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15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2-induced down-regulation of endothelial nitric oxide synthase in association with HSP70 induction

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Abstract

A natural ligand of peroxisome proliferator-activated receptor γ (PPARγ), 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), decreases endothelial nitric oxide synthase (eNOS) expression by an unknown mechanism. Here we found that 15d-PGJ_2-induced eNOS reduction is inversely associated with heat shock protein 70 (HSP70) induction in endothelial cells. Treatment of cells with 15d-PGJ_2 decreased eNOS protein expression in a concentration- and time-dependent manner, but independently of PPARγ with no effect on mRNA levels. Although 15d-PGJ_2 elicited endothelial apoptosis, inhibition of both pan-caspases and cathepsins failed to reverse reduction of eNOS protein. Interestingly, we observed that 15d-PGJ_2 induced HSP70 in a dose-dependent manner. Immunoprecipitation and heat shock treatment demonstrated that eNOS reduction was strongly related to HSP70 induction. Cellular fractionation revealed that treatment with 15d-PGJ_2 increased eNOS distribution 2.5-fold from soluble to insoluble fractions. These findings provide new insights into mechanisms whereby eNOS regulation by 15d-PGJ_2 is related to HSP70 induction.

Keywords: 15d-PGJ_2; Apoptosis; eNOS; HSP70

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear hormone receptor superfamily that modulates gene expression upon ligand binding. The cyclopentenone prostaglandin, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), is a potent natural ligand for PPARγ and exerts a variety of biological effects through PPARγ-dependent and -independent pathways [1,2]. Irrespective of well-known beneficial roles of low dose 15d-PGJ_2, high dose of 15d-PGJ_2 induce a rapid generation of reactive oxygen species (ROS) in mitochondria of endothelial cells with subsequent caspase activation, a hallmark of apoptosis [3,4].

Heat shock proteins (HSPs) are protective of cells under stress such as heat shock. Induction of HSP70 occurs via heat shock transcription factor (HSF) activation and exerts chaperoning effects on other proteins through aiding of folding, translocation, and translation [5]. Cyclopentenone prostaglandins have been shown to activate HSF1 and induce HSP70 in various cells [6–8], but this relationship in endothelial cells is not clear.

Endothelial nitric oxide synthase (eNOS) is a key molecule in normal vascular biology and pathophysiology. NO produced by eNOS mediates vasorelaxation and inhibits both platelet aggregation and leukocyte adherence to the vascular wall [9]. Regulation of eNOS is controlled by subcellular localization, eNOS-interacting protein interactions, phosphorylation, and cofactor availability [10]. Under normal conditions, eNOS is localized in the Golgi and in caveolae of the plasma membrane (PM). Stimuli such as

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estradiol [11] and vascular endothelial growth factor [12] have been shown to induce eNOS translocation from the PM to the cytoplasm.

Recently, 15d-PGJ_2 was reported to decrease eNOS expression in human umbilical vein endothelial cells (HUVECs) [13,14] by an unknown mechanism. Here, we report that 15d-PGJ_2 reduced eNOS protein in a dose- and time-dependent, but PPARγ-independent, manner in HUVECs. This phenomenon was concomitant with 15d-PGJ_2-induced apoptosis and was not prevented by apoptosis inhibitors. However, this inhibitory effect of 15d-PGJ_2 might be regulated by eNOS trafficking into a detergent-resistant fraction with HSP70 induction. Collectively, the present study provides novel evidence on 15d-PGJ_2 regulation of eNOS expression via HSP70 induction in endothelial cells.

Materials and methods

Cell culture. HUVECs and bovine aortic endothelial cells (BAECs) were maintained according to the manufacturer's instructions (Clonetics). In all experiments, HUVECs were treated with the indicated concentrations of vehicle (0.1% ethanol), PPARγ ligand [15d-PGJ_2 (Calbiochem), troglitazone, rosiglitazone, or ciglitazone (Cayman)], or PPARγ inhibitor, GW9662 (Cayman) for the indicated times. To examine the effects of apoptosis inhibition, cells were pretreated with z-Val-Ala-Asp(OMe)-CH_2F (z-VAD-fmk; Calbiochem) for 4 h and cathepsin inhibitors (Calpain inhibitor IV, CA-074 ME, and Pepstatin A; Calbiochem) for 2 h before addition of 15d-PGJ_2. Cyclopentenone ring (Sigma) and 9,10-dihydro-15-deoxy-prostaglandin J_2 (9,10-dihydro-15d-PGJ_2; Cayman) were added at the indicated concentrations. To induce heat shock, HUVECs were incubated at 37, 42, and 45 °C for 1 h. For recovery, HUVECs were returned to 37 °C for another 23 h.

Western blot analysis. After experimental treatments, cells were washed in ice-cold phosphate-buffered saline (PBS) and lysed with radio-immunoprecipitation-assay buffer containing protease inhibitors (Sigma) and 1 mM phenylmethylsulfonylfluoride (PMSF). Whole cell lysates were resolved on 4–12% Bis–Tris gels (Invitrogen), transferred onto a nitrocellulose membrane (Whatman), and probed with primary antibodies to eNOS, poly(ADP-ribose) polymerase (PARP) (BD Transduction Laboratories), caspase-3, actin (Santa Cruz), Akt (Cell signaling), or HSP70 (Stressgen). After immunodetection, relative densities of bands were quantified using an NIH image J program (http://rsb.info.nih.gov/ij/). All data were normalized to the actin content of the same sample.

Reverse transcription-polymerase chain reaction (RT-PCR). Isolation of total RNA was performed using the Trizol reagent (Invitrogen). Total RNA (1 μg) was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen). PCR was performed with the following primers: human eNOS sense 5′-CAGCACCTTGGCAGAAGAG-3′, antisense 5′-TTAGCCACGTGGAGCAGAC-3′; human β-actin sense 5′-AGAAAATCTGGCACCACACC-3′; and antisense 5′-CTCCTTAAT GTCACGCACGA-3′. Relative mRNA levels were quantified using the image J program.

Immunoprecipitation (IP). After experimental treatments, cells were washed in PBS and lysed with IP buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, and 1% NP-40] containing protease inhibitors and PMSF. Cell lysates (400 μg) were incubated with a monoclonal antibody to eNOS, and successively with protein G–agarose beads. Immunoprecipitates were blotted as described above.

Confocal immunocytochemistry. After treatment with 15d-PGJ_2, cells were rinsed with PBS, fixed with 4% paraformaldehyde (pH 7.4) and permeablized with 0.2% Triton X-100. Cells were rinsed and blocked with 3% BSA in PBS and incubated with antibodies to eNOS and HSP70. Afterward, cells were rinsed with PBS, followed by treatment with Alexafluor secondary antibody (Molecular Probes).

Cellular fractionation. Fractionation was performed as described previously with the following modifications [15]. Briefly, cells were washed in PBS and lysed with Tris/Triton buffer [50 mM Tris–HCl (pH 7.4) and 1% Triton X-100] containing protease inhibitors and PMSF. The detergent-insoluble fraction was pelleted by centrifugation at 15,000 *g* for 5 min, and the supernatant was considered the detergent-soluble fraction. The pellet was resuspended in lithium dodecyl sulfate sample buffer (Invitrogen) and sonicated. Blotting and quantification were performed as described above.

Statistical analysis. For all experiments, statistical analysis was performed using the Student's *t* test to detect differences between experimental groups; $p < 0.05$ was considered statistically significant.

Results and discussion

Previous studies [13,14] have shown that 15d-PGJ_2 reduced eNOS protein expression by an unknown mechanism. In the present study, we demonstrated that 15d-PGJ_2 reduced eNOS expression in endothelial cells by increasing HSP70 protein levels and subsequent eNOS translocation from the soluble into the insoluble fraction.

15d-PGJ_2 down-regulates eNOS protein in a dose- and time-dependent manner

To determine whether PPARγ regulates eNOS protein expression, HUVECs and BAECs were treated for 24 h with PPARγ ligands. 15d-PGJ_2 significantly decreased eNOS protein expression in HUVECs (Fig. 1A) and BAECs (data not shown), whereas other PPARγ ligands did not alter eNOS protein, suggesting a PPARγ-independent mechanism is involved. This result was specific for eNOS protein, since 15d-PGJ_2 did not significantly change levels of Akt and actin. Treating HUVECs for 24 h with graded concentrations of 15d-PGJ_2 decreased eNOS protein expression in a dose-dependent manner (Fig. 1B). Time course analysis of 15d-PGJ_2-induced eNOS reduction at a concentration of 10 μM demonstrated that 15d-PGJ_2 tended to decrease eNOS protein expression at 8 h, and apparent reduction was shown at 24 h (Fig. 1C). These findings are consistent with previous studies showing [14] that 15d-PGJ_2 decreased both expression and activity of eNOS in HUVECs by inhibiting eNOS mRNA and protein synthesis, but this decrease was observed only after long-term treatment of 15d-PGJ_2 for 48 to 72 h. However, Calnek et al. [13] reported that 15d-PGJ_2 decreased only eNOS protein expression, but not mRNA levels, after 24 h treatment of 15d-PGJ_2 in endothelial cells. The differences in time required for eNOS down-regulation with previous reports may be attributable to different culture conditions, such as cell sources and media systems.

To further examine the involvement of PPARγ in this process, the PPARγ antagonist GW9662 was added. Up to 10 μM GW9662 could not prevent 15d-PGJ_2-induced eNOS reduction (Fig. 1D), additionally confirming that eNOS reduction by 15d-PGJ_2 is not PPARγ-dependent.

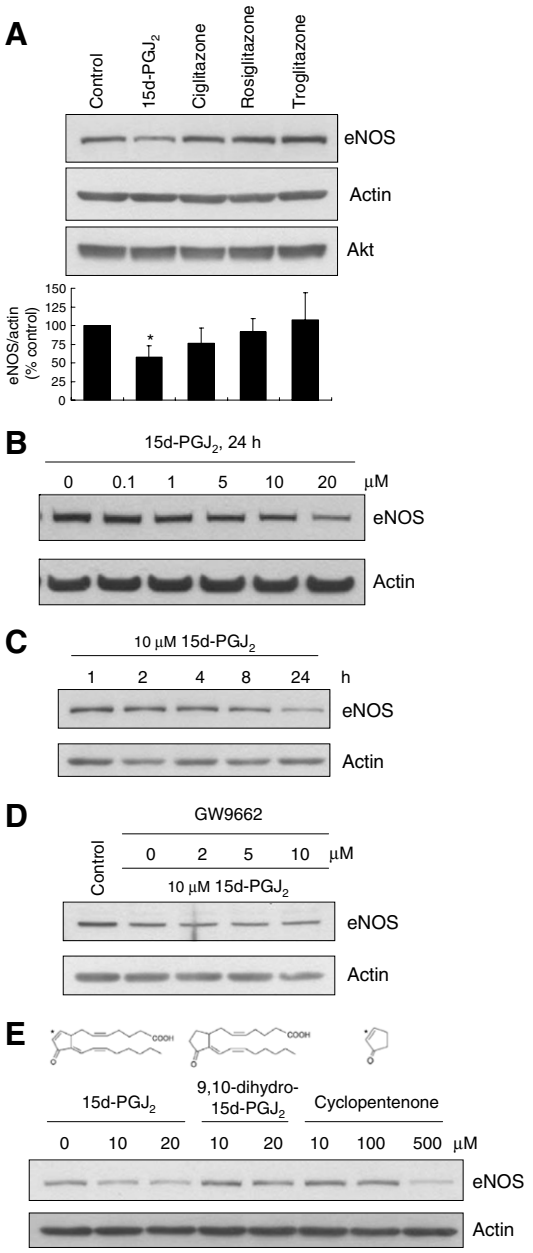

Fig. 1. Down-regulation of eNOS protein expression by 15d-PGJ_2. (A) HUVECs treated with 10 μM of various PPARγ ligands for 24 h. Each bar represents the mean ± SD ($n = 4$); $^*p < 0.05$ vs. control. (B) HUVECs treated with indicated concentrations of 15d-PGJ_2 for 24 h. (C) HUVECs incubated for indicated times with 15d-PGJ_2. (D) HUVECs treated with the indicated concentrations of GW9662 along with 15d-PGJ_2 for 24 h. (E) HUVECs incubated with the indicated concentrations of 15d-PGJ_2, 9,10-dihydro-15d-PGJ_2, and the cyclopentenone ring. *The reactive double bond.

Reactive double bond of 15d-PGJ_2 is involved in eNOS reduction

Since the α,β-unsaturated carbonyl group in the cyclopentenone ring was determined to be a prerequisite for protein turnover and apoptosis [16], we examined the role of the reactive double bond of the cyclopentenone ring. HUVECs were treated with 15d-PGJ_2 and its two analogues, 9,10-dihydro-15d-PGJ_2 (an analogue which lacks the reactive double bond, but retains properties of PPARγ agonists [17]) and the cyclopentenone ring (Fig. 1E). 9,10-Dihydro-15d-PGJ_2 did not affect eNOS levels, while the cyclopentenone ring decreased eNOS levels, although a relatively high-dose (500 μM) was required. These results indicate that the biological activities of 15d-PGJ_2 may be due to the double bond. In previous reports, cyclopentenone was shown to have the same efficacy as 15d-PGJ_2 at a 10- to 20-fold higher concentration [6,8].

eNOS reduction is not mediated by either transcriptional down-regulation or proteasomal degradation

To determine whether 15d-PGJ_2 regulates eNOS protein expression at the transcriptional level, mRNA levels after 15d-PGJ_2 treatment were examined. Since TNFα is well-known for eNOS down-regulation by decreasing mRNA stability [18], we used TNFα-treated HUVECs as a positive control. 15d-PGJ_2 did not alter eNOS mRNA levels, whereas TNFα decreased eNOS mRNA levels to 20% of control (Fig. 2A).

Since protein ubiquitination and subsequent proteasomal degradation is a well-known pathway for protein turnover, we employed the proteasome inhibitor, MG-132, to elucidate the role of the proteasome in eNOS reduction. MG-132 was added with 15d-PGJ_2 but did not block 15d-PGJ_2-induced eNOS reduction (Fig. 2B), indicating that 15d-PGJ_2-induced eNOS reduction is not due to either transcriptional regulation or proteasomal degradation. To our knowledge, there is only one report regarding the long-term effects of proteasomal inhibition on eNOS expression in bovine endothelial cells, in which relatively low-dose MG-132 treatment (>24 h) resulted in eNOS protein accumulation, an effect attributable to enhanced eNOS mRNA transcription, suggesting that eNOS was not the direct target of the proteasome [19]. Moreover, 15d-PGJ_2 was reported to inhibit rather than enhance proteasomal activity by covalent modification of a proteasomal subunit [16].

Inhibition of apoptosis failed to prevent eNOS reduction

Recently, Tesauro et al. [20] reported that caspases activated by staurosporine induced eNOS cleavage during apoptosis, suggesting that eNOS is a substrate for caspases. Therefore, we examined whether 15d-PGJ_2-induced apoptosis is responsible for eNOS reduction. Twenty micromolar 15d-PGJ_2 induced caspase-3 activation and PARP cleavage (Fig. 2C). The pan-caspases inhibitor, zVAD-fmk, significantly blocked cleavage of caspase-3 and PARP (Fig. 2C), but failed to block 15d-PGJ_2-induced eNOS reduction (Fig. 2C). In contrast to their findings that zVAD-fmk ameliorated staurosporine-induced eNOS reduction, pre-treatment with zVAD-fmk failed to recover 15d-PGJ_2-induced eNOS reduction in our system. This discrepancy with previous reports may be due to different mechanisms of causal stimuli or cell characteristics.

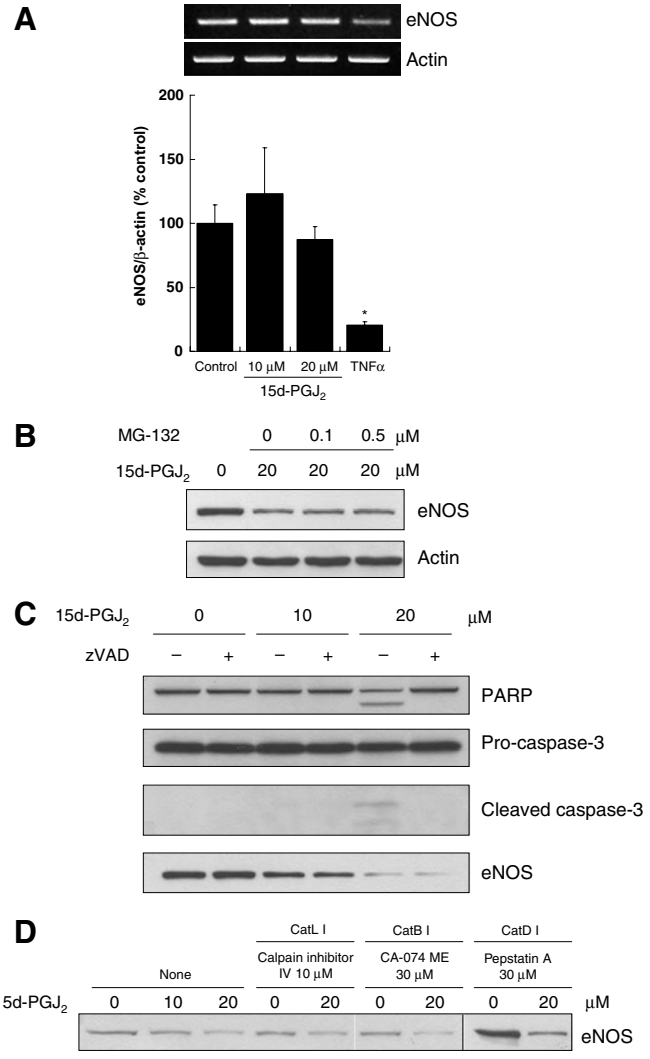

Fig. 2. Effects of 15d-PGJ_2 on eNOS down-regulation in HUVECs. (A) eNOS mRNA levels after treatment with 15d-PGJ_2 and TNFα (20 ng/mL) for 24 h. Each bar represents the mean ± SD ($n = 4$); $^*p < 0.05$ vs. control. (B) eNOS protein expression after treatment with 15d-PGJ_2 and MG-132. (C) Expression of eNOS and apoptosis markers in HUVECs pre-incubated with 100 μM zVAD-fmk for 4 h and then treated with 15d-PGJ_2 for 24 h. (D) eNOS expression in HUVECs pre-incubated with the designated cathepsin inhibitors (Cat I) for 2 h, then treated with 15d-PGJ_2 for 24 h.

Since lysosomal cysteine proteases, cathepsins, have been shown to function in autophagic cell death and caspase-independent apoptosis [21,22], we tested the possibility of cathepsin involvement in eNOS down-regulation by 15d-PGJ_2. Treatment with 15d-PGJ_2 induced expression of multiple cathepsin isoforms, but no cathepsin inhibitors were able to reverse 15d-PGJ_2-induced eNOS reduction (Fig. 2D), suggesting that eNOS reduction is not mediated by either caspase-dependent or -independent apoptosis.

15d-PGJ_2-induced HSP70 up-regulation is associated with eNOS down-regulation

There is emerging evidence that chemicals containing a cyclopentenone ring, including 15d-PGJ_2, induce HSP70 mRNA and protein expression via the activation of HSF [6–8]. HSP70 induction was exclusively observed in 15d-PGJ_2-treated HUVECs, but not in other synthetic PPARγ agonist-treated cells (Fig. 3A). The cyclopentenone ring also induced HSP70 in a dose-dependent manner accompanied by gradual eNOS reduction (Fig. 3B). Since it is reported that *de novo* protein synthesis is a prerequisite for HSP70 mRNA up-regulation by cyclopentenone [6], we investigated the effects of 15d-PGJ_2 in the presence and absence of cycloheximide, a protein synthesis inhibitor (Fig. 3C). Cycloheximide treatment not only inhibited HSP70 induction, but also eNOS down-regulated, while cycloheximide *per se* had no effects on either eNOS or HSP70 protein levels. Heat shock treatment at 45 °C for 1 h markedly increased HSP70 protein expression, and HSP70 induction was proportional to eNOS reduction (Fig. 3D). Taken together, these results demonstrate a strong inverse correlation between eNOS and HSP70 proteins in a variety of experimental conditions, suggesting a potential role of HSP70 in regulation of eNOS protein.

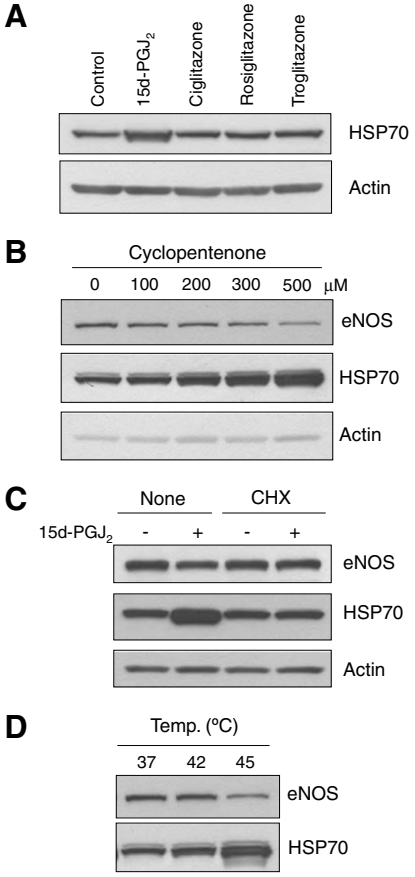

Fig. 3. Effects of 15d-PGJ_2 on eNOS and HSP70 expression in HUVECs. (A) HUVECs treated with 10 μM of various PPARγ ligands for 24 h. (B) HUVECs treated with the indicated concentrations of cyclopentenone for 24 h. (C) HUVECs treated with 20 μM 15d-PGJ_2 in the presence or absence of cycloheximide (CHX) for 24 h. (D) HUVECs incubated at 37, 42, and 45 °C for 1 h, and analyzed after 23 h of recovery.

eNOS and HSP70 were co-localized in 15d-PGJ$_2$-treated HUVEC

Regulation of eNOS can be controlled by subcellular localization as well as by synthesis and degradation. To determine whether cellular redistribution of eNOS occurs in 15d-PGJ$_2$-treated cells, we performed immunocytochemical studies. In untreated BAECs, eNOS was found mainly in the PM and Golgi apparatus (Fig. 4A); however, treatment of cells with 10 μM 15d-PGJ$_2$ disrupted the localization of eNOS to the PM and increased cytoplasmic staining, although a substantial amount of eNOS protein still remained in the Golgi (Fig. 4A). In contrast to eNOS, the intensity of HSP70 was increased by 15d-PGJ$_2$ treatment. Merged images of eNOS and HSP70 in 15d-PGJ$_2$-treated cells revealed that the preferential location of eNOS was largely cytosolic and that eNOS was co-localized with HSP70. In agreement with results from immunofluorescence, IP experiments also showed a physical interaction between HSP70 and eNOS in 15d-PGJ$_2$-treated cells (Fig. 4B).

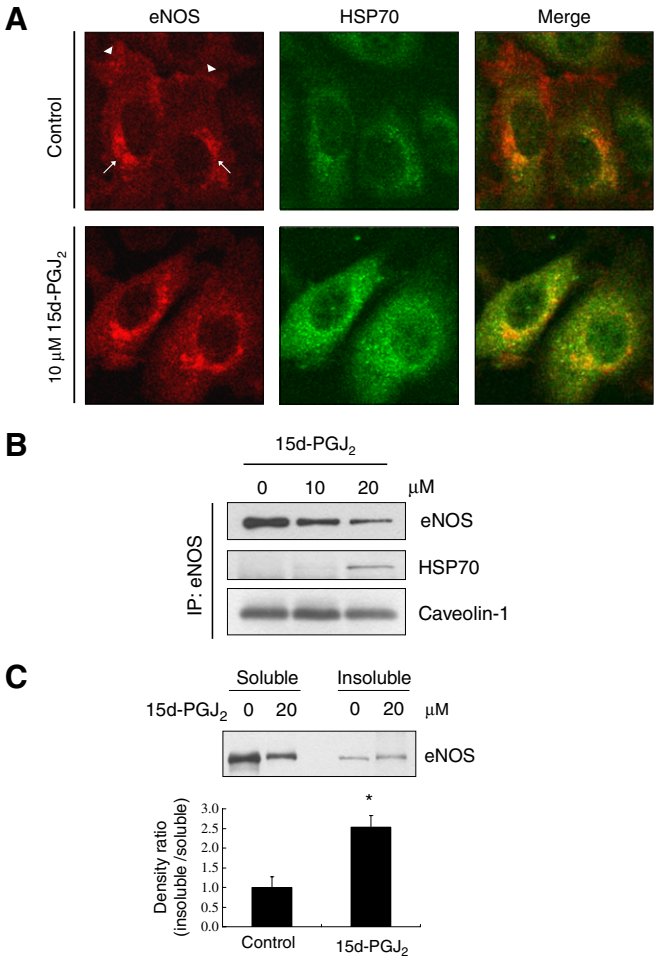

Fig. 4. HSP70 and eNOS are co-localized in 15d-PGJ$_2$-treated cells. (A) Immunocytochemistry with eNOS and HSP70 antibodies in BAECs. (Arrows: perinuclear Golgi; arrow heads: plasma membrane) (B) Expression of eNOS, HSP70, and caveolin-1 after eNOS immunoprecipitation in 15d-PGJ$_2$-treated HUVECs. (C) eNOS expression and the ratio of detergent-soluble and -insoluble fractions in HUVECs treated with 15d-PGJ$_2$ for 24 h. Each bar represents the mean ± SD of density ratio of insoluble/soluble fractions ($n = 4$); $^*p < 0.05$ vs. control.

15d-PGJ$_2$ induced eNOS translocation

Since chaperones, including HSP70, play roles in protein translocation and HSP70 could regulate eNOS localization through associations with the C-terminus of the HSP70-interacting protein (CHIP) [15], we examined whether 15d-PGJ$_2$-induced eNOS reduction was associated with eNOS redistribution. Cellular fractionation demonstrated that eNOS primarily resided in the soluble fraction in intact cells, whereas it was translocated into the insoluble fraction in 15d-PGJ$_2$-treated cells (Fig. 4C). Therefore, the ratio of insoluble/soluble fractions of eNOS was 2.5-fold higher in 15d-PGJ$_2$-treated cells than in control cells (Fig. 4C), indicating that 15d-PGJ$_2$ induces eNOS trafficking from the soluble fraction to the detergent-insoluble fraction. Accumulation of HSP70 in the insoluble fraction (data not shown) may also increase the possibility of co-localization between eNOS and HSP70 in 15d-PGJ$_2$-treated cells.

The mechanisms responsible for 15d-PGJ$_2$-induced eNOS reduction in relation to cellular localization are not clear at this point, although several known mechanisms may contribute. First, eNOS may be modified by 15d-PGJ$_2$. 15d-PGJ$_2$ has been shown to form covalent adducts with cysteine residues of cellular proteins [16,17]. Human eNOS protein has 29 cysteine residues, and several cysteine resides are critically important for dimerization and tetrahydrobiopterin-binding [23,24]. It was reported that *S*-nitrosylation of eNOS puts the enzyme in a monomeric state, and that this effect is attributable to cysteine 99 [25]. Myristoylation of glycine 2 targets eNOS to the Golgi complex and PM [26]. A myristoylation-deficient mutant showed less NO production and a diffuse cellular staining pattern, implying that defects in acylation could impair normal phosphorylation and subcellular localization. Therefore, thiol modification by 15d-PGJ$_2$ may elicit disruption of natural protein folding and cause subsequent eNOS protein aggregates in the insoluble fraction.

Second, 15d-PGJ$_2$-induced HSP70 could regulate eNOS expression and trafficking. For example, CHIP could be involved in intracellular trafficking of eNOS that accompanying with HSP70 induction [15]. Although CHIP resulted in eNOS redistribution from soluble to insoluble fractions due to its activity with the HSP90 hetero-complex, it did not induce eNOS ubiquitination and subsequent protein degradation [15]. Based on this previous report, 15d-PGJ$_2$-induced eNOS reduction in the cytosol could be attributable to eNOS translocation from the soluble to insoluble fractions in our system. 15d-PGJ$_2$ might enhance eNOS-CHIP interactions and subsequent eNOS translocation by an undefined mechanism. Immunocytochemisty supported this hypothesis by showing that treatment with 15d-PGJ$_2$ did not decrease overall immunofluorescence

intensity of eNOS (Fig. 4A). Instead of decreasing eNOS protein content, 15d-PGJ_2 re-distributed eNOS from the Golgi and PM to the cytoplasm. Irrespective of CHIP, HSP70 accumulation occurs rapidly in rat heart [27] and vascular endothelium [28] following heat shock to the whole animal, implying that HSP70 induction might be associated with endothelial function *in vivo*. Our heat shock experiment is consistent with the previous report that eNOS and HSP70 protein expression had opposite patterns after heat shock in BAECs [29].

In summary, we demonstrate that 15d-PGJ_2-induced eNOS reduction is mediated by covalent modification and HSP70 induction rather than by apoptosis and transcriptional or proteasomal regulation. Our findings provide novel evidence for a reciprocal link between 15d-PGJ_2-mediated alterations in eNOS reduction and HSP70 induction in endothelial cells; however, the molecular and regulatory mechanisms remain unclear. Ongoing studies will determine whether 15d-PGJ_2-induced eNOS reduction is directly regulated by HSP70 induction.

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