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Differential regulation of endothelial nitric oxide synthase phosphorylation by protease-activated receptors in adult human endothelial cells

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Abstract

Protease-activated receptors have been shown to regulate endothelial nitric oxide synthase through the phosphorylation of specific sites on the enzyme. It has been established that PAR-2 activation phosphorylates eNOS-Ser-1177 and leads to the production of the potent vasodilator nitric oxide, while PAR-1 activation phosphorylates eNOS-Thr-495 and decreases nitric oxide production in human umbilical vein endothelial cells. In this study, we hypothesize a differential coupling of protease-activated receptors to the signaling pathways that regulates endothelial nitric oxide synthase and nitric oxide production in primary adult human coronary artery endothelial cells. Using Western Blot analysis, we showed that thrombin and the PAR-1 activating peptide, TFLLR, lead to the phosphorylation of eNOS-Ser-1177 in human coronary artery endothelial cells, which was blocked by SCH-79797 (SCH), a PAR-1 inhibitor. Using the nitrate/nitrite assay, we also demonstrated that the thrombin- and TFLLR-induced production of nitric oxide was inhibited by SCH and L-NAME, a NOS inhibitor. In addition, we observed that TFLLR, unlike thrombin, significantly phosphorylated eNOS-Thr-495, which may explain the observed delay in nitric oxide production in comparison to that of thrombin. Activation of PAR-2 by SLIGRL, a PAR-2 specific ligand, leads to dual phosphorylation of both catalytic sites but primarily regulated eNOS-Thr-495 phosphorylation with no change in nitric oxide production in human coronary artery endothelial cells. PAR-3, known as the non-signaling receptor, was activated by TFRGAP, a PAR-3 mimicking peptide, and significantly induced the phosphorylation of eNOS-Thr-495 with minimal phosphorylation of eNOS-Ser-1177 with no change in nitric oxide production. In addition, we confirmed that PAR-mediated eNOS-Ser-1177 phosphorylation was Ca^{2+}-dependent using the Ca^{2+} chelator, BAPTA, while eNOS-Thr-495 phosphorylation was mediated via Rho kinase using the ROCK inhibitor, Y-27632, suggesting protease-activated receptor coupling to G_q and $G_{12/13}$, respectively. These data suggest a vascular bed specific differential coupling of protease-activated receptors to the signaling pathways that regulate endothelial nitric oxide synthase and nitric oxide production that may be responsible for endothelial dysfunction associated with cardiovascular disease.

Keywords: Endothelial nitric-oxide synthase, nitric oxide, endothelial dysfunction, protease-activated receptors, thrombin

Introduction

Endothelial dysfunction is the primary predictor of systemic hypertension, its persistence contributing to severe cardiovascular events.[1,2] Some of the current therapies include the manipulation of agonist/antagonist properties[3] of protease-activated receptors (PARs), which are a family of G protein-coupled receptors that are primarily expressed in cells of the vasculature and known for their involvement in regulation of vascular tone. These receptors induce endothelium-dependent relaxation via production and release of a potent vasodilator, nitric oxide (NO), and therefore, play an important role in endothelial function.[4,5] There are four members of the PAR family, PAR-1 through -4, that are uniquely irreversibly activated via cleavage by serine proteases that expose a neo-N-terminal, which acts as a self-activating peptide.[5–7] PAR-1, -3, and -4, are directly activated by thrombin, a serine protease responsible for platelet aggregation, endothelial cell activation, and other important responses in vascular biology.[4–6,8] Whereas, PAR-2 is the receptor for trypsin-like enzymes and can respond to thrombin only through transactivation by PAR-1.[5,6]

Activation of PARs via thrombin has been highly defined by its response to coagulant proteases but also stimulates an array of signal transduction pathways, which include the activation of distinct heterotrimeric G proteins (G_i, G_o, G_q, and $G_{12/13}$) and tyrosine and serine/threonine kinases.[5,7] Accordingly, these signaling pathways are essential in the maintenance of vascular homeostasis.[8–11]

Recent advances in the cardiovascular characterization of PAR signaling make them extremely attractive for drug targeting[3]; however, how PARs regulate the human eNOS/NO pathway remains unclear. Studies have shown that multiple PARs modulate the production of NO via post-translational modifications of endothelial nitric oxide synthases (eNOS) via specific phosphorylation of various regulatory sites.[6,7,12–14] Most of what is known regarding this regulation centers on the functional consequences of the phosphorylation of eNOS-Ser-1177/-1179 (human/bovine) and eNOS-Thr-495/-497 (human/bovine). When eNOS-Ser-1177/-1179 is phosphorylated, there is an increase in eNOS activity followed by an immediate increase in the production of NO. In contrast, when eNOS-Thr-495/-497 is phosphorylated, eNOS activity is down-regulated, which leads to a decrease in NO production.[9,12,13,15]

We have previously demonstrated the reciprocal regulation of the phosphorylation of these two sites on eNOS by PARs using bovine aortic endothelial cells (BAECs) and human umbilical vein endothelial cells (HUVECs). The two eNOS sites are phosphorylated by PARs via different G protein-dependent signal transduction pathways. Thrombin and a selective PAR-1 activating peptide (AP), TFLLR, modulate eNOS negatively by phosphorylating eNOS at the Thr-495 site and signaling through a Rho/ROCK-dependent pathway.[12] Activation of PAR-2 via PAR-1 transactivation or using an activating peptide specific for PAR-2, SLIGRL, phosphorylates eNOS at Ser1177 through a Ca^{2+} and PKC-δ-sensitive, but PI3K/AKT-independent pathway.[12,15] However, the role the highly co-expressed PAR-3 plays on eNOS phosphorylation still remains unknown, while PAR-4 has been shown to induce the production of NO in HUVECs.[16] Alternatively, studies have shown that low amounts of shear stress increase ERK-mediated eNOS-Thr-495 phosphorylation to enhance vasoconstriction, which was reduced in the presence of the common phenol, resveratrol.[17] While the deletion of Δ594–604 residues of the autoinhibitory element (AIE) within the FMN-binding domain of human eNOS regulates the Ca^{2+}/calmodulin-dependent domain of eNOS to produce NO via phosphorylation of eNOS-Ser-1177 and dephosphorylation of eNOS-Thr-495.[18,19]

Here, we provide evidence of a deviation from the classical role of PARs in mediating the regulation of eNOS phosphorylation in humans, which is different from that observed previously in HUVECs.[12,13] In this study, primary human coronary artery endothelial cells (HCAECs) were used as a model system to characterize the signaling potential of PARs in the critical vasculature altered in ischemic heart disease. Thrombin was observed to primarily regulate the positive site of eNOS, inducing phosphorylation of eNOS-Ser-1177. Activation of PAR-1 and PAR-2 using specific activating peptide ligands phosphorylated both

regulatory sites with evidence of dual signaling pathways. However, only PAR-1 activation resulted in an increase in NO metabolites, which favors vasodilation, while selective PAR-2 activation prevented NO production, which favors vasoconstriction. In addition, we were able to characterize the functional role of PAR-3, which induced eNOS-Thr-495 phosphorylation only whereas PAR-4 was not expressed in these cells. These findings characterizing PAR-mediated eNOS/NO signaling in relevant HACECs will provide a greater understanding of vascular signaling events that will assist in the development of more specific therapeutic agents for the prevention and treatment of cardiovascular diseases.

Materials and methods

Materials

Thrombin (bovine derived), β-actin antibody, proteinase inhibitor cocktail, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma, St. Louis, MO. TFLLR and SCH-79797 were purchased from Tocris, Minneapolis, MN. TRAGAP was purchased from Bachem, Torrance, CA. L-N^G-Nitroarginine methyl ester (L-NAME) and SCH-79797 were purchased from Cayman Chemical, Ann Arbor, MI. PAR-1, PAR-3 H-108,-2,-4 primary antibodies were purchased from Santa Cruz, Dallas, TX. eNOS-III, p-eNOS-Thr-495, p-eNOS-Ser-1177 were purchased from BD Bioscience, San Jose, CA. Primary antibodies, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetraacetoxy-methyl ester (BAPTA-AM), and ROCK inhibitor Y-27632 were purchased from Tocris, Minneapolis, MN.

Cell culture

Primary HCAECs from adult male and female (age 24-59) donors were purchased and used under the manufacturer's (PromoCell, Heidelberg, Germany) instructions. Male HCAECs were also purchased from American Type Culture Collection (ATCC), Manassas, VA, and maintained the same as the cells from PromoCell. Cells were grown in endothelial cell growth medium (ECGM) macro vascular (MV) containing fetal calf serum, endothelial growth supplement, human recombinant epidermal growth factor, heparin, and hydrocortisone. At 80–90% confluency, the cells were subcultured using a trypsin-EDTA solution and $1 \times$ phosphate-buffered saline (PBS). Experiments were performed in 6/12-well plates using confluent cells from passages 3–7, which were serum deprived in ECGM without MV for 12–18 h before each experiment to ensure a quiescent cell state.

Western blotting

Total protein was extracted from cells via lysing in ice-cold modified RIPA buffer with 1 mM Na_3VO_4, 20 mM β-glycerophosphate, sodium deoxycholate, with 1 mM PMSF, and 1% (v/v) protease inhibitor cocktail solution (Sigma, St. Louis, MO) freshly added. Up to 50 μg of protein from cell lysates was separated on 7.5% or 4–12% SDS-polyacrylamide and electrophoretically transferred to nitrocellulose

membranes (BioRad, Hercules, CA). Blots were blocked with 5% (w/v) casein in TBS containing 0.1% (v/v) tween 20 and then incubated with the appropriate primary antibodies (phospho-specific antibodies for eNOS-Thr-495 and eNOS-Ser-1177 were used at a 1:1000 concentration, control antibodies, total eNOS, and β-actin, were used at 1:1000, 1:4000, and 1:5000, respectively) followed by horseradish peroxidase-labeled secondary antibody, and the bands were visualized by enhanced chemiluminescence (Pierce/Thermo Scientific, Rockford, IL).

Nitrate/nitrite (NO measurement)

Total NO was measured via total nitrate/nitrite (colorimetric and flurometric) assay (Cayman Chemical, Ann Arbor, MI), which measured nitrate/nitrite (NO_2^-/NO_3^-) production, an indicator of NO synthesis. Detection of nitrate/nitrite production is based on colorimetric detection of nitrite as an azo dye product of the Griess reaction. Nitrate/nitrite concentrations were calculated by comparison with the optical density 540 of standard solutions as directed by the manufacturer's instructions. In brief, nitrate is converted into nitrite using nitrite reductase. The second step is the addition of the Griess reagents, which converts nitrite into a deep purple azo compound whose photometric measurement of the absorbance at 540 nm wavelength correlates total nitrite concentrations. To determine thrombin/AP-induced NO_2^-/NO_3^- accumulation, basal NO_2^-/NO_3^- accumulation was subtracted from values obtained with thrombin/AP stimulation conditions.

Statistics

The data presented in this study were representative of three or more separate experiments yielding similar results. Intensity of the bands was measured by UN-SCAN-IT gel automated digitizing system version 6.1 (Silk Scientific, Orem, UT) and/or image studio lite 3.1.4 software from (LI-COR Biosciences, Lincoln, NE). Data analysis was done with prism 6.0 statistical program software using student t-test and/or one-way ANOVA and expressed as standard error of the mean.

Results

Effects of thrombin on eNOS phosphorylation and NO production in HCAECs

We previously demonstrated that thrombin induces post-translational modification of both eNOS-regulatory sites in a time-dependent manner to regulate NO production.[12,13,15] To determine the physiological effects of thrombin in the adult coronary endothelium, HCAECs were stimulated with thrombin in a time-dependent manner from 30 s up to 30 min. The phosphorylation of eNOS at both regulatory sites was measured via Western blot. As shown in Figure 1(a), thrombin (10 U/ml) significantly stimulated phosphorylation at eNOS-Ser-1177 at the 30-s time point ($P < 0.0001$ vs. Basal), and occasional phosphorylation was detected at 2 min, which was not statistically significant. Phosphorylation at the later time points was comparable

to basal levels. Thrombin did not stimulate an increase in the phosphorylation of eNOS-Thr-495 site. Using a colorimetric or fluorometric absorbance assay to measure eNOS enzymatic activity, total NO metabolites, nitrate/nitrite, were increased maximally at 10 min ($P < 0.05$ vs. Basal) in Figure 1(b).

To confirm that the thrombin-eNOS-Ser-1177-NO response observed in Figure 1(a) and (b), was mediated via PAR-1 activation, we pretreated the HCAECs with a selective PAR-1 inhibitor, SCH-79797 (SCH) (10 μmol/ml) for 1 h prior to stimulation with thrombin (10 U/ml). As shown in Figure 1(c), SCH significantly inhibited the phosphorylation of eNOS-Ser-1177 ($P < 0.001$ vs. Vehicle); however, it had no effect on the basal levels of eNOS-Thr-495 phosphorylation observed by stimulation with thrombin (data not shown). In addition, SCH also inhibited the thrombin-induced production of NO metabolites at 10 min ($P < 0.05$) as shown in Figure 1(d). These findings demonstrate that thrombin induces the phosphorylation of eNOS-Ser-1177 site preferentially with an increase in NO production via PAR-1 activation in HCAECs.

Effects of PAR-1 activating peptide on eNOS phosphorylation and NO production in HCAECs

Thrombin most notably is the primary endogenous agonist for PAR-1, -3, and -4.[5,20] To directly assess the effect of PAR-1 activation on eNOS regulation, we stimulated cells with a selective PAR-1-AP, TFLLR (10 μmol/ml) in a time-dependent manner, and measured the phosphorylation of the two eNOS regulatory sites in HCAECs. Similar to thrombin, TFLLR significantly phosphorylated eNOS-Ser-1177 at the 30-s time point ($P < 0.0001$) (Figure 2(a)). Unlike thrombin, TFLLR significantly phosphorylated eNOS-Thr-495 starting at 30 s and the phosphorylation remained constant up to 10 min ($P < 0.001$). Nitrate/nitrite was also measured following TFLLR stimulation and there was a significant increase at the 30-min time point, ($P < 0.0001$) (Figure 2(b)), which confirms PAR-1 mediated NO production. The delay in NO production observed when the cells were stimulated with TFLLR may be due to the input of eNOS-Thr-495. We observed that the phosphorylation of this catalytic site declines over time, and therefore, its negative affect may be eventually over-ridden by the earlier activation of eNOS-Ser-1177.

To validate PAR-1 regulation of eNOS in HCAECs, we pretreated the cells with SCH (10 μmol/ml) for 1 h, which blocked TFLLR-mediated eNOS-Ser-1177 phosphorylation at 30 s ($P < 0.001$) and subsequent nitrate/nitrite production at 30 min ($P < 0.01$) (Figure 2(c) and (d). In our previous studies, we demonstrated that activation of PAR-1 by TFLLR in HUVECs leads to the phosphorylation of eNOS-Thr-495 and a decrease in NO production (29). These findings support the cell-type specific regulation of eNOS by PARs.

Effects of PAR-2 activating peptide on eNOS phosphorylation and NO production in HCAECs

Thrombin has been characterized to phosphorylate both regulatory sites of eNOS via direct activation of PAR-1
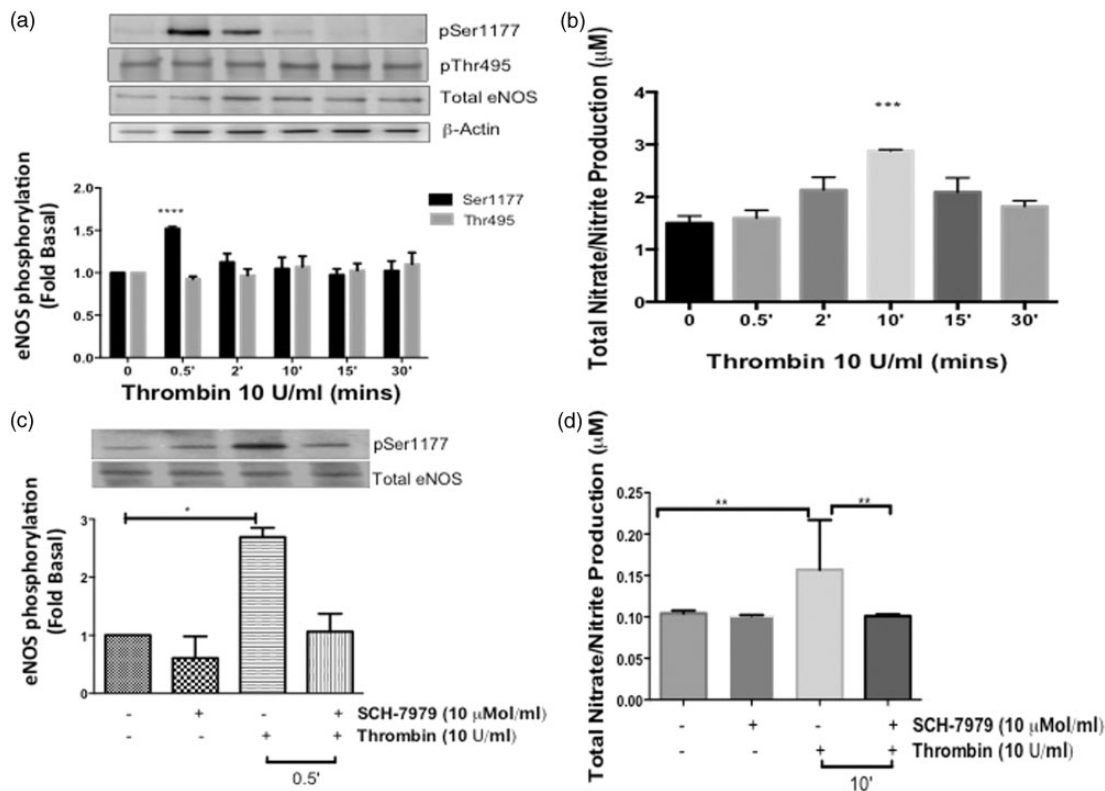
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Figure 1   Effects of thrombin on eNOS phosphorylation and NO production in HCAECs (a) HCAECs were stimulated with thrombin (10 U/ml) for the indicated time points and phosphorylated eNOS-ser1177 and eNOS-Thr495 were determined using Western blot analysis with phosphospecific antibodies. Representative immunoblots are shown with densitometric analyses. Mean $+/-$ SEM of measurements in cultures of five different passages; ****$P < 0.0001$ Thrombin 0.5 min versus Basal. (b) NO production was determined indirectly by measuring total nitrate/nitrite using media from HCAECs treated with thrombin (10 U/ml) for the indicated time points. $n = 5$, ***$P < 0.05$ Thrombin versus Basal. (c) Cells were pretreated with SCH-79797 (10 µmol/L) or vehicle (0.01% DMSO) for 1 h prior to stimulation with thrombin (10 U/ml) for 0.5 min. Phosphorylated eNOS-Ser1177 and eNOS-Thr495 were determined using Western blot analysis with phosphospecific antibodies. Representative immunoblots are shown with densitometric analyses. Mean $+/-$ SEM of measurements in cultures of four different passages; *$P < 0.001$ Thrombin versus Vehicle. (d) Total nitrate/nitrite production was measured using media from HCAECs pretreated with SCH-79797 (10 µmol/L) or vehicle (0.01% DMSO) for 1 h prior to stimulation with thrombin (10 U/ml) for 10 min $n = 3$, **$P < 0.05$ Thrombin versus Basal and SCH + Thrombin 10 min

and PAR-1 transactivation of PAR-2 in HUVECs.[12,13,15] Because thrombin was shown to preferentially modulate PAR-1 activity in HCAECs, we wanted to investigate the role of PAR-2 in this cell line. Therefore, we used a PAR-2-AP, SLIGRL, to test direct PAR-2 function in the regulation of eNOS. HCAECs were stimulated with SLIGRL (50 µmol/ml) in a time-dependent manner, which led to significant phosphorylation of eNOS-Thr-495 at 10 ($P < 0.0001$), 15 ($P < 0.05$), and 30 min ($P < 0.05$) (Figure 3(a)). We also observed a significant increase in phosphorylation at eNOS-Ser-1177 at 30 s and 2 min ($P < 0.05$ vs. Basal). Consequently, total NO metabolites were not detected during the time-course when compared to basal levels (Figure 3(b)). These data suggest that PAR-2 can modulate both regulatory sites; however, activated PAR-2 sustained the phosphorylation of eNOS-Thr-495, demonstrating for the first time a functional dysregulation of PAR-2 signaling in HCAECs.

Effects of PAR-3 activating peptide on eNOS phosphorylation and NO production in HCAECs

PAR-3 is classified as the second thrombin receptor, which is richly expressed in a wide variety of vascular cells, and is

thought to possess no independent signaling capacity.[5,20–22] In order to assess the function of PAR-3 in human endothelial cells, we stimulated the cells with a synthetic PAR-3-AP, TFRGAP (500 µmol/mL)[23] in a time-dependent manner. Maximum phosphorylation was observed at 10 min ($P < 0.05$) at the negative regulatory site, eNOS-Thr-495. The positive site, eNOS-Ser-1177 showed no significant change in phosphorylation in comparison to basal (Figure 4(a)). In addition, there was no increase in NO metabolites above the basal level during the time-dependent stimulation with TFRGAP (Figure 4(b)). These data establish for the first time that PAR-3 primarily signals to regulate eNOS-Thr-495 in primary adult HCAECs.

Effects of NOS inhibitor, L-NAME, on PAR-1 mediated NO production in HCAECs

We previously reported that a NOS inhibitor blocked the thrombin-mediated regulation of eNOS/cGMP in bovine endothelial cells.[15] Interestingly, the timing between thrombin- and TFLLR-induced eNOS-Ser-1177 phosphorylation and subsequent NO production in HCAECs is substantially delayed when compared to that observed in our previous studies, where PAR-mediated NO production occurred
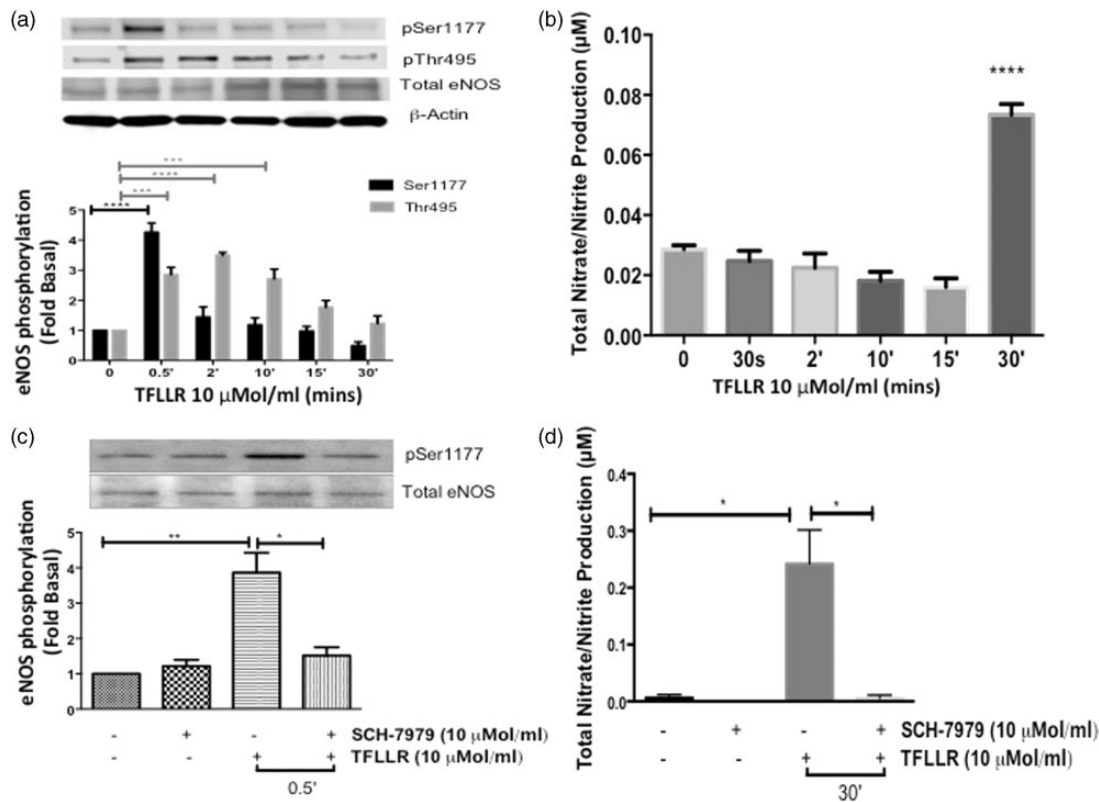
Figure 2 Effects of PAR-1 activating peptide on eNOS phosphorylation and NO production in HCAECs (a) HCAECs were stimulated with TFLLR (10 µmol/L) for the indicated time points and phosphorylated eNOS-Ser1177 and eNOS-Thr495 were determined using Western blot analysis with phosphospecific antibodies ****$P < 0.0001$ TFLLR 0.5 min versus Basal, ***$P < 0.001$ TFLLR Thr495p 0.5 min, 2 min and 10 min versus Basal. (b) NO production was determined indirectly by measuring total nitrate/nitrite using media from HCAECs treated with TFLLR (10 µmol/L) for the indicated time points $n = 3$, ****$P < 0.0001$ TFLLR 30 min versus Basal. (c) Cells were pretreated with SCH-79797 (10 µmol/L) or vehicle (0.01% DMSO) for 1 hour prior to stimulation with TFLLR (10 µmol/L) for 0.5 min. Phosphorylated eNOS-Ser-1177 was determined using Western blot analysis with phosphospecific antibody. Representative immunoblots are shown with densitometric analyses. Mean +/− SEM of measurements in cultures of six different passages; **$P < 0.001$ TFLLR 0.5 min versus Vehicle, *$P < 0.005$ TFLLR versus SCH + TFLLR 0.5 min. (d) Total nitrate/ nitrite production was measured using media from HCAECs pretreated with SCH-79797 (10 µmol/L) or vehicle (0.01% DMSO) for 1 hour prior to stimulation with TFLLR (10 µmol/ml) for 30 min $n = 3$, *$P < 0.01$ TFLLR 30 min versus Vehicle; TFLLR 30 min versus SCH + TFLLR 30 min

immediately after eNOS-Ser-1177 phosphorylation.[12,13,15] Therefore, to confirm the role of PAR-1 activation in eNOS function, HCAECs were pretreated with eNOS inhibitor, L-NAME (100 µmol/L) 1 h prior to stimulation with thrombin or TFLLR. In Figure 5(a), thrombin caused a significant increase in eNOS-Ser-1177 phosphorylation ($P < 0.05$), which was not inhibited by L-NAME ($P < 0.01$) at the 30-s time point. However, at the 10-min time point eNOS-Ser-1177 phosphorylation was undetectable under the same treatment conditions. Consequently, L-NAME did not alter the basal level of NO metabolites when the cells were stimulated with thrombin for 30 s. However, a significant increase in NO metabolites was observed when the cells were stimulated with thrombin for 10 min ($P < 0.001$), and this increase in NO metabolites was significantly inhibited by L-NAME ($P < 0.001$) (Figure 5(b)).

The PAR-1 specific activating peptide, TFLLR, produced similar results to thrombin showing a significant phosphorylation of eNOS-Ser-1177 at 30 s ($P < 0.0001$), which was not affected by the pretreatment with L-NAME ($P < 0.0001$), and TFLLR-induced eNOS-Ser-1177 phosphorylation was detectable below basal at the 30-min time point, and again was not inhibited by L-NAME in HCAECs (Figure 5(c)).

In Figure 5(d), the nitrate/nitrite metabolites were undetectable when stimulated with TFLLR for 30 s in the absence and presence of L-NAME. However, a significant amount of nitrate/nitrite was measured in cells stimulated with TFLLR for 30 min ($P < 0.0001$). In the presence of L-NAME (Figure 5(d)), there was a significant decrease in the production of these NO metabolites ($P < 0.0001$). Overall, our data demonstrate that PAR-1 activation induces immediate phosphorylation of eNOS-Ser-1177 with eNOS being activated at a later time point in adult HCAECs, which, as stated earlier, may be due to input from the activation of eNOS-Thr-495.

Effects of calcium inhibitor, BAPTA, on PAR-mediated eNOS-Ser-1177 phosphorylation in HCAECs

The Ca^{2+}/calmodulin domain regulates all isoforms of NOS,[16,18] specifically, thrombin has been shown to increase intracellular Ca^{2+} to induce eNOS-Ser-1177 phosphorylation and cGMP production.[5,15,24,25] Therefore, we wanted to determine whether calcium was involved in the differential PAR-mediated phosphorylation of eNOS-Ser-1177 in HCAECs. Cells were pretreated with the selective intracellular Ca^{2+} chelator, BAPTA (10 µmol/ml) for
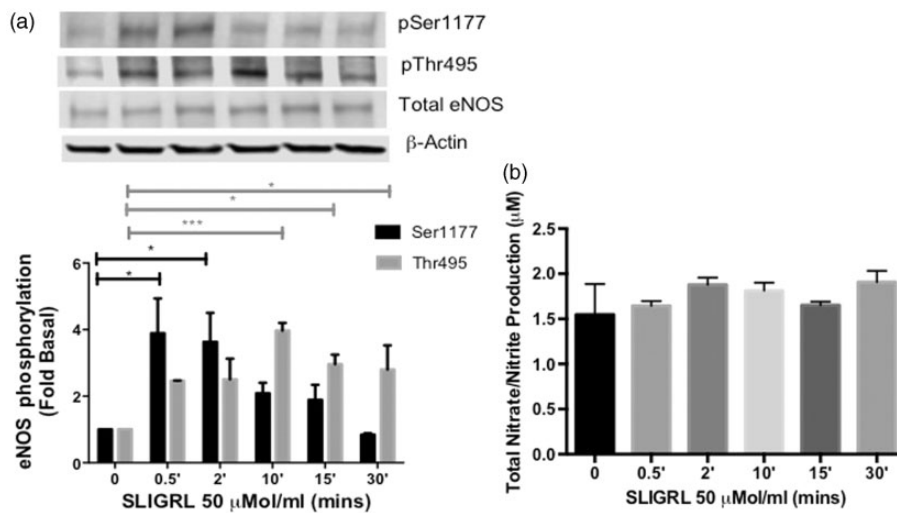
Figure 3 Effects of PAR-2 activating peptide on eNOS phosphorylation and NO production in HCAECs (a) HCAECs were stimulated with SLIGRL (50 μmol/L) for the indicated time points and phosphorylated eNOS-ser1177 and eNOS-Thr495 was determined using Western blot analysis with phosphospecific antibodies. Representative immunoblots are shown with densitometric analyses. Mean $+/-$ SEM of measurements in cultures of six different passages; ***$P < 0.001$ SLIGRL Thr495p 10 min versus Basal, *$P < 0.05$ SLIGRL 15 min and 30 min versus Basal, *$P < 0.05$ SLIGRL Ser1177p 0.5 min and 2 min versus Basal. (b) NO production was determined indirectly by measuring total nitrate/nitrite using media from HCAECs treated with SLIGRL (50 μmol/L) for the indicated time points, $n = 6$, NS
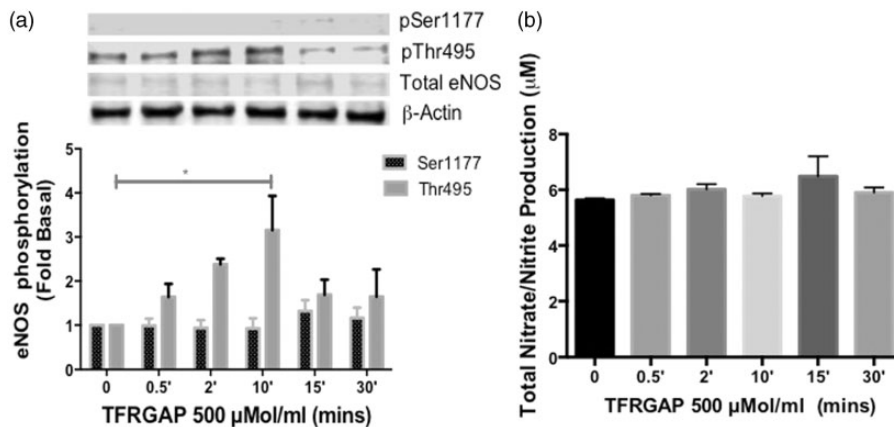

Figure 4 Effects of PAR-3 activating peptide on eNOS phosphorylation and NO production in HCAECs (a) HCAECs were stimulated with TFRGAP (500 μmol/L) for the indicated time points and phosphorylated eNOS-Ser1177 and eNOS-Thr495 was determined using Western blot analysis with phosphospecific antibodies. Representative immunoblots are shown with densitometric analyses. Mean $+/-$ SEM of measurements in cultures of four different passages; *$P < 0.05$ TFRGAP Thr495p 10 min versus Basal. (b) NO production was determined indirectly by measuring total nitrate/nitrite using media from HCAECs treated with TFRGAP (500 μmol/L) for the indicated time points, $n = 4$, NS

30 min, and then stimulated with thrombin and TFLLR for 30 s, and SLIGRL for 2 min. Immunoblot analysis showed that BAPTA significantly inhibited thrombin- ($P < 0.05$) (Figure 6(a)), TFLLR- ($P < 0.001$) (Figure 6(b)) and SLIGRL-mediated ($P < 0.001$) (Figure 6(c)) eNOS-Ser-1177 phosphorylation. These data confirm that thrombin/PAR-mediated eNOS-Ser-1177 phosphorylation is contingent upon the mobilization of intracellular Ca^{2+} stores within HCAECs.

Effects of ROCK inhibitor, Y-27632, on PAR-mediated eNOS-Thr-495 phosphorylation in HCAECs

The involvement of the RhoA/ROCK pathway in endothelial dysfunction has been a major source of controversy.[6,9,12,13]

We used Y-27632 (Y-27), a ROCK inhibitor, to elucidate the mechanism by which activated PAR-1, -2, and -3 induces eNOS-Thr-495 phosphorylation. HCAECs were pretreated with Y-27 (10 μmol/L) for 90 min, and then stimulated with thrombin for 10 min, TFLLR for 2 min, and SLIGRL and TFRGAP for 10 min. Thrombin stimulation did not alter eNOS-Thr-495 phosphorylation regardless of the presence of Y-27 (Figure 7(a)). However, pretreatment with Y-27 significantly decreased selective TFLLR-induced ($P < 0.05$) (Figure 7(b)), as well as SLIGRL-induced ($P < 0.05$) (Figure 7(c)) and TFRGAP-induced ($P < 0.05$) (Figure 7(d)) eNOS-Thr-495 phosphorylation in HCAECs. These data confirm that eNOS-Thr-495 regulation is mediated via a ROCK-dependent pathway in HCAECs.
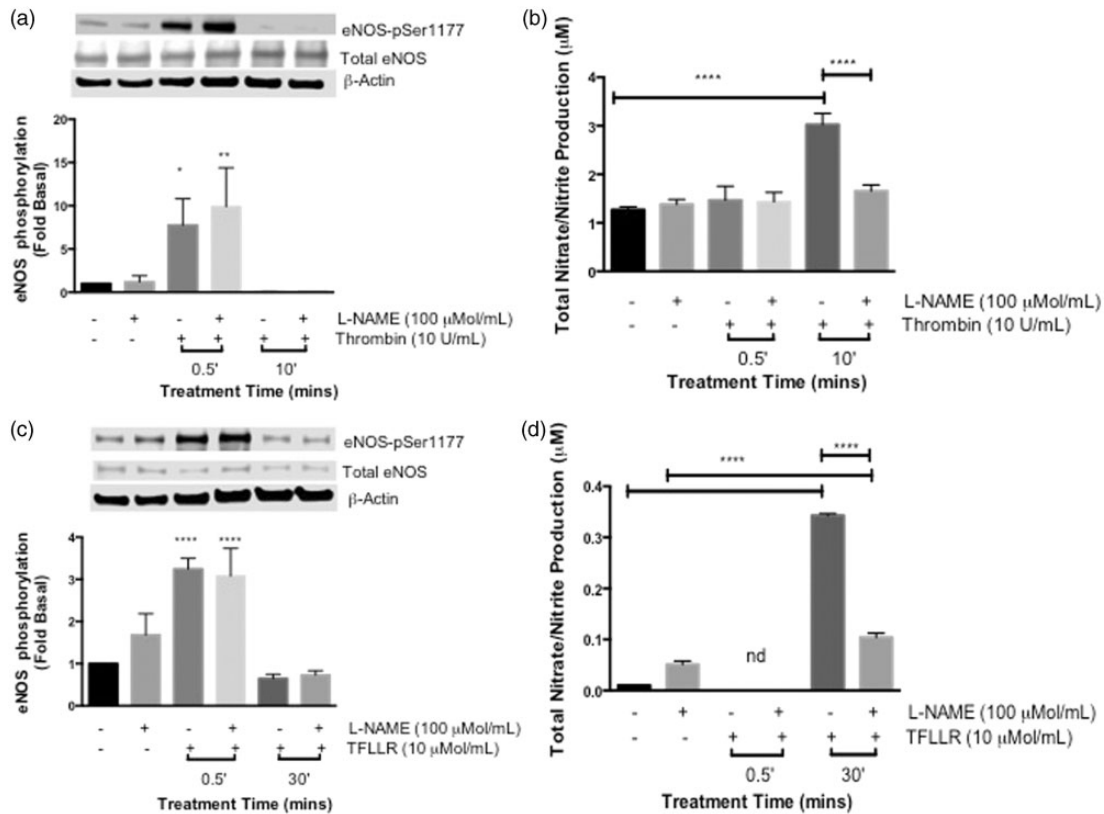
Figure 5 Effects of NOS inhibitor, L-NAME, on PAR-1 mediated NO production in HCAECs. (a) HCAECs were pretreated with L-NAME (100 μmol/L) for 1 h prior to stimulation with thrombin (10 U/ml) for 0.5 min and 10 min. Phosphorylation of eNOS-Ser1177 was determined using Western blot analysis with phosphospecific antibody. Representative immunoblots are shown with densitometric analyses. Mean $+/-$ SEM of measurements in cultures three of different passages; $*P < 0.05$ Thrombin 0.5 min versus Basal, $**P < 0.01$ L-NAME + Thrombin 0.5 min versus Basal. (b) NO production was determined indirectly by measuring total nitrate/nitrite using media from HCAECs pretreated with L-NAME (100 μmol/L) for 1 h prior to stimulation with thrombin (10 U/ml) for 0.5 min and 10 min $****P < 0.001$ Thrombin 0.5 min versus Basal, $****P < 0.001$ L-NAME + Thrombin 0.5 min versus Basal. (c) HCAECs were pretreated with L-NAME (100 μmol/L) for 1 h prior to stimulation with TFLLR (10 μmol/ml) for 0.5 min and 10 min. Phosphorylation of eNOS-Ser1177 was determined using Western blot analysis with phosphospecific antibody. Representative immunoblots are shown with densitometric analyses, $n = 3$, $****P < 0.0001$ TFLLR 0.5 min and L-NAME + TFLLR 0.5 min versus Basal. (d) Total nitrate/nitrite production was measured using media from HCAECs pretreated with L-NAME (100 μmol/L) for 1 h prior to stimulation with TFLLR (10 μmol/L) for 0.5 min and 30 min, $n = 3$, TFLLR 0.5 min, no detection (nd) $****P < 0.0001$ TFLLR 30 min and L-NAME + TFLLR 30 min versus basal

Discussion

In this study, we have identified a variance in PAR coupling to signaling pathways that regulate eNOS phosphorylation and NO production in adult HCAECs. The principal findings are as follows: (1) a PAR-mediated biphasic signaling event that regulates eNOS through dual signaling pathways that results in simultaneous phosphorylation of both eNOS regulatory sites; (2) PAR-induced eNOS-Ser-1177 phosphorylation is Ca^{2+}-dependent, which suggests a G_q-mediated mechanism,[13,15,16] and results in a delayed increase in NO production, while PAR-induced eNOS-Thr-495 phosphorylation is ROCK-dependent, suggesting $G_{12/13}$ coupling,[12] which results in a decrease in NO production in HCAECs; and finally (3) we demonstrated for the first time that the elusive PAR-3 regulates eNOS-Thr-495 via a ROCK-dependent pathway. All of which strongly supports differential PAR signaling and altered eNOS regulation in the adult coronary arterial endothelium as demonstrated in Figure 8.

The attenuation of the endothelium-dependent dilator response as a result of aging, a major risk factor for the development of atherosclerosis, coronary artery disease and other cardiovascular events, is a consequence of the alteration in expression and/or activity of NO synthase.[1,26] Our data show PAR-1 ligands, thrombin and TFLLR, induced phosphorylation of eNOS-Ser-1177 with a substantial delay in NO production in HCAECs, whereas our previous studies showed a PAR-2 ligand, SLIGRL, induced phosphorylation of eNOS-Ser-1177 followed by NO production in HUVEC,[12] which supports the role of a given vascular environment in specific eNOS regulation. NO regulates normal blood pressure and its reduced bioavailability is considered a major cause of vascular disease[27]; however, high concentrations of NO can react negatively by combining with oxidants to produce superoxide (O_2^-) and peroxynitrite ($ONOO^-$), which will lead to vascular inflammation and increased vascular permeability. Lowry et al.[28] recently found that under inflammatory conditions eNOS-Ser-1177 is phosphorylated and can contribute to high NO output in the vasculature, which is normally attributed to inducible NOS (iNOS). Alternatively, at low levels, NO promotes barrier function, reduce cytokine recruitment, and enhance vascular stability.[29] The use of L-NAME, an eNOS inhibitor, significantly blocked the thrombin/eNOS/NO signaling pathway confirming that
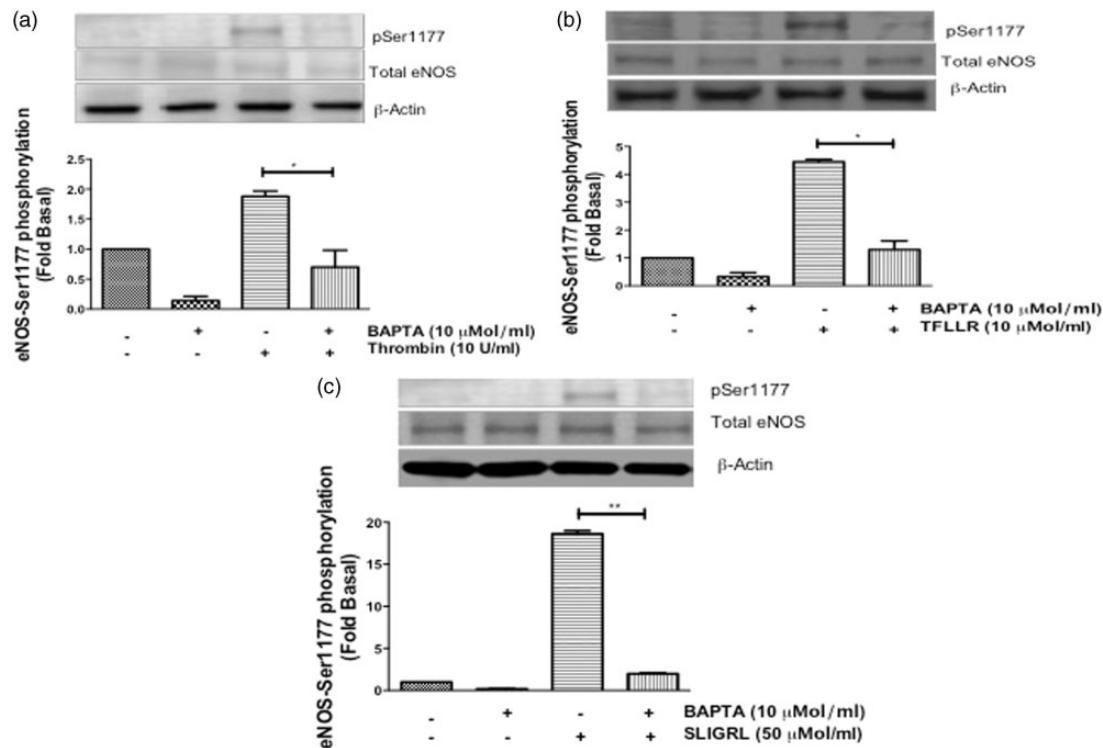
Figure 6 Effects of calcium inhibitor, BAPTA, on PAR-mediated eNOS-Ser-1177 phosphorylation in HCAECs. HCAECs were pretreated with BAPTA-AM (10 µmol/L) for 30 min and then stimulated with (a) thrombin (10 U/ml) and (b) TFLLR (10 µmol/L) for 0.5 min, and (c) SLIGRL (50 µmol/L) for 2 min. Phosphorylation of eNOS-Ser1177 was determined using Western blot analysis with phosphospecific antibody. Representative immunoblots are shown with densitometric analyses. Mean +/− SEM of measurements in cultures of three different passages; *P < 0.05 Thrombin versus BAPTA + Thrombin and TFLLR versus BAPTA + TFLLR, **P < 0.001 SLIGRL versus BAPTA + SLIGRL, n = 3
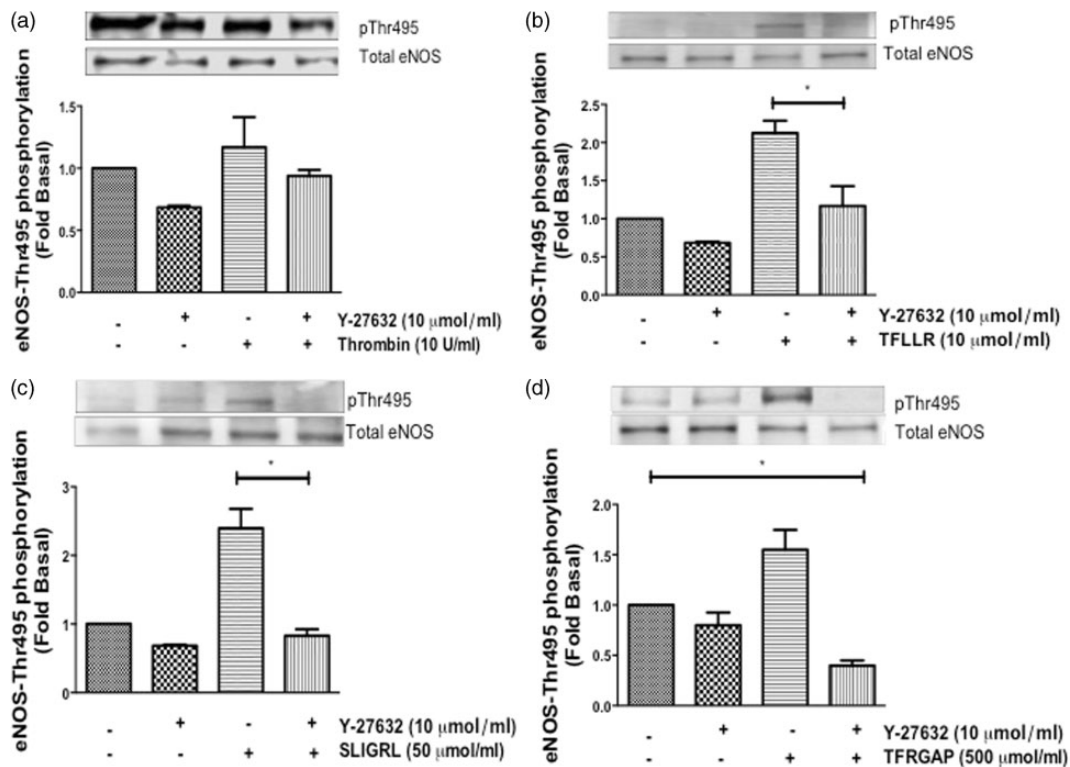

Figure 7 Effects of ROCK inhibitor, Y-27632, on PAR-mediated eNOS-Thr-495 phosphorylation in HCAECs. HCAECs were pretreated with Y-27632 (10 µmol/L) for 90 min and then stimulated with (a) thrombin (10 U/ml) for 10 min, (b) TFLLR (10 µmol/L) for 2 min, (c) SLIGRL (50 µmol/L) for 10 min and (d) TRAGAP (500 µmol/L) for 10 min. Phosphorylation of eNOS-Thr-495 was determined using Western blot analysis with phosphospecific antibody. Representative immunoblots are shown with densitometric analyses. Mean +/− SEM of measurements in cultures of three different passages; *P < 0.05 TFLLR 2 min, SLIGRL 10 min, TFRGAP 10 min versus Basal, or Y-27 versus Y-27 + TFLLR 2 min, SLIGRL 10 min, or TFRGAP 10 min, n = 3
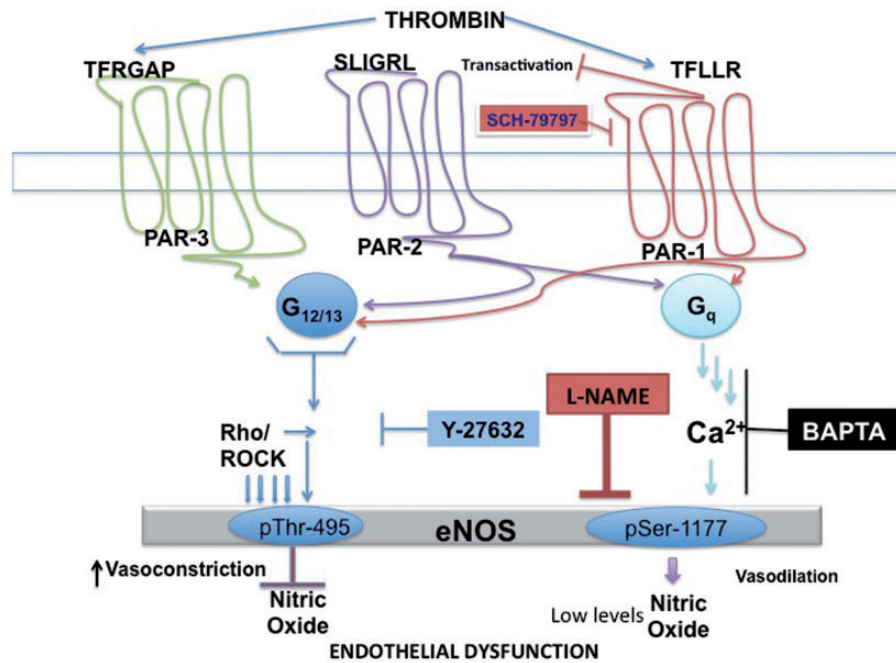
Figure 8 Proposed signal transduction cascade of PAR-1, PAR-2 and PAR-3 in Adult HCAECs. In adult HCAECs, PAR-1 and PAR-2 couple to both G_q and $G_{12/13}$ to regulate the signaling pathways that phosphorylate eNOS and cause NO production. A Ca^{2+}-dependent-mediated mechanism, which is coupled to G_q, leads to the phosphorylation of eNOS-Ser-1177 and causes an increase in NO production. A ROCK-dependent pathway, which is coupled to $G_{12/13}$, leads to eNOS-Thr-495 phosphorylation and a decrease in NO production. PAR-3 activation is coupled to $G_{12/13}$ and the ROCK signaling pathway, which leads to a suppression of NO production via phosphorylation of eNOS-Thr-495. (A color version of this figure is available in the online journal.)

eNOS is functional and responsible for NO production in HCAECs. In previous studies, we showed thrombin-induced cGMP production via eNOS activation was inhibited using an eNOS alternative L-NAME analog, N^G-nitro-L-arginine methyl ester, which attenuated thrombin-induced cGMP production.[15] The low and undetectable levels of NO shown in our studies suggest a hypertensive-like state and could be the result of cell aging, genetic modifications, and/or limited endothelial NOS regulation at the substrate and/or cofactor levels. Despite the two-fold increase in phosphorylation of eNOS-Ser-1177 shown with thrombin, TFLLR, and SLIGRL, NO production may be modulated by input from the phosphorylation of eNOS-Thr-495. The phosphorylation of this catalytic site was significantly elevated by TFLLR and SLIGRL, which resulted in a significant delay or complete inhibition in enhanced NO production, respectively. Our current methodology for NOS function is limited to NO byproduct, nitrates and nitrite, only as demonstrated in previous studies.[12] Therefore, it is also possible that the reduced NO levels may be attributed to low bioavailability of the essential cofactor, tetrahydrobiopterin (BH_4). It has been shown that trace amounts of BH_4 will lead to eNOS uncoupling to produce reactive oxygen species (ROS), superoxide (O_2^-), which will exacerbate the endothelium in a negative feedback loop increasing ROS, vascular inflammation, and a severe vascular instability.[30] It is known that eNOS uncoupling promotes "Nitroxidative Stress," an event that enhances the expression of nitrotyrosine, a reactive nitrogen marker[31]; therefore, we measured the expression of this protein and found that it was not detected in any of our

experimental conditions (data not shown). Techniques for direct measurement of other "nitro" species for eNOS function will be utilized in future studies. Low PAR-1 expression, thrombin concentration, or receptor recycling may also contribute to the difference in eNOS activity displayed in HCAECs.[4,5,10] The attenuation of the endothelium-dependent vasodilation response as a consequence of the alteration in the expression and/or activity of eNOS is associated with aging vessels or disease development.[26] *In vivo* studies support these findings showing that coronary artery occlusion triggers endothelial dysfunction and decreased eNOS-dependent vasoreactivity.[32] A more recent study demonstrates impairment in endothelial-dependent vasodilation secondary to a loss of NO production from NOS with a shift of the enzyme to the production of O_2^-.[33]

It has been confirmed that thrombin, at low concentrations, induces eNOS-Thr-495 phosphorylation through its high-affinity receptor, PAR-1, whereas high thrombin concentrations induce transactivation of PAR-2 via PAR-1 tethered ligand leading to G_q coupling which mediates eNOS-Ser-1177 phosphorylation and immediate NO production.[12,13] In HCAECs, activation of PAR-1 via thrombin has a minimal effect on eNOS-Thr-495 phosphorylation, which appears to be predominantly regulated by PAR-2, and -3. Thrombin-mediated NO production is attenuated in the presence of the PAR-1 inhibitor, SCH, confirming the specificity of PAR-1 in the production of NO. Extended PAR-1 activation via thrombin in human pulmonary endothelial cells revealed a negative regulation of key players in the NO/cGMP pathway to promote pulmonary arterial hypertension.[11] While HCAECs used in this study

were classified as normal, they could have been derived from a stressed or a hypertensive endothelium state, demonstrating a PAR-mediated eNOS/NO dysfunction in primary arterial cells.[17]

Differential coupling of PARs in other cellular systems such as in HUVECs, which are embryonic and differentiate in nature shows PAR-2 activation with SLIGRL as a positive regulator of eNOS.[12] Supporting this phenomenon, studies using BAECs revealed PAR-2 activation leads to increased phosphorylation at eNOS-Ser-1179 through a phospholipase C-dependent rapid and transient increase in intracellular Ca^{2+}.[15] *In vivo* studies have shown that systemic administration of a PAR-2 agonist yields a persistent hypotensive response due to arterial dilation with no change in heart rate.[34] Similarly, short-term activation of PAR-2 has been described to have vasodilative properties in humans and rodents.[35,36] This study challenged the established dogma of PAR-2 signaling, by demonstrating that PAR-2 activation leads to the phosphorylation of both regulatory sites in HCAECs with no increase in NO production, supporting PAR-2 could be a negative regulator of eNOS in certain human endothelial cells. Studies in smooth muscle cells attribute PAR-2 activation to increased MYPT phosphorylation and contractility.[37] In addition, prolonged PAR-2 activation was characterized to cause endothelium dysfunction, independent of systemic pro-inflammation *in vivo*, supporting the diverse signaling potential of PAR-2 in the vasculature.[35] This opposing role of PAR-1/-2 signaling demonstrated in this study provides a clue of disease initiation, an area to be explored in future studies.

Little is known about the function and signaling capacity of the second thrombin receptor, PAR-3. Initial studies in mouse platelets concluded that PAR-3 functions as a cofactor for PAR-4; mouse PAR-3 (mPAR-3) response was null to thrombin when over-expressed; however, inhibition of mPAR-3 and/or knockdown of the mPAR-3 gene inhibited mouse platelet activation at low levels of thrombin, suggesting mPAR-3 as a facilitator for mPAR-4 signaling.[21] However, unlike PAR-4, PAR-3 is significantly expressed in HCAECs and other human vascular subtypes.[5,10] Our data suggest that activated PAR-3 regulates the negative site of eNOS by increasing the phosphorylation of eNOS-Thr-495 in a time-dependent manner, via ROCK-mediated signaling; however, NO levels remain unchanged. The functional role for PAR-3 in the eNOS/NO pathway remains to be explored further. Non-classical activation of PAR-3 by Factor FXa, at Arg41 unlike Lys38 by thrombin, stabilizes tight junctions via Tie2 activation to enhance vascular stability in immortalized endothelial cells.[38] Preliminary studies in our lab demonstrated that PAR-3 can signal independently of PAR-1, and act as the primary regulator of eNOS in a PAR-1 down-regulated HCAEC-PAR-1$^{-/-}$ cell line, which caused a transient phosphorylation of eNOS-Ser-1177 and a sustained phosphorylation of eNOS-Thr-495 (data not shown) in the presence of thrombin. Thrombin and TFRGAP were shown to signal via the ERK1/2 pathway to enhance the upregulation and release of IL-8 in PAR-3 over-expressed HEK-293 cells.[6] On the contrary, McLaughlin et al.[20] observed an increase in $G_{\alpha 13}$ coupling to PAR-1/PAR-3 heterodimers rather than PAR-1

homodimers, concluding that PAR-3 alloseterically regulates PAR-1 signaling to increase endothelial cell permeability, suggesting that PAR-3 has no independent signaling potential. More studies in primary human cells, healthy and diseased, will validate the pathophysiological relevance of PAR-3, making it a potential target for therapeutic manipulation.

Activation of PARs by thrombin prompts an array of intracellular signaling pathways, including PKC and Rho/ROCK.[3,39] Studies using a specific G_q inhibitor, YM-254890, show that eNOS-Ser-1177 induced phosphorylation and NO production was blocked.[13] In BAECs, thrombin-induced positive eNOS/NO modulation occurs via a Ca^{2+}-dependent, PKCδ-sensitive, but PI3-K/AKT-independent pathway.[15] In this study, we found that the phosphorylation of eNOS-Ser-1177, a site conserved in the Ca^{2+}/calmodulin domain of eNOS, was blocked by BAPTA, an intracellular Ca^{2+} chelator, confirming the essential role of Ca^{2+} in eNOS-Ser-1177 phosphorylation and NO production.[16,18,19] Alternatively, Rho has been established to block NO production in arteries[40] and cease AKT signaling in endothelial cells to prevent eNOS-Ser-1177 phosphorylation.[9] We were able to discern eNOS-Thr-495 regulation by inhibiting the downstream kinase of Rho, ROCK, with inhibitor Y-27632, which blocked PAR-1, PAR-2, and PAR-3 mediated eNOS-Thr-495 phosphorylation. Targeting RhoA by C3 exoenzyme, which inactivates Rho by ADP-ribosylation at the asparagine 41 in the effector region of the GTPase, recovered the down-regulation of eNOS expression induced by thrombin and suppressed TFLLR-induced eNOS-Thr-495 phosphorylation.[12,39] All of which confirms that the constitutively active Rho/ROCK pathway enhances PAR-mediated negative eNOS function, likely suggesting $G_{12/13}$ activity to suppress the Ca^{2+}/calmodulin interaction of eNOS. Concluding that phosphorylated eNOS-Thr-495 down-regulates eNOS enzymatic activity; a PAR-2 and PAR-3 mediated response, indicating a reverse function in the adult human endothelium.

In conclusion, the findings in this study confound the established PAR-mediated signaling of vascular eNOS function and NO production. The well-known regulators of eNOS appear to exchange roles in human coronary endothelial cells, which could be the initiation of disease progression, establishing a novel PAR signal alteration. The expression ratio of PARs in primary HCAEC verses other subtypes/species vary and may also contribute to the reverse signaling demonstrated in this study. Endothelial derived NO and its derivatives play a critical role in regulating normal vascular function but can also be involved in exacerbating or eliminating cardiovascular diseases.[29,41] In contrast to other studies, we found that PAR-1 and -2 can couple to both Ca^{2+} and ROCK signaling pathways to increase or suppress NO production, while also discerning the role of the elusive PAR-3, which negatively regulates eNOS via a ROCK-dependent mechanism. Complementary translational studies in distinct vascular beds need to be explored to fully ascertain PAR-mediated eNOS/NO signaling. Furthermore, understanding of PAR signaling in the human vasculature will be beneficial to look for new

therapies to circumvent and treat endothelial dysfunction and other associated cardiovascular diseases.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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