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Effect of 15-lipoxygenase metabolites, 15-(S)-HPETE and 15-(S)-HETE on chronic myelogenous leukemia cell line K-562: Reactive oxygen species (ROS) mediate caspase-dependent apoptosis

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ABSTRACT

Growth inhibitory effects of 15-lipoxygenase-1 [13-(S)-HPODE and 13-(S)-HODE] and 15-lipoxygenase-2 [15-(S)-HPETE and 15-(S)-HETE] (15-LOX-1 and LOX-2) metabolites and the underlying mechanisms were studied on chronic myeloid leukemia cell line (K-562). The hydroperoxy metabolites, 15-(S)-HPETE and 13-(S)-HPODE rapidly inhibited the growth of K-562 cells by 3 h with IC_{50} values, 10 and 15 μM, respectively. In contrast, the hydroxy metabolite of 15-LOX-2, 15-(S)-HETE, showed 50% inhibition only at 40 μM by 6 h and 13-(S)-HODE, hydroxy metabolite of 15-LOX-1, showed no significant effect up to 160 μM. The cells exposed to 10 μM of 15-(S)-HPETE and 40 μM of 15-(S)-HETE showed typical apoptotic features like release of cytochrome c, caspase-3 activation and PARP-1 (poly(ADP) ribose polymerase-1) cleavage. A flow cytometry based DCFH-DA analysis and inhibitory studies with DPI, a pharmacological inhibitor of NADPH oxidase, NAC (N-acetyl cysteine) and GSH revealed that NADPH oxidase-mediated generation of ROS is responsible for caspase-3 activation and subsequent induction of apoptosis in the K-562 cell line.

1. Introduction

Lipoxygenases (LOXs) are a group of closely related non-heme iron containing dioxygenases, which catalyze the addition of molecular oxygen into polyunsaturated fatty acids (PUFAs) containing *cis*, *cis* 1-4 pentadiene structures to yield their hydroperoxy derivatives. LOXs are classified depending on their site of oxygen insertion on arachidonic acid (AA) into 5-, 8-, 12- and 15-LOXs [1] and according to the positional specificity of arachidonate oxygenation into *S* and *R* isoforms [2]. 15-LOX has two isoforms, 15-LOX-1 and 15-LOX-2. Linoleate (LA) is the preferred substrate for 15-LOX which is metabolized to 13-(S)-HPODE that eventually gets reduced to 13-(S)-HODE. 15-LOX-2, on the other hand, oxygenates mainly AA to 15-(S)-HPETE that is reduced to 15-(S)-HETE (hereafter, 13-(S)-HPODE and 13-(S)-HODE are mentioned as 15-LOX-1

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Abbreviations: AA, arachidonic acid; DAPI, 4′,6-diamidino-2-phenylindole; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DPI, diphenylene iodonium; GSH, reduced glutathione; 13-(S)-HODE, 13-(S)-hydroxyoctadecadienoic acid; 13-(S)-HPODE, 13-(S)-hydroperoxyoctadecadienoic acid; 15-(S)-HETE, 15-(S)-hydroxyeicosatetraenoic acid; 15-(S)-HPETE, 15-(S)-hydroperoxyeicosatetraenoic acid; LA, linoleic acid; 15-LOX, 15-lipoxygenase; NAC, N-acetyl cysteine

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metabolites and 15-(S)-HPETE and 15-(S)-HETE as 15-LOX-2 metabolites according to their substrate preferentiality to make a distinction, though these metabolites are not exclusively produced by the specified enzyme).

The role of various LOXs in regulating carcinogenesis was very well documented. 5-LOX, 8-LOX and 12-LOX were shown to have a procarcinogenic role, whereas 15-LOX was shown to be anti-carcinogenic [3]. Among the two isoforms, the role of 15-LOX-2 as anti-carcinogenic agent is established [4], the role of 15-LOX-1 in controlling carcinogenesis is still unclear. It was shown that 15-LOX-1 is expressed at higher levels in colorectal carcinoma and in tumors associated with prostate [5,6]. However, Shureiqi et al. reported the higher expression of 15-LOX-1 in normal tissues compared to the tumors [7]. Non-steroidal anti-inflammatory drugs (NSAIDs) and histone deacetylase (HDAC) inhibitors were shown to induce the expression of 15-LOX-1 in colorectal carcinomas and this up-regulation of 15-LOX-1 is critical for subsequent induction of apoptosis [8–10]. The expression of 15-LOX-2 and the production of its metabolite 15-(S)-HETE were found to be reduced in prostate carcinomas [11]. We have earlier shown that 15-LOX-2 metabolites [15-(S)-HPETE and 15-(S)-HETE] exert differential effects on BHK-21 cell proliferation. While 15-(S)-HPETE inhibited the proliferation of these cells potently, 15-(S)-HETE at the same concentration did not show any significant effect [12]. Recently, Maccarrone et al. demonstrated that, various LOX metabolites induce apoptosis in neuronal and leukemic cell types *in vitro* [13]. Although it is clear that 15-LOXs play a role in regulating carcinogenesis and induce apoptosis, the molecular mechanisms mediating these effects are still unknown. This study is designed to understand the molecular mechanisms mediating 15-LOX metabolite-induced apoptosis in human chronic myeloid leukemia cell line (K-562). Apoptosis induced by 15-LOX metabolites in K-562 cell line was found to be very rapid and hydroperoxy metabolites [15-(S)-HPETE and 13-(S)-HPODE] induce cell death more effectively compared to the corresponding hydroxy metabolites [15-(S)-HETE and 13-(S)-HODE]. The mechanistic aspects of 15-(S)-H(P)ETEs suggest that these metabolites activate the intrinsic cell death pathway through cytochrome *c* release and caspase-3 activation. These effects were found to be mediated by reactive oxygen species (ROS) generated through the activation of NADPH oxidase.

2. Materials and methods

2.1. Materials

Cell lines used in this study, K-562 (chronic myeloid leukemia), U-937 (human histiocytic leukemia), HL-60 (human promyelocytic leukemia) and Jurkat (human peripheral blood T cell leukemia) were obtained from the National Center for Cell Science, India. Phosphate buffered saline (PBS), RPMI medium and fetal bovine serum (FBS) were purchased from GIBCO Ltd. (BRL Life Technologies, Inc., USA). All the fine chemicals used in the study were procured from Sigma Chemical Co., USA. Nitrocellulose membranes and the ECL kit were from Amersham Biosciences, USA. Mouse monoclonal antibodies against cytochrome *c* were from Santa Cruz Biotechnology, Inc., USA. Polyclonal antibodies of poly(ADP) ribose polymerase (PARP) were from R&D systems, USA and polyclonal antibodies for caspase-3 were purchased from Cell Signaling Technology, Inc., USA. DCFH-DA was purchased from Molecular Probes, USA. Fluorogenic caspase-3 substrate—Ac-DEVD-AFC, caspase-3 inhibitor—Ac-DEVD-CHO and cell permeable caspase inhibitor—Z-VAD-FMK were from BD Biosciences, USA. Diphenylene iodonium (DPI) were procured from Calbiochem (EMD Biosciences, USA). The LOX metabolites used in this study were prepared through enzyme catalysis reactions by incubating arachidonic acid or linoleic acid with commercially procured Soybean LOX according to the procedures described elsewhere [14,15]. These metabolites were purified on straight phase HPLC (Shimadzu model equipped with SPD 6AV and CR4A chromatopac) using CLC-SIL (25 cm × 0.4 cm) column and they are confirmed by LC–MS analysis by Flow Injection Analysis in the negative ion mode employing electron spray ionization (ESI) on 1100 Series LC-MSD, Agilent Technologies.

2.2. Cell culture and treatment

The leukemic cell lines (K-562, U-937, HL-60 and Jurkat) were grown in suspension in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine in a humidified atmosphere with 5% CO_2 at 37 °C. Exponentially growing K-562 cells resuspended in fresh culture medium with 1% FBS were treated with HPLC purified 15-LOX metabolites [15-(S)-HPETE, 15-(S)-HETE, 13-(S)-HPODE and 13-(S)-HODE] dissolved in ethanol. The final concentration of the vehicle never exceeded 0.1%. Wherever the inhibitors were used in this study, toxic-profiling in terms of dosage and time was carried out and the non-toxic and efficient doses were used for studies.

2.3. Cell viability and cytotoxicity

15-LOX metabolite-induced growth inhibitory effects were assessed using the MTT assay as described [16]. For the MTT assay, 5×10^3 exponentially growing cells were plated in 100 μl of the growth medium in the presence or absence of 1–20 μM of hydroperoxy 15-LOX metabolites [15-(S)-HPETE and 13-(S)-HPODE] and 10–160 μM of hydroxy 15-LOX metabolites [15-(S)-HETE and 13-(S)-HODE] in 96 well plates and cultured at 37 °C in 5% CO_2 for 3–24 h. The cells were then incubated with 20 μl of MTT (5 mg/ml) at 37 °C for 4 h. After dissolving the crystals in a triplex solution containing 12% SDS, 5% isobutanol and 12 mM HCl, the plates were read in a microtiter plate reader at 570 nm. Each concentration was tested in three independent experiments run in four replicates. Standard errors of means were calculated and reported as %growth versus control. The concentration of the compound that inhibited cell growth by 50% (IC_{50}) was determined from cell survival plots.

2.4. DNA fragmentation assay

K-562 cells were treated with either 15-(S)-HPETE (5 and 10 μM for 3 h) or 15-(S)-HETE (20 and 40 μM for 6 h) for indicated time periods and used for the isolation of the DNA. DNA laddering was detected by isolating fragmented DNA using the SDS/