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## Nitric oxide donor [Ru(terpy)(bdq)NO]<sup>3+</sup> induces uncoupling and phosphorylation of endothelial nitric oxide synthase promoting oxidant production

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### Abstract

[Ru(terpy)(bdq)NO]<sup>3+</sup> (TERPY) is a nitric oxide (NO) donor that promotes relaxation of the mesenteric artery and aorta in rats. We sought to investigate whether it acts as both an NO donor and endothelial NO synthase (eNOS) activator, as shown previously for nitroglycerin. Human umbilical vein endothelial cells (HUVECs) and human embryonic kidney 293 cells transfected with empty vector (HEK) or eNOS cDNA (HEK-eNOS) were treated with TERPY (1 μM) for different lengths of time. eNOS expression, dimerization, and Ser<sup>1177</sup> phosphorylation, caveolin-1 (Cav-1) oligomerization, Cav-1 Tyr<sup>14</sup> phosphorylation were evaluated by Western blotting. Studies also assessed the production of reactive oxygen/nitrogen species (ROS/RNS) in HUVECs and HEK-eNOS cells. In HEK cells devoid of eNOS, TERPY released NO without additional stimulus indicating that is an NO donor. Moreover, in HEK-eNOS cells, TERPY-induced NO production that was blocked by L-NAME. In addition, TERPY increased ROS and ONOO<sup>-</sup> production which

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### Author Contributions

Conceived and designed the experiments: SRP ZC SDSO RDM.

Performed the experiments: SRP ZC SDSO.

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Provided the drugs and contributed to the writing of the manuscript: SRP LMB RS da S MGB CA RDM.

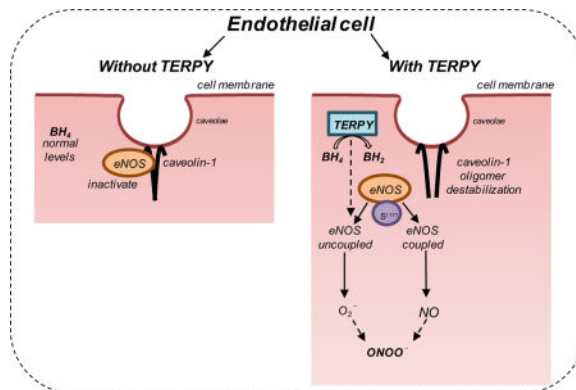
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### Conflict of interest

The authors have no conflicts of interest to declare.

were blocked by more than 80% by BH<sub>4</sub> (essential eNOS co-factor) and eNOS siRNA. These results suggest that TERPY-induced ROS and ONOO<sup>-</sup> production were originated from eNOS. HUVECs stimulated with TERPY showed increased eNOS Ser<sup>1177</sup> and Cav-1 Tyr<sup>14</sup> phosphorylation, and decreased eNOS dimerization, Cav-1 oligomerization, and Cav-1/eNOS interaction after 20 min. It suggests that TERPY induces eNOS hyperactivation and uncoupling by disrupting Cav-1/eNOS interaction and depleting BH<sub>4</sub>. Endothelium-dependent vasodilation in response to NO donor TERPY is associated with eNOS activation and uncoupling, and thereby appears to be mediated, at least in part, via eNOS-dependent ROS/RNS production.

## Graphical abstract



## Keywords

NO donor; TERPY; eNOS uncoupling; caveolin-1; eNOS hyperactivation

## 1. Introduction

Endothelial nitric oxide synthase (eNOS), comprised of reductase and oxygenase domains connected by calmodulin (CaM), functions as a dimer [1]. Nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN) are co-substrates of eNOS, whereas (6R-)5,6,7,8-tetrahydro-L-biopterin (BH<sub>4</sub>) and L-arginine are cofactors. When CaM affinity for eNOS increases, it promotes the alignment of two monomeric subunits, enabling the reductase domain of one eNOS molecule to transfer NADPH-derived electrons via FAD and FMN to the heme moiety in the oxygenase domain of a second juxtaposed eNOS molecule [2]. In the oxygenase domain, BH<sub>4</sub> facilitates electron transfer upon L-arginine oxidation forming NO and L-citrulline [3].

In blood vessels, NO is primarily produced by eNOS and it is an important modulator of vascular tone [4]. However, NO is a highly reactive free radical [5] and its half-life in biological tissues is estimated to be less than 6 seconds [6]. Therefore, NO donors have become an attractive pharmacological tool to study cellular mechanisms of NO action and commonly used therapeutically, especially in patients with cardiovascular disease characterized by a reduction in NO bioavailability.

[Ru(terpy)(bdq)NO]<sup>3+</sup> (TERPY) is a ruthenium complex NO donor that promotes a hypotensive effect with greater magnitude in hypertensive rats when compared with their normotensive controls [7]; [8]. The TERPY-induced hypotensive effect is slow, long lasting, and it does not lead to reflex tachycardia [7]. Furthermore, TERPY promotes relaxation in aorta [7]; [9] and in resistance vessels [10] from hypertensive and normotensive rats.

It was demonstrated that TERPY oxidizes BH<sub>4</sub> to dihydrobiopterin (BH<sub>2</sub>) [11]. When BH<sub>4</sub> bioavailability is limiting, electron transfer from eNOS flavins become uncoupled from L-arginine oxidation and hydrogen peroxide or superoxide anion are generated by uncoupled eNOS [12];[13];[14]; [15]. Moreover, in aortas of normotensive rats, the presence of endothelium or NOS activity attenuated the vasodilator effect of TERPY [11]. However, in aorta from spontaneously hypertensive rats (SHR), the effect of TERPY was entirely distinct from that observed in Wistar rats in that it was improved by endothelium or NOS activity [9].

In this work, we have demonstrated that TERPY increases the NO production by endothelial cells, in addition to donating NO. As the pharmacological interaction between TERPY and NOS is not completely understood, this study aimed to evaluate the effect of TERPY on NOS activity in endothelial cells.

## 2. Methods

### 2.1 Cell culture

**Human umbilical vein endothelial cells (HUVECs)**—HUVECs were purchased from Vec Technologies (Rensselaer, NY). They were cultured in growth medium (EGM-2 plus rhEGF 0.5 mL, ascorbic acid 0.5 mL, hydrocortisone 0.2 mL, heparin 0.5 mL; VEGF 0.5 mL; GA-1000 0.5 mL, R3-IGF-1 0.5 mL, rhFGF-B 2.0 mL) from Lonza (Walkersville, MD) supplemented with 10% of fetal bovine serum (FBS) from Gemini Bio-Products (West Sacramento, CA) and used at passages 4–6.

**Wild-type (WT) human embryonic kidney (HEK) 293 and HEK cells stably overexpressing eNOS cDNA (HEK-eNOS)**—HEK cells were purchased from the American Type Culture Collection (Rockville, MD). eNOS cDNA (in pcDNA3.1) was transduced in HEK 293 cells using Lipofectamine 2000 from Invitrogen (Carlsbad, CA) according to the manufacturer's instructions. They were cultured in Dulbecco's modified eagle medium (DMEM 1x) from Corning Cellgro (Manassas, VA) supplemented with 10% FBS and 1% penicillin/streptomycin from Invitrogen (Carlsbad, CA).

### 2.2 Drugs and Reagents

The NO donor compound TERPY (MW: 951.00) was synthesized in the Analytical Chemistry Laboratory at the Department of Physics and Chemistry of the Faculty of Pharmaceutical Sciences of Ribeirão Preto, as described by de Lima *et al.* [16]. The structure of TERPY was demonstrated by Bonaventura *et al.* [11] and Munhoz *et al.* [7]. eNOS si-RNA was purchased from QIAGEN (Valencia, CA). N<sub>ω</sub>-nitro-L-arginine methyl ester hydrochloride (L-NAME, non-selective NOS inhibitor), dihydroethidium (DHE, fluorescence probe for measurement of ROS), catalase-polyethylene glycol (PEG-catalase,

C4963), calcium ionophore (A23187), 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo [3, 4-*d*] pyrimidine (PP2, Src kinase inhibitor), catalase (C1345), superoxide dismutase (SOD, S8160), superoxide dismutase-polyethylene glycol (PEG-SOD, S9549) and uric acid (U0881) were obtained from Sigma-Aldrich (St. Louis, MO). Coumarin-7-boronic acid (7-CBA, fluorescent probe for detection of peroxynitrite, ONOO<sup>-</sup>) and tetrahydro-L-biopterin hydrochloride (BH<sub>4</sub>) were obtained from Cayman Chemical (Ann Arbor, MI). 4-Amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM, fluorescence probe for detection of NO) was obtained from Invitrogen (Carlsbad, CA). CuFL, a fluorescein-based Cu(II) NO sensor, was obtained from Strem Chemicals (Newburyport, MA). Rabbit anti-phospho-eNOS (Ser<sup>1177</sup>) was obtained from Cell Signaling Technology (Danvers, MA). Mouse anti-eNOS (610297), rabbit anti-Cav-1 polyclonal (610060), mouse anti-Cav-1 monoclonal (610406), mouse anti-phospho-Cav-1 (pTyr<sup>14</sup>, 611339) and mouse anti-actin (612657) were obtained from BD Biosciences (San Jose, CA). Mouse anti-GAPDH (sc-25778) was from Santa Cruz Biotechnology (Dallas, TX). Rabbit anti-phospho-eNOS (Tyr<sup>657</sup>) was obtained from ECM Biosciences (Versailles, KY).

### 2.3 Western Blotting

After serum deprivation in culture medium for 3–5 h, treatment was performed with different reagents in confluent HUVECs, HEK-WT, and HEK-eNOS cells. The samples were lysed on ice in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.4 ± 0.2) containing 1% of protease and phosphatase inhibitors cocktail (Sigma-Aldrich, St. Louis, MO). The lysates were centrifuged (20 minutes, 12,000 rpm, 4°C) and protein concentration was quantified by Lowry method [17]. Lysates were boiled in 4× Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) and 10% mercaptoethanol and after that they were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membranes. The membranes were blocked with 5% blotting-grade nonfat dry milk (Bio-Rad, Hercules, CA) in Tris-buffered saline (TBS plus 0.05% Tween-20, 60 min) and incubated with primary antibodies overnight at 4°C, followed by subsequent incubation with secondary antibodies (mouse or rabbit) for 1 hour at room temperature. Proteins were visualized with enhanced chemiluminescence substrate (Super Signal West Pico or Femto, Thermo Scientific, Waltham, MA).

**Low-Temperature SDS-PAGE analyses of eNOS dimer/monomer and Cav-1 oligomer/monomer**—Cultured cells were lysed on ice in buffer (RIPA) and lysates were under non-denaturing conditions. Samples were prepared with 5x SDS loading buffer (250 mM Tris, 10% SDS, 20% glycerol, 0.02% bromophenol blue) plus 10% dithiothreitol (DTT 1M) and were loaded on 8–12% polyacrylamide gels. During the electrophoresis process and transfer of proteins to nitrocellulose membrane, buffers were placed in an ice-water bath and the whole apparatus was kept at 4°C. The monomer and dimer forms of eNOS and oligomer and monomer forms of Cav-1 were incubated with specific polyclonal antibodies and detected by chemiluminescence substrate (Pierce).

## 2.4 Co-immunoprecipitation (co-IP)

Co-immunoprecipitation (co-IP) was performed as previously described [18]; [19], with the following modifications. We performed the treatment with TERPY in confluent HUVECs plated on 100 mm dishes. Cells were lysed in 2% octyl-D-glucoside (ODG) buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1% protease inhibitor cocktail, 1 mM sodium orthovanadate) and centrifuged (20 minutes, 13,000 rpm, 4°C). Supernatants were collected and rotated overnight at 4°C with specific antibodies (anti-rabbit immunoglobulin IgG, rabbit anti-eNOS polyclonal or mouse anti-Cav-1 monoclonal). After that, the lysates were rotated for 2 hours at 4°C with protein A/G PLUS-agarose beads. Samples containing beads were washed eight times in Tris-buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 0.5% NP-40). The proteins were eluted with Laemmli sample buffer and then the samples were boiled for 10 minutes at 100°C and eNOS or Cav-1 were detected by Western blot analysis.

## 2.5 siRNA-mediated eNOS depletion

HUVECs seeded in 60 mm wells were grown to ~40% confluence and transfected with siRNA specific for eNOS or scrambled siRNA at the final concentration of 100 nM. After 72 hours, cells were stimulated and lysed at 4°C for Western blot analysis.

## 2.6 Analysis of eNOS activity by DAF-FM or NO sensor CuFL fluorescence probes

Cells were grown in 96-well black assay plates (Corning Incorporated, Corning, NY), washed twice with HBSS, and incubated with media containing 2.5 µM DAF-FM or 1 µM CuFL plus 1 mM L-arginine for 60 minutes. Before stimulation, the cells were pretreated with 100 µM BH<sub>4</sub> (30 minutes), 100 µM L-NAME (30 minutes) or 1 mM L-NAME (30 minutes). Then, treatment of the same cells was performed with different reagents (vehicle, 1 µM TERPY or 5 µM A23187) for times indicated. Fluorescence produced by benzotriazole or CuFL was read using the bottom-read mode of a SpectraMax M5 Microplate Reader (Molecular Advances, Sunnyvale, CA) and fluorescence of DAF-FM was measured using Excitation: 488 nm/Emission: 530 nm.

## 2.7 Measurement of superoxide anion using DHE probe

Cells were grown in 96-well black assay plates (Corning Incorporated, Corning, NY), washed twice with HBSS, and incubated with media containing 2.5 µM DHE plus 1 mM L-arginine for 60 minutes. Before stimulation, the cells were pretreated with 100 µM BH<sub>4</sub> (30 minutes), 100 µM L-NAME (30 minutes), 1 mM L-NAME (30 minutes), 300 U/mL SOD (60 minutes), 300 U/mL PEG-SOD (60 minutes) or 300 µM uric acid (120 minutes). Then, treatment of the same cells was performed with different reagents (vehicle or 1 µM TERPY) for times indicated. Fluorescence produced by DHE was measured using wavelength 370 nm/420 nm (Excitation/Emission) in SpectraMax M5 Microplate Reader (Molecular Advances, Sunnyvale, CA).

## 2.8 Measurement of ONOO<sup>-</sup> production by coumarin-7-boronate acid (7-CBA)

Cells were grown in 6-well assay plates (Corning Incorporated, Corning, NY), washed twice with HBSS, and incubated with media containing 20 µM 7-CBA plus 1 mM L-arginine for

60 minutes. Before stimulation, some samples were pretreated with 100  $\mu\text{M}$   $\text{BH}_4$ , 300 U/mL Catalase (30 minutes), 300 U/mL PEG-cat (30 minutes), 300 U/mL SOD (60 minutes), 300 U/mL PEG-SOD (60 minutes) or 300  $\mu\text{M}$  uric acid (120 minutes). Then, treatment with different reagents (vehicle or 1  $\mu\text{M}$  TERPY) was performed for the times indicated. The supernatant was collected, placed in 96-well assay plates, and fluorescence analysis of the oxidation product 7-OH-coumarin (COH) [20] was performed on a SpectraMax M5 Microplate Reader (Molecular Advices, Sunnyvale, CA). Fluorescence was measured using 350 nm (excitation) and 450 nm (emission). The results were normalized to total cellular protein.

## 2.9 Densitometry and statistics

Densitometry of protein bands was performed with ImageJ software (<http://rsbweb.nih.gov/ij/>). Comparison of two groups was conducted using Student's *t* test, and three or more groups were compared using one-way ANOVA with Tukey's *post hoc* testing. Experimental data are presented as mean  $\pm$  standard error of the mean (SEM). All statistical tests were performed two-sided and non-blinded using GraphPad Prism software for Mac (GraphPad Software, La Jolla, CA). *P* values  $<0.05$  were considered statistically significant.

## 3. Results

### NO production induced by TERPY is dependent, in part, on eNOS

It is known that eNOS phosphorylation on Ser<sup>1177</sup> promotes NO production. Thus, we examined NO production stimulated by TERPY in HUVECs, WT-HEK and HEK-eNOS cells. These results are shown in two different ways: using DAF-FM diacetate probe in HUVECs and via Cu(II) fluorescein-based NO sensor (CuFL) in WT-HEK and HEK-eNOS cells. TERPY induced an increase in DAF-FM fluorescence in HUVECs which was blocked by approximately 50% in cells pretreated with L-NAME (100  $\mu\text{M}$  or 1 mM) (Figure 1A). Also, we stimulated HUVECs with A23187 (5  $\mu\text{M}$ ) as a positive control (Figure 1B) and NO production was blocked by L-NAME (100  $\mu\text{M}$ ). Using the CuFL fluorescent copper probe, TERPY increased NO level in WT-HEK cells (Figure 1C) which may be promoted by its direct effect as a NO donor. In non-treated HEK-eNOS cells, we observed time-dependent basal NO production which was significantly increased after addition of A23187 (5  $\mu\text{M}$ ) or TERPY (1  $\mu\text{M}$ ) and partially blocked by L-NAME (1 mM) (Figure 1D). These data indicate that NO production in HUVECs and HEK-eNOS stimulated by TERPY is partially dependent on eNOS.

### TERPY-induced eNOS dysfunction: eNOS uncoupling, ROS and ONOO<sup>-</sup> production

HUVECs were exposed to TERPY (1  $\mu\text{M}$ ) for 1 to 60 minutes. As shown in Figures 2A and 2B, the ratio of monomeric to dimeric eNOS was significantly higher after 5 minutes of treatment with TERPY. Accumulation of eNOS in its monomeric form was observed for up to 60 minutes, indicative of eNOS uncoupling. After TERPY treatment, no difference was observed in total eNOS expression (Figure 2C). Next, to evaluate whether TERPY-induced eNOS uncoupling is associated with ROS and ONOO<sup>-</sup> production, we incubated HUVECs with DHE and 7-CBA respectively, in the absence (control) or presence of TERPY. TERPY (1  $\mu\text{M}$ ) induced an increase ROS (Figures 3A, 3B and 3F) and ONOO<sup>-</sup> (Figure 3D)

production. The source of ROS and ONOO<sup>-</sup> was confirmed to be eNOS, since L-NAME (100  $\mu$ M or 1 mM) blocked the increase in DHE fluorescence (Figures 3A and 3F) induced by TERPY. Moreover, in absence of eNOS (HUVECs treated with eNOS siRNA), the fluorescence intensity of 7-CBA following stimulation with TERPY was abolished (Figure 3C), as well as no fluorescent products in the presence of DHE were observed in WT-HEK after TERPY's stimulation (Figure 3E). Moreover, the both superoxide scavengers SOD and PEG-SOD were able to decrease the fluorescence intensity of DHE (Figure 3B) or 7-CBA (Figure 3D), showing that TERPY-induced eNOS uncoupling were followed by ROS and ONOO<sup>-</sup> production. However, uric acid did not change the fluorescence intensity of DHE (Figure 3B) or 7-CBA (Figure 3D) stimulated by TERPY.

### Treatment with BH<sub>4</sub> prevented eNOS uncoupling induced by TERPY

Oxidation, and thus functional depletion of BH<sub>4</sub>, is associated with eNOS dysfunction and uncoupling [13–15]. To determine whether eNOS uncoupling occurs directly or indirectly in response to treatment with TERPY, cells were treated with 100  $\mu$ M BH<sub>4</sub> for 30 minutes prior to stimulation with TERPY. In non-boiled gels, eNOS dimerization (>250 kDa) was reduced and eNOS monomers (140 kDa) accumulated (Figure 4A and 4B) consistent with TERPY-induced eNOS uncoupling in HUVECs. However, TERPY-induced eNOS uncoupling was prevented in presence of BH<sub>4</sub> (Figures 4A and 4B). In absence of TERPY, BH<sub>4</sub> had no effect indicating basal levels were not limiting and eNOS was fully coupled in untreated HUVECs (Figure 4A and 4B). In boiled samples, neither TERPY nor BH<sub>4</sub> had an effect on total eNOS protein (Figure 4C). To assess eNOS function, we measured the production of ROS (Figure 4D) and ONOO<sup>-</sup> (Figure 4E) in HUVECs stimulated by TERPY with and without pretreatment with BH<sub>4</sub>. As shown in Figure 4D, TERPY induced a significant increase in DHE fluorescence which was abolished in cells pretreated with BH<sub>4</sub> suggesting TERPY increases ROS production via uncoupled eNOS. In addition, we observed that TERPY promoted a significant increase in ONOO<sup>-</sup> production which was blocked by BH<sub>4</sub> (Figure 4E). Zielonka *et al.* [21] showed through the HPLC analysis the stoichiometry of the reaction, indicating that one molecule of CBA reacts with a molecule of ONOO<sup>-</sup>, producing COH with the overall yield of ~81%. Thus, ONOO<sup>-</sup> reacts with the coumarin boronate at least a million times faster than hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Since 7-CBA can also detect hydroperoxides, we used catalase (300 U/mL) and PEG-Cat (300 U/mL) to remove H<sub>2</sub>O<sub>2</sub> and we observed no additional effect on blocked of ONOO<sup>-</sup> level stimulated by TERPY (Figure 4E).

### TERPY-induced Cav-1 oligomer destabilization

Next, we evaluated whether TERPY had an effect on Cav-1 oligomer stability, which we had shown previously to be rapidly modulated upon NO-mediated s-nitrosylation of critical Cys residues and subsequent phosphorylation of Cav-1 Tyr<sup>14</sup> [22]. As shown in Figures 5A and 5B, treatment of HUVECs with TERPY for 20 minutes reduced Cav-1 oligomer expression which was associated with an increase in Cav-1-Tyr<sup>14</sup> phosphorylation (Figures 5C and 5D), indicative of cav-1 oligomers destabilization. On the other hand, there was no change in total Cav-1 expression (denatured samples heated to 100°C) (Figures 5G and 5H). Moreover, we showed that phosphorylation of Cav-1 (Tyr<sup>14</sup>) was following by phosphorylation of Src (Tyr<sup>418</sup>) and eNOS (Ser<sup>1177</sup>). Src inhibitor PP2 blocked Src and Cav-1 phosphorylation.

## TERPY reduced eNOS/Cav-1 interaction and promoted eNOS-Ser<sup>1177</sup> hyperphosphorylation

Cav-1 oligomer destabilization induced by TERPY may modulate the interaction between eNOS and Cav-1 and thereby affect eNOS activity. To assess this, we immunoprecipitated Cav-1 and blotted for Cav-1 and eNOS in HUVECs treated with TERPY. As shown in Figures 6A and 6B, TERPY, in a time-dependent manner, reduced eNOS/Cav-1 interaction. Next, HUVECs and HEK-eNOS were treated with TERPY for 5 to 60 minutes and eNOS phosphorylation on active site Ser<sup>1177</sup> was assessed with a peak effect at about 20 minutes in both HUVECs (Figures 6C and 6D) and HEK-eNOS cells (Figures 6E and 6F). TERPY reduced the association between eNOS and Cav-1 and this effect was associated with an increase in eNOS phosphorylation. Moreover, it is known that eNOS phosphorylation at Tyr<sup>657</sup> residue regulates negatively eNOS activity. However, we did not observe changing at eNOS Tyr<sup>657</sup> phosphorylation (Figures 6C and 6D).

## 4. Discussion

Besides the well-known effect of TERPY as a NO donor [7];[8];[9];[10]; [11], our work elucidates a secondary effect of TERPY as an eNOS activator and uncoupling factor. This effect of TERPY was associated with NO, ONOO<sup>-</sup>, and ROS production, BH<sub>4</sub> depletion, Cav-1-Tyr<sup>14</sup> phosphorylation and oligomer destabilization, and a decrease in Cav-1/eNOS interaction. In its phosphorylated form, Cav-1 might impair the localization of Cav-1 in the plasma membrane and thereby, render it less able to bind and inactivate eNOS. Alternatively, uncoupled eNOS may not be able to bind or be inhibited by Cav-1, leading to sustained eNOS phosphorylation and sustained NO production. In fact, in cells treated with TERPY, eNOS Ser<sup>1177</sup> phosphorylation persisted for 20 minutes and it was associated with NO production. Thus, TERPY is not simply an NO donor, rather, it also promotes sustained NO, ONOO<sup>-</sup>, and ROS production, consistent with eNOS activation and uncoupling.

Kojima *et al.*, [23] developed di-amino-fluorescein (DAF), 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) and the cell-permeable DAF-FM diacetate to detect NO in many different cells types and using several methodologies. Other molecules as the NO<sub>2</sub> and N<sub>2</sub>O<sub>3</sub> are intermediate that activates DAF-FM through the formation of one electron oxidation product of DAF, which then combines with NO to form DAF-T [24]. Despite some disadvantages in using DAF-FM diacetate to detect NO, different studies have been using this probe to measure NO [25]; [26]. Unlike existing fluorescent sensors, the construct - a Cu(II) complex of a fluorescein modified with an appended metal-chelating ligand (FL) - can directly and immediately pick up NO formed or released rather than a derivative reactive nitrogen species. The NO-induced fluorescence involves reduction of the complex to Cu(I) with release of the nitrosated ligand, which occurs irreversibly. NO production was detected through nanomole concentrations of NO in both constitutive and inducible NO synthases (cNOS and iNOS, respectively) in mammalian cultured cells in a concentration- and time-dependent manner [27]; [28]. In our study, we used two different NO sensors (DAF-FM diacetate and CuFL) to confirm that TERPY is a NO donor and to show that NO production induced by TERPY is dependent, in part, on eNOS (Figure 1A and D).



Sullivan and Pollock [29] suggested that eNOS can exist in two forms: coupled and uncoupled, with the uncoupled enzyme residing mainly in the cytosol and the coupled dimeric form associated with the membrane. Schmidt *et al.*, [30] confirmed this hypothesis by showing that HUVECs exhibit basal BH<sub>4</sub> levels of 0.3 pmol/mg, and as a consequence, eNOS may not be fully coupled. When we analyzed the eNOS dimer/monomer ratio in HUVECs under basal conditions, we observed that eNOS exists primarily as dimers (Figure 2B) and that addition of BH<sub>4</sub> did not have a statistically significant effect on dimerization (Figure 4B). When we treated HUVECs with TERPY for 1 to 60 minutes, we observed a remarkable increase in eNOS monomerization (<50% decrease in dimer/monomer ratio) after 5 minutes of treatment indicating eNOS was becoming uncoupled, with a fraction of the total eNOS pool remaining coupled (Figure 2A–B). eNOS uncoupling induced by TERPY was reversed by pretreatment of cells with BH<sub>4</sub>, and thus dependent on BH<sub>4</sub> depletion (Figure 4).

Ravi *et al.* [31] demonstrated in bovine aortic endothelial cells (BAECs) that exogenous NO can promote S-nitrosylation and act as an inhibitor of eNOS activity associated with decrease in eNOS dimer levels. However, eNOS could be maintained in a completely dimeric state after increasing the concentration of thioredoxin and thioredoxin reductase system or in the presence of the reducing agent DTT (5 mM). Also, Chen *et al.* [32] demonstrated that two cysteine residues, Cys<sup>689</sup> and Cys<sup>908</sup>, can be S-glutathionylated modifying their own conformation that would disrupt FAD–FMN alignment, interrupting electron transfer between the flavins resulting in S-glutathionylation dependent-eNOS-uncoupling, changing NO to superoxide anion generation. The eNOS uncoupling induced by S-glutathionylation in BAECs treated with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) was blocked by DTT.

In our study, the eNOS monomerization was not due to S-nitrosylation or S-glutathionylation, since we used DTT (10%) in all samples to perform western blot in non-denaturing conditions. Furthermore, S-glutathionylation is increased in hypertensive vessels, resulting in impaired endothelium-dependent vasodilation [32]. However, we showed previously that vasodilator effect of TERPY is improved by endothelium only in hypertensive rats [9].

TERPY-induced eNOS monomerization can be explained by the fact that TERPY releases an active metabolic ([Ru(H<sub>2</sub>O)(bdq)(terpy)<sup>2+</sup>) that is able to oxidize BH<sub>4</sub> to BH<sub>2</sub> [11]. Several studies have shown that reduced levels of BH<sub>4</sub> increase eNOS oxidase activity [13]; [14]; [15], and in fact we demonstrated superoxide anion production and ONOO<sup>-</sup> formation in HUVECs stimulated with TERPY (Figure 3) suggesting that eNOS oxidase activity and dysfunction may be induced by TERPY in healthy endothelial cells. When we treated the HUVECs with BH<sub>4</sub>, the eNOS uncoupling was prevented (Figure 4A and 4B).

SOD (cell-impermeable) and PEG-SOD (cell-permeable) are superoxide scavengers, while uric acid is a natural scavenger of ONOO<sup>-</sup>. For the constant formation of ONOO<sup>-</sup> is necessary the presence of superoxide and NO. When we treated HUVECs with SOD or PEG-SOD, the fluorescence intensity of DHE and 7-CBA stimulated by TERPY was reduced as expected (Figure 3). However, uric acid did not change the fluorescence intensity

of DHE or 7-CBA induced by TERPY. We believed that the concentration of uric acid (300  $\mu\text{M}$ ) or the treatment-time (2 hours) were not enough to decrease, at least, the fluorescence intensity of 7-CBA. Uric acid (in the concentration higher than 300  $\mu\text{M}$  and in treatment-time higher than 6 hours) decreased expression and activity of eNOS, reduced NO bioavailability, enhanced ROS generation and induced the apoptosis in HUVECs [33];[34]; [35]; [36]. Park *et al.* [37] showed that attenuated NO production induced by uric acid could be related with decreasing the interaction between eNOS and calmodulin. On the other hand, Papežíková *et al.* [38] showed that the treatment of HUVECs with uric acid (300  $\mu\text{M}$ ) for 2 hours did not change NO bioavailability or eNOS Ser<sup>1177</sup> phosphorylation, but a higher concentration (600  $\mu\text{M}$ ) was able to reduce NO production and decrease eNOS Ser<sup>1177</sup> phosphorylation. In this way, we used uric acid 300  $\mu\text{M}$  (physiological concentration) to avoid the unwanted effects on eNOS activity.

It was previously demonstrated that BH<sub>2</sub> competes with BH<sub>4</sub> for binding to eNOS, however, BH<sub>2</sub> does not provide electrons for reductive oxygen activation [39]; [40]. Moreover, Karuppiah *et al.* [41] using a combination of gene silencing and pharmacological approaches, demonstrated that reduced levels of pterin bioavailability serves to suppress eNOS uncoupling. Thus, the competition between BH<sub>2</sub> and BH<sub>4</sub> for specific sites on eNOS could suppress eNOS uncoupling. We observed that part of the total eNOS pool remained coupled, or in dimeric form, as shown in Figure 2A–B. In this configuration, dimeric eNOS is still able to be activated and to synthesize NO. The activity of eNOS is regulated by its subcellular localization, phosphorylation on multiple residues, by post-translational lipid modifications, and through its interaction with different proteins [42], including Cav-1 which was shown previously to maintain eNOS in an inactive state [43].

Caveolin-1 degradation induced by oxidative stress [44]; [45] and NO-dependent destabilization of Cav-1 oligomers [22];[46]; [47] was previously observed. TERPY induced the destabilization of Cav-1 oligomers in association with an increase in Cav-1-Tyr<sup>14</sup> phosphorylation, as shown in Figure 5. Chen *et al.* [48] showed that the NO molecule derived from NO donor DEA NONOate induces Src activation and thereby allowing Cav-1 phosphorylation on Tyr<sup>14</sup> as we observed in our results in Figure 5E–F. The Src-dependent Cav-1-Tyr<sup>14</sup> phosphorylation induced by the NO donor TERPY was inhibited by Src inhibitor PP2. Unlike observed by Chen *et al.* [48], we showed that TERPY decreased eNOS/Cav-1 interaction that could be associated with TERPY-induced Cav-1 oligomer destabilization. Cav-1 phosphorylation and monomerization [22,41] might impair its ability to remain in the plasma membrane and thereby, deter association of eNOS with Cav-1 as demonstrated in Figure 6A–B. In fact, various studies have shown that loss of caveolin-1 [22];[49]; [51] or disruption of eNOS/Cav-1 binding [43];[52]; [53] promotes the hyperactivation of eNOS. We showed that TERPY promoted sustained phosphorylation of the Ser<sup>1177</sup> residue in eNOS (Figure 6C–F) and furthermore, the increase in DAF-FM fluorescence in HUVECs and HEK-eNOS stimulated by TERPY was decreased in presence of L-NAME (Figure 1A and 1C). These results suggest that eNOS is activated by TERPY. Although the phosphorylation of eNOS at tyrosines is less investigated, mutation analyses revealed that Tyr<sup>657</sup> phosphorylation attenuates eNOS enzyme activity, presumably in order to limit the detrimental consequences of maintained high NO output in situations of redox

stress [54];[55]; [56]. In this way, we analyzed TERPY-induced eNOS-phosphorylation at Tyr<sup>657</sup>. Our result showed that eNOS Tyr<sup>657</sup> phosphorylation is not modulate by TERPY.

Mao *et al.* [57] showed that nitroglycerin, another NO donor, results in Cav-1 modification and depletion leading to eNOS hyperactivation and uncoupling. In the present study, our data support the hypothesis that TERPY promotes the oxidization of BH<sub>4</sub> to BH<sub>2</sub> resulting in eNOS uncoupling prior to the destabilization of Cav-1 oligomers, but ultimately also promote eNOS hyperactivation and NO production by endothelial cells.

## 5. Conclusion

Taken together, these results suggest that in addition to being an NO donor, TERPY also increases ROS and RNS production by affecting eNOS activity. We demonstrate that TERPY promotes eNOS uncoupling and Cav-1 oligomer destabilization and consequently, promotes sustained eNOS Ser<sup>1177</sup> phosphorylation in endothelial cells. Thus, chemical and biological molecules that are being developed to increase NO bioavailability may do so, at least in part, by increasing eNOS activity. Future studies should consider the possibility that these agents may also promote eNOS uncoupling, ROS and RNS production, and ultimately endothelial dysfunction.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## List of nonstandard abbreviations

|              |                                   |
|--------------|-----------------------------------|
| <b>TERPY</b> | [Ru(terpy)(bdq)NO] <sup>3+</sup>  |
| <b>eNOS</b>  | endothelial nitric oxide synthase |
| <b>SHR</b>   | spontaneously hypertensive rats   |
| <b>Cav-1</b> | caveolin-1                        |
| <b>NO</b>    | nitric oxide                      |
| <b>CaM</b>   | calmodulin                        |
| <b>FAD</b>   | flavin adenine dinucleotide       |
| <b>FMN</b>   | flavin mononucleotide             |

|                                   |   |
|-----------------------------------|---|
| <b>BH<sub>4</sub></b>             | (6R-)5,6,7,8-tetrahydro-L-biopterin   |
| <b>BH<sub>2</sub></b>             | dihydrobiopterin  |
| <b>HUVECs</b>                     | human umbilical vein endothelial cells  |
| <b>WT-HEK</b>                     | wild-type human embryonic kidney 293 cell line  |
| <b>HEK-eNOS</b>                   | HEK cells stably expressing eNOS  |
| <b>co-IP</b>                      | co-immunoprecipitation  |
| <b>L-NAME</b>                     | N <sub>ω</sub> -nitro-L-arginine methyl ester hydrochloride                           |
| <b>DHE</b>                        | dihydroethidium   |
| <b>7-CBA</b>                      | coumarin-7-boronic acid   |
| <b>DAF-FM</b>                     | 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate                             |
| <b>CuFL</b>                       | Cu(II) fluorescein-based NO sensor  |
| <b>A23187</b>                     | calcium ionophore   |
| <b>H<sub>2</sub>O<sub>2</sub></b> | hydrogen peroxide   |
| <b>PP2</b>                        | 4-amino-5-(4-chlorophenyl)-7-( <i>t</i> -butyl) pyrazolo [3, 4- <i>d</i> ] pyrimidine |
| <b>COH</b>                        | 7-OH-coumarin   |
| <b>DTT</b>                        | dithiothreitol  |
| <b>PEG-catalase</b>               | catalase polyethylene glycol  |
| <b>ONOO-</b>                      | peroxynitrite   |
| <b>SOD</b>                        | superoxide dismutase  |
| <b>PEG-SOD</b>                    | superoxide dismutase-polyethylene glycol  |

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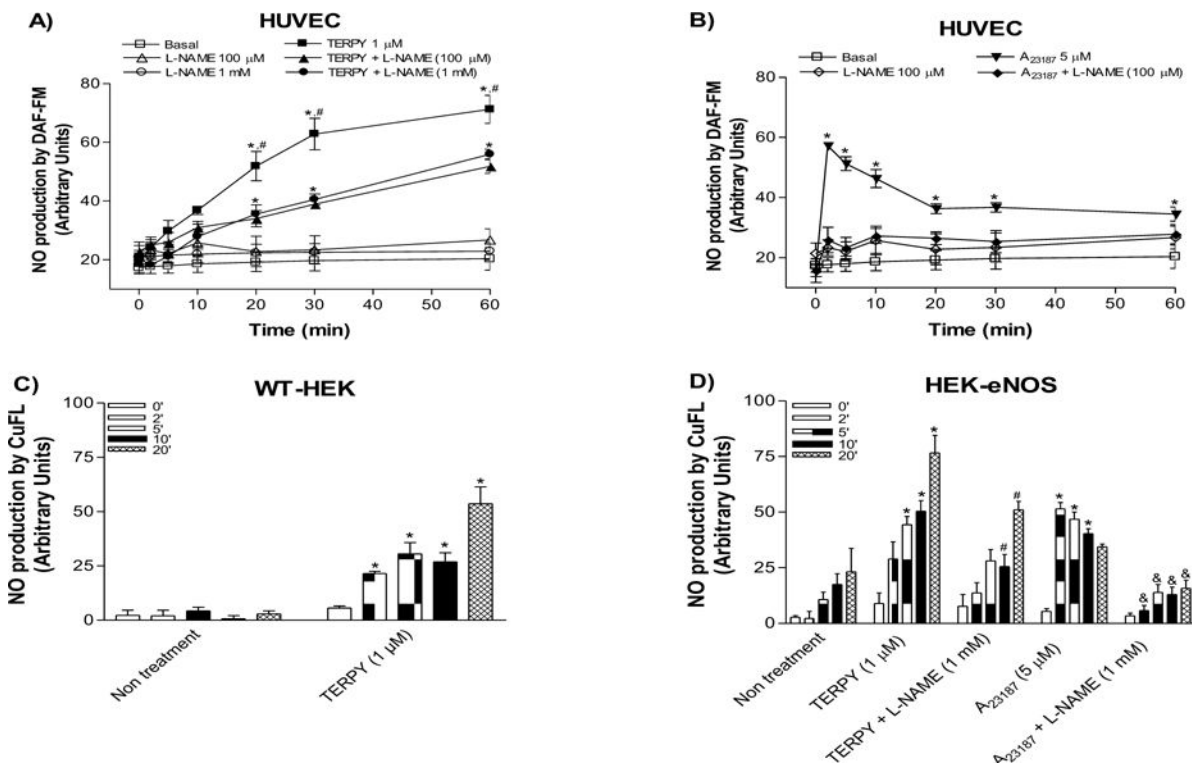
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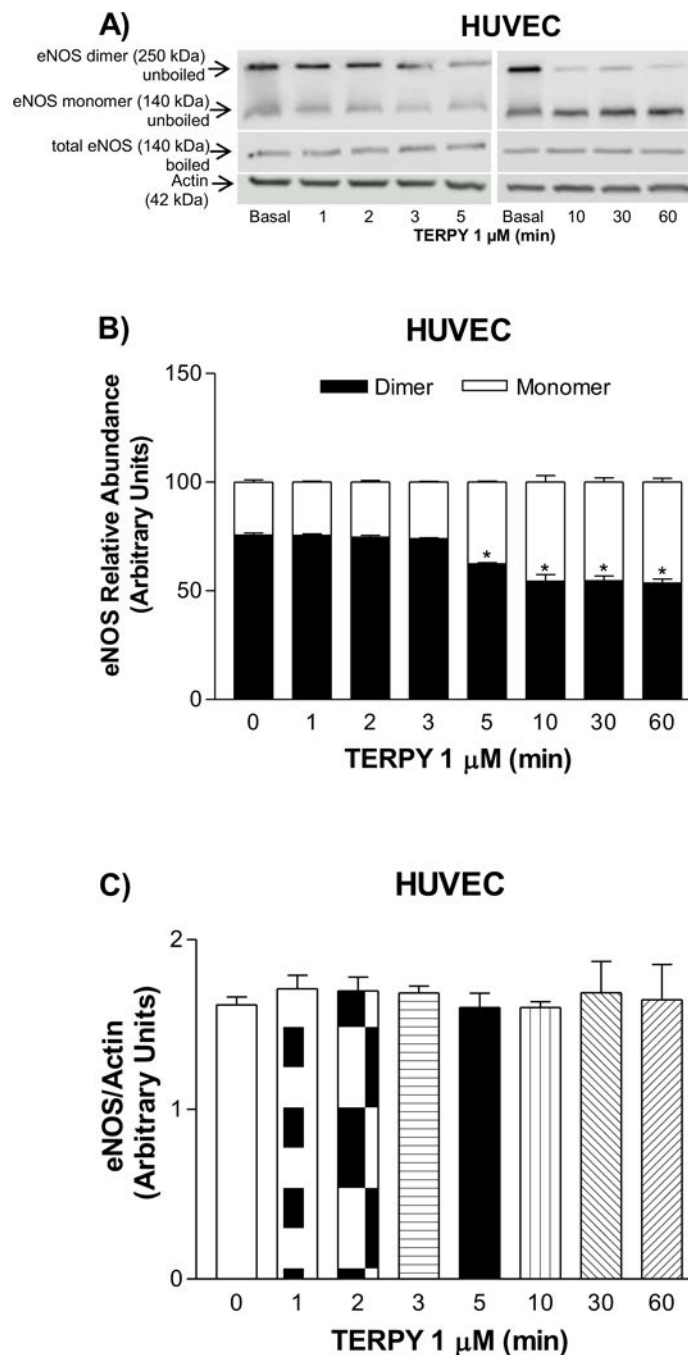
**Highlights**

- TERPY promotes eNOS uncoupling
- TERPY increases oxidant production
- TERPY promotes Cav-1 and Src phosphorylation
- TERPY decreases Cav-1/NOS interaction leading to eNOS hyperphosphorylation
- TERPY is a NO-donor that can modulate eNOS activity

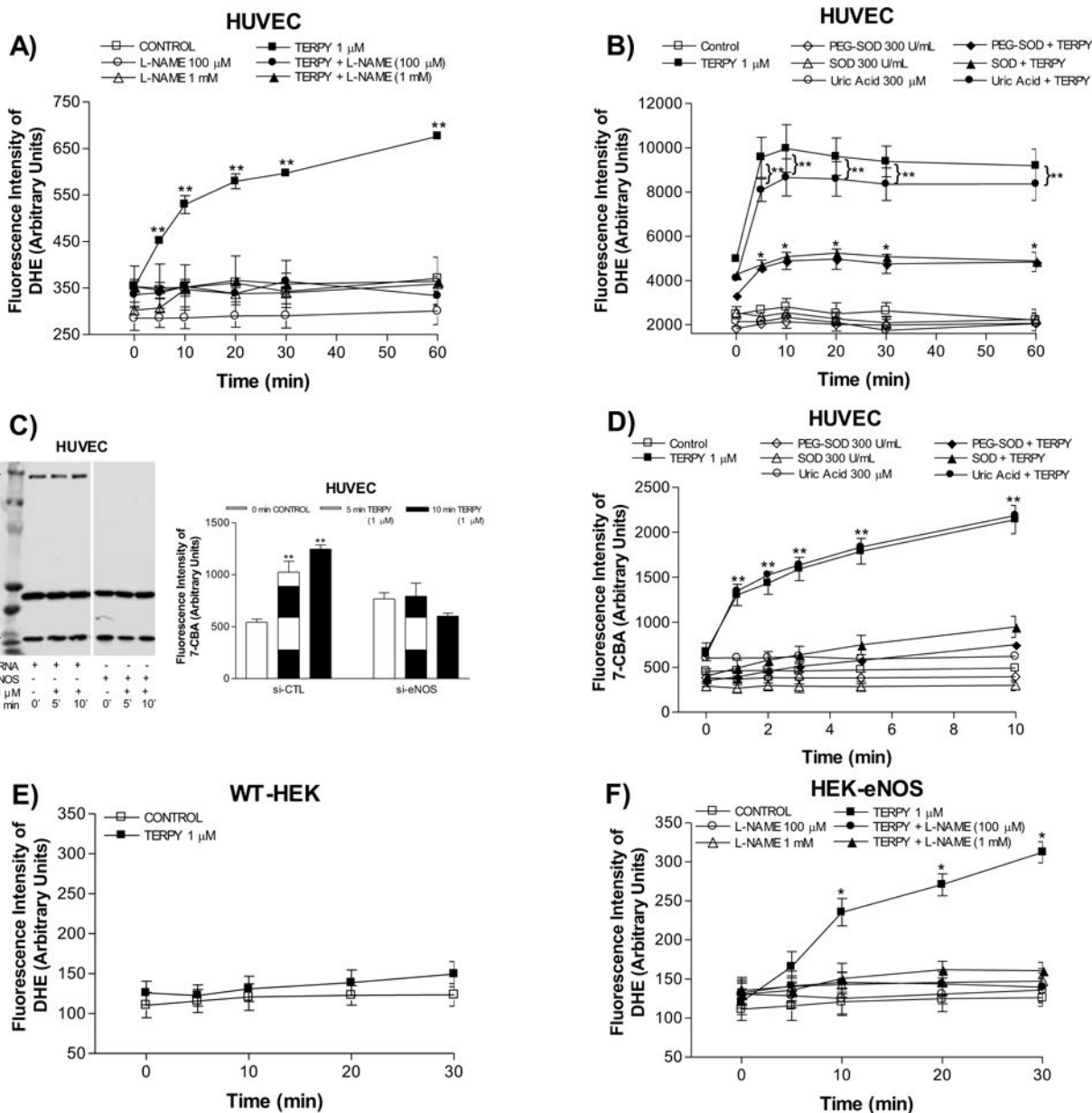




**Figure 1. NO production induced by TERPY is dependent in part on eNOS**  
 (A) Nitric oxide (NO) was measured by DAF-FM probe in human umbilical vein endothelial cells (HUVECs) stimulated by TERPY 1  $\mu$ M (A) and A23187 5  $\mu$ M (B) as a positive control. TERPY-induced NO production was inhibited in part by L-NAME (100  $\mu$ M or 1 mM) (A). CuFL NO sensor was used in wild-type human embryonic kidney 293 cells (WT-HEK) and in HEK cells stably transduced with eNOS cDNA (HEK-eNOS) to detect NO level directly liberated by TERPY (C,D). HEK-eNOS, in absence or presence of L-NAME (1 mM), were stimulated with TERPY (1  $\mu$ M) or A23187 (5  $\mu$ M) (D). \* $p$ <0.05 indicates statistical difference versus basal condition (non- treatment or basal,  $n$ =3–5). # $p$ <0.05 indicates statistical difference between TERPY versus TERPY + L-NAME ( $n$ =3–5). & $p$ <0.05 indicates statistical difference between A23187 versus A23187 + L-NAME ( $n$ =3–5). “ $n$ ” indicates independent experiments.



**Figure 2. TERPY-induced changes in eNOS dimerization in HUVECs**  
 (A) Representative Western blot of eNOS dimer/monomer distribution in unboiled samples (top blot), total eNOS expression observed in boiled samples (middle blot), and actin (bottom blot; loading control) in human umbilical vein endothelial cells (HUVECs). (B) Basal and time-dependent changes in eNOS dimer and monomer induced by TERPY (1 μM) as compared to (C) total eNOS expression in boiled samples. \*Indicates p < 0.05 versus basal condition (n=3–5). “n” indicates independent experiments.



**Figure 3. TERPY-induced eNOS dysfunction in HUVECs and HEK-eNOS**  
 Reactive oxygen species were measured using dihydroethidium (DHE) in human umbilical vein endothelial cells (HUVECs) (A,B), wild-type human embryonic kidney 293 cells (HEK-WT) (E) and HEK cells stably transduced with eNOS cDNA (HEK-eNOS) (F) in absence (Control) or presence of TERPY (1  $\mu$ M) for times indicated. (C) and (D) Peroxynitrite was measured using coumarin-7-boronic acid (7-CBA) in HUVECs. (C) Representative Western blot of total eNOS, actin, and total Cav-1 in control si-RNA (right lane) and eNOS si-RNA treated cells (left lane). (A) and (F) L-NAME (100  $\mu$ M or 1 mM) inhibited the TERPY-stimulated fluorescence intensity of DHE. (B) and (D) SOD (300 U/mL) and PEG-SOD (300 U/mL) decreased the fluorescence intensity of DHE and 7-CBA stimulated by TERPY, but not uric acid (300  $\mu$ M). \* $p$ <0.05 indicates difference between

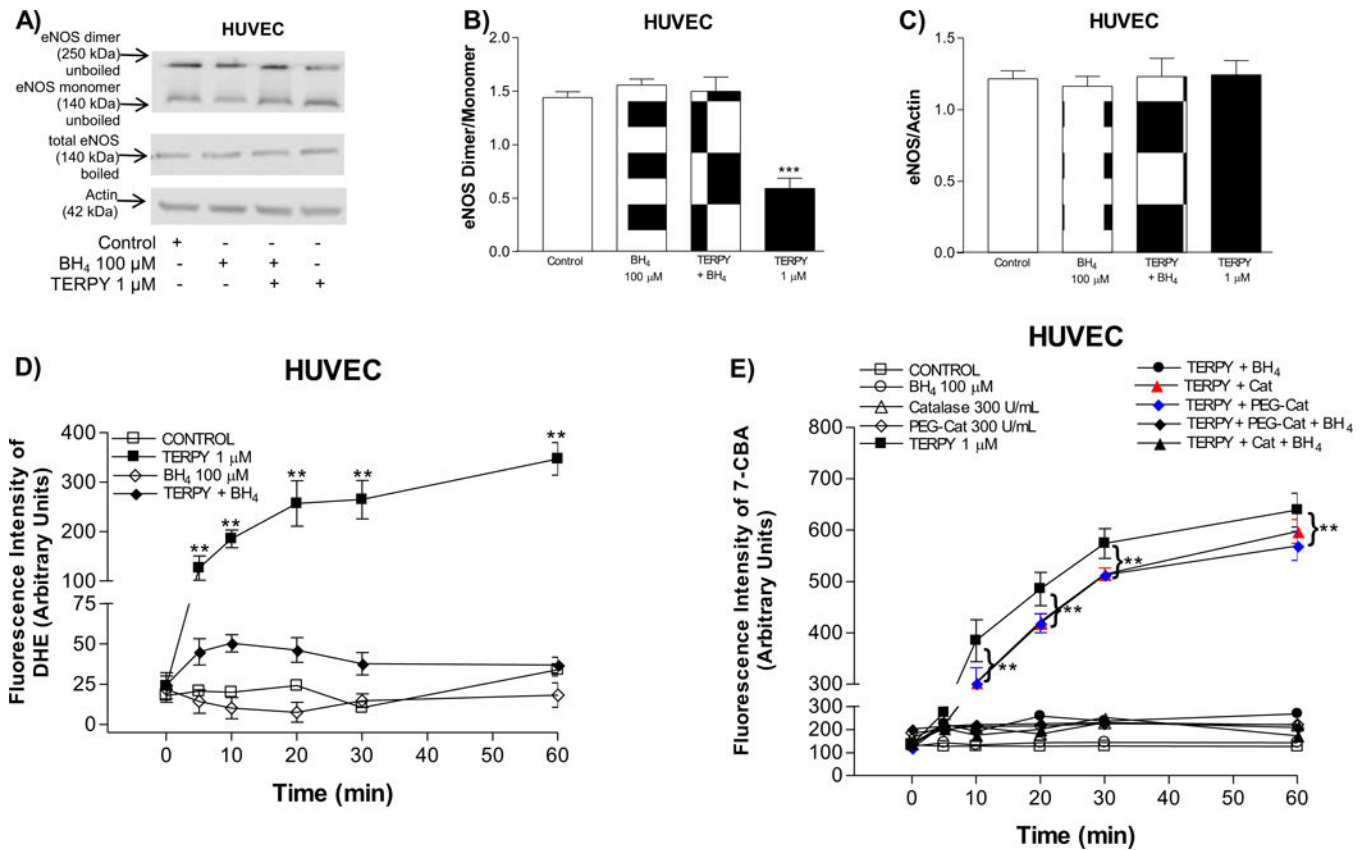
TERPY, TERPY + SOD or TERPY + PEG-SOD versus basal condition (control, n=3–5) and \*\* $p < 0.01$  indicates difference between TERPY or TERPY + uric acid versus basal condition (control, n=3–5). “*n*” indicates independent experiments.

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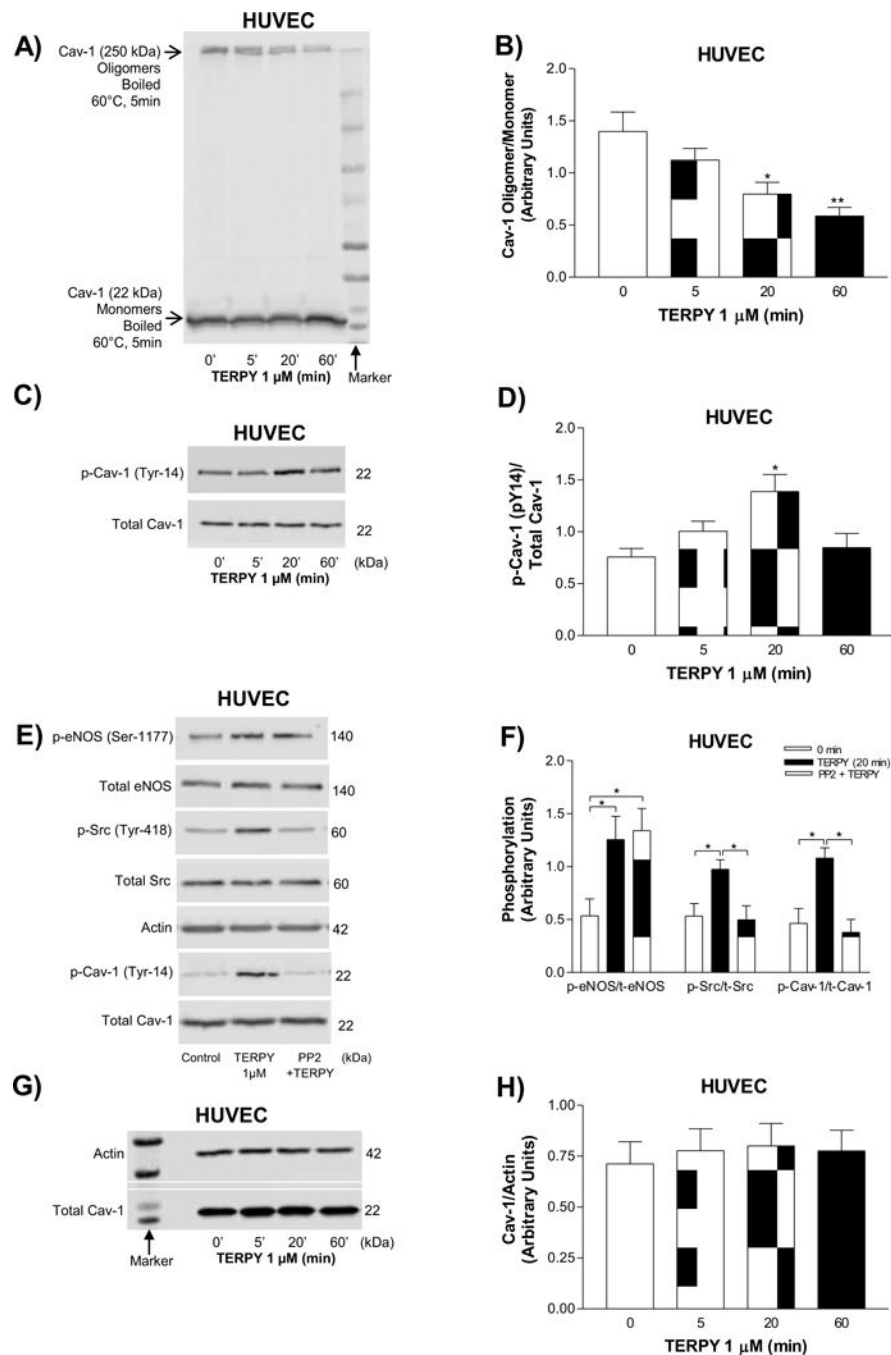
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**Figure 4. Treatment with BH<sub>4</sub> prevented eNOS uncoupling induced by TERPY**

(A) Representative Western blot of eNOS dimer/monomer distribution in unboiled samples (top blot), total eNOS expression (middle blot) and actin (bottom blot) in boiled samples of human umbilical vein endothelial cells (HUVECs) in absence (Control) or presence of BH<sub>4</sub> (100 μM). TERPY (1 μM) was added for the times indicated before lysis. (B) and (C) summarized data. (D) Reactive oxygen species measurement by dihydroethidium (DHE). (E) Peroxynitrite measurement by coumarin-7-boronic acid (7-CBA). Catalase (Cat, 300 U/mL) and PEG-Catalase (PEG-Cat, 300 U/mL) were used to remove hydrogen peroxide. \*\*Indicates  $p < 0.01$  between TERPY, TERPY + PEG-Cat or TERPY + Cat versus basal conditions ( $n = 3-5$ ). \*\*\*Indicates  $p < 0.001$  between TERPY versus basal conditions ( $n = 3-5$ ). “ $n$ ” indicates independent experiments.



**Figure 5. TERPY-induced destabilization of Cav-1 oligomers** (A) Representative Western blot of Cav-1 oligomer/monomer distribution and (B) summarized data. (C) Representative Western blot of Cav-1 Tyr-14 phosphorylation and (D) summarized data. (E) Representative Western blot of phosphorylation of eNOS-Ser<sup>1177</sup>, Src-Tyr<sup>418</sup>, and Cav-1-Tyr<sup>14</sup> in absence or presence of PP2 (Src kinase inhibitor) for times indicated and (F) summarized data. (G) Representative Western blot of total Cav-1 expression and (H) summarized data. All experiments were performed in human umbilical vein endothelial cells (HUVECs) exposed to the TERPY (1  $\mu$ M) for times indicated.

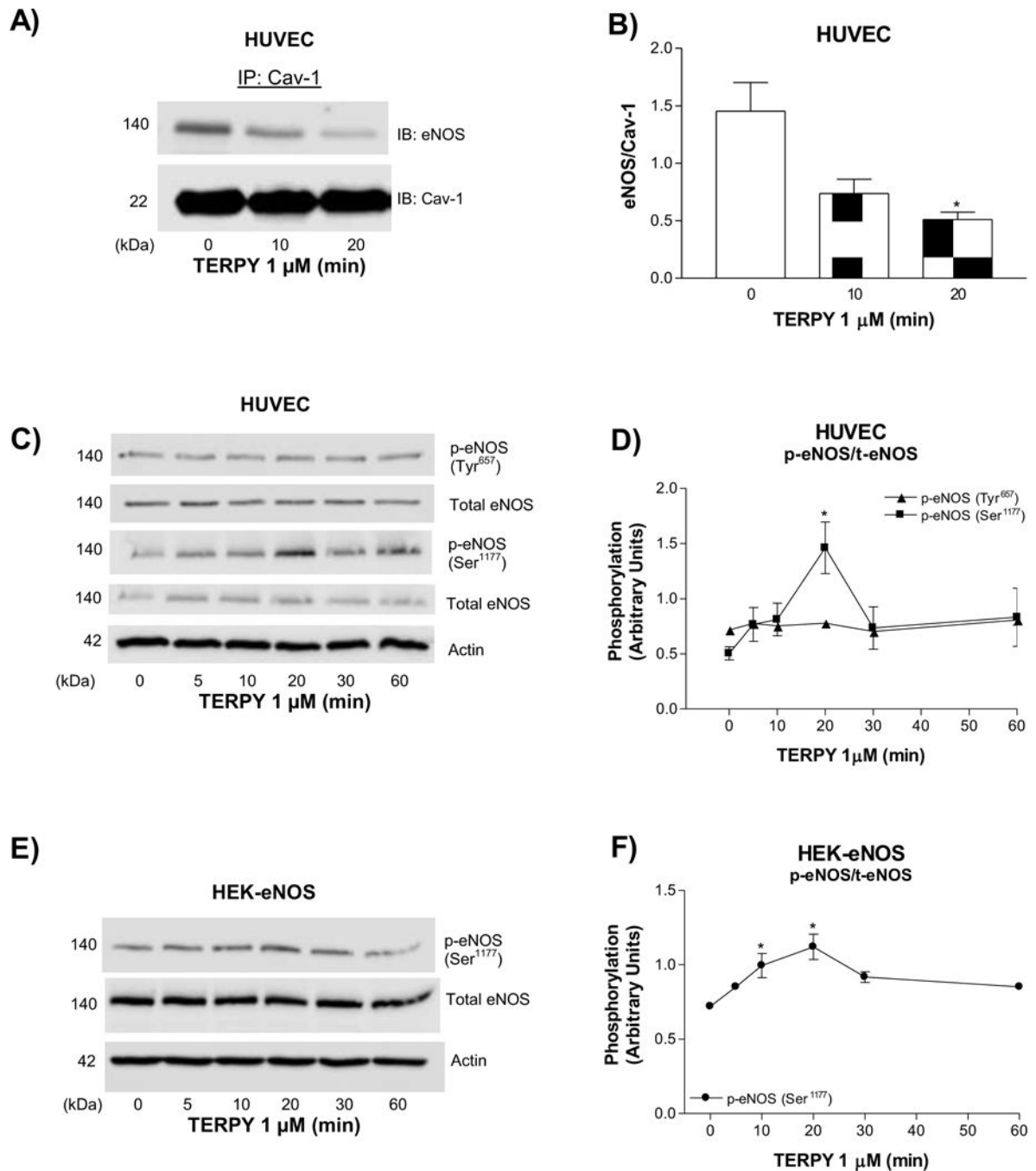
\* $p < 0.05$  and \*\* $p < 0.01$  indicate statistical difference versus basal condition ( $n=3-5$ ). “ $n$ ” indicates independent experiments.

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**Figure 6. TERPY decreases eNOS/Cav-1 interaction and promotes eNOS hyperphosphorylation at Serine<sup>1177</sup>**

(A) Interaction of Cav-1 and eNOS determined by co-immunoprecipitation (Co-IP) in human umbilical vein endothelial cells (HUVECs) exposed to TERPY (1  $\mu$ M). (C) Phosphorylation of eNOS Ser<sup>1177</sup> and eNOS Tyr<sup>657</sup> in HUVECs stimulated with TERPY (1  $\mu$ M) for times indicated and (D) summarized data. (E) Phosphorylation of eNOS Ser<sup>1177</sup> in human embryonic kidney 293 cells transduced with eNOS cDNA (HEK-eNOS) and (F)



summarized data. \* $p < 0.05$  indicates statistical difference versus basal condition ( $n=3-5$ ).  
“ $n$ ” indicates independent experiments.

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