Free Radical Biology and Medicine 112 (2017) 587–596

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original article

Nitric oxide donor $[Ru(terpy)(bdq)NO]^{3+}$ induces uncoupling and phosphorylation of endothelial nitric oxide synthase promoting oxidant production

Simone R. Potje[a,d], Zhenlong Chen[d], Suellen D'Arc S. Oliveira[d], Lusiane M. Bendhack[b], Roberto S. da Silva[b], Marcelo G. Bonini[c], Cristina Antoniali[a,*], Richard D. Minshall[d,e,**]

[a] Programa Multicêntrico de Pós-graduação em Ciências Fisiológicas, Department of Basic Sciences, School of Dentistry, São Paulo State University, Araçatuba, Brazil
[b] Department of Physics and Chemistry, School of Pharmaceutical Sciences, University of São Paulo, Ribeirão Preto, Brazil
[c] Department of Medicine, University of Illinois at Chicago, Chicago, IL, USA
[d] Department of Anesthesiology, University of Illinois at Chicago, Chicago, IL, USA
[e] Department of Pharmacology, University of Illinois at Chicago, Chicago, IL, USA

ARTICLE INFO

Keywords:
NO donor
TERPY
eNOS uncoupling
Caveolin-1
eNOS hyperactivation

ABSTRACT

$[Ru(terpy)(bdq)NO]^{3+}$ (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^{1177} phosphorylation, caveolin-1 (Cav-1) oligomerization, Cav-1 Tyr^{14} 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– production which were blocked by more than 80% by BH_4 (essential eNOS co-factor) and eNOS siRNA. These results suggest that TERPY-induced ROS and ONOO– production were originated from eNOS. HUVECs stimulated with TERPY showed increased eNOS Ser^{1177} and Cav-1 Tyr^{14} 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_4. 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.

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_4) 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_4 facilitates electron transfer upon L-arginine oxidation forming NO and L-citrulline [3].

Abbreviations: TERPY, $[Ru(terpy)(bdq)NO]^{3+}$; eNOS, endothelial nitric oxide synthase; SHR, spontaneously hypertensive rats; Cav-1, caveolin-1; NO, nitric oxide; CaM, calmodulin; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; BH_4, (6R-)5,6,7,8-tetrahydro-L-biopterin; BH_2, dihydrobiopterin; HUVECs, human umbilical vein endothelial cells; WT-HEK, wild-type human embryonic kidney 293 cell line; HEK-eNOS, HEK cells stably expressing eNOS; co-IP, co-immunoprecipitation; L-NAME, N_{ω}-nitro-L-arginine methyl ester hydrochloride; DHE, dihydroethidium; 7-CBA, coumarin-7-boronic acid; DAF-FM, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate; CuFL, Cu(II) fluorescein-based NO sensor; A23187, calcium ionophore; H_2O_2, hydrogen peroxide; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo [3, 4-*d*] pyrimidine; COH, 7-OH-coumarin; DTT, dithiothreitol; PEG-catalase, catalase polyethylene glycol; ONOO–, peroxynitrite; SOD, superoxide dismutase; PEG-SOD, superoxide dismutase-polyethylene glycol

* Correspondence to: Faculdade de Odontologia de Araçatuba – UNESP/Departamento de Ciências Básicas, Rua: José Bonifácio 1193, CEP: 16015-050 Araçatuba, SP, Brazil.
** Correspondence to: Departments of Anesthesiology and Pharmacology, 835 S Wolcott Avenue, E709C MSB, Chicago, IL 60612, USA.
E-mail addresses: crisant@foa.unesp.br (C. Antoniali), rminsh@uic.edu (R.D. Minshall).

http://dx.doi.org/10.1016/j.freeradbiomed.2017.09.004
Received 29 March 2017; Received in revised form 5 September 2017; Accepted 6 September 2017
Available online 09 September 2017
0891-5849/

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 s [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.

$[Ru(terpy)(bdq)NO]^{3+}$ (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_4 to dihydrobiopterin (BH_2) [11]. When BH_4 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 [12–15]. 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

2.1.1. 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.

2.1.2. 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 siRNA was purchased from QIAGEN (Valencia, CA). N_ω-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–) and tetrahydro-L-biopterin hydrochloride (BH_4) 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^{1177}) 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 ($pTyr^{14}$, 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^{657}) 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 min, 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 h at room temperature. Proteins were visualized with enhanced chemiluminescence substrate (Super Signal West Pico or Femto, Thermo Scientific, Waltham, MA).

2.3.1. 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 1 M) 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 min, 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 h 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

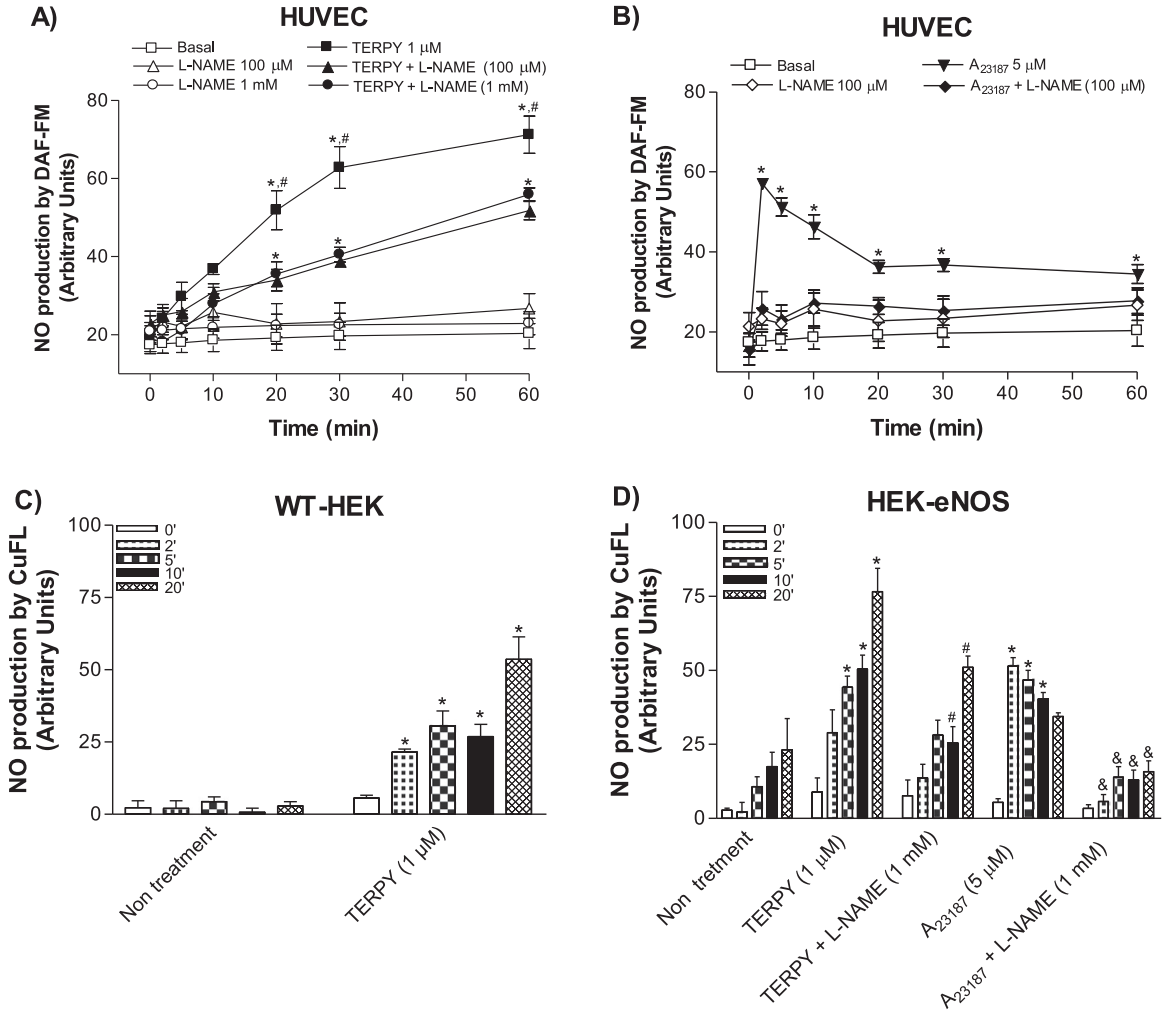

Fig. 1. NO production induced by TERPY is dependent in part on eNOS. (A) Nitric oxide (NO) was measured by DAF-FM probe in human umbilical vein endothelial cells (HUVECs) stimulated by TERPY 1 μM (A) and A23187 5 μM (B) as a positive control. TERPY-induced NO production was inhibited in part by L-NAME (100 μM 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 μM) or A23187 (5 μM) (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.

samples were boiled for 10 min 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 h, cells were stimulated and lysed at 4° 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 min. Before stimulation, the cells were pretreated with 100 μM BH_4 (30 min), 100 μM L-NAME (30 min) or 1 mM L-NAME (30 min). 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 Advices, 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 min. Before stimulation, the cells were pretreated with 100 μM BH_4 (30 min), 100 μM L-NAME (30 min), 1 mM L-NAME (30 min), 300 U/mL SOD (60 min), 300 U/mL PEG-SOD (60 min) or 300 μM uric acid (120 min). 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 Advices, Sunnyvale, CA).

2.8. Measurement of ONOO– 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 min. Before stimulation, some samples were pretreated with 100 μM BH_4, 300 U/mL

Catalase (30 min), 300 U/mL PEG-cat (30 min), 300 U/mL SOD (60 min), 300 U/mL PEG-SOD (60 min) or 300 μM uric acid (120 min). Then, treatment with different reagents (vehicle or 1 μM 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 ± 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

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

It is known that eNOS phosphorylation on Ser^{1177} 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 μM or 1 mM) (Fig. 1A). Also, we stimulated HUVECs with A23187 (5 μM) as a positive control (Fig. 1B) and NO production was blocked by L-NAME (100 μM). Using the CuFL fluorescent copper probe, TERPY increased NO level in WT-HEK cells (Fig. 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 μM) or TERPY (1 μM) and partially blocked by L-NAME (1 mM) (Fig. 1D). These data indicate that NO production in HUVECs and HEK-eNOS stimulated by TERPY is partially dependent on eNOS.

3.2. TERPY-induced eNOS dysfunction: eNOS uncoupling, ROS and ONOO– production

HUVECs were exposed to TERPY (1 μM) for 1–60 min. As shown in Fig. 2A and B, the ratio of monomeric to dimeric eNOS was significantly higher after 5 min of treatment with TERPY. Accumulation of eNOS in its monomeric form was observed for up to 60 min, indicative of eNOS uncoupling. After TERPY treatment, no difference was observed in total eNOS expression (Fig. 2C). Next, to evaluate whether TERPY-induced eNOS uncoupling is associated with ROS and ONOO– production, we incubated HUVECs with DHE and 7-CBA respectively, in the absence (control) or presence of TERPY. TERPY (1 μM) induced an increase ROS (Fig. 3A, B and F) and ONOO– (Fig. 3D) production. The source of ROS and ONOO– was confirmed to be eNOS, since L-NAME (100 μM or 1 mM) blocked the increase in DHE fluorescence (Fig. 3A and F) 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 (Fig. 3C), as well as no fluorescent products in the presence of DHE were observed in WT-HEK after TERPY's stimulation (Fig. 3E). Moreover, the both superoxide scavengers SOD and PEG-SOD were able to decrease the fluorescence intensity of DHE (Fig. 3B) or 7-CBA (Fig. 3D), showing that TERPY-induced eNOS uncoupling were followed by ROS and ONOO– production. However, uric acid did not change the fluorescence intensity of DHE (Fig. 3B) or 7-CBA (Fig. 3D) stimulated by TERPY.

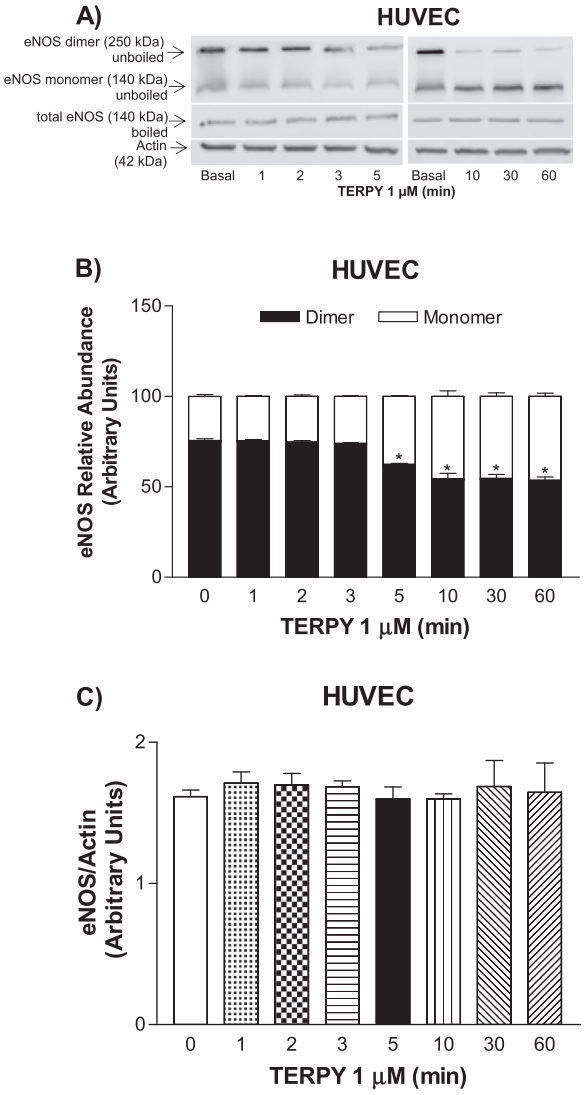

Fig. 2. TERPY-induced changes in eNOS dimerization in HUVECs. (A) Representative Western blot of eNOS dimer/monomer distribution in unboiled samples (top blot), total eNOS expression observed in boiled samples (middle blot), and actin (bottom blot; loading control) in human umbilical vein endothelial 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.

3.3. Treatment with BH_4 prevented eNOS uncoupling induced by TERPY

Oxidation, and thus functional depletion of BH_4, 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 μM BH_4 for 30 min prior to stimulation with TERPY. In non-boiled gels, eNOS dimerization (> 250 kDa) was reduced and eNOS monomers (140 kDa) accumulated (Fig. 4A and B) consistent with TERPY-induced eNOS uncoupling in HUVECs. However, TERPY-induced eNOS uncoupling was prevented in presence of BH_4 (Fig. 4A and B). In absence of TERPY, BH_4 had no effect indicating basal levels were not limiting and eNOS was fully coupled in untreated HUVECs (Fig. 4A and B). In boiled samples, neither TERPY nor BH_4 had an effect on total eNOS protein (Fig. 4C). To assess eNOS function, we measured the production of ROS (Fig. 4D) and ONOO– (Fig. 4E) in HUVECs stimulated by TERPY with and without

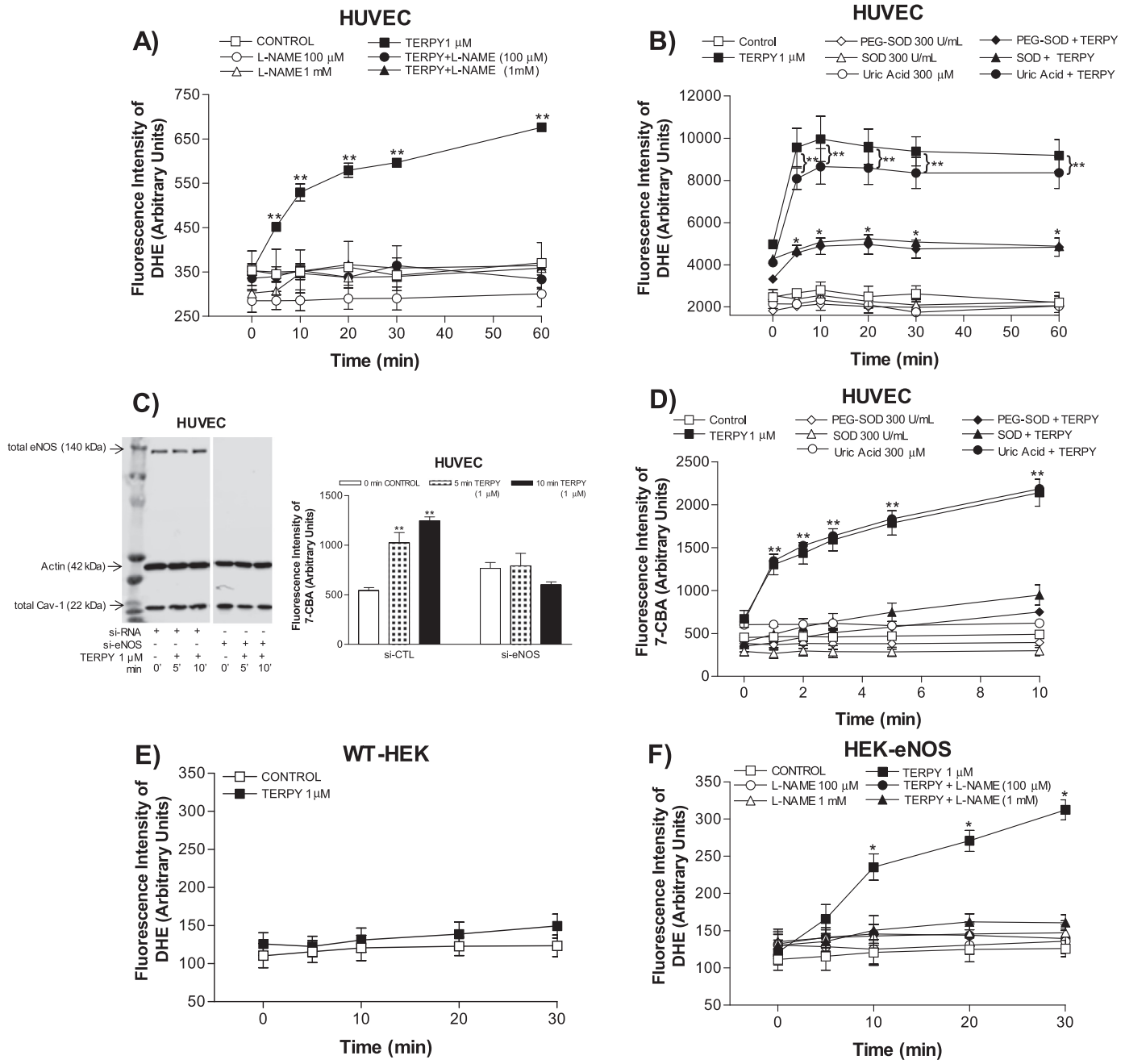

Fig. 3. TERPY-induced eNOS dysfunction in HUVECs and HEK-eNOS. Reactive oxygen species were measured using dihydroethidium (DHE) in human umbilical vein endothelial cells (HUVECs) (A,B), wild-type human embryonic kidney 293 cells (HEK-WT) (E) and HEK cells stably transduced with eNOS cDNA (HEK-eNOS) (F) in absence (Control) or presence of TERPY (1 µM) 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 µM 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 µM). *$p < 0.05$ 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.

pretreatment with BH_4. As shown in Fig. 4D, TERPY induced a significant increase in DHE fluorescence which was abolished in cells pretreated with BH_4 suggesting TERPY increases ROS production via uncoupled eNOS. In addition, we observed that TERPY promoted a significant increase in ONOO– production which was blocked by BH_4 (Fig. 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–, producing COH with the overall yield of ~ 81%. Thus, ONOO– reacts with the coumarin boronate at least a million times faster than hydrogen peroxide (H_2O_2). Since 7-CBA can also detect hydroperoxides, we used catalase (300 U/mL) and PEG-Cat (300 U/mL) to remove H_2O_2 and we observed no additional effect on blocked of ONOO– level stimulated by TERPY (Fig. 4E).

3.4. 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^{14} [22]. As shown in Fig. 5A and B, treatment of HUVECs with TERPY for 20 min reduced Cav-1 oligomer expression which was associated with an increase in Cav-1-Tyr^{14}

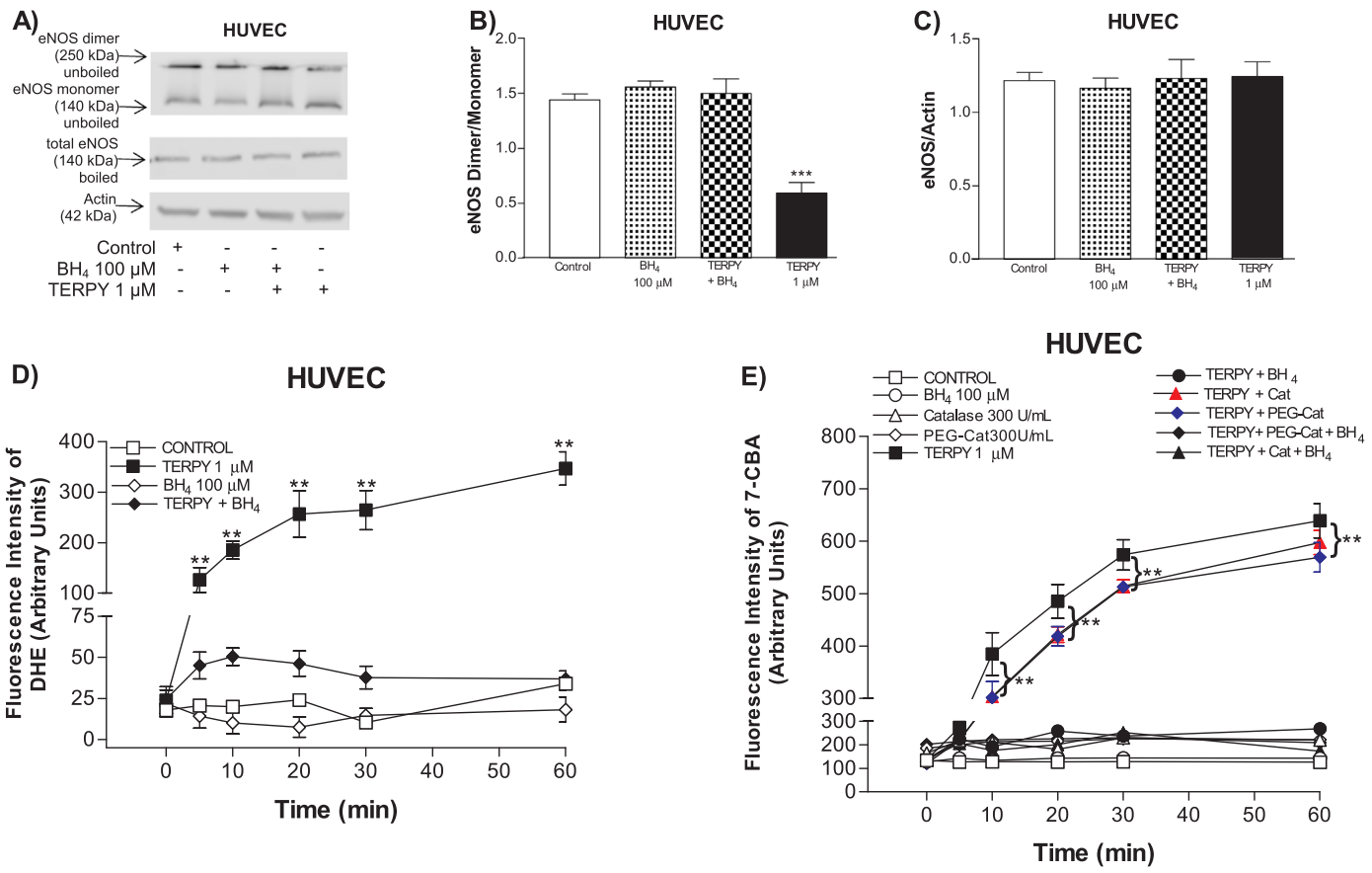

Fig. 4. Treatment with BH_4 prevented eNOS uncoupling induced by TERPY. (A) Representative Western blot of eNOS dimer/monomer distribution in unboiled samples (top blot), total eNOS expression (middle blot) and actin (bottom blot) in boiled samples of human umbilical vein endothelial cells (HUVECs) in absence (Control) or presence of BH_4 (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.

phosphorylation (Fig. 5C and D), 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) (Fig. 5G and H). Moreover, we showed that phosphorylation of Cav-1 (Tyr^{14}) was following by phosphorylation of Src (Tyr^{418}) and eNOS (Ser^{1177}). Src inhibitor PP2 blocked Src and Cav-1 phosphorylation.

3.5. TERPY reduced eNOS/Cav-1 interaction and promoted eNOS-Ser^{1177} 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 Fig. 6A and B, TERPY, in a time-dependent manner, reduced eNOS/Cav-1 interaction. Next, HUVECs and HEK-eNOS were treated with TERPY for 5–60 min and eNOS phosphorylation on active site Ser^{1177} was assessed with a peak effect at about 20 min in both HUVECs (Fig. 6C and D) and HEK-eNOS cells (Fig. 6E and F). TERPY reduced the association between eNOS and Cav-1 and this effect was associated with an increase in eNOS phosphorylation. Moreover, is known that eNOS phosphorylation at Tyr^{657} residue regulates negatively eNOS activity. However, we did not observe changing at eNOS Tyr^{657} phosphorylation (Fig. 6C and D).

4. Discussion

Besides the well-known effect of TERPY as a NO donor [7–11], our work elucidates a secondary effect of TERPY as an eNOS activator and uncoupling factor. This effect of TERPY was associated with NO, ONOO–, and ROS production, BH_4 depletion, Cav-1-Tyr^{14} 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^{1177} phosphorylation persisted for 20 min and it was associated with NO production. Thus, TERPY is not simply an NO donor, rather, it also promotes sustained NO, ONOO–, 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_2 and N_2O_3 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)

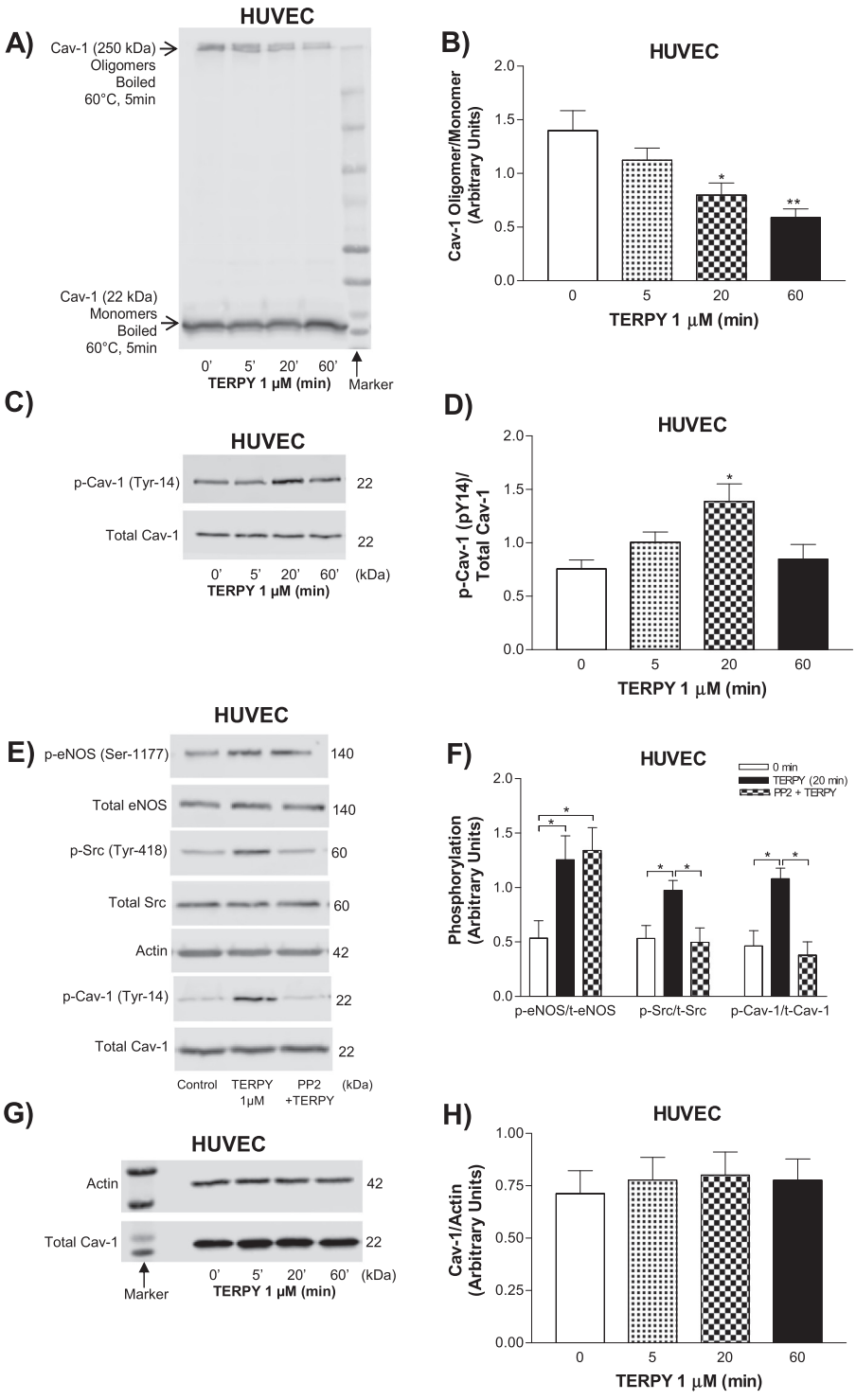

Fig. 5. TERPY-induced destabilization of Cav-1 oligomers. (A) Representative Western blot of Cav-1 oligomer/monomer distribution and (B) summarized data. (C) Representative Western blot of Cav-1 Tyr-14 phosphorylation and (D) summarized data.
(E) Representative Western blot of phosphorylation of eNOS-Ser^{1177}, Src-Tyr^{418}, and Cav-1-Tyr^{14} 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 μM) for times indicated. *$p < 0.05$ and **$p < 0.01$ indicate statistical difference versus basal condition ($n = 3–5$). "n" indicates independent experiments.

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 (Fig. 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_4 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 (Fig. 2B) and that addition of BH_4 did not have a statistically significant effect on dimerization (Fig. 4B). When we treated HUVECs with TERPY for 1–60 min, we observed a remarkable increase in eNOS monomerization (< 50% decrease in dimer/monomer ratio) after 5 min of treatment indicating eNOS was becoming uncoupled, with a fraction of the total eNOS pool remaining coupled (Fig. 2A and B). eNOS uncoupling induced by TERPY was reversed by pretreatment of cells with BH_4, and thus dependent on BH_4 depletion (Fig. 4).

Ravi et al. [31] demonstrated in bovine aortic endothelial cells

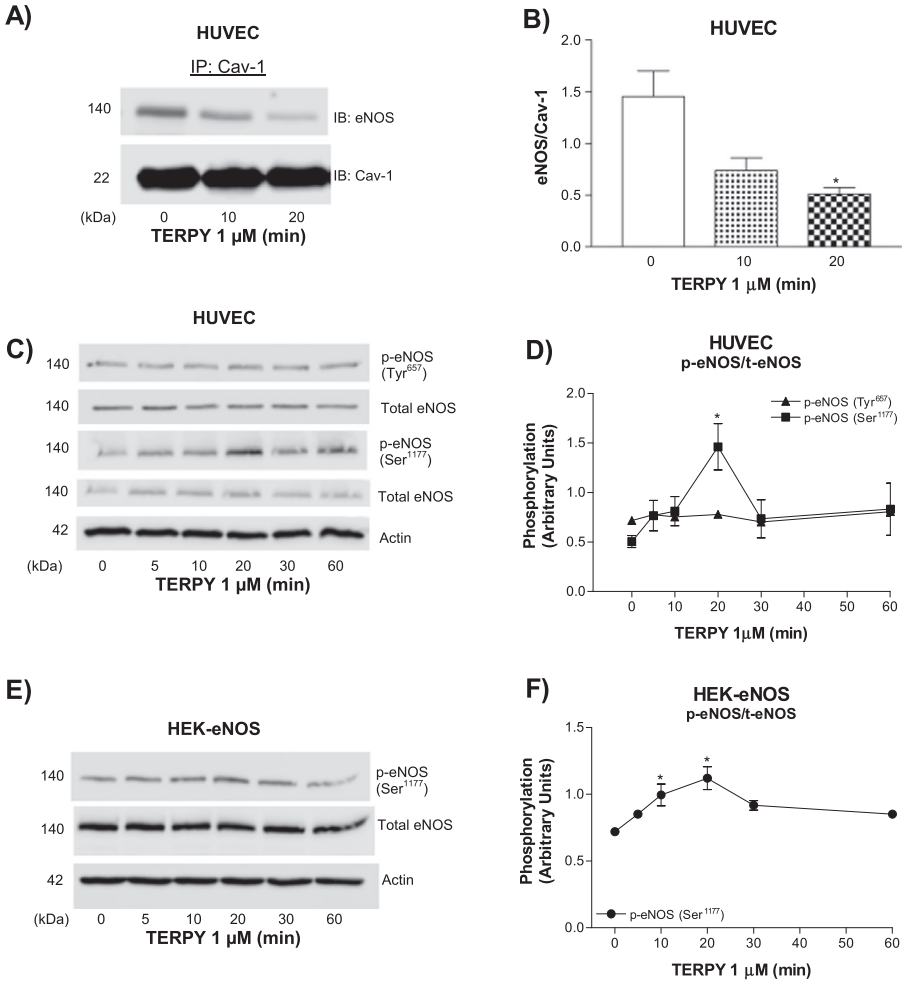

Fig. 6. TERPY decreases eNOS/Cav-1 interaction and promotes eNOS hyperphosphorylation at Serine^{1177}. (A) Interaction of Cav-1 and eNOS determined by co-immunoprecipitation (Co-IP) in human umbilical vein endothelial cells (HUVECs) exposed to TERPY (1 μM). (C) Phosphorylation of eNOS Ser^{1177} and eNOS Tyr^{657} in HUVECs stimulated with TERPY (1 μM) for times indicated and (D) summarized data. (E) Phosphorylation of eNOS Ser^{1177} 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.

(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^{689} and Cys^{908}, 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 ($[Ru(H_2O)(bdq)(terpy)^{2+}$) that is able to oxidize BH_4 to BH_2 [11]. Several studies have shown that reduced levels of BH_4 increase eNOS oxidase activity [13–15], and in fact we demonstrated superoxide anion production and ONOO– formation in HUVECs stimulated with TERPY (Fig. 3) suggesting that eNOS oxidase activity and dysfunction may be induced by TERPY in healthy endothelial cells. When we treated the HUVECs with BH_4, the eNOS uncoupling was prevented (Fig. 4A and B).

SOD (cell-impermeable) and PEG-SOD (cell-permeable) are superoxide scavengers, while uric acid is a natural scavenger of ONOO–. For the constant formation of ONOO– 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 (Fig. 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 μM) or the treatment-time (2 h) were not enough to decrease, at least, the fluorescence intensity of 7-CBA. Uric acid (in the concentration higher than 300 μM and in treatment-time higher than 6 h) decreased expression and activity of eNOS, reduced NO bioavailability, enhanced ROS generation and induced the apoptosis in HUVECs [33–36]. 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 μM) for 2 h did not change NO bioavailability or eNOS Ser^{1177} phosphorylation, but a higher concentration (600 μM) was able to reduce NO production and decrease eNOS Ser^{1177} phosphorylation. In this way, we used uric acid 300 μM (physiological concentration) to avoid the unwanted effects on eNOS activity.

It was previously demonstrated that BH_2 competes with BH_4 for binding to eNOS, however, BH_2 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_2 and BH_4 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 Fig. 2A and 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^{14} phosphorylation, as shown in Fig. 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^{14} as we observed in our results in Fig. 5E and F. The Src-dependent Cav-1-Tyr^{14} 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 Fig. 6A and 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 hyper-activation of eNOS. We showed that TERPY promoted sustained phosphorylation of the Ser^{1177} residue in eNOS (Fig. 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 (Fig. 1A and C). These results suggest that eNOS is activated by TERPY. Although the phosphorylation of eNOS at tyrosines is less investigated, mutation analyses revealed that Tyr^{657} phosphorylation attenuates eNOS enzyme activity, presumably in order to limit the detrimental consequences of maintained high NO output in situations of redox stress [54–56]. In this way, we analyzed TERPY-induced eNOS-phosphorylation at Tyr^{657}. Our result showed that eNOS Tyr^{657} 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_4 to BH_2 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^{1177} 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.

Author contributions

Conceived and designed the experiments: SRP ZC SDSO RDM.

Performed the experiments: SRP ZC SDSO.

Analyzed the data: SRP ZC SDSO RDM.

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

Final approval of the version to be submitted: SRP ZC SDSO RS da S LMB MGB CA RDM

Funding source

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico [Grant numbers 400164/2014-0; 232217/2014-9 (LMB, CA)] and National Institute of Health [Grant numbers HL60678 and HL125356 (RDM)].

Conflict of interest

The authors have no conflicts of interest to declare.

Acknowledgments

We would like to thank Professor Dr. Randall O. Dull for allowing the use of his research lab.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2017.09.004.

References

[1] P.J. Andrew, B. Mayer, Enzymatic function of nitric oxide synthases, Cardiovasc. Res. 43 (1999) 521–531.
[2] U. Förstermann, W.C. Sessa, Nitric oxide synthases: regulation and function, Eur. Heart J. 33 (2012) 829–837.
[3] B. Mayer, B. Hemmens, Biosynthesis and action of nitric oxide in mammalian cells, Trends Biochem. Sci. 22 (1997) 477–481.
[4] P. Valance, N. Chan, Endothelial function and nitric oxide: clinical relevance, Heart 85 (2001) 342–350.
[5] L. Lorente, M.A. Aller, J.L. Arias, J. Arias, Nitric oxide (NO) is a highly reactive free radical with a multitude of organ specific regulatory functions, Ann. Surg. 224 (1996) 688–689.
[6] M. Kelm, M. Feelisch, A. Deussen, J. Schrader, B.E. Strauer, The role of nitric oxide in the control of coronary vascular tone in relation to partial oxygen pressure, perfusion pressure and flow, J. Cardiovasc. Pharmacol. 17 (1991) S95–S99.
[7] F.C. Munhoz, S.R. Potje, A.C. Pereira, M.G. Daruge, R.S. da Silva, L.M. Bendhack, C. Antoniali, Hypotensive and vasorelaxing effects of the new NO-donor [Ru(terpy)(bdq)NO(+)](3+) in spontaneously hypertensive rats, Nitric Oxide 26 (2012) 111–117.
[8] G.J. Rodrigues, A.C. Pereira, J.A. Vercesi, R.G. de Lima, R.S. da Silva, L.M. Bendhack, Long-lasting hypotensive effect in renal hypertensive rats induced by nitric oxide released from a ruthenium complex, J. Cardiovasc. Pharmacol. 60 (2012) 193–198.
[9] S.R. Potje, F.C. Munhoz, L.A. Perassa, M.E. Graton, A.A. Pereira, A.C. Nakamune, R.S. da Silva, L.M. Bendhack, D.H. Sumida, C. Antoniali, Mechanisms underlying the hypotensive and vasodilator effects of Ru(terpy)(bdq)NO$]^{3+}$, a nitric oxide donor, differ between normotensive and spontaneously hypertensive rats, Eur. J. Pharmacol. 15 (2014) 222–229.
[10] A.V. Araújo, A.C. Pereira, M.D. Grando, R.S. da Silva, L.M. Bendhack, The new NO donor Terpy induces similar relaxation in mesenteric resistance arteries of renal hypertensive and normotensive rats, Nitric Oxide 30 (2013) 47–53.
[11] D. Bonaventura, C.N. Lunardi, G.J. Rodrigues, M.A. Neto, J.A. Vercesi, R.G. de Lima, R.S. da Silva, L.M. Bendhack, Endothelium negatively modulates the vascular relaxation induced by nitric oxide donor, due to uncoupling NO synthase, J. Inorg. Biochem. 103 (2009) 1366–1374.
[12] J. Vásquez-Vivar, B. Kalyanaraman, P. Martásek, N. Hogg, B.S. Masters, H. Karoui, P. Tordo, K.A. Pritchard Jr, Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors, Proc. Natl. Acad. Sci. USA 95 (1998) 9220–9225.
[13] R.M. Wever, T. van Dam, H.J. van Rijn, F. de Groot, T.J. Rabelink, Tetrahydrobiopterin regulates superoxide and nitric oxide generation by recombinant endothelial nitric oxide synthase, Biochem. Biophys. Res. Commun. 237 (1997) 340–344.
[14] U. Landmesser, S. Dikalov, S.R. Price, L. McCann, T. Fukai, S.M. Holland, W.E. Mitch, D.G. Harrison, Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension, J. Clin. Investig. 111 (2003) 1201–1209.
[15] J.B. Laursen, M. Somers, S. Kurz, L. McCann, A. Warnholtz, B.A. Freeman, M. Tarpey, T. Fukai, D.G. Harrison, Endothelial regulation of vasomotion in apoE-deficient mice: implications for interactions between peroxynitrite and tetrahydrobiopterin, Circulation 103 (2001) 1282–1288.
[16] R.G. de Lima, M.G. Sauaia, D. Bonaventura, A.C. Tedesco, L.M. Bendhack, R.S. da Silva, Influence of ancillary ligand L in the nitric oxide photo release by the [Ru(L)(terpy)NO]$^{3+}$ complex and its vasodilator activity based on visible light irradiation, Inorg. Chim. Acta 359 (2006) 2543–2549.

[17] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randal, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[18] L. Busconi, T. Michel, Endothelial nitric oxide synthase. N-terminal myristoylation determines subcellular localization, J. Biol. Chem. 268 (1993) 8410–8413.
[19] O. Feron, J.B. Michel, K. Sase, T. Michel, Dynamic regulation of endothelial nitric oxide synthase: complementary roles of dual acylation and caveolin interactions, Biochemistry 37 (1998) 193–200.
[20] J. Zielonka, A. Sikora, M. Hardy, J. Joseph, B.P. Dranka, B. Kalyanaraman, Boronate probes as diagnostic tools for real time monitoring of peroxynitrite and hydroperoxides, Chem. Res. Toxicol. 25 (2012) 1793–1799.
[21] J. Zielonka, A. Sikora, J. Joseph, B. Kalyanaraman, Peroxynitrite is the major species formed from different flux ratios of co-generated nitric oxide and superoxide, direct reaction with boronate-based fluorescent probe, J. Biol. Chem. 285 (2010) 14210–14216.
[22] F.R. Bakhshi, M. Mao, A.N. Shajahan, T. Piegeler, Z. Chen, O. Chernaya, T. Sharma, W.M. Elliot, R. Szulcek, H.J. Bogaard, S. Comhair, S. Erzurum, G.P. van Nieuw Amerongen, M.G. Bonini, R.D. Minshall, Nitrosation-dependent caveolin-1 phosphorylation, ubiquitination, and degradation and its association with idiopathic pulmonary arterial hypertension, Pulm. Circ. 3 (2013) 816–830.
[23] H. Kojima, N. Nakatsubo, K. Kikuchi, S. Kawahara, Y. Kirino, H. Nagoshi, Y. Hirata, T. Nagano, Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins, Anal. Chem. 70 (1998) 2446–2453.
[24] P. Wardman, Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: progress, pitfalls, and prospects, Free Radic. Biol. Med. 43 (2007) 995–1022.
[25] X. Zhou, P. He, Improved measurements of intracellular nitric oxide in intact microvessels using 4,5-diaminofluorescein diacetate, Am. J. Physiol. Heart Circ. Physiol. 301 (2011) H108–H114.
[26] J.A. Xian, H. Guo, B. Li, Y.T. Miao, J.M. Ye, S.P. Zhang, X.B. Pan, C.X. Ye, A.L. Wang, X.M. Hao, Measurement of intracellular nitric oxide (NO) production in shrimp haemocytes by flow cytometry, Fish Shellfish Immunol. 35 (2013) 2032–2039.
[27] M.H. Lim, Preparation of a copper-based fluorescent probe for nitric oxide and its use in mammalian cultured cells, Nat. Protoc. 2 (2007) 408–415.
[28] M.H. Lim, D. Xu, S. Lippard, Visualization of nitric oxide in living cells by a copper-based fluorescent probe, Nat. Chem. Biol. 2 (2006) 375–380.
[29] J.C. Sullivan, J.S. Pollock, Coupled and uncoupled NOS: separate but equal? Uncoupled NOS in endothelial cells is a critical pathway for intracellular signaling, Circ. Res. 98 (2006) 717–719.
[30] K. Schmidt, B. Kolesnik, A.C. Gorren, E.R. Werner, B. Mayer, Cell type-specific recycling of tetrahydrobiopterin by dihydrofolate reductase explains differential effects of 7,8-dihydrobiopterin on endothelial nitric oxide synthase uncoupling, Biochem. Pharmacol. 90 (2014) 246–253.
[31] K. Ravi, L.A. Brennan, S. Levic, P.A. Ross, S.M. Black, S-nitrosylation of endothelial nitric oxide synthase is associated with monomerization and decreased enzyme activity, Proc. Natl. Acad. Sci. USA 101 (2003) 2619–2624.
[32] C.-A. Chen, T.-Y. Wang, S. Varadharaj, L.A. Reyes, C. Hemann, M.A. Talukder, Y.-R. Chen, L.J. Druhan, J.L. Zweier, S-glutathionylation uncouples eNOS and regulates its cellular and vascular function, Nature 468 (2010) 1115–1118.
[33] I. Papezikova, M. Pekarova, A. Lojek, L. Kubala, The effect of uric acid on homocysteine-induced endothelial dysfunction in bovine aortic endothelial cells, Neuro Endocrinol. Lett. 30 (2009) 112–115.
[34] P. Li, L. Zhang, M. Zhang, C. Zhou, N. Lin, Uric acid enhances PKC-dependent eNOS phosphorylation and mediates cellular ER stress: a mechanism for uric acid-induced endothelial dysfunction, Int. J. Mol. Med. 37 (2016) 989–997.
[35] W. Cai, X. Duan, Y. Liu, J. Yu, Y. Tang, Z. Liu, S. Jiang, C. Zhang, J. Liu, J. Xu, Uric acid induces endothelial dysfunction by activating the HMGB1/RAGE signaling pathway, Biomed. Res. Int. 2017 (2017) 4391920.
[36] Z. Huang, Q. Hong, X. Zhang, W. Xiao, L. Wang, S. Cui, Z. Feng, Y. Lv, G. Cai, X. Chen, D. Wu, Aldose reductase mediates endothelial cell dysfunction induced by high uric acid concentrations, Cell Commun. Signal. 15 (2017) 3.
[37] J.H. Park, Y.M. Jin, S. Hwang, D.H. Cho, D.H. Kang, I. Jo, Uric acid attenuates nitric oxide production by decreasing the interaction between endothelial nitric oxide synthase and calmodulin in human umbilical vein endothelial cells: a mechanism for uric acid-induced cardiovascular disease development, Nitric Oxide 1 (2013) 36–42.
[38] I. Papežíková, M. Pekarová, H. Kolářová, A. Klinke, D. Lau, S. Baldus, A. Lojek, L. Kubala, Uric acid modulates vascular endothelial function through the down regulation of nitric oxide production, Free Radic. Res. 47 (2013) 82–88.
[39] A. Presta, U. Siddhanta, C. Wu, N. Sennequier, L. Huang, H.M. Abu-Soud, S. Erzurum, D.J. Stuehr, Comparative functioning of dihydro- and tetrahydropterins in supporting electron transfer, catalysis, and subunit dimerization in inducible nitric oxide synthase, Biochemistry 37 (1998) 298–310.
[40] M.J. Crabtree, C.L. Smith, G. Lam, M.S. Goligorsky, S.S. Gross, Ratio of 5,6,7,8-tetrahydrobiopterin to 7,8-dihydrobiopterin in endothelial cells determines glucose-elicited changes in NO vs. superoxide production by eNOS, Am. J. Physiol. Heart Circ. Physiol. 294 (2008) H1530–H1540.
[41] K. Karuppiah, L.J. Druhan, C.A. Chen, T. Smith, J.L. Zweier, W.C. Sessa, A.J. Cardounel, Suppression of eNOS-derived superoxide by caveolin-1: a biopterin-dependent mechanism, Am. J. Physiol. Heart Circ. Physiol. 301 (2011) H903–H911.
[42] D. Fulton, J.P. Gratton, W.C. Sessa, Post-translational control of endothelial nitric oxide synthase: why isn't calcium/calmodulin enough? J. Pharmacol. Exp. Ther. 299 (2001) 818–824.
[43] H. Ju, R. Zou, V.J. Venema, R.C. Venema, Direct interaction of endothelial nitric-oxide synthase and caveolin-1 inhibits synthase activity, J. Biol. Chem. 272 (1997) 18522–18525.
[44] S. Luanpitpong, S.J. Talbott, Y. Rojanasakul, U. Nimmannit, V. Pongrakhananon, L. Wang, P. Chanvorachote, Regulation of lung cancer cell migration and invasion by reactive oxygen species and caveolin-1, J. Biol. Chem. 285 (2010) 38832–38840.
[45] A. Mougeolle, S. Poussard, M. Decossas, C. Lamaze, O. Lambert, E. Dargelos, Oxidative stress induces caveolin-1 degradation and impairs caveolae functions in skeletal muscle cells, PLos One 10 (2015) e0122654.
[46] H. Li, S. Brodsky, M. Basco, V. Romanov, D.A. De Angelis, M.S. Goligorsky, Nitric oxide attenuates signal transduction, Circ. Res. 88 (2001) 229–236.
[47] A.M. Zimnicka, Y.S. Husain, A.N. Shajahan, M. Sverdlov, O. Chaga, Z. Chen, P.T. Toth, J. Klomp, A.V. Karginov, C. Tiruppathi, A.B. Malik, R.D. Minshall, Src-dependent phosphorylation of caveolin-1 Tyr-14 promotes swelling and release of caveolae, Mol. Biol. Cell 27 (2016) 2090–2106.
[48] Z. Chen, F.R. Bakhshi, A.N. Shajahan, T. Sharma, M. Mao, A. Trane, P. Bernatchez, G.P. Amerongen, M.G. Bonini, R.A. Skidgel, A.B. Malik, R.D. Minshall, Nitric oxide–dependent Src activation and resultant caveolin-1 phosphorylation promote eNOS/caveolin-1 binding and eNOS inhibition, Mol. Biol. Cell 23 (2012) 1388–1398.
[49] W. Schubert, P.G. Frank, S.E. Woodman, H. Hyogo, D.E. Cohen, C.W. Chow, M.P. Lisanti, Microvascular hyperpermeability in caveolin-1 (-/-) knock-out mice. Treatment with a specific nitric-oxide synthase inhibitor, L-NAME, restores normal microvascular permeability in Cav-1 null mice, J. Biol. Chem. 277 (2002) 40091–40098.
[50] Y.Y. Zhao, Y.D. Zhao, M.K. Mirza, J.H. Huang, H.H. Potula, S.M. Vogel, V. Brovkovych, J.X. Yuan, J. Wharton, A.B. Malik, Persistent eNOS activation secondary to caveolin-1 deficiency induces pulmonary hypertension in mice and humans through PKG nitration, J. Clin. Investig. 119 (2009) 2009–2018.
[51] M.R. Siddiqui, Y.A. Komarova, S.M. Vogel, X. Gao, M.G. Bonini, J. Rajasingh, Y.Y. Zhao, V. Brovkovych, A.B. Malik, Caveolin-1-eNOS signaling promotes p190RhoGAP-A nitration and endothelial permeability, J. Cell Biol. 193 (2011) 841–850.
[52] O. Feron, F. Saldana, J.B. Michel, T. Michel, The endothelial nitric-oxide synthase-caveolin regulatory cycle, J. Biol. Chem. 273 (1998) 3125–3128.
[53] V. Rizzo, D.P. McIntosh, P. Oh, J.E. Schnitzer, In situ flow activates endothelial nitric oxide synthase in luminal caveolae of endothelium with rapid caveolin dissociation and calmodulin association, J. Biol. Chem. 273 (1998) 34724–34729.
[54] G. García-Cardeña, R. Fan, D.F. Stern, J. Liu, W.C. Sessa, Endothelial nitric oxide synthase is regulated by tyrosine phosphorylation and interacts with caveolin-1, J. Biol. Chem. 271 (1996) 27237–27240.
[55] A.E. Loot, J.G. Schreiber, B. Fisslthaler, I. Fleming, Angiotensin II impairs endothelial function via tyrosine phosphorylation of the endothelial nitric oxide synthase, J. Exp. Med. 206 (2009) 2889–2896.
[56] E.H. Heiss, V.M. Dirsch, Regulation of eNOS enzyme activity by posttranslational modification, Curr. Pharm. Des. 220 (2014) 3503–3513.
[57] M. Mao, S. Varadarajan, T. Fukai, F.R. Bakhshi, O. Chernaya, S.C. Dudley Jr, R.D. Minshall, M.G. Bonini, Nitroglycerin tolerance in caveolin-1 deficient mice, PLoS One 9 (2014) e104101.