Biochimie 95 (2013) 2157–2167

Contents lists available at ScienceDirect

Biochimie

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

Research paper

Antitumour activity on extrinsic apoptotic targets of the triterpenoid maslinic acid in p53-deficient Caco-2 adenocarcinoma cells

Fernando J. Reyes-Zurita [a], Eva E. Rufino-Palomares [a], Pedro P. Medina [a], E. Leticia García-Salguero [a], Juan Peragón [b], Marta Cascante [c,**], José A. Lupiáñez [a,*]

[a] Department of Biochemistry and Molecular Biology I, University of Granada, 18071 Granada, Spain
[b] Biochemistry and Molecular Biology Section, Department of Experimental Biology, University of Jaen, 23071 Jaen, Spain
[c] Department of Biochemistry and Molecular Biology, University of Barcelona, 08028 Barcelona, Spain

ARTICLE INFO

Article history:
Received 9 April 2013
Accepted 6 August 2013
Available online 20 August 2013

Keywords:
Maslinic acid
Triterpenes
Colon cancer cells
Extrinsic apoptotic pathway

ABSTRACT

We report that a novel triterpenoid, (2a,3b)-2,3-dihydroxyolean-12-en-28-oic acid (maslinic acid), isolated from olive pomace from *Olea europaea*, triggers primarily the extrinsic and later the intrinsic apoptotic pathways in Caco-2 human colon-cancer cells. Apoptosis induced by maslinic acid was confirmed by FACS analysis using annexine-V FICT staining. This induction of apoptosis was correlated with the early activation of caspase-8 and caspase-3, the activation of caspase-8 was also correlated with higher levels of Bid cleavage and decreased Bcl-2, but with no change in Bax expression. Maslinic acid also induced a sustained activation of c-Jun N-terminal kinase (JNK). Incubation with maslinic acid also resulted in the later activation of caspase-9, which, together with the lack of any Bax activation, suggests that the mitochondrial pathway is not required for apoptosis induced by maslinic acid in this cell line. In this study we found that the mechanism of apoptotic activation in p53-deficient Caco-2 cells differs significantly from that found in HT-29 cells. Natural agents able to activate both the extrinsic and intrinsic apoptotic pathways by avoiding the mitochondrial resistance mechanisms may be useful for treatment against colon cancer regardless of its aetiology.

1. Introduction

The progression of cancer is characteristically linked to resistance to apoptosis, which is generally mediated by the intrinsic mitochondrial pathway but which may be naturally disabled by blockage signals that trigger mitochondrial perturbations. Although the intrinsic mitochondrial pathway is fundamental to breaking down the resistance of human colon-cancer cells, there also exists a second apoptotic pathway, known as the extrinsic apoptotic pathway, which may achieve the same effect.

The mechanism of action of maslinic acid, in this cell line, related with the receptors and with its potential targets is now not well known. Nevertheless, this could be related with the activation of death receptors pathway as described in [1,2]. The activation of the cell surface receptor Fas (CD95/APO-1) whit its ligand, Fas-ligand (FasL) produce apoptosis activation very fast and efficiently. Binding Fas-L promotes Fas oligomerization and FADD recruits, resulting in caspase-8 activation (FLICE). Also, has been report [2] that chemotherapeutic drugs increase Fas receptor expression in the surface of colon carcinoma cell lines and sensitize the apoptotic respond of cells from different anti-cancer treatment. With respect to death receptor activation, has been described that compounds as cisplatin or topoisomerase II inhibitors can activate the Fas pathways in a FasL-independent manner [3].

When activated, caspase-8can also prompt downstream events such as cleavage of the BH_3 domain of Bid to its active form, truncated Bid (tBid), resulting in translocation to the mitochondria. This in turn triggers the intrinsic or mitochondrial pathway, releasing cytochrome c into the cytoplasm. Cytochrome-c, released in the

Abbreviations: CDDO, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FACS, fluorescence-activated cell sorter; FCS, foetal calf serum; MA, maslinic acid; PI, propidium iodide; Rh123, rhodamine; MMP, mitochondrial membrane potential.

* Corresponding author. Department of Biochemistry and Molecular Biology I, Faculty of Sciences, University of Granada, Avenida Fuentenueva s/n, 18071 Granada, Spain. Tel.: +34 958 243089; fax: +34 958 249945.
** Corresponding author. Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Barcelona, Avenida Diagonal 645, 08028 Barcelona, Spain. Tel.: +34 934 021593; fax: +34 934 021219.
E-mail addresses: ferjes@ugr.es (F.J. Reyes-Zurita), evaevae@ugr.es (E.E. Rufino-Palomares), pedromedina@ugr.es (P.P. Medina), elgarcia@ugr.es (E. Leticia García-Salguero), jperagon@ujaen.es (J. Peragón), martacascante@ub.edu (M. Cascante), jlcara@ugr.es (J.A. Lupiáñez).

0300-9084/$ – see front matter © 2013 Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.biochi.2013.08.017

presence of (d)ATP, binds to and activates the adaptor protein Apaf-1, which in turn recruits caspase-9, leading to the formation of apoptosome.Caspase-9 then proteolytically activates caspase-3 downstream, this latter enzyme being responsible for the apoptotic phase. The secondary activation of the mitochondrial apoptotic pathway increases and amplifies the initial extrinsic apoptotic signalling.

In addition, the sustained activation of c-Jun N-terminal kinase (JNK) is directly related to the induction of apoptosis [4–6]. Nevertheless, the activation of Bid has also been described as being mediated by JNK [6,7]. Activated Bid targets the mitochondria to modulate other Bcl-2-like factors such as Bax [8]. It is not clear, however, whether Bid is the only target of JNK pro-apoptotic signalling. Furthermore, JNK may act directly upon the Bcl-2 protein family, thus stimulating the mitochondrial pathway. JNK phosphorylates members of the Bcl-2 family of proteins, such as Bcl-2, and inactivates their apoptotic function [9]. Although the activation of JNK has been described as necessary to induce apoptosis in different cell types [10,11], it does not appear to be required for maslinic-acid-induced apoptosis in the Caco-2 cell type which is clearly triggered by the initial activation of caspase-8 and caspase-3.

The activation of the extrinsic apoptotic pathway and the caspase-8 has been described previously in response to triterpene derivatives, such as betulinic acid [12] and its derivatives [13] or the oleanolic derivative CDDO (2- cyano-3,12-dioxooleana-1,9,-dien-28-oic) [14–16]. In the case of CDDO and its derivatives (CDDO-Me and CDDO-Im), the activation of the extrinsic apoptotic pathway has been described as a direct apoptotic pathway induced by caspase-8-mediated caspase-3 or induced by mitochondrial disruption in response to the generation of t-Bid mediated by caspase-8 [16,17].

For example, the induction of apoptosis by CDDO in osteosarcoma cell lines has been put down to the activation of caspase-8, and thence caspase-3, by a cytochrome-c-independent mechanism, although a belated release of cytochrome c by the caspase-8-dependent cleavage of Bid has also been reported [15]. Finally, the secondary activation of JNK in caspase-8-dependent triterpene apoptosis has also been reported [16,17]. CDDO-Me activates caspase-8 apoptosis whatever the status of p53. The activation of JNK prior to caspase-8 has been reported in apoptosis mediated by CDDO-Me DR5 [18]. Apoptosis mediated by betulinic acid has been described as being initiated by the activation of caspase-8, which than induces a direct downstream cleavage of caspase-3 [12]. Alternatively, the activation of the intrinsic apoptotic pathway mediated by the cleaving of Bid by caspase-8 has also been reported [19].

Maslinic acid, a novel oleanane triterpenoid, known to have therapeutic activity and promising anti-cancer potential as a chemopreventive, is one of the main components of the protective wax-like coating of olive fruit and leaf. Maslinic acid has been shown to induce anti-tumoural effects in several types of tumour cells, including colon, ovary and melanoma, as well as cells belonging to the central nervous system and non-small lung-cancer cells [20]. We have demonstrated in a previous publication that maslinic acid induces apoptosis in HT29 colon-cancer cells via the mitochondrial apoptotic pathway, controlled by the Bcl-2 family of proteins, causing mitochondrial disturbances, cytochrome-c release, and the activation of caspase-9 and caspase-3 [21]. In HT29 cells, maslinic acid inhibits the expression of Bcl-2 while increasing that of Bax; it also stimulates the release of mitochondrial factors and activates caspases. In these studies we have not found any activation or involvement of caspase-8 similar to that which we proposed as being responsible for apoptosis induced by maslinic acid in HT29 cells [22].

Here we have used flow-cytometry to show the genotoxicity and apoptotic effects of maslinic acid in Caco-2 p53-deficient colon-cancer cells and suggest the plausible molecular mechanism responsible for its activity. Our results show that apoptosis is brought about directly via the extrinsic apoptotic pathway, which is related to the activation of caspase-8. The down-regulation of Bid cleavage goes on to induce the secondary activation of the mitochondrial apoptotic intrinsic pathway, thus increasing the overall apoptotic response.

The expression of a functional, though mutated, p53 in HT29 cells may explain the major differentiation and late activation of apoptosis observed in the HT29 cell line. The fact that in Caco-2 cells the p53 gene contains deleted and mutated alleles and no detectable accumulation of the corresponding protein [23,24] might explain the lower differentiation and immediate induction of apoptosis in this cell line.

Our results suggest a different mechanism for the apoptosis caused by maslinic acid in the Caco-2 cell line compared to that found in the HT29 cell line. Firstly, the treatment of Caco-2 cells with maslinic acid prompted immediate apoptosis and secondly, caspase-8 was highly activated in Caco-2, whilst it was not detected in HT29 cells, and finally, low differentiation was found in the Caco-2 cell line.

The differences in the mechanisms proposed in Caco-2 and HT29 cells cannot be attributed exclusively to the p53 status, since this protein is expressed in HT29 cells in muted and inactivated form [25]. Moreover, caspase-8 activation in HT29 cells have been described when apoptosis is induced by the topoisomerase I inhibitor, camptothecin [1]. We report here on our further investigations into the apoptotic mechanism of maslinic acid in the Caco-2 cell line.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), bisbenzimide (Hoechst 33258), low-melting-point agarose (LMP), high-melting-point agarose (HMP), phosphate-buffered saline (PBS) and propidium iodide (PI) all came from Sigma, St. Louis, MO, USA; foetal calf serum (FCS) and penicillin/streptomycin were from Gibco-BRL, Eggenstein, Germany; 3-(4,5-dimethylthiazol-2yl)-2,5,-diphenyltetrazolium bromide (MTT, Sigma, St. Louis, MO, USA); primary antibodies, rabbit polyclonal anti-caspase-3 and anti-caspase-9 and mouse monoclonal anti-JNK, were from Cell Signalling Technology, Danvers, USA; rabbit polyclonal anti-caspase-8 and anti-Bid were from BD Biosciences, Erembodegem, Belgium; rabbit polyclonal anti-Bax, mouse monoclonal anti-Bcl-2, and secondary antibodies anti-rabbit and anti-mouse were from Santa Cruz Biotechnology, Santa Cruz, California, USA; culture flasks and well-plates were from Techno Plastic Products, Trasadingen, Switzerland, and DAPI (4′,6-diamidino-2-phenylindole) from Molecular Probes, Invitrogene, Eugene, OR, USA. All reagents were of analytical grade.

2.2. Drugs

Maslinic acid, a natural hydroxyl pentacyclic derivate from the skin of the olive (*Olea europaea* L.) (Fig. 1), was obtained from olive pomace using the method described by Garcia-Granados et al. [26]. Its molecular weight is 472.7 g/mol. The extract used was a white powder composed of 98% maslinic acid and 2% oleanolic acid, which is stable when stored at 4 °C. It was dissolved before use at 10 mg/mL in a 25% DMSO and 75% PBS solution. A stock solution was frozen and stored at −20 °C. Prior to experiments the solution was diluted in cell-culture medium (see below). All experiments were conducted at $IC_{50} = 39.7 \pm 0.4$ μg/mL and $IC_{80} = 56.8 \pm 0.1$ μg/mL, the maslinic-acid concentrations required for 50% and 80%