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Camptothecin promotes the production of nitric oxide that triggers subsequent S-nitrosoproteome-mediated signaling cascades in endothelial cells

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ABSTRACT

Camptothecin (CPT) has been used for colorectal cancer therapy. At low concentration of 10^{-9} M, CPT modulates endothelial nitric oxide production following the phosphorylation of LKB1 Ser431, AMPK-α Thr172, eNOS Ser633 and Ser1177. Elevated nitric oxide (NO) was observed by FA-OMe fluorescent probe. 726 S-nitrosoproteins were identified by iTRAQ quantitative proteomics. IPA analysis indicated that ERK/MAPK was closely linked in the signaling network. Further studies showed that CPT phosphorylated p38 MAPK Thr180/Tyr182 and dephosphorylated Tau Ser199/202. CPT also suppressed the TNF-α-induced expression of the inflammasome and cyclooxygenase 2. All this suggests that in addition to the original character of CPT in attenuating the binding of topoisomerase I and DNA in cancer cells, the role of CPT in triggering NO production and the subsequent S-nitrosylated signaling including anti-inflammatory effects in endothelial cells are proposed here. CPT, therefore, provides a potential application addition in preventing vascular disorders.

Abbreviations: CPT, camptothecin; MMTS, methyl methanethiosulfonate; LKB1, liver kinase B1; AMPK, 5′ AMP-activated protein kinase; eNOS, endothelial nitric oxide synthase; FA-OMe, 5-amino-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid methyl ester; iTRAQ, isobaric tags for relative and absolute quantitation; IPA, ingenuity pathway analysis; p38 MAPK, p38 mitogen-activated protein kinases; COX-2, cyclooxygenase-2.

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1. Introduction

Camptothecin (CPT) is an inhibitor of topoisomerase I (TOP I) that suppresses DNA replication, and in the combination of topoisomerase II inhibitors such as doxorubicin or the other compounds, it has been clinically applied as a therapeutic drug for cancer treatment [1–3]. In a recent study, however, at the very low concentration of 10^{-9} M, CPT was reported to promote the biological activity of endothelial nitric oxide synthase (eNOS) by phosphorylating its Ser1177 residue in endothelial cells (ECs) [4]. This observation attracted much attention since nitric oxide (NO) was shown to be beneficial to the vascular system.

NO, a gaseous molecule, is a mild reactive oxygen species (ROS) that can compete with other, more potent ROS, and protects the cardiovascular system from irreversible oxidative stress [5]. NO can trigger numerous endothelial signaling pathways that prevents the progression of atherosclerosis and thrombosis [6]. In clinical therapy, NO-releasing drugs such as nitrostat (nitroglycerin) increases vasorelaxation and can be used to treat patients with acute myocardial infarction. The proposed molecular mechanisms to explain NO's ability to promote cardiovascular protection mainly comprises posttranslational S-nitrosylation, where NO binds to cysteine residues and preserves the structural conformation and activity of enzymes [7,8]. In annotated studies, phosphorylation of Ser1177 represents a major switch for the activation of eNOS. However, an increasing number of studies suggest that phosphorylation of Ser633 represents an alternative route for sustained activation of eNOS [9,10]. As a result, the phosphorylation of eNOS Ser633 and Ser1177 that was confirmed by Western blot is regarded as a plausible mechanism for the generation of NO. However, due to the labile character of NO, the Griess reagent, which is the most frequently used fluorescent probe for detecting NO, is greatly affected by the presence of peroxinitrite ($ONOO^-$) [11]. Therefore, 5-amino-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl) benzoic acid methyl ester (FA-OMe), a new fluorescent probe that can specifically bind to endogenous NO, was developed and utilized [12,13]. As a result, CPT-mediated increases in the production of NO can be determined not only by eNOS phosphorylation through Western blot but also by direct quantification using FA-OMe.

NO-mediated protein S-nitrosylation can be purified preliminarily by the biotin-switch method [14]. Isobaric tagging for relative and absolute quantitation (iTRAQ) is a labeling method that uses stable-isotope-labeled molecules that are covalently bonded to the N-terminus and side chain amines of proteins [15]. We purified the S-nitrosoproteins by a biotin-switch method, labeled them with the iTRAQ reagent, and identified them by tandem mass spectrometry. Ingenuity Pathway Analysis (IPA) software can predict the network and interplay of proteins, in particular within signaling pathways to allow further investigation into the roles of these proteins in the indicated physiological processes [16]. In the present study, the identified S-nitrosoproteome modulated by CPT was analyzed by IPA. The involvement of subsequent signaling components such as p38 MAPK and Tau and inflammatory responses were investigated.

Taken together, these observations showed that low concentrations of CPT triggered the LKB1–AMPK-eNOS signaling cascade to produce NO which led to the posttranslational S-nitrosoproteome. With this omics information, the modulation of vascular homeostasis by CPT through the p38 MAPK, Tau, and inflammatory response pathways was further investigated. The above suggests for further effect evaluations of a conventional drug to obtain vascular homeostasis.

2. Materials and methods

2.1. Cell culture and CPT treatment

Human umbilical vein endothelial cells (HUVECs) were obtained from Cell Applications, Inc. (San Diego, CA, USA). HUVECs were cultured in M199 medium (Gibco, Thermo Fisher Scientific, Carlsbad, CA, USA) supplemented with 100 U/ml penicillin/streptomycin, 2.5 mg/ml amphotericin B, 20% fetal bovine serum (FBS), and 20% endothelial cell growth medium (Lonza, Walkersville, MD, USA). CPT was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in DMSO. ECs were incubated with different concentrations of CPT (10^{-5}–10^{-9} M) and for different times (1, 6, and 12 h).

2.2. Cell lysis and protein extraction

ECs were washed with cord buffer (0.14 M NaCl, 4 mM KCl, 11 mM glucose, 10 mM HEPES pH 7.4) after treatment, and then lysed with 100 μL of lysis buffer (250 mM HEPES pH 7.7, 1 mM EDTA, 0.1 mM neocuproine, 0.4% (w/v) CHAPS). After centrifugation, the supernatant was collected and protein concentrations were determined using a BCA reagent (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. Western blot analysis

Forty micrograms of cell lysate resulting from different treatments resolved by SDS-PAGE, were transferred to PVDF membranes (Millipore, Temecula, CA, USA). The membranes were immunoblotted with antibodies against the following proteins: eNOS (1:3000); peNOS Ser1177 (1:2000); AMPK (1:3000); pAMPK Thr172 (1:1000); p38 MAPK (1:1000); pp38 MAPK Thr108/Tyr182 (1:1000); Caspase 1 (1:1000); cyclooxygenase 2 (COX2, 1:1000); Tau (1:1000) (all from Cell Signaling Technology, Beverly, MA, USA); LKB (1:1000, Millipore); pLKB1 Ser431 (1:1500, Millipore); pTau Ser199/202 (1:500, Sigma-Aldrich); peNOS Ser633 (1:500, BD Biosciences, San Jose, CA, USA); and NALP3 (Abcam, San Francisco, CA, USA). The blotted membranes were visualized with the SuperSignal West Femto reagent (Thermo Fisher Scientific) on X-ray films. The resulting images were scanned using a digital scanner (Microtek International, Hsinchu, Taiwan). The density was calculated using Progenesis Samespots v2.0 software (NonLinear Dynamics, Newcastle, UK).

2.4. Application of fluorescent probes and imaging conditions

ECs treated with CPT were co-incubated with 10 μM FA-OMe for 4 h prior to imaging. After washing with PBS buffer for three times, images were obtained (λex 460 nm, λem 524 nm) using an Axiovert 40CFL fluorescence microscope (Zeiss, Oberkochen, Germany). For confocal fluorescence images, ECs were seeded at a density of 2×10^5 cells/well on cover glasses (24 × 24 mm) and grown for 24 h. The cells incubated with 10 μM FA-OMe were fixed with 4% formaldehyde. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Cover glasses containing fixed ECs were mounted in a mixture of PBS and glycerol (1:1) on a microscopic slide. The cells were observed using a Fluoview300 confocal imaging system (Olympus, Tokyo, Japan), consisting of an Olympus BX51 microscope and a 20-mW argon ion laser.

2.5. Flow cytometry assay

After fluorescence microscopic study, the ECs were washed twice with PBS buffer and detached using trypsin. ECs were collected by centrifugation (400 g, 3 min, room temp.), and then resuspended in PBS buffer. The fluorescence was immediately measured using an Accuri C6 flow cytometer (BD Biosciences) with excitation and emission wavelengths of 488 and 530 nm, respectively. The fluorescence intensity measurements were obtained using 1×10^4 cells and statistically calculated from three replicates.

2.6. Biotin-switch and the purification of S-nitrosoproteins

The biotin-switch protocol was adapted from a previously reported method [14]. Briefly, cell lysates were obtained by sonication and