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Research Article

Tannin 1-α-*O*-galloylpunicalagin induces the calcium-dependent activation of endothelial nitric-oxide synthase *via* the phosphatidylinositol 3-kinase/Akt pathway in endothelial cells

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Many polyphenols have been found to increase endothelial nitric oxide (NO) production. In our present study, we investigated the effects of 1-α-*O*-galloylpunicalagin upon endothelial nitric oxide synthase (eNOS) activity in endothelial cells (ECs). Both 1-α-*O*-galloylpunicalagin and punicalagin induced NO production in a dose-dependent manner in ECs. Despite having similar chemical structures, punicalagin induced lower levels of NO production than 1-α-*O*-galloylpunicalagin. After 1-α-*O*-galloylpunicalagin addition, a rise in the intracellular Ca^{2+} concentration preceded NO production. The Ca^{2+} ionophore A23187 stimulated eNOS phosphorylation and augmented NO production. Pretreatment with Ca^{2+} chelators inhibited 1-α-*O*-galloylpunicalagin-induced eNOS phosphorylation and NO production. Treatment with 1-α-*O*-galloylpunicalagin did not alter the eNOS protein levels but, unlike punicalagin, induced a sustained activation of eNOS Ser^{1179} phosphorylation. 1-α-*O*-galloylpunicalagin was also found to activate ERK1/2, JNK and Akt in ECs. Moreover, simultaneous treatment of these cells with specific phosphatidylinositol-3-kinase inhibitors significantly inhibited the observed increases in eNOS activity and phosphorylation levels. In contrast, the inhibition of (ERK)1/2, JNK and p38 had no influence on eNOS Ser^{1179} phosphorylation. Our present results thus indicate that the 1-α-*O*-galloylpunicalagin-induced calcium-dependent activation of eNOS is primarily mediated *via* a phosphatidylinositol 3-kinase/Akt-dependent increase in eNOS activity, and occurs independently of the eNOS protein content.

Keywords: Akt / Endothelial cells / 1-α-*O*-galloylpunicalagin / Nitric oxide / Nitric oxide synthase

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1 Introduction

Nitric oxide (NO) is a relaxing factor produced in the endothelium *via* the activation of endothelial NO synthase (eNOS) and acts as a vasodilator [1]. NO also protects blood vessels from thrombosis by inhibiting platelet aggregation and adhesion, and endothelial NO further exerts a protective role during atherogenesis. Based on these protective effects of endothelial NO, an enhancement in its production could be of considerable prophylactic or therapeutic interest.

Terminalia calamansanai (Blanco) Rolf is a Combretaceae plant that has a broad distribution in tropical and subtropical beach areas. *T. calamansanai* is also used medicinally in the Philippine Islands as a lithontripic [2]. Other plants in the same genus have additionally been found to show therapeutic effects; extracts of the leaves and bark of *Terminalia catappa* show anti-inflammatory activity [3] and protect against hepatotoxicity [4, 5]. In addition, the bark extracts of *Terminalia arjuna* show antioxidant activity [6] and cardioprotective effects [7]. However, the biological

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Abbreviations: **EC**, endothelial cell; **EGCG**, (−)-epigallocatechin-3-gallate; **eNOS**, endothelial NO synthase; **MAPK**, mitogen-activated protein kinase; **NO**, nitric oxide

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activities of leaf extracts from *T. calamansanai* have not yet been fully analyzed.

The polyphenolic tannins known as the punicalagins are abundant in the leaves of *T. catappa* [8]. Punicalagin itself is also present in large quantities in pomegranate fruit extracts, which have been shown to have increased NO-production levels [9, 10]. We thus hypothesized that the punicalagins may up-regulate NO production in endothelial cells (ECs). Numerous studies have now demonstrated that polyphenols enhance NO production [11 – 18]. Moreover, the activity of eNOS during cardiovascular homeostasis is regulated post-translationally by both a rapid increase in intracellular Ca^{2+} and/or phosphorylation, and at the transcriptional level *via* changes in its gene expression [19]. Several polyphenols have also been found to influence NO production by increasing the eNOS expression levels, including the red wine polyphenol, resveratrol [15], the anthocyanin pigment, cyanidin-3-glucoside [11], and the flavonoids luteolin and cynaroside [14]. Other mechanisms by which NO production is up-regulated *via* increased eNOS activity may occur through eNOS phosphorylation, the modulation of the intracellular calcium levels ($[Ca^{2+}]_i$), or induction of heat shock protein 90 by constituents of green tea, epigallocatechin-3-gallate, black tea polyphenols, and isoflavones from soy beans [13, 16 – 18]. We thus designed our current study to investigate the effects of the natural plant-derived polyphenols, the punicalagins, on eNOS expression and activity in ECs, and we have elucidated many of the underlying molecular mechanisms involved in these pathways.

2 Materials and methods

2.1 Materials

Antibodies raised against eNOS, phospho-Ser^{1179}eNOS and phospho-Ser^{473} Akt1 were purchased from BD Biosciences (San Jose, CA, USA). Anti-phospho-ERK2 (Thr^{202}/Tyr^{204}) was purchased from Transduction Laboratories (Lexington, KY, USA). Anti-phospho-JNK (Thr^{183}/Tyr^{185}) and anti-phospho-p38 (Thr^{180}/Tyr^{182}) were purchased from Cell Signaling Technology (Beverly, MA, USA). Luciferase assay kits were purchased from Promega (Madison, WI, USA). Peroxidase-conjugated anti-rabbit and anti-mouse antibodies were obtained from Amersham (Arlington Heights, IL, USA) and nitrocellulose was obtained from Schleicher & Schuell (Dassel, Germany). PD98059, SB203580, SP600125, LY294002, and genistein were purchased from Calbiochem (San Diego, CA, USA). All other reagents were purchased from Sigma (St. Louis, MO, USA).

2.2 Preparation of 1-α-*O*-galloylpunicalagin and punicalagin

Leaves of the *T. calamansanai* plant were collected from the grounds of National Chiayi University, Chiayi, Taiwan, in July 2002. Positive identification of the plant species was performed by Prof. Fu-Yuen Lu, Department of Forestry, National Chiayi University. A voucher specimen (NCYU-H0101) is deposited in the Graduate Institute of Biopharmaceutics, National Chiayi University. The dried leaves of *T. calamansanai* (1.0 kg) were homogenized in 70% aqueous acetone and filtered. The concentrated filtrate was then evaporated and freeze-dried to yield a 70% acetone extract. A part of the extract was chromatographed over a Diaion HP-20 column with H_2O, and H_2O-MeOH (from 20% MeOH, to 40%, 60% or 100%). The 40% MeOH eluate was then chromatographed over a Toyopearl HW-40C column and developed step by step with H_2O, 60% MeOH, 70% MeOH, MeOH/H_2O/acetone (7:2:1) and MeOH/H_2O/acetone (8:1:1). The MeOH/H_2O/acetone (7:2:1) eluate was rechromatographed over a LiChroprep RP-18 column with 0.05% TFA/ACN (88:12) to yield 1-α-*O*-galloylpunicalagin (900 mg, 0.235%). The 70% MeOH eluate was rechromatographed over LiChroprep RP-18 with 0.05% TFA/ACN (92:8) to yield punicalagin (189 mg, 0.049%). All chemical structures were estimated by ^{1}H- and ^{13}C-NMR, including ^{2}D-NMR techniques, and also by comparison of these data with authentic compounds [2]. The purity of each compound was determined by HPLC and was shown to exceed 95% in each case.

2.3 EC cultures

Bovine aortic ECs (BAECs) were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were maintained at 37°C in a humidified atmosphere of air and 5% CO_2 and grown in petri dishes for 3 days to reach confluence [20]. The culture medium was then replaced with serum-free DMEM and the cells were incubated for 12 h prior to experimental treatments.

2.4 Measurement of eNOS activity in intact cells

The production of intracellular NO was determined using DAF-2/DA (10 μmol/L) [21]. Briefly, cells were stimulated as described, washed twice with PBS, and incubated with PBS containing 100 μM L-arginine for 10 min at 37°C. Subsequently, 0.1 μM DAF-2 was added into the buffer and cells were further incubated in the dark for 30 min at 37°C. Cell supernatants were then transferred into 2-mL reagent tubes and spun down quickly to remove debris. Fluorescence was measured by spectrofluorophotometry (Shimadzu, Rf-5301PC) with excitation and emission wavelengths of 490 and 500 nm, respectively. Production of nitrite and nitrate in the cultured medium was measured using the Griess method. Briefly, the samples were mixed with Griess reagent [50 μL 1% sulfanilamide containing 5% phosphoric acid and 50 μL 0.1% *N*-(1-naphthyl)ethyle-

nediamine] and incubated for 10 min at room temperature. The absorbance was then measured at 540 nm using a μQuant plate-spectrophotometer (Bio-Tek Instr.).

2.5 Determination of eNOS promoter activity

An eNOS-3500-Luc construct containing a 3.5-kb human eNOS promoter fragment was introduced into the pGL3 promoter plasmid (Promega Corp) as described previously [22]. All subsequent transfection experiments were performed using lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. For luciferase assays, the cell lysate was first mixed with luciferase substrate solution (Promega), and the resulting luciferase activity was measured using a luminometer. For each experiment, luciferase activity was determined in triplicate and normalized with β-galactosidase activity.

2.6 Western blotting

Whole lysates of ECs were prepared as previously described [23]. Cells (1×10^6) were lysed on ice in lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and a protease inhibitor mixture) and whole-cell extracts were boiled for 5 min prior to separation on 10% SDS-PAGE, in which the protein samples were evenly loaded. The proteins were then transferred to a nitrocellulose filter (Millipore) in Tris-glycine buffer at 100 V for 1.5 h. The membranes were then blocked with PBS containing 5% nonfat milk and incubated with the target antibodies for 2 h at 4°C, with gentle shaking. The results were visualized by chemiluminescence using ECL (Amersham Pharmacia Biotech), according to the manufacturer's instructions.

2.7 Calcium influx determination with fluorescent indicators

Cells were loaded with Fura-2/AM (5 μM) and then incubated 45 min at 37°C in culture medium. Fura 2-AM-loaded cells were washed and placed in HEPES-buffered balanced salt solution [24]. $[Ca^{2+}]_i$ was determined by fluorimetric readings performed by spectrofluorophotometry (Shimadzu, Rf-5301PC) with excitation and emission wavelengths of 340 and 510 nm, respectively.

2.8 Measurement of intracellular H_2O_2

Cells were cultured at 37°C in the presence or absence of reagents as indicated in the corresponding figures, washed with PBS, and incubated with the peroxide-sensitive fluorescent probe 5-(and-6)-carboxy-2,7,dichlorodihydro fluorescein diacetate (carboxy-H_2DCFDA; Molecular Probes, Eugene, OR, USA; 20 μM) for 30 min at 37°C. After two washes with PBS, the cells were solubilized with 1% SDS and 5 mM Tris-HCl (pH 7.4). Fluorescence was measured by spectrofluorophotometry (Shimadzu, Rf-5301PC) with excitation and emission wavelengths of 450 and 520 nm, respectively. Samples were assayed in triplicate.

2.9 Statistical analysis

Data for all experiments were obtained from at least three independent experiments, each performed in triplicate. These data were then analyzed by the analysis of variance (ANOVA), and subsequently, for significant ANOVAs, pairwise comparisons between the control group and treatment groups were performed by post hoc comparison using the Tukey test (SPSS software package, Chicago, IL, USA). The confidence limit of $p < 0.05$ was considered as statistically significant. All graphs indicate the means ± SD.

3 Results

3.1 Both 1-α-*O*-galloylpunicalagin and punicalagin increase NO production in ECs

Both 1-α-*O*-galloylpunicalagin and punicalagin share a common basic tannin structure (Fig. 1A). To examine whether 1-α-*O*-galloylpunicalagin and punicalagin could each activate eNOS in BAECs, we first examined NO production in these cells. The results showed that both compounds induced significant increases in NO production, compared with untreated or N^G-nitro-L-arginine methyl ester (L-NAME)-treated BAECs, at a dose of 50 μmol/L for 12 h (Fig. 1B). Despite their similar chemical structures, punicalagin induced less NO production than 1-α-*O*-galloylpunicalagin, suggesting an important role for the galloyl groups in this process. The Griess reaction only detected nitrite and nitrate in the culture medium. Thus, a complementary experiment using DAF-2/DA fluorescence was performed to evaluate the eNOS activity levels in 1-α-*O*-galloylpunicalagin-treated ECs. As shown in Fig 1C, the 1-α-*O*-galloylpunicalagin significantly increased levels of intracellular NO after 3 h of treatments. The tea-derived catechin (–)-epigallocatechin-3-gallate (EGCG), which also containing a galloyl group, was shown to have a greater up-regulatory effect upon NO production [13]. After 12 h of treatment, the 1-α-*O*-galloylpunicalagin-increased intracellular NO levels was very similar to those of the EGCG-treated ECs.

To investigate whether treatment with 1-α-*O*-galloylpunicalagin or punicalagin influenced the eNOS protein expression levels, we incubated BAECs with increasing doses of these agents for 12 h; however, using Western blotting, we found no effects on the eNOS protein levels in either case, regardless of the concentration used (Fig. 2A). To test whether these compounds altered eNOS transcription, we performed luciferase reporter assays but found no effects on eNOS promoter activity (Fig. 2B).

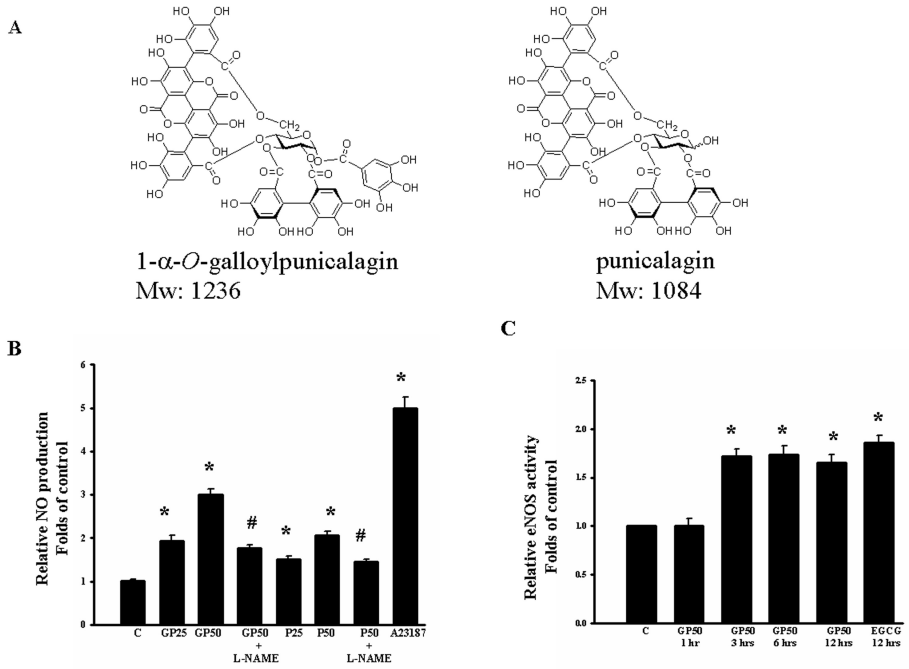

Figure 1. Both 1-α-*O*-galloylpunicalagin and punicalagin increase NO production in ECs. (A) 1-α-*O*-galloylpunicalagin and punicalagin share a common basic tannin structure. The galloyl side chains in 1-α-*O*-galloylpunicalagin promote its ability to activate eNOS activity. (B) BAECs were treated for 12 h with increasing concentrations of 1-α-*O*-galloylpunicalagin (GP) or punicalagin (P) in the presence or absence of 100 μM L-NAME or 1 μM A23187. Cell lysates were then harvested for the determination of eNOS activity. Data are the mean ± SE values from five independent experiments. $^{*}p < 0.05$, compared with control and $^{\#}p < 0.05$ compared with L-NAME. (C) ECs were incubated with 50 μM 1-α-*O*-galloylpunicalagin (GPun) for the indicated times. NO release was then quantified using the 4,5-diaminofluorescein fluorescence assay as described previously. Fluorescence values were normalized to the cell numbers and to the activity of the control cells. EGCG (50 μM) served as a positive control. $^{*}p < 0.05$, compared with the control.

3.2 Involvement of $[Ca^{2+}]_i$ in eNOS phosphorylation and activation

Because eNOS activity is strictly Ca^{2+} dependent [25], we explored the role of Ca^{2+} in NO production in response to 1-α-*O*-galloylpunicalagin. First, the effects of 1-α-*O*-galloylpunicalagin upon $[Ca^{2+}]_i$ were assessed in BAECs. Cells were loaded with the Ca^{2+} indicator dye fura 2-AM, and then stimulated with 50 mM 1-α-*O*-galloylpunicalagin. Fluorescence emission of intracellular fura 2-AM was measured simultaneously. As shown in Fig. 3A, 1-α-*O*-galloylpunicalagin induced a rapid rise in $[Ca^{2+}]_i$. The peak levels of Ca^{2+} were reached within 1 h of incubation with 1-α-*O*-galloylpunicalagin and then decreased, but were still higher than the basal levels within 6 h.

The increased $[Ca^{2+}]_i$ may stimulate eNOS activity through a Ca^{2+}/calmodulin-dependent mechanism, whereas the possible contribution to the phosphorylation of eNOS remains uncertain. We observed that A23187, a Ca^{2+} ionophore, stimulated eNOS phosphorylation at Ser^{1179} in BAECs (Fig. 3B). As shown in Fig. 3B, we found a critical role for Ca^{2+} in the 1-α-*O*-galloylpunicalagin-induced phosphorylation of eNOS. Pretreatment with Ca^{2+} chelator EGTA partially inhibits eNOS phosphorylation. We next studied the role of Ca^{2+} on 1-α-*O*-galloylpunicalagin-induced NO production. BAECs were loaded with the Ca^{2+} chelator EGTA or the Ca^{2+} ionophore A23187, and then stimulated with 1-α-*O*-galloylpunicalagin and processed for the analysis of NO production. As shown in Fig. 3C, EGTA completely blocked the effects of 1-α-*O*-galloylpunicalagin on NO production, but A23187 augmented these effects. These data suggest that Ca^{2+} are required for Ser^{1179} phosphorylation and activation of eNOS by 1-α-*O*-galloylpunicalagin.

3.3 Time course assessment of 1-α-*O*-galloylpunicalagin- and punicalagin-induced eNOS Ser^{1179} phosphorylation in ECs

eNOS has been shown to be post-translationally activated by phosphorylation [19]. To determine whether this phosphorylation of eNOS is involved in the up-regulation of its activity by 1-α-*O*-galloylpunicalagin and punicalagin, we assessed the levels of phosphorylation of bovine eNOS Ser^{1179}. As shown in Fig. 4A, 1-α-*O*-galloylpunicalagin (at 25, 50 and 100 μM) or punicalagin (at 50 and 100 μM) induced the phosphorylation of eNOS on Ser^{1179}. We then examined whether this phosphorylation occurs in a time-dependent manner for either compound, and found that

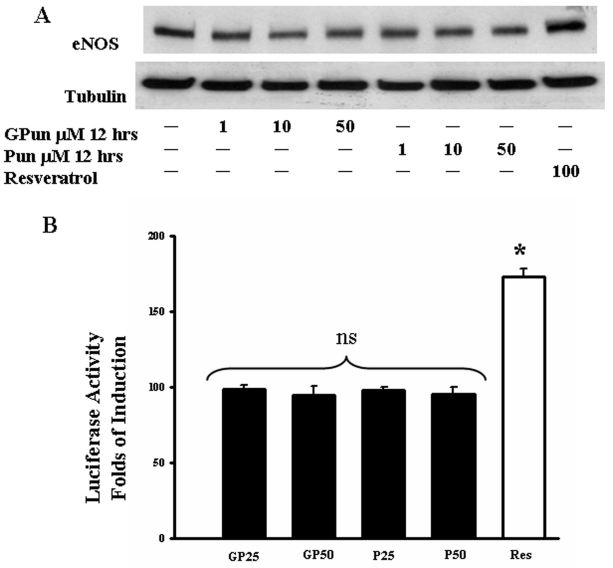

Figure 2. The eNOS expression levels in 1-α-*O*-galloylpunicalagin or punicalagin-treated BAECs. (A) BAECs were treated with 1-α-*O*-galloylpunicalagin (GPun) or punicalagin (Pun) at the indicated concentrations for 12 h. Protein expression levels were determined by Western blot analysis with antibodies against eNOS or tubulin as indicated. The tubulin band intensities indicate equal loading of each well. (B) Cells were transfected with an eNOS-luciferase construct for 12 h, maintained in low serum medium for a further 6 h, and then stimulated with 25 or 50 μM 1-α-*O*-galloylpunicalagin (GPun) or punicalagin (Pun) for an additional 12 h. The cells were lysed and analyzed for luciferase activity. Induction is indicated by increased normalized luciferase activity in the treated ECs, relative to the control. Resveratrol (100 μM) served as a positive control. *$p <$ 0.05; ns, not significant (ANOVA/Tukey). The data shown (mean ± SD) are from three independent experiments performed in triplicate.

50 mM 1-α-*O*-galloylpunicalagin increased eNOS Ser^{1179} phosphorylation from 10 min and that this persisted for 12 h (Fig. 4B). In contrast, although the punicalagin-induced eNOS Ser^{1179} phosphorylation followed a similar initial time course that commenced at 10 min, this was only maintained for 1 h (Fig. 3C). This suggests that the galloyl structure of 1-α-*O*-galloylpunicalagin may function to stabilize the activation of eNOS phosphorylation.

3.4 1-α-*O*-galloylpunicalagin-induced activation of eNOS in BAECs is Akt/phosphatidylinositol 3-kinase dependent

Because Akt has been shown to phosphorylate eNOS and is known to be activated by *via* phosphatidylinositol 3-kinase (PI3K) [26], we investigated whether these upstream signaling pathways are involved in 1-α-*O*-galloylpunicalagin-dependent eNOS activation. BAECs were pretreated with the protein tyrosine kinase inhibitor, genistein (50 and 100 μM) or with the PI3K inhibitors, wortmannin (1 and

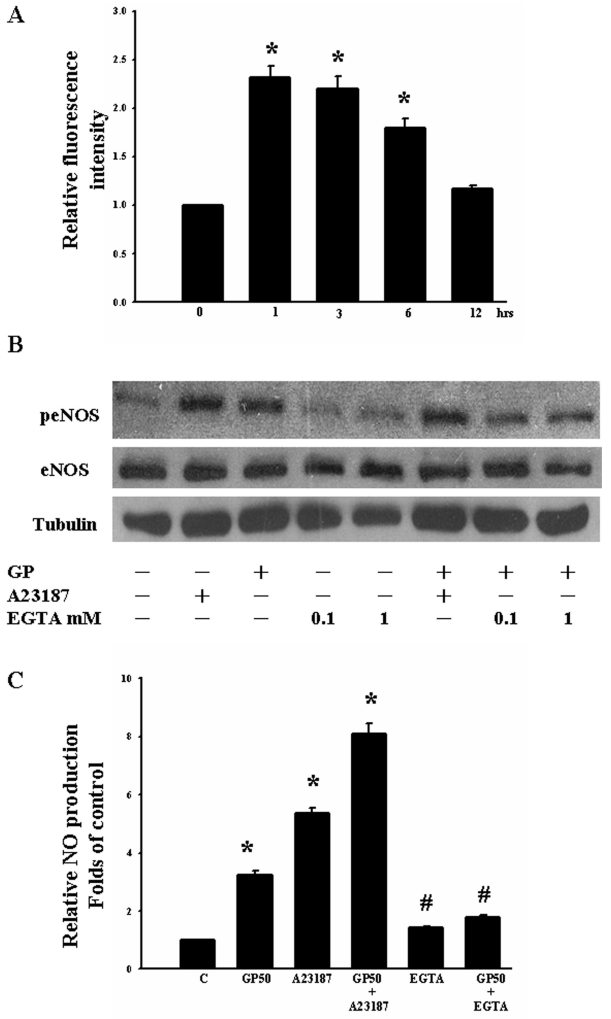

Figure 3. Involvement of $[Ca^{2+}]_i$ in eNOS phosphorylation and activation. (A) BAECs were incubated with the calcium indicator dye fura 2-AM and processed for $[Ca^{2+}]_i$ measurements, as described in Section 2. The cells were treated for indicated time with 1-α-*O*-galloylpunicalagin. Cell lysates were then harvested for determination of fluorescence intensity. Data are the mean ± SE. *$p < 0.05$, compared with the control. (B) BAECs were stimulated with 10 μmol/L A23187, a Ca^{2+} ionophore, for 30 min, and eNOS phosphoprylation at Ser^{1179} was observed or 0.1 or 1 mM EGTA, a Ca^{2+} chelator, for 30 min and stimulated with or without 50 μM 1-α-*O*-galloylpunicalagin for 60 min. eNOS phosphorylation at Ser^{1179} was observed by immunoblot analysis with a phosphospecific antibody. (C) BAECs were treated for 12 h with increasing concentrations of 1-α-*O*-galloylpunicalagin in the presence or absence of 1 mM EGTA or 1 μM A23187. Cell lysates were then harvested for the determination of the eNOS activity. Data are the mean ± SE. *$p < 0.05$, compared with control and $^{\#}p < 0.05$ compared with 1-α-*O*-galloylpunicalagin-treated cells.

5 μM) and LY294002 (5 and 25 μM) for 30 min. As shown in Fig. 5A, the inhibition of PI3K completely blocked 1-α-*O*-galloylpunicalagin-induced eNOS phosphorylation, demonstrating the requirement of this kinase for eNOS activation by 1-α-*O*-galloylpunicalagin.

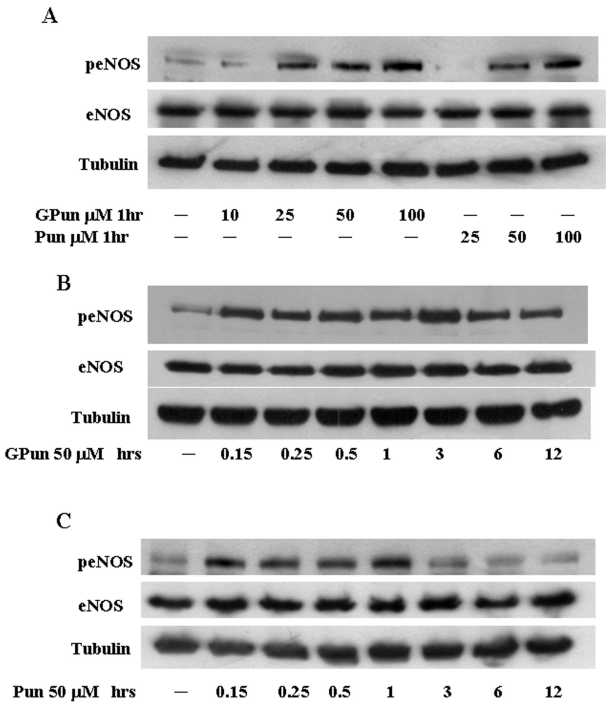

Figure 4. Time course of 1-α-*O*-galloylpunicalagin- and punicalagin-induced eNOS Ser^{1179} phosphorylation in ECs. (A) BAECs were treated for 1 h with the indicated concentrations of 1-α-*O*-galloylpunicalagin or punicalagin. Western blots were probed with phospho-specific antibodies against eNOS Ser^{1179}. Anti-eNOS and tubulin antibodies served as equal loading controls. The results shown are representative of three separate experiments. (B, C) BAECs were treated for the indicated times with 50 μM 1-α-*O*-galloylpunicalagin (GPun) or punicalagin (Pun) as indicated. Western blots were probed with phospho-specific antibodies against eNOS Ser^{1179}. Results are representative of three separate experiments.

Previous studies have also demonstrated that the activation of the mitogen-activated protein kinase (MAPK) pathways, which can be induced by tyrosine kinases, contributes to the induction of eNOS phosphorylation [27]. We tested whether 1-α-*O*-galloylpunicalagin-induced eNOS phosphorylation occurs through an MAPK pathway using the specific pharmacological inhibitors, PD098059, an inhibitor of MEK1/2, which is the upstream activator of ERK1/2, SB203580, a p38 MAPK inhibitor, and SP600125, a JNK inhibitor. As shown in Fig. 5B, no inhibitory effects of these agents were evident upon 1-α-*O*-galloylpunicalagin-induced eNOS phosphorylation.

To further elucidate whether additional kinases are involved in the upregulation of eNOS phosphorylation by 1-α-*O*-galloylpunicalagin, we examined the Akt, ERK1/2 and JNK phosphorylation profile in our EC system. 1-α-*O*-galloylpunicalagin, at concentrations of 50 μM, was found to induce the transient phosphorylation of ERK1/2 and JNK after 30-min treatments, which returned to basal levels after 3 and 1 h, respectively (Fig. 5C). Additionally, whereas the activation of ERK1/2 by 1-α-*O*-galloylpunicalagin was more sustained than JNK, the phosphorylation of Akt remained at elevated levels for at least 12 h following exposure to this compound. To confirm the role of the PI3K/Akt pathway in eNOS induction by 1-α-*O*-galloylpunicalagin, we inhibited PI3K with either wortmannin or LY294002 and found that eNOS activity was attenuated (Fig. 5D). Our data thus indicate that the PI3K/Akt pathway participates in the induction of eNOS phosphorylation in ECs.

3.5 Induction of H_2O_2 by 1-α-*O*-galloylpunicalagin

Since the PI3K/Akt pathway has been shown to play a role during H_2O_2-induced NO production [28], we tested its impact upon the intracellular H_2O_2 levels at various concentrations using dichlorofluorescein fluorescence. However, we could not detect any increase in H_2O_2 in ECs following treatment with 10 – 100 mM 1-α-*O*-galloylpunicalagin (Fig. 6). To exclude any contribution of H_2O_2, we performed these eNOS activity assays in the presence of catalase. Preincubation with catalase (1000 U/mL) had no influence on the obtained results, indicating that the effects of 1-α-*O*-galloylpunicalagin are not due to the production of H_2O_2 (data not shown).

4 Discussion

Our present study demonstrates that the up-regulation of NO production by the *T. calamansanai*-derived tannin 1-α-*O*-galloylpunicalagin occurs in response to a potent, dose-dependent activation of eNOS in BAECs. 1-α-*O*-galloylpunicalagin, was found to up-regulate $[Ca^{2+}]_i$ prior to NO production. We further showed that the resulting increase in eNOS activity was modulated by a post-translational regulatory mechanism. Using established pharmacological inhibitors, we further demonstrated the involvement of the PI3K/Akt-dependent pathway in this induction process.

In our present experiments, the punicalagins used were derived from extracts of *T. calamansanai* leaves, but these bioactive compounds are also present in large quantities (1.8 mg/mL) in pomegranate fruit extracts [29]. The safety and bioavailability of punicalagins from pomegranate fruit extracts have been identified in previous studies by other investigators. Cerda *et al*. [29] found no toxic effects of these compounds in rats upon repeated oral administration (6% of the diet) for 37 days, and identified punicalagin and related metabolites in plasma, liver, and kidney. Heber *et al*. [30] previously demonstrated the safety of a pomegranate ellagitannin-enriched polyphenol dietary supplement in humans. Seeram *et al*. [31] reported that maximal plasma concentrations of a punicalagin-related metabolite, ellagic acid, were reached after 1 h, and of the ellagic acid metabolite urolithin after 6 h, following the consumption of pome-

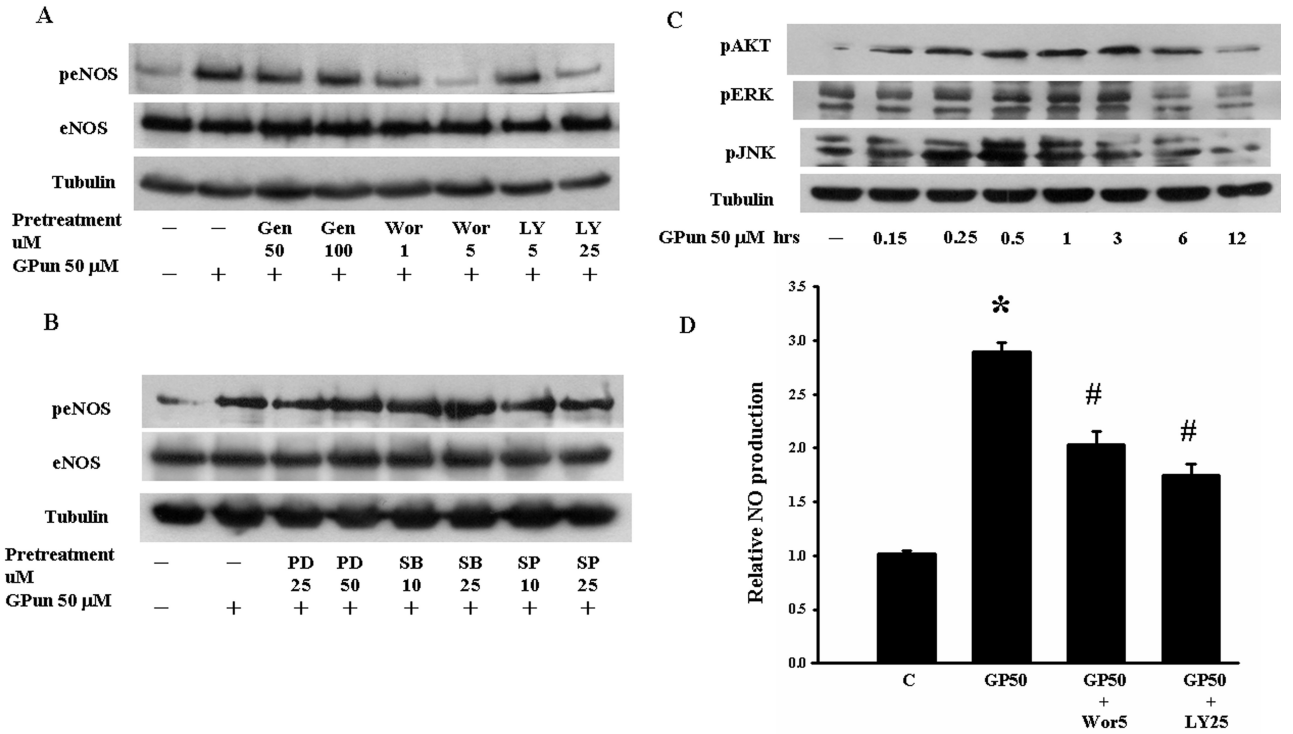

Figure 5. 1-α-*O*-galloylpunicalagin-induced eNOS activation in BAECs is PI3K/Akt dependent. (A) Western blot analysis of BAECs pretreated with the protein tyrosine kinase inhibitor, genistein (Gen, 50 and 100 μM) or with the PI3K inhibitors, wortmannin (Wor, 1 and 5 μM) and LY294002 (LY, 5 and 25 μM) for 30 min, then incubated with 50 μM 1-α-*O*-galloylpunicalagin for 1 h. (B) BAECs were pretreated with the MEK1/2 inhibitor PD098059 (PD, 25 and 50 μM), the p38 MAPK inhibitor SB203580 (SB, 10 and 25 μM) or a JNK inhibitor SP600125 (SP, 10 and 25 μM), followed by 50 μM 1-α-*O*-galloylpunicalagin for 1 h. Western blot analysis was then performed. (C) BAECs were treated for the indicated times with 50 μM 1-α-*O*-galloylpunicalagin. Western blots were probed with phospho-specific antibodies against ERK1/2 (Thr^{202}/Tyr^{204}), Akt (Ser^{473}), and JNK (Thr^{183}/Tyr^{185}). Results are representative of three separate experiments. (D) BAECs were pretreated or not with the PI3K inhibitor wortmannin (5 μM) or LY294002 (25 μM) for 30 min. After stimulation with 50 μM 1-α-*O*-galloylpunicalagin (GP) for 12 hs, the eNOS activity in intact cells was measured. Data are the mean ± SD from five separate experiments. $^{*}p < 0.05$, compared with control and $^{\#}p < 0.05$ compared with 1-α-*O*-galloylpunicalagin treatment.

granate fruit juice. These results suggested that these metabolites contribute to the bioactivity of pomegranate fruit juice. Since many intracellular enzymes are likely to digest punicalagines, their effects upon the activation of eNOS may be due to their derived metabolites. However, whether punicalagin-related metabolites can indeed induce eNOS activity requires further investigation.

Many polyphenolic compounds are known to be activators of eNOS in various cell types [11 – 18]. Our current results show that the pattern of activation differs for 1-α-*O*-galloylpunicalagin and punicalagin, indicating that even subtle changes in chemical structures can significantly affect the potency of these compounds to up-regulate eNOS. As shown in Fig. 1B, 1-α-*O*-galloylpunicalagin had a more potent effect than punicalagin on increasing NO production in ECs, suggesting that galloyl groups are important for the efficiency of this process. Consistent with this, the tea-derived catechin EGCG, also containing a galloyl group, was shown to have greater up-regulatory effects upon NO production compared with tea catechins without gallate [13]. In addition, as shown in Figs. 3A and B, we found in our current analyses that the prominent increase in eNOS phosphorylation in ECs following 1-α-*O*-galloylpunicalagin exposure persisted for 12 h, whereas in the case of punicalagin this was maintained for only 1 h. Kajiya *et al*. [32] have demonstrated that the presence of the galloyl moiety in tea catechins governs the hydrophobicity and affinity of these compounds for lipid bilayers. Therefore, the galloyl structure of 1-α-*O*-galloylpunicalagin may have a similar effect on the induction of eNOS phosphorylation and thus sustain higher enzyme activity.

Numerous data have now been reported indicating that eNOS is activated in association with changes in the cytosolic calcium concentration in response to agonists such as bradykinin, estradiol, and shear stress [25]. The data presented here also add to our understanding of 1-α-*O*-galloylpunicalagin-induced eNOS activity with regards to calcium. Although there is a discrepancy in the time course of the Ca^{2+} response and the time course of NO production, 1-α-*O*-galloylpunicalagin-induced eNOS stimulation was

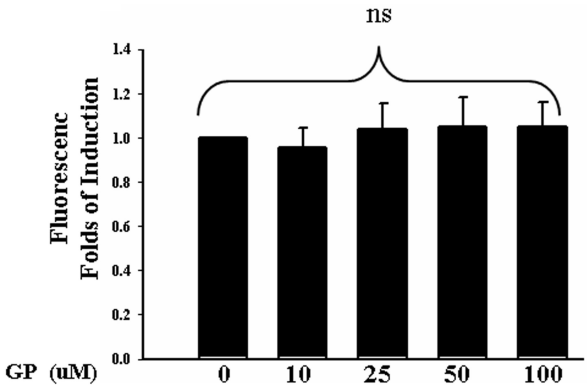

Figure 6. The induction of H_2O_2 by 1-α-*O*-galloylpunicalagin. ECs were subjected to 1-α-*O*-galloylpunicalagin treatment (10, 25, 50 and 100 μM) for 1 h, followed by measurement of the intracellular H_2O_2 levels as described in Section 2. The results shown are the mean ± SEM of at least three separate experiments. ns, not significant (ANOVA/Tukey). $^*p < 0.05$ as compared with ECs treated with vehicle.

found to be calcium dependent in our experiments. eNOS can be activated by certain stimuli without a sustained increase in $[Ca^{2+}]_i$ being necessary, the most important example being fluid shear stress [33]. There are other polyphenols (*e.g.*, equol and delphinidin) that also affect NO production, but the signal transduction cascades that are activated do not require an increase in $[Ca^{2+}]_i$ [18, 24]. However, black tea polyphenol-induced eNOS activation is dependent on calcium [16]. The different structures of the polyphenols may thus result in apparently mutually exclusive mechanisms.

To examine the possible mechanisms underlying eNOS activation in BAECs after treatment with 1-α-*O*-galloylpunicalagin, we investigated the potential role of several protein kinases activated by this polyphenol. Our data demonstrated that ERK1/2, JNK and Akt are involved in the cellular response to 1-α-*O*-galloylpunicalagin stimulation. This is consistent with the earlier report of Lorenz et al. [13] showing that EGCG increased eNOS phosphorylation *via* the activation of the PI3K/Akt cascade pathways. Several other studies of phytochemical-regulated eNOS phosphorylation have also focused on the role of the MAPK pathways [16, 18]. As shown in Fig. 4A, the treatment of ECs with specific kinase inhibitors implicates PI3K in eNOS phosphorylation. With the use of specific antibodies against phosphorylated ERK1/2, JNK and Akt, we further found that 1-α-*O*-galloylpunicalagin promotes the phosphorylation of these three kinases (Fig. 5C).

Although our results indicate that ERK, JNK and PI3 kinase are activated by 1-α-*O*-galloylpunicalagin, we found that these kinases do not participate equally in the induction of eNOS phosphorylation in ECs. Our results show that 1-α-*O*-galloylpunicalagin-induced eNOS phosphorylation can be completely blocked by PI3K inhibitors, but is not fully abrogated by the inhibition of ERK and JNK (Figs. 5A and B). On the other hand, Akt phosphorylation was found to be sustained for far longer than either ERK or JNK phosphorylation following 1-α-*O*-galloylpunicalagin treatment of ECs. A sustained activation of the PI3K/Akt pathway has also been demonstrated previously during H_2O_2-induced NO production [28]. Taken together, these data suggest that the PI3K/Akt is the major pathway mediating the effects of 1-α-*O*-galloylpunicalagin.

The normal endothelium plays a critical role in regulating vasomotor tone, and it is well established that impaired endothelium-derived NO activity is associated with the progression of atherosclerosis. We have demonstrated in an earlier study that endothelial NO acts as a negative regulator of the endothelial responses to hemodynamic forces, by suppressing Rac-dependent pathways [34]. We have also demonstrated that resveratrol suppresses ICAM-1 gene induction *via* NO [35]. The release of NO exerts its inhibitory effects by attenuating specific signaling pathways [36, 37]. Endogenous increases in NO production have also been observed to promote protein *S*-nitrosylation, which is a reversible protective mechanism against intracellular oxidative stress. Under these conditions, increased NO levels would be expected to have beneficial protective effects in ECs.

In summary, we postulate that the novel plant-derived tannin, 1-α-*O*-galloylpunicalagin, acts as a naturally occurring activator of eNOS in ECs by promoting its protein phosphorylation.

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