Prostaglandins & other Lipid Mediators 91 (2010) 30–37

Contents lists available at ScienceDirect

Prostaglandins and Other Lipid Mediators

Prostaglandin A_2 activates intrinsic apoptotic pathway by direct interaction with mitochondria in HL-60 cells

Sun-Young Lee[a,1], Ji-Hyun Ahn[b,1], Kyoung Won Ko[a], Jaetaek Kim[b], Seong Whan Jeong[a], In-Kyung Kim[a], Jin Kim[c], Ho-Shik Kim[a,*]

[a] Department of Biochemistry, College of Medicine, The Catholic University of Korea, Seoul 137-701, Republic of Korea
[b] Department of Internal Medicine, College of Medicine, Chung-Ang University, Seoul 156-755, Republic of Korea
[c] Department of Anatomy, College of Medicine, The Catholic University of Korea, Seoul 137-701, Republic of Korea

ARTICLE INFO

Article history:
Received 13 March 2009
Received in revised form
10 December 2009
Accepted 18 December 2009
Available online 5 January 2010

Keywords:
Apoptosis
Caspase
Cytochrome c
Mitochondria
Prostaglandin A_2

ABSTRACT

HL-60 cells treated by prostaglandin (PG) A_2 showed characteristics of apoptosis such as accumulation of hypodiploid and annexin V positive cells, condensed and fragmented nuclei, cytochrome c (Cyt C) release from mitochondria and activation of caspase-1, -2, -3, -7 and -9. PGA_2-induced cell death was rescued by inhibitors of caspase-9 and -3, but PGA_2-induced Cyt C release was not prevented by caspase inhibitors. During Cyt C release by PGA_2, mitochondrial transmembrane potential was maintained and mitochondrial permeability transition pore was not formed. In addition, anti-apoptotic BCL-2 family proteins like BCL-2 and BCL-XL, and ROS scavengers including ascorbic acid and 2,2,6,6-tetramethyl-1-piperidinyloxy were not able to inhibit Cyt C release as well as apoptosis by PGA_2. Finally, it was shown that PGA_2-induced Cyt C release *in vitro* from purified mitochondria in the absence of cytosolic components. Furthermore, thiol-containing compounds such as N-acetylcysteine, L-cysteine and monothioglycerol prevented Cyt C release, and hence induction of apoptosis. Taken together, these results suggest that PGA_2 activates intrinsic apoptotic pathway by directly stimulating mitochondrial outer membrane permeabilization to release Cyt C, in which thiol-reactivity of PGA_2 plays a pivotal role.

1. Introduction

Prostaglandins (PGs) of A and J series which are dehydration products of PGE_2 and PGD_2, respectively, contain α,β-unsaturated ketone moieties in their cyclopentane ring, and thus called cyclopentenone PGs [1]. Cyclopentenone PGs have various biological functions related to inflammation and cancer. At the site of inflammation, these PGs are produced from their precursors via non-enzymatic dehydration process and exert anti-inflammatory activity to resolve inflammation [2]. The anti-inflammatory effect of these PGs can be explained by chemical reactivity of their cyclopentenone structure. Because unsaturated ketone moieties of these PGs are susceptible to nucleophilic addition reaction [3], they can bind to critical cysteine residues of IκB kinase and inhibit its kinase activity, leading to prevention of inflammatory signal propagation [4].

Cyclopentenone PGs were also known to induce apoptosis in cancer cell lines [5,6]. Although the molecular mechanisms involved were not clarified in detail yet, it was suggested that these PGs induce apoptosis by regulating stress proteins such as p53, p21WAF1/CIP1, c-Myc and HSP-70 which are critical players in some models of cell death. For example, p53 protein was suggested to play a pivotal role in the activation of cell death by cyclopentenone PGs in SK-HEP1 hepatocellular carcinoma cells [7]. Meanwhile, in Hep3B cells harboring mutated p53, c-Myc and HSP-70 were shown to mediate and prevent cell death by cyclopentenone PGs, respectively [8]. It was also demonstrated that p21WAF1/CIP1 blocked the induction of cell death by PGA_2 in colon cancer cells [9].

In contrast, we reported that PGA_2 induced apoptosis and c-*myc* down-regulation in HL-60 cells, which were not prevented by cycloheximide suggesting the *de novo* protein synthesis-independent [10]. In addition, the apoptosis by PGA_2 was dependent on caspase-3 activity associated with cytochrome c (Cyt C) release. However, it was not determined, yet, whether the mitochondrial apoptotic pathway is indispensable and how Cyt C is released during PGA_2-induced apoptosis in HL-60 as well.

Abbreviations: AA, ascorbic acid; BKA, bongkrekic acid; CsA, cyclosporine A; Cyt C, cytochrome c; MAC, mitochondrial apoptosis-induced channel; MIM, mitochondrial inner membrane; MOM, mitochondrial outer membrane; MOMP, mitochondrial outer membrane permeabilization; MPTP, mitochondrial permeability transition pore; MTG, monothioglycerol; MTP, mitochondrial transmembrane potential; NAC, N-acetyl-L-cysteine; PG, prostaglandin; TEMPO, 2,2,6,6-tetramethyl-1-piperidinyloxy; VDAC, voltage-dependent anion channel.

* Corresponding author. Tel.: +82 2 590 1181; fax: +82 2 596 4435.
E-mail address: hoshik@catholic.ac.kr (H.-S. Kim).

[1] These authors contributed equally to this work.

1098-8823/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.prostaglandins.2009.12.003

To address these issues, we analyzed the effect of PGA_2 on mitochondrial transmembrane potential (MTP) and formation of mitochondrial permeability transition pore (MPTP), and identified the upstream caspase that activated caspase-3 to characterize the signal transduction pathway leading to PGA_2-induced apoptosis in HL-60 cells. The effects of anti-apoptotic BCL-2 family proteins like BCL-2 and BCL-XL, and ROS scavengers on apoptosis and Cyt C release by PGA_2 were also analyzed.

2. Materials and methods

2.1. Chemicals

PGA_2 was purchased from BIOMOL International Inc. (Plymouth Meeting, PA). Caspase inhibitors were obtained from Tocris Bioscience (Ellisville, MO). Protease inhibitor cocktail was from Roche (Pezberg, Germany). Unless otherwise specified, all chemicals were of molecular biology grade and from Sigma–Aldrich (St Louis, MO).

2.2. Cell culture

HL-60 cells were cultured with RPMI 1640 media supplemented with 20 mM Hepes (USB Corporation, Cleveland, OH), 25 mM sodium bicarbonate, 50 μg/ml gentamicin and 10% heat-inactivated FBS (Hyclone Laboratories Inc., Logan, UT) at 37 °C in a humidified 5% CO_2 incubator.

2.3. Apoptosis assessment

HL-60 cells treated by PGA_2 for 6 h were fixed in ice-cold 70% ethanol and stained with propidium iodide (PI, 0.2 mg/ml) followed by flow cytometric analysis (FACSCalibur, BD bioscience, San Jose, CA). Cell cycle distribution was analyzed by Cell Quest and Modfit software (BD bioscience). For determination of phosphatidylserine translocation in plasma membranes, HL-60 cells were stained with PI and annexin V using AnnexinV-FLOUS Staining Kit (Roche) following the manufacturer's instruction. Stained cells were analyzed as reported before [11]. To measure caspase activities, CaspACE™ Assay System, colorimetric (Promega, Madison, WI) was used following the manufacturer's instruction.

2.4. Immunoblot analysis

Cells treated as indicated were lysed with RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.25% Na-deoxycholate, 1% NP-40, 1 mM EDTA) containing protease inhibitor cocktail. Protein concentrations were measured by bicinchoninic acid method. Equal amount of proteins were separated by SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Millipore, Billerica, MA). After hybridization with primary and secondary antibodies, proteins were detected using enhanced chemiluminescence (ECL, GE Healthcare, UK). Mouse anti-caspase-3, mouse anti-caspase-2, and mouse anti-Cyt C were purchased from BD Bioscience. Rabbit anti-caspase-1, rabbit anti-caspase-9 and mouse anti-GAPDH were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-caspse-8 was from LABVISION Co. (Fremont, CA). Mouse anti-porin was from Calbiochem (San Diego, CA).

2.5. Subcellular fractionation

Cytosolic and mitochondrial fractions were separated following the previous report [12]. Briefly, cells treated as indicated were resuspended in ice-cold hypotonic buffer (10 mM Hepes, 10 mM $MgCl_2$, 42 mM KCl) containing protease inhibitor cocktail. Plasma membranes were ruptured by passing cells through a 30-gauge syringe. After centrifugation at 1000 rpm for 5 min 4 °C, the supernatant was collected and centrifuged at 13,000 rpm for 15 min 4 °C again. The clear fraction was saved as the cytosolic fraction and the pellet, heavy membrane fraction, was used as the mitochondrial fraction.

2.6. Assay of MTP and MPTP

The change of MTP and formation of MPTP during PGA_2-induced apoptosis was analyzed using MitoProbe™ JC-1 Assay Kit (Invitrogen, Carlsbad, CA) and MitoProbe™ Transition Pore Assay Kit (Invitrogen), respectively, following the manufacturer's instruction.

2.7. Overexpression of BCL-2 and BCL-XL

Human BCL-2 and BCL-XL expressing plasmids, pCAGGS/Flag-BCL-2 and pCAGGS/Flag-BCL-XL were kindly donated from Professor Jeong-Hwa Lee. To express BCL-2 and BCL-XL in HL-60 cells, these constructs were electro-transfected into HL-60 cells with 1270 V, 30 ms and 1 pulse using microporator (Digital-Bio, Suwon, Korea).

2.8. In vitro Cyt C release assay

The effect of PGA_2 on mitochondrial Cyt C release *in vitro* was assayed following the report [13]. Briefly describing, mitochondria were isolated from HL-60 cells and resuspended in mitochondria resuspension buffer (200 mM mannitol, 50 mM sucrose, 10 mM succinate, 5 mM potassium phosphate, pH 7.4, 10 mM Hepes-KOH, pH 7.0, 0.1% BSA) in the absence or presence of PGA_2. After indicated times, resuspended mitochondrial mixtures were then centrifuged at 13,000 rpm for 15 min 4 °C. Supernatants and pellets were separately collected and subjected to immunoblot analysis.

3. Results

3.1. PGA_2 induces typical apoptosis in HL-60 cells

PGA_2 induced sub-G0/G1 peak in cell cycle indicating DNA fragmentation in HL-60 cells at 6 h (Supplementary Fig. 1). The sub-G0/G1 peak level gradually increased according to PGA_2 concentrations up to 15 μg/ml, above which the peak level tended to slightly decrease (Supplementary Fig. 1). So, it can be assumed that PGA_2 induces cell death to the maximum extent at 15 μg/ml for 6 h, and thus 15 μg/ml of PGA_2 was used afterwards in this study.

To confirm the apoptosis by PGA_2 in HL-60 cells, morphological analyses were performed. As shown in Fig. 1A, HL-60 cells positive for annexin V indicating translocation of phosphatidylserine in the inner leaflet to outer leaflet of plasma membranes were augmented by PGA_2 treatment. Electron microscopic examination showed apoptotic characteristics such as condensed and fragmented nuclei, and vacuolar changes in PGA_2-treated HL-60 cells (Fig. 1B). Therefore, it can be suggested that PGA_2 induces typical apoptosis in HL-60 cells.

3.2. PGA_2-induced apoptosis is dependent on caspases of intrinsic apoptotic pathway

Although PGA_2-induced apoptosis was dependent on caspase-3 which seemed to be activated by caspase-9 in the previous report [10], the activation mechanism of caspase-3 by PGA_2 was not elucidated yet. To this end, we checked activation of all initiator caspases in this apoptosis. According to immunoblot analysis, initiator procaspase-1, -2, and -9, but not procaspase-8, as well