Laminar flow activation of ERK5 leads to cytoprotective effect via CHIP-mediated p53 ubiquitination in endothelial cells

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Abstract: Atherosclerosis is readily observed in areas where disturbed flow is formed, while the atheroprotective region is found in areas with steady laminar flow (L-flow). It has been established that L-flow protects endothelial cells against endothelial dysfunction, including apoptosis and inflammation. It has also been reported that extracellular signal-regulated kinase 5 (ERK5) regulated endothelial integrity and protected endothelial cells from vascular dysfunction and disease under L-flow. However, the molecular mechanism by which L-flow-induced ERK5 activation inhibits endothelial apoptosis has not yet been determined. Transcription factor p53 is a major pro-apoptotic factor which contributes to apoptosis in various cell types. In this study, we found that 15-deoxy-Δ(12,14)-prostaglandin J$_2$ induced p53 expression and that endothelial apoptosis was reduced under the L-flow condition. This anti-apoptotic response was reversed by the biochemical inhibition of ERK5 activation. It was also found that activation of ERK5 protected endothelial apoptosis in a C terminus of Hsc70-interacting protein (CHIP) ubiquitin ligase-dependent manner. Moreover, molecular interaction between ERK5-CHIP and p53 ubiquitination were addressed with a CHIP ubiquitin ligase activity assay. Taken together, our data suggest that the ERK5-CHIP signal module elicited by L-flow plays an important role in the anti-apoptotic mechanism in endothelial cells.

Key words: Laminar flow, Endothelial apoptosis, ERK5, CHIP, p53

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Introduction

Atherosclerosis is a localized vascular disease characterized by the accumulation of lipids, leukocytes, and fibrous elements that go on to form arterial plaques [1, 2]. It has been well established that arterial plaque develops in certain areas such as bifurcations and curvatures, which has been proven by examining differences in blood flow dynamics [3]. Atheroprotective regions contain high, unidirectional and steady laminar flow (L-flow) while atheroprone regions develop in low and disturbed flow areas [4]. The developmental process of atherosclerosis can be initiated by endothelial dysfunction, which includes activation of inflammatory responses and compromised barrier function due to endothelial apoptosis [5]. Manifestations of dysfunctional endothelial cells (ECs) were readily observed in certain areas of the arterial tree, while endothelial apoptosis was found to be much lower in legions exposed to L-flow [6]. Many atheroprotective molecules are regulated in response to L-flow [7]. However, what is lacking in current research is a plausible mechanistic relationship between L-flow-mediated anti-apoptotic effects and any of the known regulators of

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endothelial apoptosis that play a role in accelerating atherosclerosis formation.

Extracellular signal-regulated kinase 5 (ERK5) is one of major modulator in L-flow-mediated cytoprotective responses in ECs. ERK5 belongs to the mitogen activated protein kinase (MAPK) family, which has dual phosphorylation sites characterized by a TEY motif. MAPK/ERK kinase 5 (MEK5) phosphorylates the TEY motif and activates ERK5 kinase activity. In contrast to ERK1/2, ERK5 has unique transactivation domain, suggesting that its regulation and function may be different from ERK1/2 [8, 9]. Like many MAPK family members, ERK5 plays a significant role in cell growth and differentiation, although emerging evidence suggests that it has unique functional characteristics. The redox activation of ERK5 has been documented as having an anti-apoptotic effect [10] and it was found that ERK5 knockout mice had impaired vascular development due to endothelial apoptosis [11], suggesting that ERK5 is an important regulator in endothelial survival. It has been reported that ERK5 is involved in the cytoprotective effect that occurs in ECs in response to L-flow [12]. Endothelial apoptosis elicited by serum deprivation was markedly diminished under exposure to L-flow, but this protective response was reversed by transducing the dominant negative form of ERK5, indicating the cytoprotective role of ERK5 in L-flow signaling pathways. Garin et al. [13] reported that an L-flow antagonized tumor necrosis factor alpha (TNFα)-induced endothelial apoptosis via inhibiting the caspase-dependent signaling pathway. These results implied that ERK5 acts as a key upstream molecule in L-flow-mediated anti-apoptotic responses via inhibiting the caspase-dependent pro-apoptotic pathway.

15-deoxy-Δ(12,14)-prostaglandin J_2 (15d-PGJ$_2$) is a member of the cyclopentenone prostaglandins and is synthesized by cyclooxygenase pathways in various cell types. Because of the strong relationship between cyclooxygenase-dependent inflammatory responses and vascular inflammation, its lipid mediators might be involved in inflammatory vascular diseases, including atherosclerosis. For instance, oxidized low-density lipoprotein (LDL) increases the intracellular level of 15d-PGJ$_2$ in a cyclooxygenase-dependent manner. In addition, expression of Cox-2, oxidized LDL, and 15d-PGJ$_2$ were induced in atherosclerotic lession of ApoE$^{-/-}$ mice [14]. Unlike other prostaglandins, 15d-PGJ$_2$ has no corresponding receptors on the cell surface. 15d-PGJ$_2$ can be actively transported into cells and acts through direct interactions with its selective nuclear targets, including the nuclear transcriptional factor peroxisome proliferator-activated receptor gamma (PPARγ) [15]. Interestingly, its cyclopentenone moiety provides an electrophilic carbon that mediates covalent modification with nucleophiles such as the free sulfhydryls of glutathione or the cysteine residues in target proteins [16]. These results suggest that 15d-PGJ$_2$ is not only a ligand of PPARγ, but also an inducer of covalent modifications of cellular target proteins, and is regulated in a PPARγ-independent manner. Several studies have demonstrated that 15d-PGJ$_2$ induced apoptosis in ECs as well as in various cancer cell lines. Some of these suggested that 15d-PGJ$_2$-mediated apoptosis was regulated through the activation of PPARγ [17, 18]. However, recent reports have indicated that 15d-PGJ$_2$-induced endothelial apoptosis is independent on PPARγ [19, 20]. For example, many of proapoptotic signal pathways induced by 15d-PGJ$_2$ have been shown to be independent of PPARγ in cancer cell lines. Although the involvement of PPARγ in 15d-PGJ$_2$-induced endothelial apoptosis is yet to be determined, p53 is a well established proapoptotic target in 15d-PGJ$_2$-induced endothelial apoptosis [18, 21]. Ho et al. [22] reported that 15d-PGJ$_2$ induced endothelial apoptosis via stabilizing p53 [22], suggesting the possible involvement of a ubiquitin-dependent proteasome pathway. The protein stability of p53 was mainly regulated by E3 ubiquitin ligase, including a mouse double minute 2 homolog (MDM2) and the C terminus of Hsc70-interacting protein (CHIP) [23-25]. Recently, we found that ERK5 activation of CHIP ubiquitin ligase activity protected cardiomyocytes via CHIP-mediated ubiquitination and degradation of the inducible cAMP early repressor [26]. Clearly, p53 expression regulates 15d-PGJ$_2$-induced endothelial apoptosis, but the molecular mechanism by which L-flow activation of ERK5 downregulates p53 protein expression via the ubiquitin-proteasome pathway remains largely unknown.

In this study, we found that ERK5 regulated L-flow-mediated anti-apoptotic responses in ECs. In addition, we discovered that ERK5 activation protects ECs from 15d-PGJ$_2$-induced endothelial apoptosis via CHIP-mediated p53 ubiquitination. Thus, these data demonstrate that L-flow-mediated ERK5-CHIP signal cascade may play a critical role of anti-apoptotic responses.

Materials and Methods

Reagents and antibodies

15d-PGJ$_2$ was purchased from Cayman (Ann Arbor, MI, USA). BIX 02189, a specific inhibitor of MEK5, was purchased from Selleck Chemicals (Houston, TX, USA). MTT reagents, H$_2$O$_2$ and DMSO, were purchased from Sigma (St. Louis, MO, USA). Antibodies were purchased from the following vendors: cleaved caspase 3 and phospho-ERK5 (Cell Signaling Technology, Danvers, MA, USA); tubulin (Sigma); poly (ADP-ribose) polymerase (PARP), CHIP, and p53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Cell culture and laminar flow

Human umbilical vein endothelial cells (HUVECs) were grown on 0.2% gelatin coated dish using an endothelial growth medium LSGS (Cascade Biologics, Portland, OR, USA) and 5% fetal bovine serum. Confluent HUVECs cultured in 100 mm dishes were exposed to laminar flow (12 dynes/cm^2) unidirectional flow for 24 hours using a cone flow system with 5% CO$_2$ and at 37°C, as described previously [27].

Transfection of the siRNAs and transduction of adenoviral vectors

Small interfering RNAs against human p53 and CHIP were purchased from Santa Cruz Biotechnology (sc-29435 and sc-43555, respectively). For our transient expression experiments, HUVECs were transiently transfected with 100 pM of control RNA or specific siRNAs against p53 or CHIP and using Lipofectamine 2000 reagent (#11668-019, Invitrogen, Carlsbad, CA, USA) following manufacturer protocols. A non-specific control siRNA from Invitrogen was used as a negative control. The adenoviral vector encoding constitutively active form of MEK5α (Ad-CA-MEK5α) was described in a previous report [28].

Western blot analysis and Immunoprecipitation analysis

HUVECs were lysed and extracts were incubated on ice for 15 minutes and then centrifuged (15,000 g for 15 minutes at 4°C). The concentrations in samples were determined using a Bradford assay. Primary antibodies were used to detect respective proteins, and visualized by using enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech) according to the manufacturer's instructions. To conduct Immunoprecipitation analysis, cell lysates were incubated with an anti-CHIP antibody (Santa Cruz Biotechnology) or an anti-p53 antibody (Santa Cruz Biotechnology) overnight at 4°C as we described in a previous report [26].

Quantitative real time reverse transcriptase-polymeratse chain reaction (RT-PCR)

To analyze mRNA expression of p53, a quantitative real time RT-PCR was performed. Briefly, the total RNA was isolated by TRIzol reagent (Invitrogen) and then a reverse transcription reaction was conducted by using Taq-Man reverse transcription reagents (Applied Biosystems, Carlsbad, CA, USA), following the manufacturer's instructions. Quantitative real time RT-PCR was conducted using 1 μl of template cDNA and Power SYBR Green (Applied Biosystems). Quantification was carried out using the efficiency-corrected ΔΔCq method. The specific oligonucleotide sequences for target genes were the following: *TP53* (p53) forward 5'-GCCTGAGGTTGGCTCTGA-3' and reverse 5'-GTGGTGAGGCTCCCCTTT-3', glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) forward 5'-GGAGCCAAAAGGGTCATCAT-3' and reverse 5'-GTGATGGCATGGACTGTGGT-3'.

In vitro ubiquitination assay with GST-p53

Ubiquitination activity was determined by *in vitro* ubiquitination assay, using GST-fused p53 recombinant protein as a substrate (Ubiquitin-Protein Conjugation kit, Boston-Biochem, Cambridge, MA, USA) as described previously [26]. Briefly, the lysates were applied for Immunoprecipitation analysis with an anti-CHIP antibody. Immunoprecipitated CHIP was incubated with recombinant proteins, including ubiquitin and an E1/E2 enzyme mixture, in energy buffer for 60 minutes at 37°C. The reaction was stopped by adding sodium dodecyl sulfate loading buffer and then followed by immunoblotting with an anti-ubiquitin antibody.

MTT assay

HUVECs were exposed to 10 μM of 15d-PGJ$_2$ for 6 hours and then further incubated with MTT reagent for 2 hours in a 37°C incubator. Precipitants were washed with phosphate buffered saline and eluted by adding a 500 μl mixture solution of DMSO and absolute ethanol (1 : 1). Cell viability was then measured by microplate reader (BIO-RAD, Hercules, CA, USA) at 570 nm, as described in a previous study [29].

Statistical analysis

Data are reported as the mean±SD using Student's *t*-testing. A probability value of <0.05 was considered as significant. *P*-values of less than 0.01 are indicated by *.

Results

Laminar flow inhibits 15d-PGJ₂-induced endothelial apoptosis

We examined whether L-flow protects ECs from 15d-PGJ₂-induced apoptosis. Confluent HUVECs were incubated under the L-flow (12 dynes/cm²) or static condition for 24 hours and then followed by exposure to 10 μM PGJ₂ for 8 hours. As shown in Fig. 1A, L-flow inhibited PARP cleavage and caspase 3 cleavage in response to 15d-PGJ₂. In addition to the protein expression of proapoptotic molecules, we determined cell viability by using MTT assay. Treatment of 15d-PGJ₂ significantly reduced cell viability, but this reduction was completely reversed under the L-flow condition (Fig. 1B). Consistent with the viability assay results, we observed the same tendency with regards to morphological changes under control 15d-PGJ₂, and 15d-PGJ₂ under L-flow conditions (Fig. 1C-E). These results suggest that L-flow protects HUVECs from 15d-PGJ₂-induced apoptosis.

L-flow inhibits 15d-PGJ₂-induced endothelial apoptosis via p53 downregulation

To examine the involvement of p53 in L-flow-mediated cytoprotective responses against 15d-PGJ₂, we examined p53 protein expression with the same condition. Western blot analysis showed that 15d-PGJ₂-induced p53 protein expression was markedly reduced in HUVECs cultured under L-flow (Fig. 2A). To assess the role of p53 induction
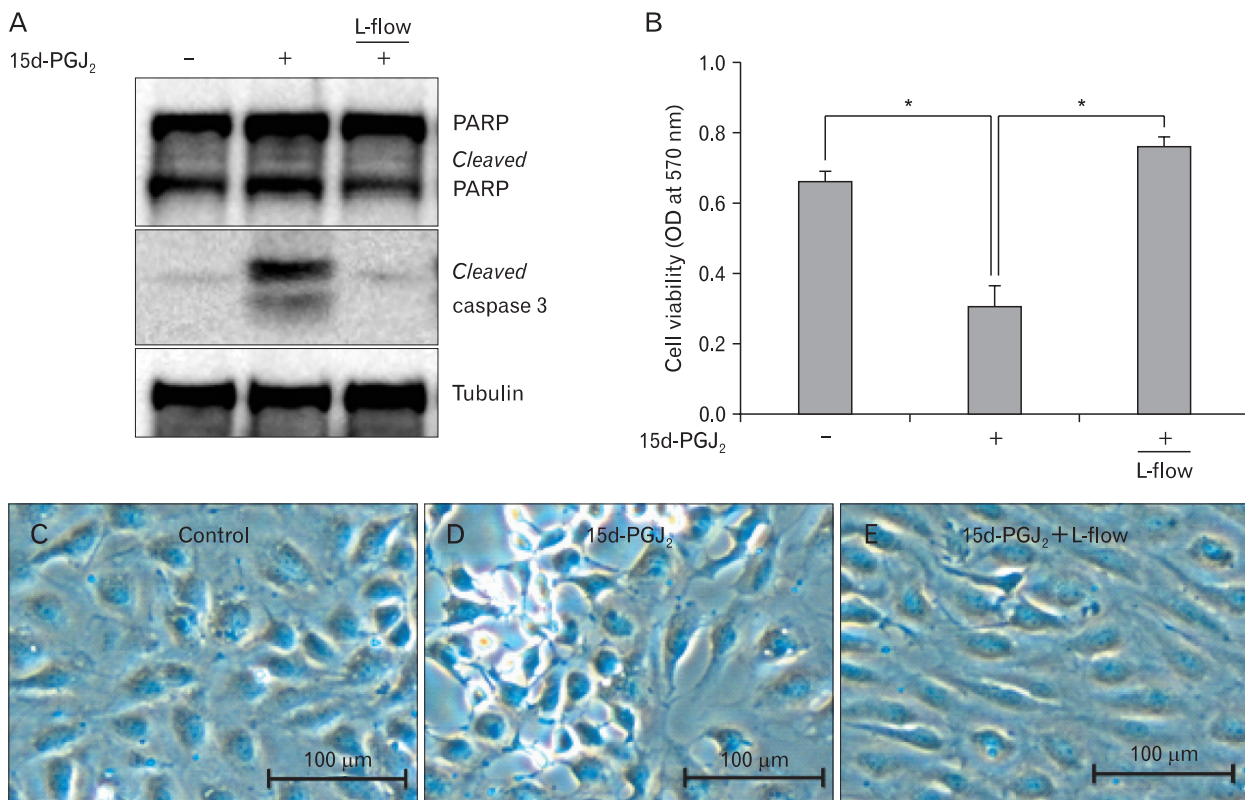

Fig. 1. Laminar flow inhibits 15d-PGJ₂-induced endothelial apoptosis. Confluent HUVECs were cultured under L-flow (12 dyne/cm²) or a static condition for 24 h. Cells were then exposed to 10 μM 15d-PGJ₂ for 8 h. (A) Cell lysates were applied for immunoblotting with anti-PARP, anti-cleaved caspase 3, and anti-tubulin antibodies. Data are representative of results from three separate experiments. (B) Cell viability was determined by using MTT assay. Data are expressed as the mean±SD from three independent experiments. *P<0.01. Morphological changes of HUVECs were examined by microscopy under control (C), 15d-PGJ₂ (D), or 15d-PGJ₂ under L-flow (E). Scale bars=100 μm (C-E). 15d-PGJ₂, 15-deoxy-Δ(12,14)-prostaglandin J₂; HUVECs, human umbilical vein endothelial cells; L-flow, steady laminar flow; PARP, poly (ADP-ribose) polymerase; OD, optical density.

in 15d-PGJ$_2$-induced endothelial apoptosis, we performed genetic depletion of p53 with siRNA against human p53. As shown in Fig. 2B, depletion of p53 reduced the level of cleaved caspase 3 in response to 15d-PGJ$_2$. In line with this observation, we found that 15d-PGJ$_2$-mediated reduction of cell viability was reversed by the depletion of p53 (Fig.

2C). These experimental results imply that L-flow-mediated reduction of p53 might be a key part of the protection of ECs from 15d-PGJ$_2$-induced apoptosis.
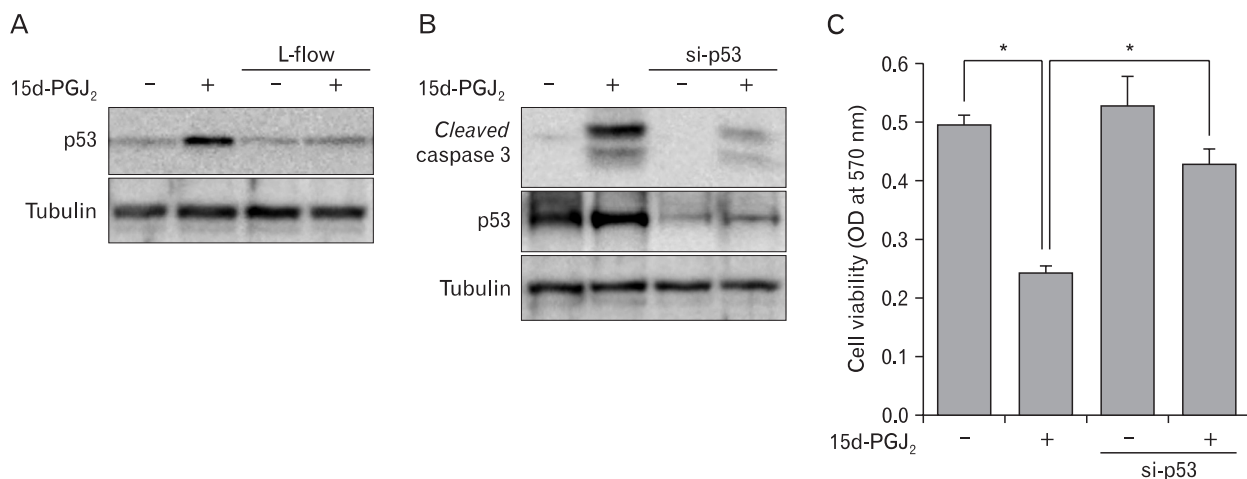

Fig. 2. L-flow inhibits 15d-PGJ$_2$-induced endothelial apoptosis via p53 downregulation. (A) HUVECs were exposed to L-flow followed by treatment with 15d-PGJ$_2$ for 8 h. Protein expression was determined by Western blotting using anti-p53 and anti-tubulin antibodies. (B) HUVECs were transfected with either control or p53 siRNA (si-p53) for two days. Cells were then exposed to 10 μM 15d-PGJ$_2$ for 8 h. The expression of cleaved caspase 3, p53, and tubulin was detected by Western blotting with the respective antibodies. (C) Cell viability was determined by MTT assay. Data are expressed as the mean±SD from three independent experiments. *P<0.01. L-flow, steady laminar flow; 15d-PGJ$_2$, 15-deoxy-Δ(12,14)-prostaglandin J$_2$; HUVECs, human umbilical vein endothelial cells.
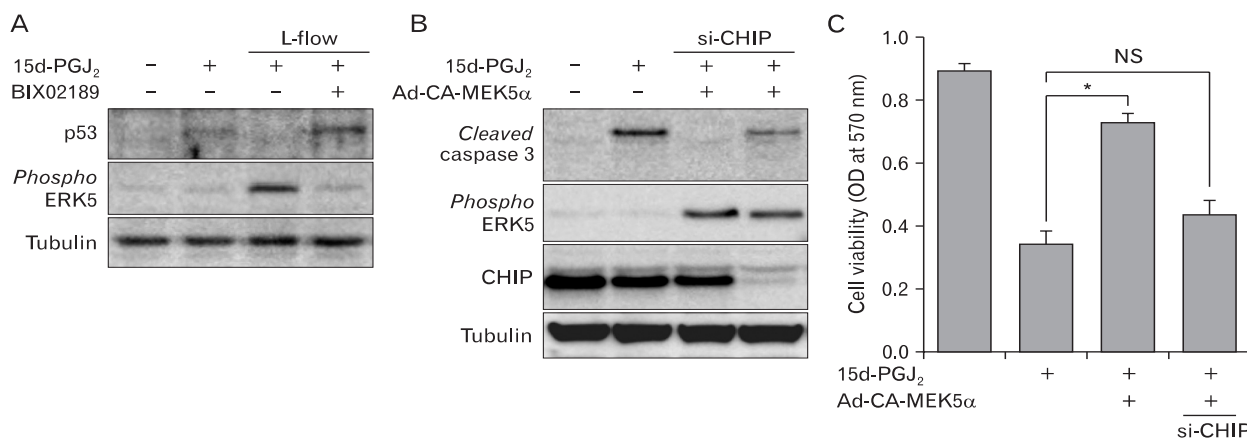

Fig. 3. The ERK5-CHIP signal module protects endothelial apoptosis in response to 15d-PGJ$_2$. (A) HUVECs were pretreated with DMSO or 10 μM BIX02189, a specific inhibitor of MEK5, before applying L-flow. Cells were then exposed to 10 μM 15d-PGJ$_2$ for 8 h. The protein expression of p53, phosphorylated ERK5, and tubulin were determined by immunoblotting with specific antibodies. (B) HUVECs were transfected with siRNA against human CHIP (si-CHIP) or control RNA and then followed by transducing the adenovirus encoding constitutively active form of MEK5 alpha (Ad-CA-MEK5α). Cells were then exposed to 10 μM 15d-PGJ$_2$ for 8 h. Cell lysates were subjected to Western blot analysis with anti-cleaved caspase 3, anti-phospho ERK5, anti-CHIP, and anti-tubulin antibodies. (C) Cell viability was determined by MTT assay. Data are expressed as the mean±SD from three independent experiments. *P<0.01. ERK5, extracellular signal-regulated kinase 5; CHIP, C terminus of Hsc70-interacting protein; 15d-PGJ$_2$, 15-deoxy-Δ(12,14)-prostaglandin J$_2$; HUVEC, human umbilical vein endothelial cells; OD, optical density; NS, non-significant.

ERK5-CHIP signal module protects endothelial apoptosis induced by 15d-PGJ$_2$

Next, we assessed the role of ERK5 in regulating p53 expression induced by 15d-PGJ$_2$ with biochemical inhibition. The pretreatment of a BIX02189 compound, which is a specific inhibitor of MEK5, suppressed L-flow-mediated reduction of p53 expression in response to 15d-PGJ$_2$, suggesting the potential role of ERK5 in the regulation of p53 expression (Fig. 3A). To confirm the specific role of ERK5 activation in endothelial apoptosis, we utilized an adenoviral system to express the constitutively active form of MEK5 alpha (Ad-CA-MEK5α). As shown in Fig. 3B, the level of cleaved caspase 3 was remarkably reduced by overexpressing CA-MEK5α. This reduction of cleaved caspase 3 was reversed by the depletion of CHIP ubiquitin ligase with siRNA against human CHIP. Additionally, we found that a similar pattern was observed using a cell viability assay (Fig. 3C). Along with previous evidence that has suggested that CHIP is a known ubiquitin ligase of p53 and that ERK5 activates the ubiquitin ligase activity of CHIP, our results sugges that L-flow activation of ERK5 inhibits 15d-PGJ$_2$-induced endothelial apoptosis via

CHIP-mediated p53 ubiquitination.

ERK5 activation induces p53 ubiquitination via increasing CHIP ubiquitin ligase activity

To determine the involvement of transcriptional regulation of p53 by ERK5, the mRNA level of p53 was determined by quantitative RT-PCR. Neither 15d-PGJ$_2$ treatment nor Ad-CA-MEK5α transduction resulted in any significant changes (Fig. 4A). This may be because p53 can be regulated by ubiquitination at the post-translation level. As shown in Fig. 4B, p53 ubiquitination was increased by transduction of Ad-CA-MEK5α, suggesting the involvement of post-translation modification in ERK5-mediated p53 downregulation (Fig. 4B). To address whether ERK5 activation induced p53 ubiquitination via increasing the ubiquitin ligase activity of CHIP, we utilized BIX02189 in the presence or absence of Ad-CA-MEK5α, and then detected CHIP ubiquitin ligase activity using *in vitro* ubiquitination assay with GST-fused p53 recombinant protein as substrate. As shown in Fig. 4, transduction with Ad-CA-MEK5α markedly increased the level of p53 ubiquitination, which was inhibited by bio-
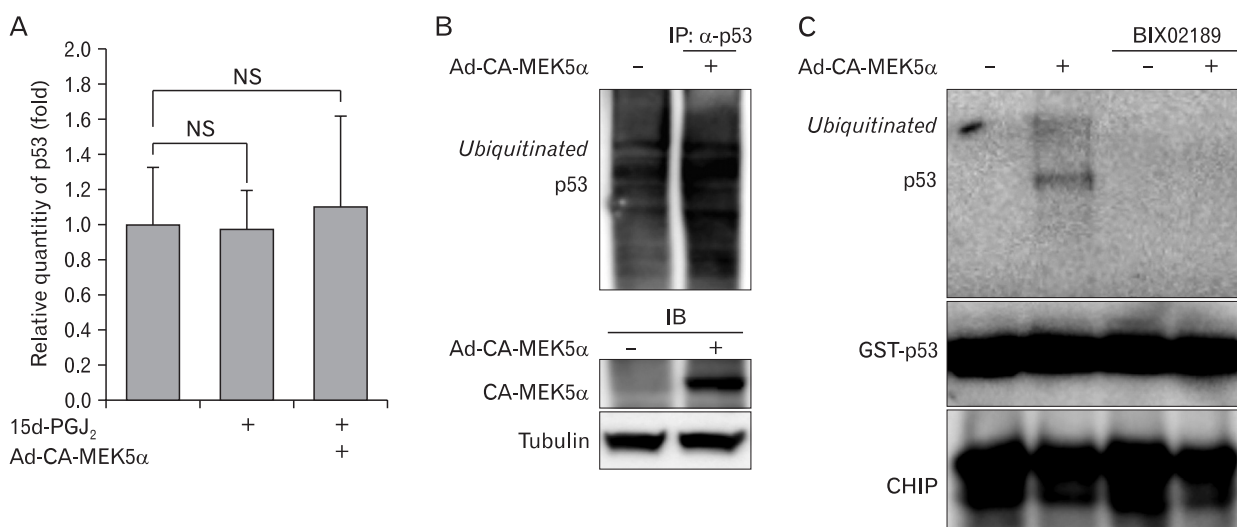

Fig. 4. ERK5 activation induces p53 ubiquitination via increasing CHIP ubiquitin ligase activity. (A) After transduction with Ad-CA-MEK5α or the Ad-LacZ control, HUVECs were exposed to 15d-PGJ$_2$ for 4 h. The level of p53 mRNA was determined by using real time quantitative RT-PCR as described in the Materials and Methods section. (B) The endogenous level of p53 ubiquitination was determined by immunoprecipitation analysis with anti-p53 antibody, followed by immunoblotting with an anti-ubiquitin antibody. (C) CHIP ubiquitin ligase activity was determined by an *in vitro* ubiquitination assay kit (BostonBiochem) with a GST-fused p53 recombinant protein as a substrate. HUVECs were transduced with Ad-CA-MEK5α and then followed by treatment with 10 μM BIX02189. Cell lysates were applied for immunoprecipitation analysis with an anti-CHIP antibody. Immunoprecipitated CHIP was incubated with recombinant proteins including ubiquitin and E1/E2 enzyme mixture, for 60 min at 37°C. CHIP ubiquitin ligase activity was determined by immunoblotting with an anti-ubiquitin antibody. The protein amount of GST-p53 and immunoprecipitated CHIP was detected by Western blot analysis with anti-p53 and anti-CHIP antibodies. Data are representative of three independent experiments. ERK5, extracellular signal-regulated kinase 5; CHIP, C terminus of Hsc70-interacting protein; Ad-CA-MEK5α, adenovirus encoding constitutively active form of MEK5 alpha; 15d-PGJ$_2$, 15-deoxy-Δ(12,14)-prostaglandin J$_2$; RT-PCR, reverse transcriptase-polymeratse chain reaction; HUVEC, human umbilical vein endothelial cells; IP, immunoprecipitation; IB, immunoblot; NS, non-significant.

chemical inhibition of ERK5 with BIX02189. These results indicated that ERK5 activation reduced p53 protein expression via CHIP-mediated ubiquitination of p53.

Discussion

The proapoptotic transcription factor p53 has been recognized as a key regulator of cell fate, acting as a sensor for DNA damage. Recent studies have revealed that p53 is involved in cytotoxic cyclopentenone prostaglandin 15d-PGJ$_2$-induced apoptosis in ECs, but the molecular mechanism that contributes to endothelial apoptosis has only been partially understood. Particularly, the relationship between blood flow dynamics-dependent anti-apoptotic responses and p53-dependent apoptotic regulation remains to be determined. Our data demonstrated that L-flow-induced ERK5 activation exerts cytoprotective responses via anti-apoptotic signaling pathways carried on p53 downregulation. In the present study, we provide evidence that L-flow inhibits 15d-PGJ$_2$-induced endothelial apoptosis in both a p53- and ERK5-dependent manner (Fig. 1). Our results showed that L-flow-mediated reduction of p53 expression and endothelial apoptosis was reversed by BIX02189, which is a specific inhibitor of MEK5 (Fig. 2). In addition to L-flow, overexpression of CA-MEK5α using an adenoviral system reduced 15d-PGJ$_2$-induced endothelial apoptosis, and this reduction of apoptosis was reversed by depletion of CHIP ubiquitin ligase (Fig. 3). Furthermore, transduction of Ad-CA-MEK5α increased the ubiquitin ligase activity of CHIP, which was determined by detecting p53 ubiquitination (Fig. 4). Based on these results, we propose that the major molecular mechanism by which L-flow activation of ERK5 downregulates p53 protein expression is via increasing the CHIP-mediated ubiquitination of p53.

The regulation of apoptosis by p53 has been suggested to take place because of the activation of transcriptional activity, which in turn leads to the induction of proapoptotic genes, including B cell lymphoma/leukemia-2 (Bcl-2) family proteins [30]. However, recent studies have also revealed non-transcriptional pro-apoptotic activities. For instance, genotoxic stress induced not only p53-dependent gene expression but also the nuclear export of p53 [30, 31]. In a resting state, p53 is mainly located in the nucleus, but cytosolic p53 directly interacts with the mitochondrial anti-apoptotic Bcl-2 family proteins such as Bcl-xL and Bcl-2 [31]. It has been well accepted that p53 stability is regulated by ubiquitin ligases,

including MDM2 and CHIP. Ubiquitin ligase-mediated polyubiquitination decreases p53 protein expression via proteasomal degradation. Esser et al. [23] reported that p53 interacted with the heat shock protein Hsp70, which is a key factor in CHIP-mediated protein ubiquitination and the proteasomal degradation of target proteins. Since Hsp70 was induced by 15d-PGJ$_2$, induced Hsp70 might affect a triple complex consisting of Hsp70, p53, and CHIP. Carter et al. [32] proposed a possible molecular mechanism for p53 nuclear export and stabilization. In the condition of low level of MDM2, MDM2 causes monoubiquitination and subsequent sumoylation of p53, which leads to p53 nuclear export. It is particularly interesting that 15d-PGJ$_2$ induces the nuclear export of p53 and increases binding affinity to prosurvival Bcl-2 family proteins.

We addressed the relationship between ERK5 activation and p53 downregulation in 15d-PGJ$_2$-induced endothelial apoptosis. Here, we showed that L-flow reduced 15d-PGJ$_2$-induced p53 expression and subsequent endothelial apoptosis, suggesting that L-flow affected signaling events downstream of p53. Since EKR5 is a major regulator in L-flow-mediated cytoprotective responses and that ERK5 activates CHIP ubiquitin ligase activity, it is relevant to note that ERK5 activation downregulates p53 protein expression via CHIP-mediated ubiquitination of p53. In agreement with this concept, ERK5 activation induced by transduction of Ad-CA-MEK5α induced CHIP-dependent p53 ubiquitination, indicating that p53 is a substrate of CHIP and that p53 downregulation contributes to the L-flow-mediated cytoprotective responses. It is not yet clear how ERK5 activation increases CHIP-mediated p53 ubiquitination. In our previous report, we showed that ERK5 bound to the C-terminus U-box domain of CHIP which is important for ubiquitinination activity, suggesting that ERK5 binding to CHIP may affect CHIP enzyme activity through a conformational change [26]. Several apoptosis regulating molecules have been recognized as the target of ERK5 in endothelial apoptosis in response to L-flow. For example, Pi et al. [12] showed that L-flow activation of ERK5 induced phosphorylation of proapoptotic Bad, which contributes to the inhibition of endothelial apoptosis. However, it was not proved whether ERK5 directly phosphorylate Bad or not. The same research group suggested that TNFα-induced c-jun kinase (JNK) activation was inhibited by the biochemical inhibition of ERK5 with BIX02188 [33]. Since apoptosis signal-regulating kinase 1 (ASK1) is an upstream molecule of the JNK pathway and is

known to be a substrate to CHIP ubiquitin ligase, it is possible to speculate that ERK5 activation might affect the CHIP-mediated downregulation of the ASK-JNK signaling pathway in L-flow-mediated cytoprotective responses.

In conclusion, we have identified ERK5 as a novel negative regulator of p53-depedent endothelial apoptosis in response to 15d-PGJ$_2$. ERK5 activation exerts the downregulation of p53 and the inhibition of subsequent endothelial apoptosis in a CHIP-dependent manner. Additionally, the ERK5 activation of CHIP ubiquitin ligase was linked to L-flow-mediated EC protection from 15d-PGJ$_2$-induced endothelial apoptosis. Our results suggest that the ERK5-CHIP signal module could be a potential target for pharmacological intervention that could prevent endothelial dysfunction-related vascular diseases, including atherosclerosis.

Acknowledgements

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