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Research Paper

Tert-butyl hydroperoxide (t-BHP) induced apoptosis and necroptosis in endothelial cells: Roles of NOX4 and mitochondrion

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ABSTRACT

Oxidative stress causes endothelial death while underlying mechanisms remain elusive. Herein, the pro-death effect of tert-butyl hydroperoxide (t-BHP) was investigated with low concentration (50 μM) of t-BHP (t-BHP_L) and high concentration (500 μM) of t-BHP (t-BHP_H). Both t-BHP_L and t-BHP_H induced endothelial cell death was determined. T-BHP_L induced caspase-dependent apoptosis and reactive oxygen species (ROS) generation, which was inhibited by N-acetyl-L-cysteine (NAC). Furthermore, NADPH oxidase inhibitor diphenyleneiodonium (DPI), NOX4 siRNA, and NOX4 inhibitor GKT137831 reduced t-BHP_L-induced ROS generation while mitochondrial respiratory chain inhibitors rotenone (Rot), 2-thenoyltrifluoroacetone (TTFA), and antimycin A (AA) failed to do so. NOX4 overexpression resulted in increased ROS generation and Akt expression but decreased sensitivity to t-BHP_L. In contrast, T-BHP_H induced LDH release, PI uptake, and cell translucent cytoplasm. RIP1 inhibitor necrostatin-1 (Nec-1), MLKL inhibitor necrosulfonamide (NSA) and silencing RIP1, RIP3, and MLKL inhibited t-BHP_H-induced cell death while pan-caspase inhibitor Z-VAD-FMK showed no effect. T-BHP_H-induced ROS production was inhibited by TTFA, AA and Rot while DPI showed no effect. T-BHP_H induced RIP1/RIP3 interaction, which was decreased by Rot, TTFA, and AA. Silence RIP1 and RIP3 but not MLKL inhibited t-BHP_H-induced mitochondrial membrane potential (MMP) decrease and ROS production. Moreover, P38MAPK inhibitor SB203580 reversed both t-BHP_L and t-BHP_H-induced cell death while inhibitors for ERKs and JNKs showed no obvious effect. These data suggested that t-BHP induced both apoptosis and necroptosis in endothelial cells which was mediated by ROS and p38MAPK. ROS derived from NADPH oxidase and mitochondria contributed to t-BHP_L and t-BHP_H-induced apoptosis and necroptosis, respectively.

1. Introduction

Cell death is a fundamental feature in the lifespan of all the metazoans. Both passive cell death caused by severe structural damage and active cell death owing to confined biological disruptions could happen. The former is uncontrolled while the latter is tightly regulated [1]. Apoptosis is the first-identified form of regulated cell death and was considered as the sole form of regulated cell death for decades [2]. However, accumulated research has identified a few previously unrecognized, regulated cell death models such as regulated necrosis, autophagic cell death. Regulated necrosis is defined as a genetically controlled necrosis with characterized morphology of cytoplasmic granulation, organelle and/or cellular swelling [3]. Although parthanatos, oxytosis, ferroptosis, NETosis, pyronecrosis, and pyroptosis have been considered as different types of regulated necrosis [3], necroptosis is the most understood form of regulated necrotic cell death routine. Though it is morphologically difficult to distinguish necrosis and necroptosis, necroptosis is tightly regulated and genetically controlled by receptor-interacting protein kinase 1 (RIP1), RIP3, and mixed lineage kinase domain-like protein (MLKL) pathways [2,4].

Endothelial cells, the single layer of cells lining all the vasculatures, exert multiple actions such as regulation of vascular permeability and tone, blood fluid and flow, coagulation and fibrinolysis, leukocyte activation, inflammatory, immune surveillance, and cell growth [5,6].

Abbreviations: AA, Antimycin A; ALL, Allopurinol; ASK1, Apoptosis-signaling kinase 1; $DCFH_2$-DA, 5-(6)-carboxy-2′, 7′-dichlorodihydrofluorescein diacetate; DPI, Diphenyleneiodonium; ERK, Extracellular signal regulated kinase; H_2O_2, Hydrogen peroxide; JNK, c-Jun-N-terminal kinase; MAPKs, Mitogen-activated protein kinases; MLKL, Mixed lineage kinase domain-like protein; MMP, Mitochondrial membrane potential; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; NAC, N-acetyl-L-cysteine; NOX4, NADPH oxidase 4; Nec-1, Necrostatin-1; NSA, Necrosulfonamide; PI, Propidium iodide; ROS, Reactive oxygen species; Rot, Rotenone; RIP1, Receptor-interacting protein 1; RIP3, Receptor-interacting protein 3; t-BHP, Tert-butyl hydroperoxide; t-BHP_L, Low concentration of t-BHP (50 μM); t-BHP_H, High concentration of t-BHP (500 μM); TTFA, 2-thenoyltrifluoroacetone;; XO, Xanthine oxidase; Z-VAD-FMK, Z-Val-Ala-DL-Asp-fluoromethylketone

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Endothelial dysfunction caused by endothelial injury and/or death is the initial step for a panel of vascular related diseases. Hyperglycemia, hyperlipidemia, hyperhomocysteinemia, smoking, inflammatory cytokines, shear stress, and environmental toxins etc are common inducers of endothelial death. Oxidative stress resulted from the overproduction of reactive oxygen species (ROS) might serve as one of the common mechanisms for them. In endothelial cells, multiple sources of ROS such as mitochondria, NADPH oxidase, xanthine oxidase (XO) etc have been identified [7,8]. The toxic roles of ROS in endothelial apoptosis has been widely recognized and deeply investigated. ROS activated apoptosis-signaling kinase 1 (ASK1) resulted in sustained JNK activation, which is a key step in initiating caspase-dependent apoptosis [5]. Recent evidence showed that ROS also contributes to TNF induced necroptosis in human colon adenocarcinoma HT-29 cells [9] while its role in endothelial cells remain unclear.

Hydrogen peroxide (H_2O_2), one of the major type of ROS, has been reported to induce necrosis dependent on PARP1 [10] while the role of RIP1 and RIP3 in H_2O_2 trigged necrosis remains controversial [11]. Although larger amount of exogenous H_2O_2 caused oncotic death in cultured endothelial cells, the cell death under this condition remains elusive. Still much less is known about the contribution of organelle-based ROS production in endothelial apoptosis and necroptosis. H_2O_2 is thermodynamically unstable and can easily decompose to form water and oxygen. Tert-butyl hydroperoxide (t-BHP), an organic peroxide widely used in a variety of oxidation processes, is widely used as a better alternative for H_2O_2 in oxidative stress studies. Herein, the pro-death effect of t-BHP on endothelial cells was studied and the underlying mechanisms were explored.

2. Materials and methods

2.1. Reagents

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT), 5-(6)- carboxy-2′, 7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA), dimethyl sulfoxide (DMSO), Hoechst 33342, N-acetyl cysteine (NAC), diphenyleneiodonium chloride (DPI), rotenone (Rot), 2-thenoyltrifluoroacetone (TTFA), antimycin A (AA), allopurinol (ALL), and Akt Inhibitor VIII were purchased from Sigma-Aldrich (USA). LipofectamineTM 3000 was purchased from Life Technology (USA). Antibodies for NOX4, p22phox, and protein A/G PLUS-Agarose were purchased from Santa Cruz (USA). Antibodies for cleaved caspase 3 and caspase 7, phosphorylated ERK1/2 (p-ERK1/2), ERK1/2, phosphorylated p38MAPK (p-p38MAPK), p38MAPK, phosphorylated Akt (p-Akt), Akt, GAPDH, Histone H3, Bcl-2, RIP1, phosphorylated MLKL (p-MLKL), MLKL and Na^+/K^+ ATPase were purchased from Cell Signaling Technology (USA). RIP3 antibody was purchased from Abnova (USA). SiRNA for NOX4, RIP1, RIP3, and MLKL were purchased from Gene Pharma Company (China). Overexpression NOX4 plasmid was purchased from GeneChem Company (China). P5 Primary Cell 4D-Nucleofector® X Kit L (24 RCT) was purchased from Lonza Company (Switzerland). JC-1 kit and LDH Assay Kit were purchased from Beyotime (China). MitoSOX and MitoTracker were purchased from Invitrogen (USA). AlamarBlue Cell Viability Reagent was purchased from Thermo Fisher Scientific (USA).GKT137831 was purchased from BioChemPartner (China). FractionPREP™ Cell Fractionation Kit (for cell fraction extractions) was purchased from BioVision (USA). All other chemicals were purchased from Sigma-Aldrich (USA).

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) were cultured in F-12K medium with 1.5 g/L sodium bicarbonate, 100 μg/ml heparin, 2 mM L-glutamine, 30 μg/mL endothelial cell growth supplement and 10% FBS at 37 °C in a humidified atmosphere of 5% CO_2. Tissue culture flasks, 96-well plates and 6-well plates were pre-coated with 0.2% gelatin. All assays were conducted using low cell passage cells (2–5 passages).

2.3. Cell viability assay

The cell viability was determined with MTT and alamarBlue assay. Cells were seeded in 96-well plates at a density of 1×10^4 cells/well and treated with t-BHP. For MTT assay, after treatment with t-BHP, cells were washed twice with PBS and incubated with 20 μL MTT (5 mg/ml in PBS) for 4 h. Then the culture medium was removed and DMSO (100 μL/well) was added. The absorbance was measured at 570 nm using a microplate reader (PerkinElmer, USA). For alamarBlue assay, t-BHP-treated cells were loaded with AlamarBlue Cell Viability Reagent (Thermo Fisher) [12] and incubated at 37 °C. After 6 h, plates were measured at 545 nm/590 nm (Ex/Em) using a Varioskan™ Flash Multimode Plate Reader (USA).

2.4. Apoptosis assay

Apoptosis was examined by human Annexin V/7AAD kit (BD Biosciences, USA) according to the manufacturer's instructions. Briefly, cells (2.5×10^5) seeded in 6-well plates were treated with t-BHP with or without Z-VAD-FMK (1 μM) pretreatment. Then cells were harvested by incubation with Annexin V/FITC solution for 15 min at room temperature. The samples were immediately analyzed by flow cytometry using a FACSCanto™ system (BD Biosciences, USA). At least 1×10^4 cells were analyzed for each sample.

2.5. Caspase activity measurement

The activity of caspase 3/7 was measured by a commercial Caspase-Glo® 3/7 Assay Kit (Promega) following manufacturer's instructions.

2.6. Immunoprecipitation assay

After determination of the protein concentrations, the cell extracts were incubated with anti-RIP1 antibody (2 μg) for 2 h at 4 °C and followed by incubation with 20 μL of proteinA/G plus-agarose beads overnight. Then the beads were washed 3 times with ice-cold radio immunoprecipitation assay (RIPA) buffer and the bound proteins were extracted by adding 20 μL 2×SDS sample buffer and boiled for 5 min. The complexes were subjected to Western blotting.

2.7. Measurement of intracellular ROS

The production of t-BHP-induced ROS was measured by $DCFH_2$-DA probe as our previous report [13]. To explore the sources of ROS, cells were pretreated with DPI (1 μM), Rot (20 μM), TTFA (10 μM), AA (5 μM), or All (10 μM) for 1 h followed by t-BHP treatment for 30 min.

2.8. Mitochondrial membrane potential assay

The mitochondrial membrane potential (MMP) was assessed using a commercial kit following the manufacturer's instructions.

2.9. Measurement of mitochondrial ROS

Cells (1×10^4 cells/well) cultured in 96-well plates were incubated with MitoTracker Green FM (100 nM) for 1 h at room temperature in an atmosphere of 5% CO_2. After treatment with t-BHP, cells were incubated with MitoSOX reagent working solution (1 μM) for 20 min at 37 °C. Fluorescence was observed with IN Cell Analyzer 2000 and quantified by a FACScan flow cytometer using the PE channel.