated to protein and converts arachidonic acid in PGH₂ and TXAS and mPGES-1 will transform it in PGE₂ and TXA₂. Both prostanoids through TP and EP1/EP3 increase TN-C expression and would activate EGFR (EGFR transactivation) that will lead to ERK1/2, JNK, CaM/pCaMKII activation ending in cell migration and vascular remodeling. Inhibitors used in the experiments are showed in the scheme with their protein targets marked with bar-headed lines. Dashed lines indicate non-investigated mechanisms.

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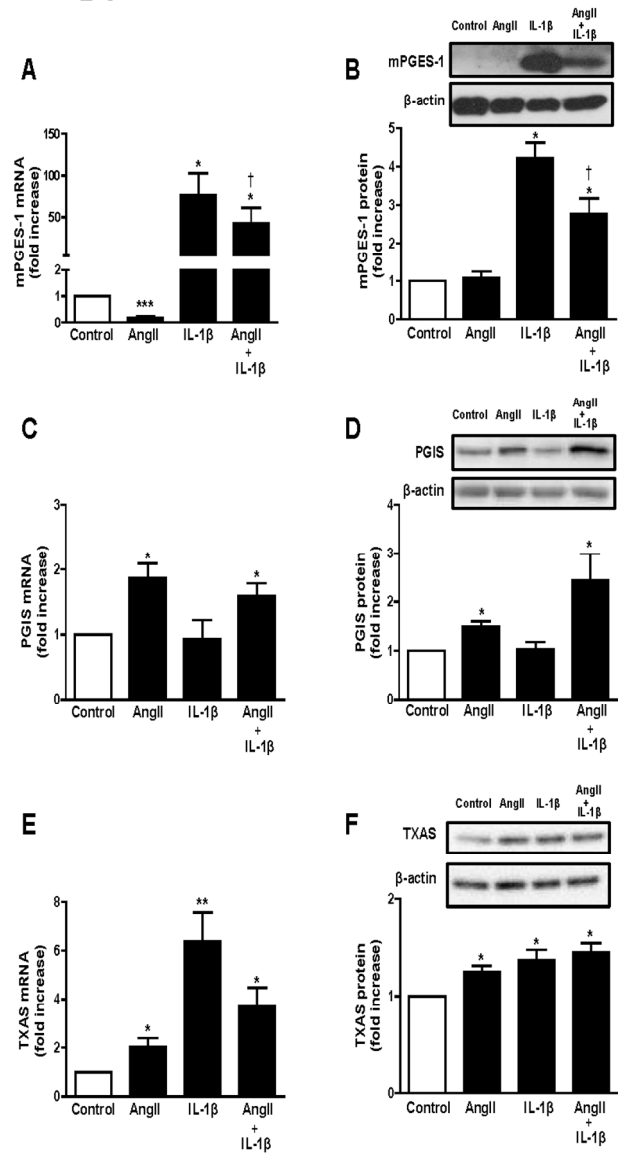


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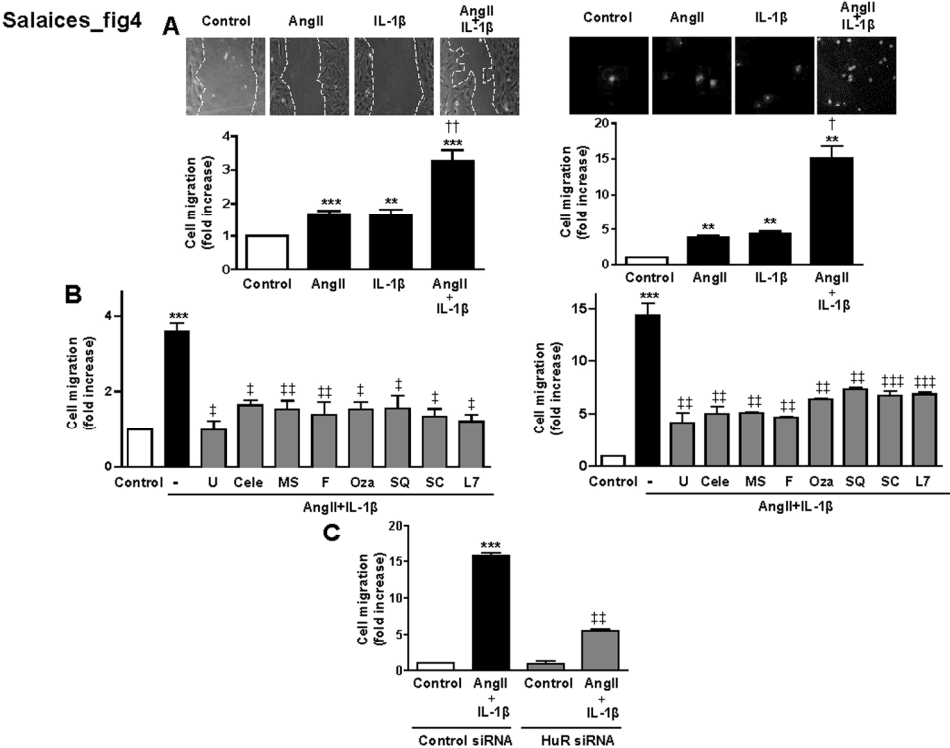


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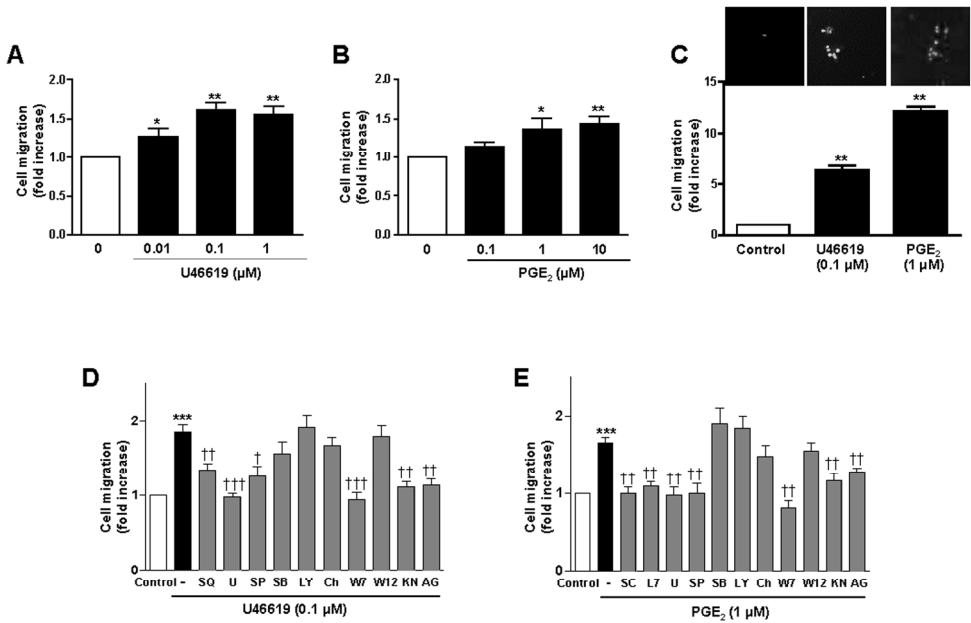


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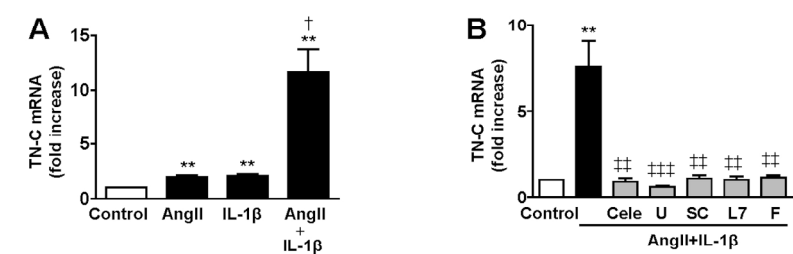
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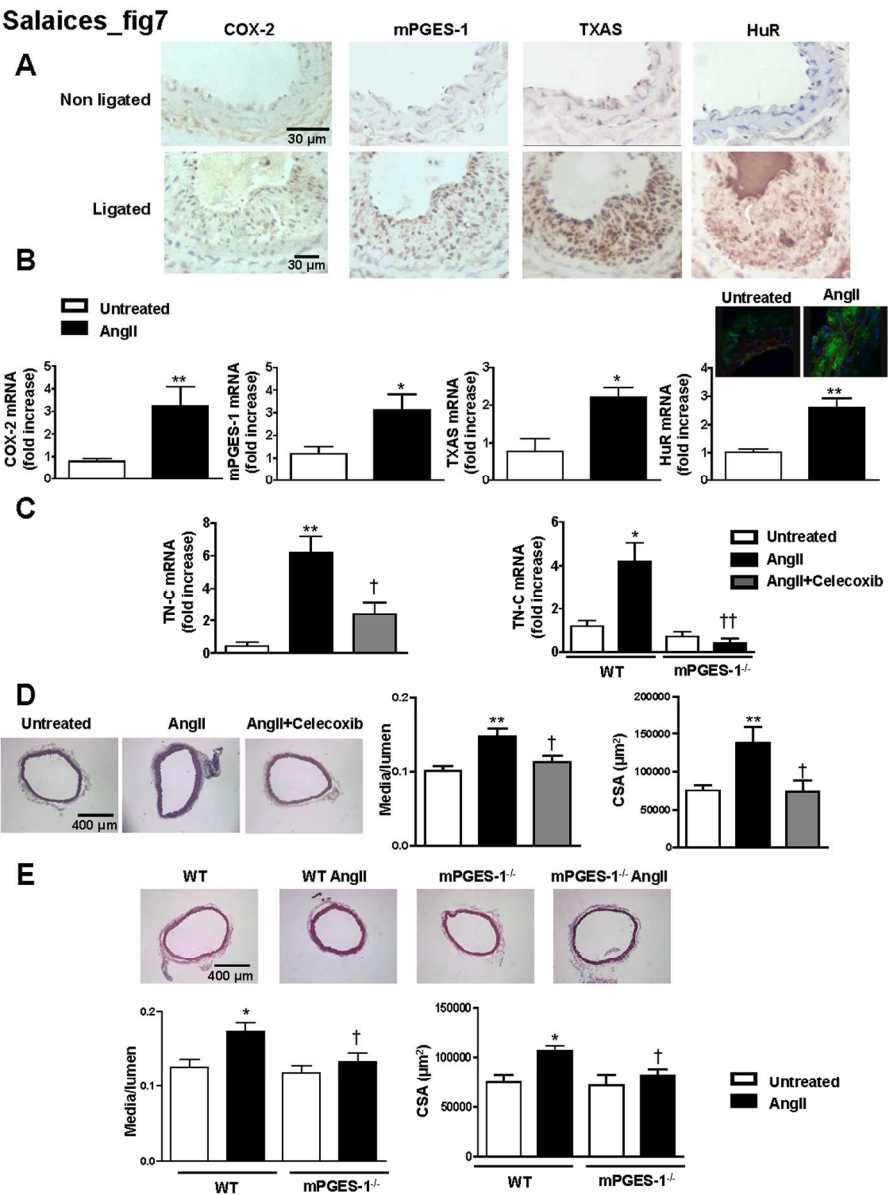


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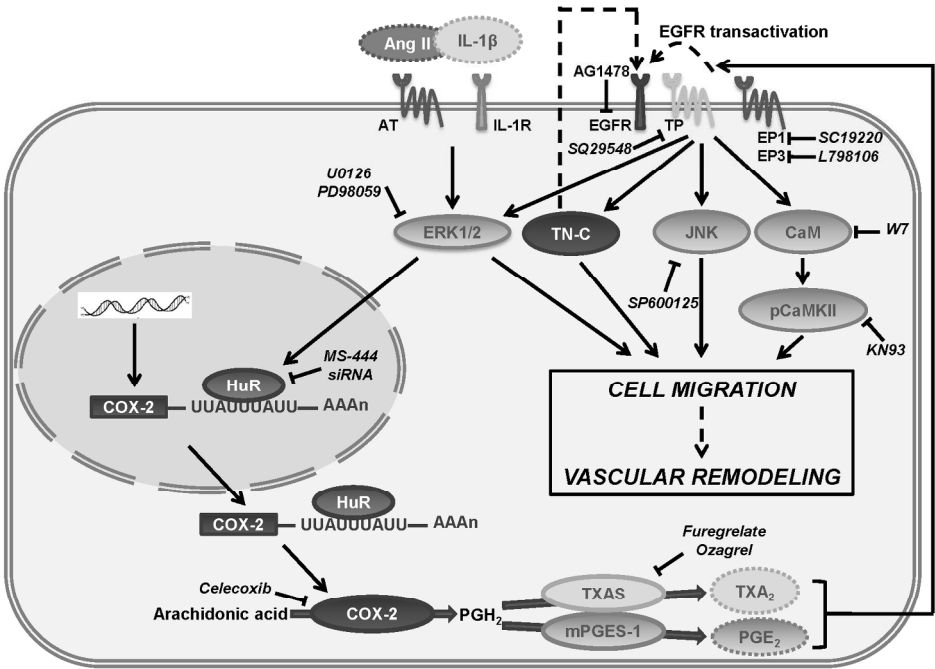


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Salaices_fig8

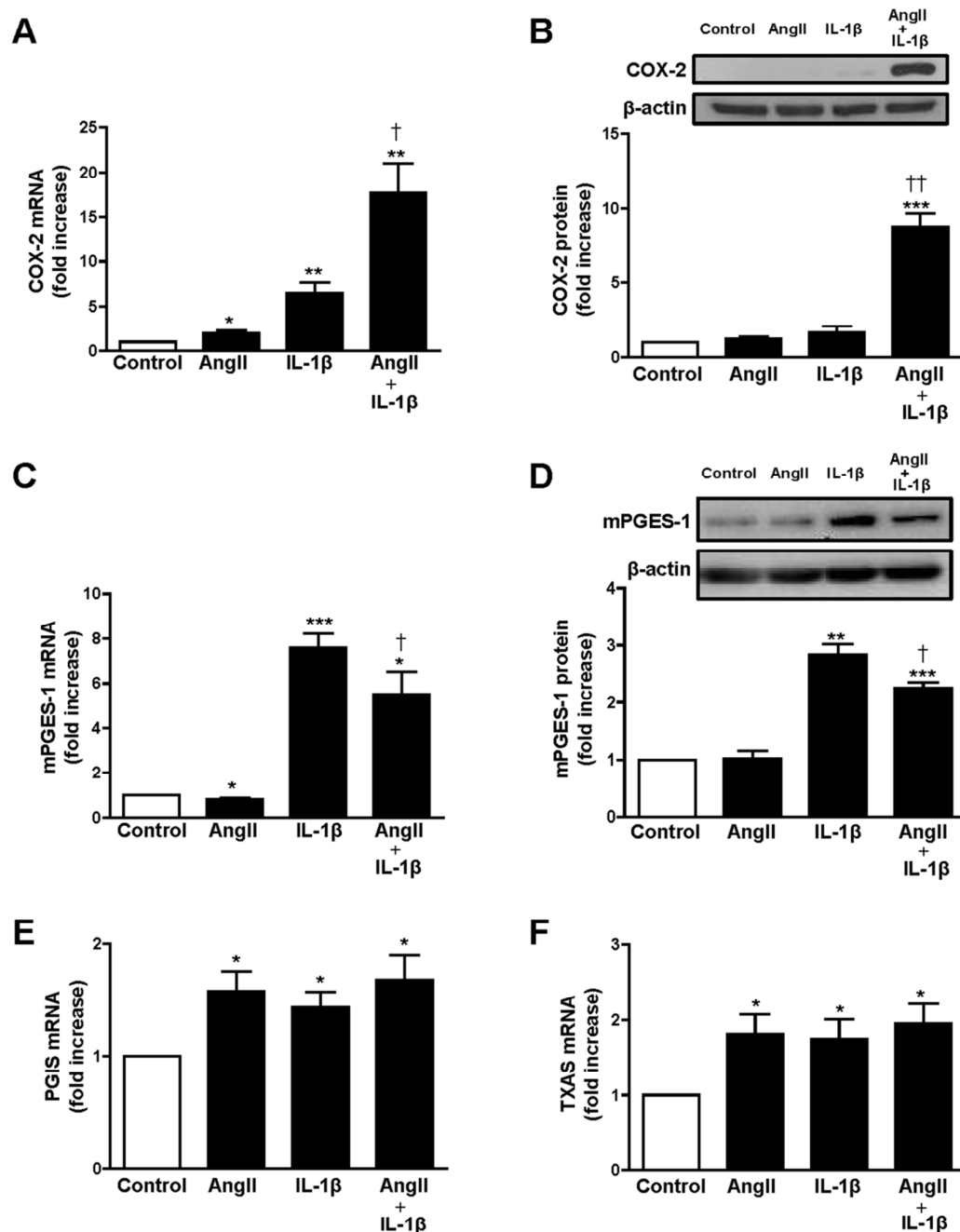


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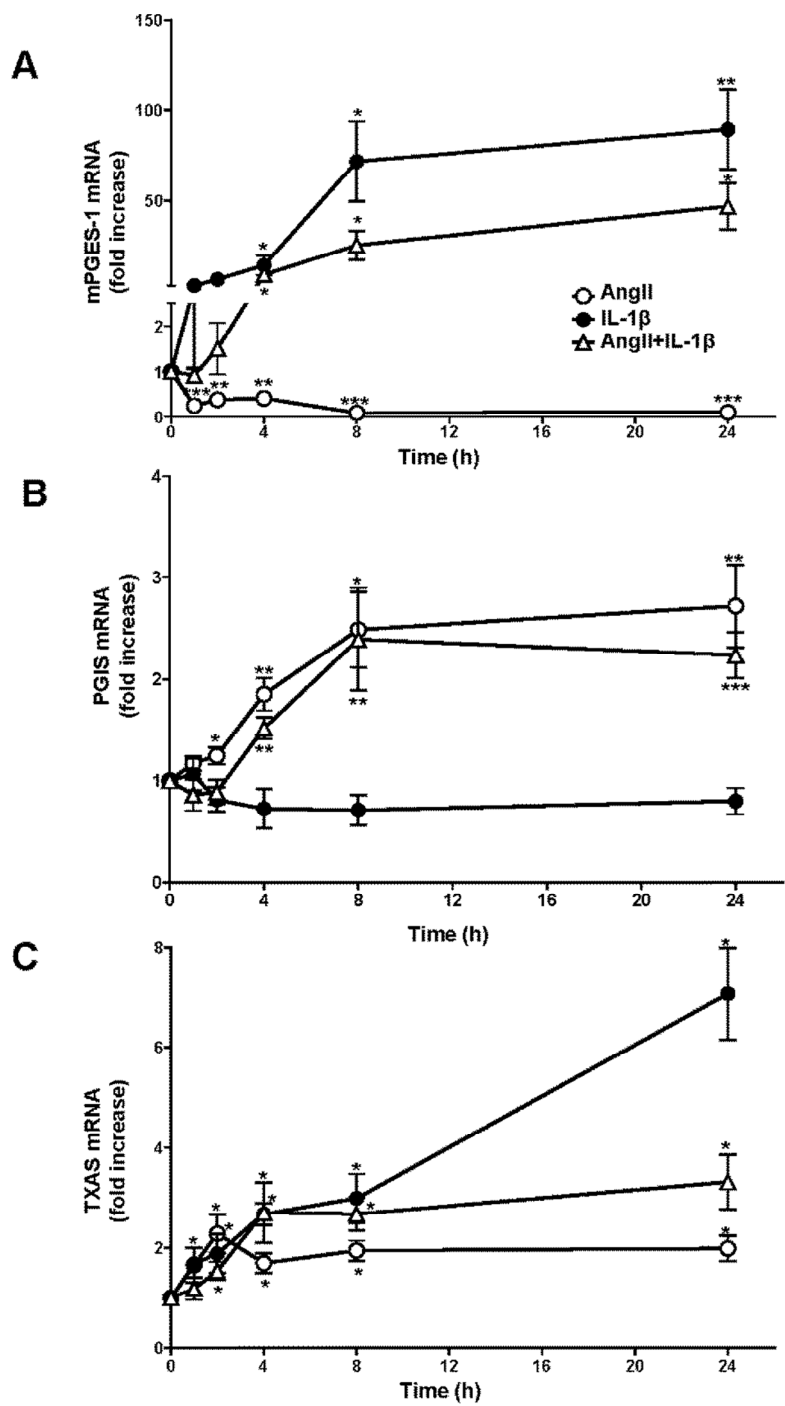
Supporting Data**HuR mediates the synergistic effects of angiotensin II and interleukin 1 β on vascular COX-2 expression and cell migration**

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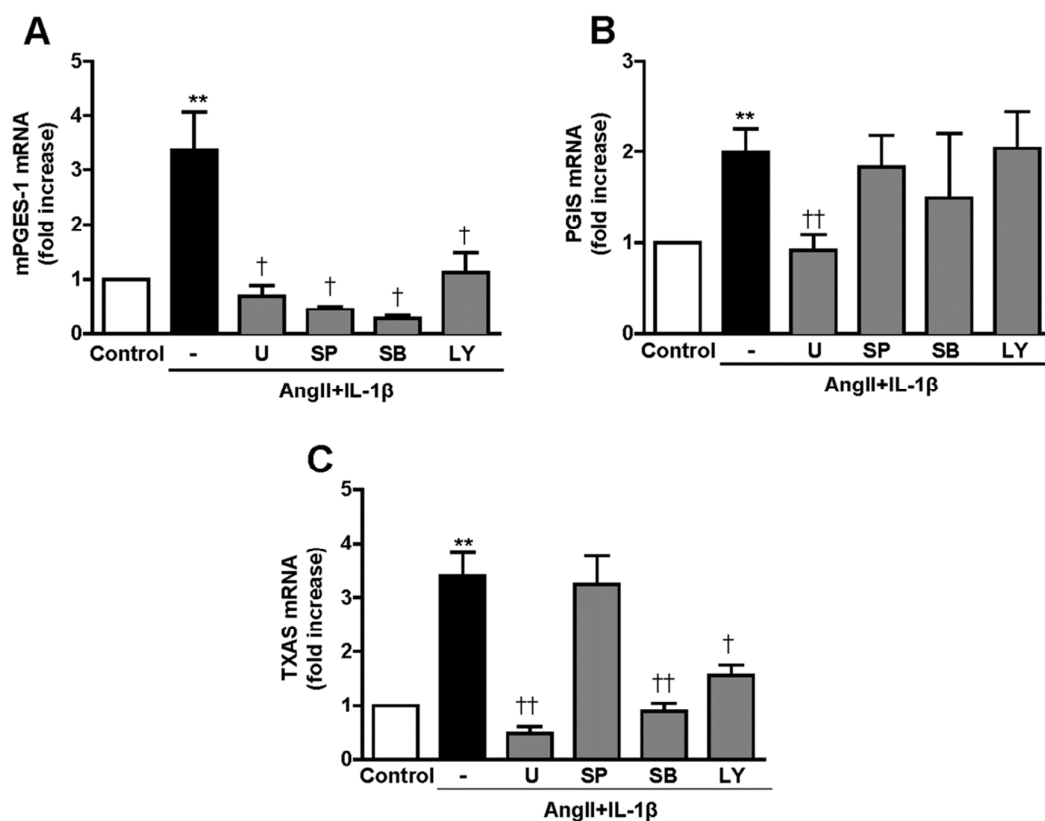
For Peer Review



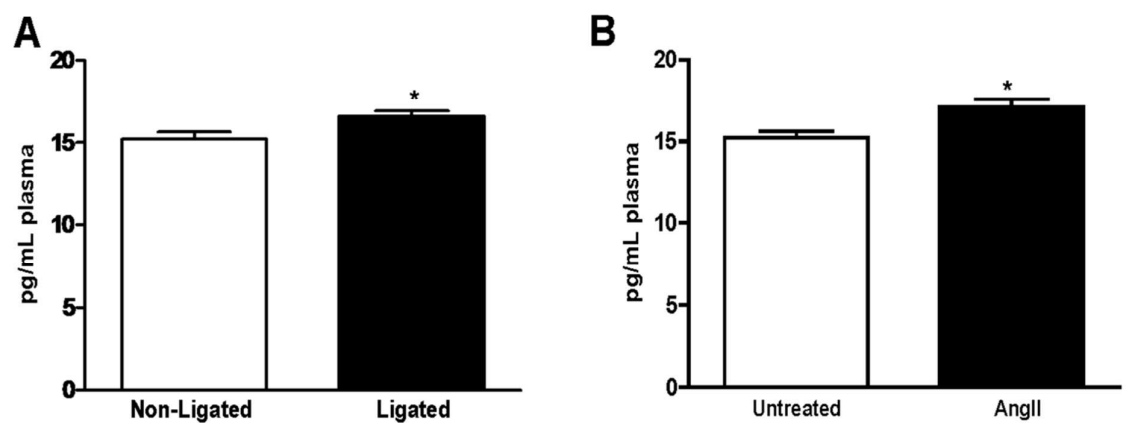
Supporting Figure 1. AngII and IL-1β regulate differentially COX-2, mPGES-1, PGIS and TXAS expressions. Effect of AngII, IL-1β or AngII+IL-1β (24 h) on COX-2 mRNA (A) and protein (B) levels, mPGES-1 mRNA (C) and protein (D) levels, and PGIS (E) and TXAS (F) mRNA levels in human VSMC. Data are expressed as mean ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs Control; † $P < 0.05$ vs AngII or IL-1β. n=5-7.



Supporting Figure 2. Effect of AngII, IL-1β and AngII+IL-1β on mPGES-1, PGIS and TXAS expressions. Time course of mPGES-1 (A), PGIS (B) and TXAS (C) mRNA levels in rat VSMC. Data are expressed as mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001 vs unstimulated cells. n=4-7.



Supporting Figure 3. Different signaling pathways are involved in mPGES-1, PGIS and TXAS expression. Effect of ERK1/2 (U0126, U), JNK (SP600125, SP), p38 MAPK (SB203580, SB) and PI3K (LY294002, LY) inhibitors on mPGES-1 (A), PGIS (B), TXAS (C) mRNA levels in rat VSMC stimulated with AngII+IL-1 β . Data are expressed as mean \pm SEM. ** P <0.01 vs Control; † P <0.05, †† P <0.01 vs AngII+IL-1 β . n=5-8.



Supporting Figure 4. PGE₂ levels are increased in two models of vascular damage. PGE₂ levels in plasma of AngII-infused (A) and carotid ligation (B) mouse models. Data are expressed as mean \pm SEM. **P*<0.05 vs non-ligated or untreated mice. n=5-8.

Supporting Table 1

Gene	Specie	FW	RV
COX-2	mouse	TTCGGGAGCACAAACAGAGT	TAACCGCTCAGGTGTTGCAC
HuR	mouse	ATGAAGACCACATGGCGGAAGAC	AGTTCACAAAACCGTAGCCCAAGC
mPGES-1	mouse	AGGATGCGCTGAAACGTGGAG	CCGAGGAAGAGGAAAGGATAG
TN-C	mouse	TGTGAAGGCATCCACGGAAGAA	TGCAGTCCAGTTGAGTCTGA
COX-2	rat	AAGGGAGTCTGGAACATTGTGAAC	CAAATGTGATCTGGACGTCAACA
HuR	rat	TGTACATCAGTGGGCTTCCA	CTGCTTCAGACCGTTTGTC
mPGES-1	rat	AGGAGTGACCCAGATGTG	ATGTATCCAGGCGATGAGA
PGIS	rat	CCATCAACAGCATCAAACAGTTT	CAAAGCCATATCTGCTAAGGTCAA
TN-C	rat	ACCTCTCTGGAATTGCTCCCA	CATCTGAAACTAGAAGGTTGTC
β_2 -microglobulin	Rat mouse	ACCCTGGTCTTTCTGGTGCTT	TAGCAGTTCAGTATGTTCCGGCTT