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MAC related mitochondrial pathway in oroxylin A induces apoptosis in human hepatocellular carcinoma HepG2 cells

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ABSTRACT

Oroxylin A is a flavonoid isolated from the root of *Scutellaria baicalensis* Georgi. Our previous work demonstrated that the anti-tumor activity of oroxylin A was mainly attributed to its apoptosis inducing effect in cells. The present study explores the exact molecular mechanism of oroxylin A-induced apoptosis in tumor cells. We showed that oroxylin A-induced apoptosis in HepG2 cells was achieved through mitochondrial pathway. We also investigated which mitochondrial channels, PTP or MAC or both, were involved in the permeabilization of the mitochondrial outer membrane after treatment with oroxylin A. The results showed that oroxylin A-induced apoptosis in a PTP-independent manner; therefore, we focused our attention on MAC. As Bax is an essential constituent of MAC in certain systems, we examined the activation, subcellular location, oligomeric structure of Bax in HepG2 cells treated with oroxylin A. Moreover, our results showed that overexpression of Bcl-2 inhibited oroxylin A-induced apoptosis. In summary, we have demonstrated that opening of MAC, but not PTP, played a key role in oroxylin A-induced activation of mitochondrial apoptotic pathway in HepG2 cells.

1. Introduction

Oroxylin A ($C_{16}H_{12}O_5$, Fig. 1) is a flavonoid isolated from the root of *Scutellaria baicalensis* Georgi, a conventional herbal medicine widely used as an antipyretic, analgesic, anti-tumor, and anti-inflammatory agent. Previous reports have revealed that oroxylin A has anti-oxidative, anti-inflammatory and anti-viral activities. For example, it suppresses superoxide and nitric oxide generation [1] and inhibits lipopolysaccharide-induced inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) gene expression by restraining nuclear factor-kappa B activation [2]. It was reported that oroxylin A possessed antagonistic properties at the γ-aminobutyric acid ($GABA_A$) receptor [3] and ameliorates memory dysfunction induced by hypoperfusion, scopolamine and Abeta(25–35) in mice [4–6]. We found that oroxylin A also exerted pro-apoptotic effect on human hepatocellular carcinoma cell line HepG2. However, the mechanisms of oroxylin A-induced apoptosis are complex and remain to be elucidated.

Apoptosis is mediated through two major pathways, the death receptor pathway and the mitochondrial pathway. The mitochondrial pathway is a complex course with mitochondria as central gateway controllers and the Bcl-2 family of proteins [7,8] as executioners. The Bcl-2 proteins can be divided into anti-apoptotic (Bcl-2, Bcl-XL, Bcl-w, Mcl-1, A1, Nr-13, and others) and pro-apoptotic members [9,10]. The pro-apoptotic members are represented by two subgroups: the multidomain or Bax subfamily (Bax, Bak, and Bok) that contains multiple BH domains and the BH3-only subfamily (Bad, Bid, Bim, Noxa, Hrk, and others) [11,12]. It is clear that the molecules upstream, like the BH3-only proteins, capture various apoptotic stimuli or signals and

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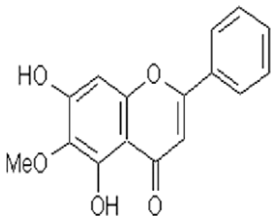

Fig. 1. Molecular structure of oroxylin A ($C_{16}H_{12}O_5$, MW: 284).

subsequently activate the multi-domain pro-apoptotic proteins. Activation of the intrinsic pathway involves release of pro-apoptotic factors, like cytochrome *c*, Smac/Diablo, Endonuclease G, and AIF, from the mitochondrial intermembrane space, which amplifies the apoptotic cascades to the final destruction of the cell [13,14]. The apoptosome/caspase 9 pathway is a signaling route downstream of the mitochondrion. The release of cytochrome *c* triggers formation of the apoptosome complex and activation of caspase-9 [15]. Two proteins, Endonuclease G and AIF, released after permeabilization of the outer membrane, can induce apoptosis in a caspase-independent manner [16,17] .

The crucial step in the mitochondrial apoptotic pathway is permeabilization of the mitochondrial outer membrane. Two mechanisms, involving opening of two different mitochondrial channels, have been proposed to be responsible for the permeabilization; the permeability transition pore (PTP) in the inner membrane and the mitochondrial apoptosis-induced channel (MAC) in the outer membrane [18]. Both channels are regulated by Bcl-2 family proteins [19,20]. Opening of PTP would lead to matrix swelling, subsequent rupture of the outer membrane, and an unspecific release of intermembrane proteins into the cytosol [18]. The multi-domain pro-apoptotic protein Bax and Bak are putative components of MAC [19,21]. MAC forms in the mitochondrial outer membrane early in apoptosis and directly provides a pathway for the release of cytochrome *c* from the intermembrane space to the cytosol. Transient opening of either MAC or PTP may independently or jointly, depending on cell type and death stimulus, result in remodeling of the cristae and maximal cytochrome *c* release to amplify the death signal and ensure completion of apoptosis [21,22]. In healthy cells, Bax is cytosolic, however in response to apoptotic stimuli the protein undergoes conformational changes and mitochondrial translocation, leading to the formation of oligomers and perhaps higher order structures that promote the release of cytochrome *c* and other apoptogenic factors [23,24]. Oligomeric Bax forms slightly cation-selective, voltage-independent channels with a variable conductance from a few pS up to several nS [25,26]. The diameter estimated for the Bax channels from the peak conductance of 1–5.4 nS is 2.7–5.4 nm, indicating many Bax channels could easily allow the passage of ~3 nm cytochrome *c* [19]. One study showed that Bax tetramers inserted into liposomes and formed channels large enough to allow the release of cytochrome *c*, which demonstrated that Bax alone was able to create channels for the passage of mitochondrial proteins through membranes [27]. Bax channels and the release of various compounds from liposomes are blocked by the anti-apoptotic proteins Bcl-2 and Bcl-xL [25]. The electrophysiological characteristics of MAC are very similar to Bax channels and depletion of Bax significantly diminishes MAC activity, suggesting that Bax is an essential constituent of MAC in some systems [18].

In this study, we explored the mechanisms involved in oroxylin A-induced apoptosis. We showed that oroxylin A-induced apoptosis in HepG2 cells was achieved through mitochondrial pathway. We also investigated which mitochondrial channels, PTP or MAC or both, were involved in the permeabilization of the mitochondrial outer membrane after treatment with oroxylin A. The results showed that oroxylin A-induced apoptosis in a PTP-independent manner; therefore, we focused our attention on MAC. As Bax is an essential constituent of MAC in certain systems, we examined the activation, subcellular location, oligomeric structure of Bax in HepG2 cells treated with oroxylin A.

2. Materials and methods

2.1. Materials

Oroxylin A was isolated from the root of *Scutellaria baicalensis* according to the protocols reported previously [28]. Samples containing 99% or higher in oroxylin A were used in all experiments unless otherwise indicated. Oroxylin A was dissolved in DMSO to 200 mM and stored at −20 °C. The concentrations used in this study were 50, 100, and 200 μM, and freshly diluted using the basal medium with a final DMSO concentration of 0.1%. Controls were always treated with the same amount of DMSO as used in the corresponding experiments.

Antibodies to caspase-3(sc-56052), caspase-9 (sc-56073), caspase-8 (sc-56070), Bax (sc-526), Bcl-2 (sc-7382) were obtained from Santa Cruz (Santa Cruz, CA); antibody to cytochrome *c* was from CALBIOCHEM (Merck, Darmstadt, Germany); antibodies to PARP (catalog number 9542) and AIF (catalog number 4642) were from Cell Signaling (Danvers, MA); antibody to Cox IV(ab14744) was from Abcam (UK); and antibody to β-actin (BM0627) was from Boster (Wuhan, China). The 6A7 active Bax antibody was from Sigma. IRDye™800 conjugated secondary antibodies were obtained from Rockland Inc. (Philadelphia, PA) Mitotracker Red was from Molecular Probes (Invitrogen, Carlsbad, CA). Lipo-fectamine reagents were obtained from Invitrogen (Carlsbad, CA). Cyclosporins A, N-acetyl-L-cysteine, and other chemicals were all obtained from Sigma unless otherwise stated.

2.2. Cell lines

Human hepatocellular carcinoma HepG2 cells were purchased from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in 90% DMEM (lot No. 1313804, Gibco, USA) supplemented with 10% FBS (Sijiqing, Hangzhou, China), 100 U/ml penicillin, and 100 μg/ml streptomycin. Exponentially growing cultures