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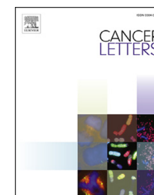

Original Article

RIP1 and RIP3 contribute to shikonin-induced DNA double-strand breaks in glioma cells via increase of intracellular reactive oxygen species

Zijian Zhou [a, b], Bin Lu [a, b], Chen Wang [a, b], Zongqi Wang [a, b], Tianfei Luo [b, c], Meihua Piao [d], Fankai Meng [e], Guangfan Chi [f], Yinan Luo [a], Pengfei Ge [a, b, *]

[a] Department of Neurosurgery, First Hospital of Jilin University, Changchun 130021, China
[b] Research Center of Neuroscience, First Hospital of Jilin University, Changchun 130021, China
[c] Department of Neurology, First Hospital of Jilin University, Changchun 130021, China
[d] Department of Anesthesiology, First Hospital of Jilin University, Changchun 130021, China
[e] Department of Neurosurgery, People's Hospital of Jilin Province, Changchun 130021, China
[f] Key Laboratory of Pathobiology, Ministry of Education, Jilin University, Changchun 130021, China

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ABSTRACT

Shikonin has been reported to induce glioma cell death via necroptosis, a type of programmed necrosis primarily mediated by RIP1 and RIP3. Although RIP1 and RIP3 are found to regulate some features of necrosis such as energy depletion and cellular membrane disruption, it remains unclear whether RIP1 and RIP3 could modulate DNA double strand breaks (DSBs), which is a crucial event leading to chromatinolysis. In this study, we used glioma cell lines and mice model of xenograft glioma to investigate the roles of RIP1 and RIP3 in shikonin-induced DNA DSBs. We found that shikonin induced upregulation of RIP1 and RIP3, necrosome formation and DNA DSBs in vitro and in vivo. In vitro investigation showed that the DNA DSBs and the reduction of cellular viabilities induced by shikonin were both prevented when RIP1 or RIP3 was pharmacologically inhibited by specific inhibitor or genetically knocked down with siRNA. Then, we proved that suppression of intracellular ROS with antioxidant NAC inhibited DNA DSBs caused by either hydrogen peroxide or shikonin, suggesting that ROS played a crucial role in regulation of DNA DSBs of glioma cells induced by shikonin. Further, we found that RIP1 and RIP3 regulated shikonin-induced overproduction of ROS via causing excessive generation of mitochondrial superoxide and depletion of GSH, indicating that ROS was the downstream signal of RIP1 and RIP3. Taken together, we demonstrated that RIP1 and RIP3 contributed to shikonin-induced DNA DSBs in glioma cells via increase of intracellular ROS levels.

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Introduction

Malignant glioma is the most common type of primary malignant brain tumor with an average survival not longer than one year, even though the patients accepted surgery in combination with postoperative chemotherapy and radiotherapy [1]. The insensitivity of glioma cell to currently-used chemotherapeutic agents and radiotherapeutic regimes is attributed to their resistance to apoptosis [2]. However, induction of necroptosis is found to be an effective strategy to kill the cancer cells resistant to apoptosis [3].

Different with apoptosis, necroptosis is a type of caspase-independent programmed cell death with morphological similarities to necrosis and is primarily regulated by receptor interacting serine-threonine protein kinases1 (RIP1) and 3 (RIP3). Upon the induction of necroptosis, the activated RIP1 interacts with its downstream signal RIP3 via their RIP homotypic interaction motifs to form a protein complex named necrosome, in which RIP3 is activated by RIP1 or via auto-phosphorylation [4]. Sequential activation of RIP1 and RIP3 were found to lead to energy depletion and cellular membrane disruption which are regard as critical biochemical events causing necroptotic cell death [5,6]. However,

* Corresponding author. Department of Neurosurgery, First Hospital, Jilin University, 71 Xinmin Avenue, Changchun 130021, Jilin Province, China.
E-mail addresses: gepf@jlu.edu.cn, pengfeige@gmail.com (P. Ge).

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the roles of RIP1 and RIP3 remain unclear in regulation of DNA double strand breaks (DSBs), another hallmark of programmed necrosis [7].

DNA double-strand breaks (DSBs) may arise spontaneously during DNA replication or following exposure to ionizing radiation (IR), chemotherapeutic drugs or oxidative stress [8]. Moreover, DNA DSBs represent the most dangerous DNA damage, because a single DSB can be lethal when not be repaired or misrepaired [9]. Notably, recent studies reveal that chromatinolysis is closely associated with DNA DSBs [7]. Upon DSB generation, ataxia telangiectasia mutated (ATM) or DNA-dependent protein kinase catalytic subunit (DNA-PKcs) are activated, and then phosphorylate histone variant H2AX at serine139 to produce γH2AX [10]. γH2AX specifically accumulates at the sites of DSBs that occur either in vitro or in vivo, and is thus regarded as a sensitive molecular maker of DNA DSBs [11,12]. Although γH2AX serves as a platform for ordered recruitment of the proteins involved in DNA repair, it recruits the AIF translocated from mitochondria to form DNA-degrading complex during the process of necroptosis [7]. Thus, generation of DNA DSBs is a crucial step leading to chromatinolysis in the cells undergoing necroptosis.

Shikonin is a natural naphthoquinone isolated from Lithospermum Erythrorhizon and has been demonstrated to induce necroptosis not only in glioma cells [13], but also in other types of cancer cells such as breast cancer, multiple myeloma and osteosarcoma [14–16]. Additionally, shikonin could also cause DNA damage and inhibit DNA synthesis [17,18]. However, it remains unclear whether shikonin-induced DNA damage is regulated by the RIP1 and RIP3. Therefore, we used rat and human glioma cell lines and mice model of xenograft glioma in this study to investigate the roles of RIP1 and RIP3 in shikonin-induced DNA DSBs in glioma cells and the underlying mechanism.

Materials & methods

Reagents

Shikonin, Nec-1(necrostatin-1), H_2O_2, NAC (N-acetyl-L-cysteine) and MG-132 were all purchased from Sigma (St.Louis, MO, USA). GSK-872 was from Calbiochem (Billerica, MA, USA). Shikonin was dissolved in PBS to a storage concentration of 50 mmol/L. Anti-RIP1, anti-RIP3, anti-CYLD, anti-phosphor-H2AX at S139, anti-phosphor-ATM at S1981, anti-H2A and anti-CD68 antibodies were from Abcam (Cambridge, MA, USA). Anti-caspase 8, anti-caspase-3, anti-HSP70, anti-Calreticulin, anti-HMGB1 antibodies were from Cell Signaling Technology (Danvers, MA). Anti- β-Actin antibodiy was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other reagents were purchased from Sigma (St. Louis, MO). BALB/c nude mice were obtained from the Animal Center of the Chinese Academy of Medical Science (Beijing, China).

Cell line and cell viability assay

Rat C6, human SHG-44, U87 and U251 glioma cells were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). They were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, penicillin (100 U/mL) and streptomycin (100 μg/mL), and maintained at 37 °C and 5% CO2 in a humid environment. Cells in the mid-log phase were used in the experiments. HL-7702 (human normal hepatocyte) and HUVEC (human umbilical vein epithelial cells) were kindly provided by Dr. Chao Niu (Research Center for Translational Medicine, first hospital of Jilin University) and cultured as per their instructions.

C6(1×10^5 cells/well), SHG-44 (5×10^4 cells/well), U87(7×10^4 cells/well) and U251 (7×10^4 cells/well) glioma cells were seeded onto 96-well microplate and cultured 24 h, and then treated with shikonin at indicated concentrations for 3 h. Two normal cell lines, HL-7702 (hepatic immortal cell line) and HUVEC (human umbilical vein endothelial cell line) were used as control. The cellular viability was assessed using an MTT assay, and was expressed as a ratio to the absorbance value at 570 nm of the control cells.

Assessment of cell death by flow cytometry

SHG-44 and U251 glioma cells were collected at indicated time after the target compounds treatment. Then, the Annexin V-FITC detection kit (Invitrogen, Grand Island, NY) was used for assessment of cell death modality as described by the manufacture's instruction. The collected cells were washed twice with PBS, and resuspended in 400 μL 1 × binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). Cells (100 μL) were transferred to a 5-mL culture tube containing 5 μL of annexin V-FITC and 10 μL of propidium iodide, and then incubated for 15 min at room temperature in the dark. After 1 × binding buffer was added into each tube, the stained cells were analyzed by flow cytometry (FACScan, Becton Dickinson, San Jose, CA). The rate of cell death was analyzed using CELLquest software (Becton Dickinson). Data acquisition was conducted by collecting 20,000 cells per tube and the numbers of viable and dead cells were determined for each experimental condition.

Detection of DNA damage by neutral and alkaline comet assay

Neutral comet assay was performed as previously described [19]. The cells were analyzed at the indicated time after incubation with shikonin in the presence or absence of Nec-1, GSK-872 and NAC. Briefly, the collected glioma cells (2×10^5cells/mL) were suspended in 1 ml of low-melting agarose and 80 μL were deposited on comet slides prelayered with 1% regular agarose, covered with coverslips and allowed to gel on a cold plate at 4 °C for 10 min. Then, the coverslip was moved and slide was covered with 80 μL low-melting agarose. Slides were immersed in lysing solution (2.5 mol/L NaCl, 100 mmol/L Na_2EDTA, 10 mmol/L Tris, pH 10, and 10% DMSO with 1% Triton X-100) in darkness at 4 °C for 1 h, and washed for 10 min in TBE buffer (0.445 mol/L Tris–HCl, 0.445 mol/L Boric acid and 0.01 mol/L EDTA). The slides were placed horizontally on an electrophoresis tray that was filled TBE buffer and electrophoresed for 20 min at 25 V, with a subsequent wash in 0.9% NaCl for 2 min. Cells were neutralized using 0.4 mol/L Tris (pH 7.5) and stained with 8.0 μg/mL of acridine orange (8.0 μg/mL) for 5 min. The slides were analyzed using a fluorescence microscope (Olympus IX71, Tokyo, Japan). The cell number with DNA comets and the DNA percent content in comet tail region were measured using ImageJ and OpenComet 1.3 software (three assays, each with about 100 cells analyzed).

For the alkaline Comet assay, the slide was incubated in a denaturing solution (0.3 mol/L NaOH and 1 mol/L Na_2EDTA, Ph > 13) for 1 h at 4 °C, and electrophoresed for 20 min at 25 V. Both neutral and alkaline slides were then incubated in the neutralizing solution (0.4 M Tris–HCl, pH 7.5) for 5 min, in TBE for 2 min and finally dehydrated in an ethanol series of 70, 90 and 100% for 2 min each.

Measurement of intracellular reactive oxygen species and mitochondrial superoxide

C6 (1×10^5 cells/well) and SHG-44 (5×10^4 cells/well) glioma cells were seeded onto 96-well microplate and cultured 24 h, and then treated with target compounds for 3 h. The average level of intracellular ROS was evaluated by using redox-sensitive dye DCFH-DA (Beyotime Biotech, Nanjing, China). All the experimental cells were washed twice in PBS and stained in the dark for 30 min with 20 μmol/L DCFH-DA. After the cells were dissolved with 1% Triton X-100, the fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength 530 nm using a fluorescence spectrometer (HTS 7000, Perkin Elmer, Boston, MA). The ROS levels were expressed as arbitrary unit/mg protein, then as the percentage of control.

Mitochondrial superoxide was assayed by using MitoSOX red (Invitrogen company, Eugene, OR) as described by manufacture's instruction. The cells were incubated 10 min with 2.0 ml MitoSOX reagent working solution at 5 μmol/L at 37 °C in dark, and washed with PBS. The red fluorescence density was measured at an excitation wavelength of 510 nm and an emission wavelength at 580 nm, and was expressed as a ratio to the fluorescence in control cells.

Other groups of C6 and SHG-44 glioma cells were seeded onto a culture dish in a diameter of 3 cm and cultured 24 h. After being treated 3 h with pristimerin, the cells were stained with DCFH-DA or MitoSox red as described above, and observed under fluorescence microscope (Olympus IX71, Tokyo, Japan).

Measurement of intracellular glutathione (GSH)

Intracellular total GSH was measured by using the DTNB-GSSH reductase recycling assay kit (Beyotime Biotechnology, Nanjing, China) as described by manufacture. Briefly, the cells treated with pristimerin for the indicated time were collected by centrifugation at 800 × g for 5 min, washed twice with PBS. The cell pellets were re-suspended in protein-removing buffer S and lysed by repeated cycles of freezing and thawing under liquid nitrogen. The cell lysates were centrifuged at 10,000 × g for 10 min at 4 °C to get the supernatant used for intracellular total GSH assay. GSH content was expressed as a ratio to the absorbance value at 412 nm of the control cells.

Transfection of small interfering RNA (siRNA)

SHG-44 (15×10^5 cells/ml) and U251 (15×10^5 cells/ml) cells were seeded onto a culture dish in a diameter of 10 cm. Transfection of siRNA was performed by using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. The RIP1 siRNA (5′- GCCAGCUGCUAAGUACCA ATT -3′), RIP3 siRNA (5′- CCAGCA-CUCUCGUAAUGAUTT -3′), and Srambled siRNA (5′-UUCUCCGAACGUGUCACGUTT-3′) were purchased from GenePharma Company (Suzhou, China). After siRNA transfection overnight, the cells were incubated with shikonin at indicated dose for subsequent experiments.

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