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The autotaxin–LPA_2 GPCR axis is modulated by γ-irradiation and facilitates DNA damage repair

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ABSTRACT

In this study we characterized the effects of radiation injury on the expression and function of the autotaxin (ATX)–LPA_2 GPCR axis. In IEC-6 crypt cells and jejunum enteroids quantitative RT-PCR showed a time- and dose-dependent upregulation of lpa2 in response to γ-irradiation that was abolished by mutation of the NF-κB site in the lpa2 promoter or by inhibition of ATM/ATR kinases with CGK-733, suggesting that lpa2 is a DNA damage response gene upregulated by ATM via NF-κB. The resolution kinetics of the DNA damage marker γ-H2AX in LPA-treated IEC-6 cells exposed to γ-irradiation was accelerated compared to vehicle, whereas pharmacological inhibition of LPA_2 delayed the resolution of γ-H2AX. In LPA_2-reconstituted MEF cells lacking $LPA_{1\&3}$ the levels of γ-H2AX decreased rapidly, whereas in Vector MEF were high and remained sustained. Inhibition of ERK1&2 or PI3K/AKT signaling axis by pertussis toxin or the $C_{311}A/C_{314}A/L_{351}A$ mutation in the C-terminus of LPA_2 abrogated the effect of LPA on DNA repair. LPA_2 transcripts in $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ enriched for bone marrow stem cells were 27- and 5-fold higher than in common myeloid or lymphoid progenitors, respectively. Furthermore, after irradiation higher residual γ-H2AX levels were detected in the bone marrow or jejunum of irradiated LPA_2-KO mice compared to WT mice. We found that γ-irradiation increases plasma ATX activity and LPA level that is in part due to the previously established radiation-induced upregulation of TNFα. These findings identify ATX and LPA_2 as radiation-regulated genes that appear to play a physiological role in DNA repair.

1. Introduction

Radiation induced DNA double-strand breaks (DSB) are detected by ataxia telangiectasia mutated kinase (ATM), which induces the activation of cell-cycle check points to allow DNA damage repair, cell survival, and stress response pathways [1,2]. The decision between survival and apoptosis of a cell exposed to a genotoxic insult depends on the stress signals and also on inputs from the cell microenvironment [3]. Radiation protectors are compounds that prevent radiation injury when applied before exposure whereas, radiation mitigators are compounds that can be administered after radiation exposure to attenuate injury. Mechanistically, radiation mitigator compounds are aimed at enhancing those innate signaling pathways which lead to DNA damage repair (DDR), inhibition of apoptosis, and enhancement of cell survival.

The lysophosphatidic acid G protein coupled receptor subtype 2 (LPA_2 GPCR) is a member of endothelial differentiation gene (EDG) family showing more than 80% homology to LPA_1 and LPA_3 [4,5]. The natural ligand of LPA_2 is lysophosphatidic acid (LPA) a growth factor like molecule abundantly present in biological fluids. LPA is produced primarily from lysophosphatidylcholine (LPC) by the lysophospholipase D enzyme designated autotaxin (ATX) [6–8]. LPA_2 is the most sensitive GPCR to LPA stimulation with an EC_{50} of ~1.4 nM. LPA_2 is expressed in a wide range of cell types including hematopoietic [9,10] and embryonic stem cells [11]. LPA_2 was shown to prevent and also to mitigate apoptosis elicited by serum withdrawal, or genotoxic stressors including chemotherapeutics, and radiation-induced DNA damage [12–14]. LPA_2 is overexpressed in different tumors thereby conferring resistance to radiation- and chemotherapy [15–17].

Our group has synthesized LPA_2-specific agonist compounds with the ultimate goal of developing drugs that can prevent and/or mitigate radiation-injury resulting from exposure to high levels of radiation

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that elicit the hematopoietic (HE) and the gastrointestinal (GI) acute radiation syndromes (ARS) [13,18–22]. Stimulation of LPA_2 among others leads to activation of MAPK/ERK, PI3K/AKT and NF-κB signaling pathways resulting in enhanced cell survival, proliferation, and migration that are important events in radiation injury repair [4,14,23–25].

The objective of the present study was to characterize the effects of radiation injury on expression and function of the ATX–LPA_2 axis in cultured cells and in mice exposed to total body γ-irradiation (TBI) from a ^{137}Cs source. Specifically, we examined the transcriptional regulation of *lpa2* in IEC-6 crypt-derived cells and in crypts isolated from the small intestine of mice in response to ionizing radiation and evaluated the impact of such regulation on the DNA damage response (DDR). Radiation-induced upregulation of *lpa2* was mediated by ATM-dependent activation of NF-κB transcription. We found that LPA_2 was dose- and time-dependently upregulated in response to γ-radiation. LPA_2 expression and activation augmented the repair of DSB monitored by the resolution of phosphorylated histone 2AX (γ-H2AX) in vitro and in vivo. In addition, we evaluated the effect of radiation on LPA production via ATX in blood, white adipose tissue (WAT), and the liver of wild type (WT) mice. We found that mice exposed to 6 Gy TBI γ-irradiation ATX activity increased within 4 h, resulting in an increase in plasma LPA levels that favors a radiation-induced acute regenerative tissue response. We also found that generation of TNFα accompanying radiation exposure upregulated ATX expression in IEC-6 cells. γ-H2AX resolution was delayed in LPA_2 knockout (KO) mice compared to WT C57BL/6 mice. These results indicate that ATX and LPA_2 are regulated by γ-irradiation and play a role in the endogenous DNA damage response and repair pathways.

2. Methods

2.1. Materials

LPA 18:1, 1-heptadecanoyl-LPC (17:0), and 1-heptadecanoyl-LPA (17:0) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). A stock solution of LPA (2 mM) was prepared with equimolar complex with charcoal-stripped, fatty acid-free bovine serum albumin (BSA, Sigma-Aldrich; St. Louis, MO, USA) in phosphate-buffered saline (PBS). The LPA_2-specific antagonist Amgen compound 35 reported by Beck et al. and the LPA_2-specific agonist compound 11 d (designated radioprotectin 1, RP-1) were synthesized as described previously [21,26]. The FS-3 ATX substrate was from Echelon Biosciences (Salt Lake City, UT, USA). LY294002 was purchased from Cell Signaling Technology (Danvers, MA, USA), U0126 from Promega (Madison, WI, USA), pertussis toxin from LIST Biological Laboratories, Inc. (Campbell, CA, USA), CGK-733 from Calbiochem (San Diego, CA, USA).

2.2. Culture and irradiation of IEC-6 cells

IEC-6 non-transformed crypt-derived rat embryonic intestinal epithelial cells at passage 17 were plated in 6-well plates at a density of 10^5 cells/well in 1.5 ml complete culture medium as described previously [18]. The next day, cells were irradiated with 5, 10, or 15 Gy γ-irradiation from a ^{137}Cs source at a dose rate of 4.4 Gy/min. After irradiation, the culture medium was replaced with fresh complete culture medium. Cells were harvested for RNA isolation using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) at 12, 24, or 36 h after irradiation.

2.3. Generation of mouse embryonic fibroblast (MEF) cells from $LPA_1 \times LPA_2$ double KO mice reconstituted with the human LPA_2 ortholog

Very few cell lines lack LPA GPCR. To generate a radiation-sensitive cell platform that lacks the EDG family LPA GPCR we generated a MEF cell line from $LPA_1 \times LPA_2$ double KO mice that also lack endogenous LPA_3 expression and transduced them with lentiviral constructs of the human LPA_2 ortholog (LPA_2 MEF) or an empty vector (Vector MEF) [24]. We used LPA_2 and Vector MEF cells to evaluate the effects of LPA_2 activation on DDR as we described [19,22].

2.4. Isolation and culture of intestinal crypts

The animal protocols used in the present study were reviewed and approved by the Institutional Animal Use and Care Committee of the University of Tennessee Health Science Center Memphis. The small intestine was removed from euthanized 8 week-old C57BL/6 mice and washed with cold PBS. After opening the small intestine, villi were scraped off and discarded before the tissue was cut into small pieces. After several rinses with cold PBS, the tissue was incubated at 4 °C with 2 mM EDTA in PBS for 30 min and passed through a cell strainer with 70 μm pore size (BD Biosciences, San Jose, CA, USA). Subsequently, the crypts that passed through the strainer were collected by centrifugation at 400 ×*g* for 5 min and washed with enteroid medium (Gibco, Grand Island, NY, USA) including 1 × Glutamax, 20 units/ml penicillin, 20 μg/ml streptomycin, 10 mM HEPES (all supplements from Gibco) without growth factors and centrifuged at 400 ×*g* for 2 min. After discarding the supernatant, the isolated crypts were resuspended with Matrigel (BD Biosciences) and plated in 24-well-plates. Enteroid medium (500 μl) supplemented with 1 × B27 (Gibco) 1 × N2 (Gibco), 1 mM N-acetyl cysteine (Sigma-Aldrich), 100 ng/ml Noggin (PeproTech, Rocky Hill, NJ, USA), 1 μg/ml R-spondin-1 (R&D Systems, Minneapolis, MN, USA) and 50 ng/ml EGF (R&D Systems) were added to the crypts in the hardened Matrigel. The enteroids were incubated at 37 °C in the presence of 5% CO_2–95% air atmosphere for 24 h and irradiated with 4 Gy at a dose rate of ~0.85 Gy/min.

2.5. Quantitative RT-PCR (qPCR)

Total RNA (1.5 μg) was used for the synthesis of cDNA using the ThermoScript RT-PCR system for first strand synthesis (Invitrogen — Life Technologies, Grand Island, NY, USA). qPCR reactions were performed using cDNA mix (cDNA corresponding to 35 ng RNA) with 300 nmol of the primers in a final volume of 25 μl of 2 × concentrated RT2 Real-Time SYBR Green/ROX master mix (Qiagen) in an Applied Biosystems 7300 Real-Time PCR instrument (Norwalk, CT, USA). The cycle parameters were: 50 °C for 2 min, one denaturation step at 95 °C for 10 min and 40 cycles of denaturation at 95 °C for 10 s followed by annealing and elongation at 60 °C. Relative gene expression of each transcript was normalized to GAPDH using the ΔΔCt method. Primer sequences for GAPDH were: forward: 5′-CTGCACCACCAACTGCTTAG-3′, reverse: 5-GGGCCATCCACAGTCTTCT-3, and for LPA_2: forward 5-CCAG CCTGCTTGTCTTCCTA-3, and reverse: 5-GTGTCCAGCACACCACAAAT-3.

For ATX gene product qPCR measurements 0.1×10^6 IEC-6 cells were plated per well of a 6-well plate in complete growth media. The following day, cells were serum-starved for 24 h prior to stimulation with 10 ng/ml of rat TNFα (R&D Systems) for 15 min, 3 h and 6 h. The ATX forward primer was 5′-ATTACAGCCACCAAGCAAGG-3′ and the reverse 5′-GGCAGAGAAAGCCACTGAAG-3′.

2.6. Construction and assay of a luciferase reporter plasmid containing the human LPA_2 promoter

The human LPA_2 promoter sequence between base pairs −965/+139 was amplified from 50 ng of human genomic DNA with Kod hot start polymerase (Novagen, Madison, WI, USA) with the primers: LPA_2-forward: 5-GTAGAGACGGGGTTTCAGCATG-3 and LPA_2-reverse: TATAAGCTTCTGGGCCTCCAGTCACGCC, with an added HindIII restriction site to the reverse primer. The PCR product was cloned into pGL 4.10(luc2) (Promega) between the EcoRV and HindIII restriction sites. Site-directed mutagenesis of the NF-κB sites was done with the Quick Change kit (Stratagene, La Jolla, CA, USA). The consensus binding site: GGGGCTCCCC was changed into GTGATTCTCC with the forward primer 5-GCCGTGGAGGCGTGATTCTCCCAGGTGGCGGG-3 and reverse primer