European Journal of Pharmacology 823 (2018) 58–64

Contents lists available at ScienceDirect

European Journal of Pharmacology

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

Full length article

Aspirin induces Beclin-1-dependent autophagy of human hepatocellular carcinoma cell

Zhenjun Huang[a,b,1], Weilun Fang[a,1], Weihua Liu[a], Li Wang[a], Bin Liu[a], Shiming Liu[a], Shaojun Liu[a,*]

[a] Guangzhou Institute of Cardiovascular Disease, The Second Affiliated Hospital of Guangzhou Medical University, Guangzhou, Guangdong 510260, PR China
[b] Experiment Center, The Fifth Affiliated Hospital of Guangzhou Medical University, Guangzhou, Guangdong 510700, PR China

ARTICLE INFO

Keywords:
aspirin
autophagy
hepatocellular carcinoma cells
Beclin-1
AMP-activated protein kinase
mammalian target of rapamycin

ABSTRACT

Aspirin not only reduces the incidence of hepatocellular carcinoma (HCC) but also plays a synergistic role with chemotherapy for HCC treatment. However, the underlying mechanisms remain incompletely elucidated. Given that autophagy triggers cancer cell death, the present study examined the autophagic effect of aspirin on HCC cells. Results showed that aspirin increased LC3II/LC3I ratio, decreased p62 expression, and enhanced autophagic flux (autophagosome and autolysosome puncta) in Hep3B, HepG2, or SMMC-7721 cells, reflecting the autophagy of HCC cells. The autophagic effects of aspirin depended on Beclin-1 expression. Aspirin disrupted the interaction between Bcl-2 and Beclin-1. In addition to activating the AMP-activated protein kinase, c-Jun N-terminal kinase, and Glycogen synthase kinase-3 pathways, aspirin inhibited the mammalian-target-of rapamycin-S6K1/4E-BP1 signaling. Aspirin induced autophagy of HCC cell. This study contributes to understanding the chemoprotective and inhibitory effects of aspirin on HCC development.

1. Introduction

Liver cancer, particularly hepatocellular carcinoma (HCC), is a common and dangerous malignancy. This disease is the fifth most frequently occurring cancer worldwide and the third most common cause of cancer mortality (Torre et al., 2015). Given the high morbidity and poor survival rate, effective therapies are urgently needed for HCC prevention.

As a widely used drug, aspirin significantly reduces the risk of cardiovascular events and death. In recent years, evidence for the antitumor effects of aspirin has emerged from cellular and animal experiments, epidemiological investigations, and randomized clinical trials. Research data suggest that aspirin works effectively in the primary prevention of various tumors, such as colon (Drew et al., 2016; Zong et al., 2016), breast (Moris et al., 2016), lung (Mc Menamin et al., 2015), and prostate tumors (Skriver et al., 2016). More recently, the US Preventive Services TaskForce recommended initiating low-dose aspirin use for the primary prevention of cardiovascular diseases and colorectal cancer (Bibbins-Domingo, 2016). This statement considers the growing evidence of the chemopreventive effects of low-dose aspirin against colorectal and other cancers. Notably, aspirin was found to reduce the incidence of HCC (Petrick et al., 2015; Sitia et al., 2013; Liu et al., 2017), and to also play a synergistic role with chemotherapy in treatment of this type of cancer (Li et al., 2016a; Li et al., 2013).

The mechanisms underlying the anticancer effects of aspirin involve both cyclooxygenase (COX) inhibition and COX-independent properties (Drew et al., 2016; Zong et al., 2016). COX consists of two major isoforms, COX-1 and COX-2. COX-1 is constitutively expressed in most tissues, whereas COX-2 is induced in several tissues in response to specific stimuli, such as pro-inflammatory cytokines and growth factors. Aspirin is a classic pharmacological agent that targets COX-1 at low or regular doses and COX-2 at regular doses. Given that the COX-2 pathway plays a key role in cellular proliferation and cancer migration and invasiveness, the anticancer effect of aspirin is traditionally assumed to rely on COX-2 inhibition. However, some reports suggested that the clinical benefit of aspirin depends on other factors and not on COX-2 expression. In COX-2-negative colon cancer cells, aspirin increases mismatch repair proteins and mediates the subsequent growth inhibition and apoptosis (Goel et al., 2003). The *Nurses' Health Study* reported that the survival benefit of aspirin is not associated with COX-2 expression in patients with breast cancer (Holmes et al., 2011). Several non-COX pathway anticancer mechanisms of aspirin have been confirmed *in vitro* studies. For example, aspirin combines with IκB (Inhibitor of Nuclear Factor-κB (NF-κB)) and suppresses the

* Corresponding author.
E-mail address: shaojunliu@gzhmu.edu.cn (S. Liu).
[1] Contributed equally to this work.

https://doi.org/10.1016/j.ejphar.2018.01.031
Received 28 April 2017; Received in revised form 20 January 2018; Accepted 23 January 2018

phosphorylation level of this factor, inhibiting NF-κB activity (Liao et al., 2015). This effect of aspirin may reduce the risk of Hodgkin lymphoma (Chang et al., 2009). In other tumors, aspirin prevents tumor growth by stabilizing the DNA repair system (Wood et al., 2007) and reducing hydroxyl radical formation (Chen et al., 2009). Aspirin also directly induces apoptosis of tumor cells through inhibition of Bcl-2 and VEGF (vascular endothelial growth factor), and upregulation of Bax and caspase-3 (Ding et al., 2014; Li et al., 2016b). Aspirin also triggers the death of cancer cells through regulation of Wnt/β-catenin (Bos et al., 2006), TRAIL (Pennarun et al., 2013), or AMP-activated protein kinase (AMPK) (Gao et al., 2016; King et al., 2015) signaling.

Therefore, the chemopreventive or chemotherapeutic function of aspirin possibly transpires via both COX and non-COX pathways (Drew et al., 2016; Zong et al., 2016). Recently, non-COX pathway mechanisms drew the attention of researchers. Interestingly, aspirin features an autophagic effect on colorectal cancer cells through inhibition of mammalian target of rapamycin (mTOR) signaling and activation of AMPK pathway (Din et al., 2012). The current study examined the autophagic role of aspirin on human HCC cells. The possible mechanism of action was also determined.

2. Materials and Methods

2.1. Reagents

Aspirin, rapamycin, and bafilomycin A1 were purchased from Biological Engineering Company (Shanghai). Lipofectamine 3000 Transfection Reagent Kit was obtained from Life Technologies. Antibody against LC3B (#L7543) and Flag (#F3165) were purchased from Sigma. Antibodies against p62 (#5114), Caspase-3 (#9662), cleaved Caspase-3 (#9661), Beclin-1 (#3495), pThr172-AMPK (#2535), pSer79-ACC (#3661), pSer65-4EBP1 (#9451), pThr389-S6K1 (#9234), pThr183/Tyr183-JNK (#9251), pSer73-c-Jun (#3270), pSer70-Bcl-2 (#2827), pThr308-AKT (#9275), pSer473-AKT (#9271), and pSer21/9-GSK3 (#9331) were purchased from Cell Signaling Technology. The antibody against proliferating cell nuclear antigen (PCNA) originated from Abcam (#ab92552). V5-Probe antibody (sc-83849) and β-Tubulin mouse anti-human monoclonal antibody (#M20005) were acquired from Santa Cruz Biotechnology and Abmart respectively. HRP-labeled anti-rabbit and anti-mouse IgG were purchased from Jackson ImmunoResearch.

2.2. Cell culture and treatment

The human hepatocellular carcinoma Hep3B, HepG2, and SMMC-7721 cells, and human embryonic kidney 293 (HEK293) cells were purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). HCC cells were maintained at 37 °C in a humidified condition of 95% air and 5% CO_2 in DMEM media supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and 1% penicillin-streptomycin. Hep3B cells were treated without or with aspirin (0.63–5 mM) or rapamycin (100 nM) for 16 h. Alternatively, the cells were simulated using 5 mM aspirin for the indicated time.

2.3. Cell viability assay

The Hep3B cells were incubated in 96-well plates at a density of 5×10^3 cells at a volume of 100 μl for 24 h before aspirin treatment. The Hep3B cells were treated with or without the indicated concentrations of aspirin, or combined with 100 nM rapamycin for 16 h, and then incubated using Cell Counting Kit-8 (CCK-8) (Dojindo) for 2 h at 37 °C. Absorbance was measured at 450 nm by using a microplate reader.

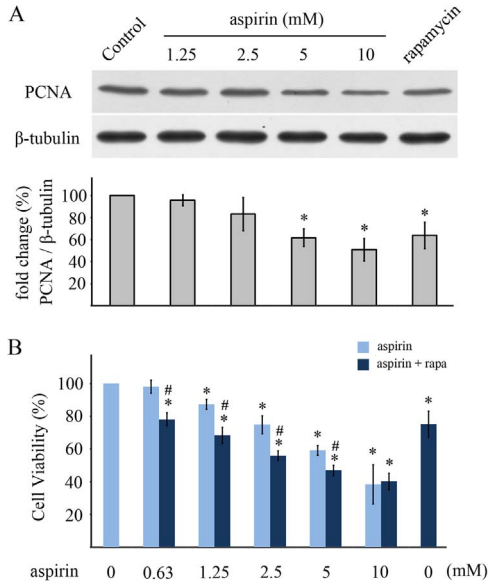

Fig. 1. Aspirin inhibits the proliferation of hepatocellular carcinoma Hep3B cells. (A) Aspirin decreased PCNA protein expression. Hep3B cells were incubated with aspirin at various concentrations (0, 1.25, 2.5, 5, and 10 mM) for 16 h. Cell lysates were subjected to immunoblotting assay with antibody against PCNA or β-tubulin. The upper panel shows the representative data. The lower panel presents the band quantification of three experiments. * $P < 0.05$, the aspirin treatment was compared with the control. (B) Cell viability assay under aspirin treatment alone or with rapamycin. Hep3B cells were treated with aspirin in the presence or absence of 100 nM rapamycin for 16 h and then subjected to CCK-8 assay. Notably, aspirin exerted a synergistic effect with rapamycin. Data are presented as the mean ± S.E.M. of 3–5 experiments. * $P < 0.05$, aspirin treatment or combination treatment with rapamycin was compared with the control. # $P < 0.05$, aspirin plus rapamycin compared with aspirin-alone treatment.

2.4. Immunoblotting analysis

The Hep3B cells were grown in six-well plates at approximately 60% to 70% confluence for 24 h and then treated with aspirin at indicated concentrations or at indicated times. The cells were harvested and subjected to immunoblotting analysis. Immunoblotting was performed according to our method described previously (Liu et al., 2016). Briefly, equal amounts of total proteins were separated using 8% or 12% SDS-PAGE, and transferred to PVDF membranes (GE Healthcare). The membranes were blocked in 5% nonfat milk (Bio-Rad Laboratories) for 1 h and then incubated with primary antibodies overnight at 4 °C. Goat anti-rabbit or anti-mouse IgG conjugated with HRP served as secondary antibodies. Finally, the immunoreactive bands were detected using the exposure method.

2.5. Constructs

Plasmid Flag-Bcl-2 (#18003) and pcDNA3-Beclin-1 (#21150) were acquired from Addgene. The obtained pcDNA3-Beclin-1 plasmid from Addgene contains a missense mutation (103Ala→Val) which was assessed by DNA sequencing. TaKaRa MutanBEST kit was first used to correct this mutated Beclin-1 coding sequence into WT (103Val→Ala). Then, WT Bcl-2 or WT Beclin-1 was subcloned into the pcDNA3.1-3×Flag (Ma and Liu et al., 2012) or pcDNA3.1-V5-his (Invitrogen) vector respectively. All constructs were confirmed by DNA sequencing.

2.6. Immunoprecipitation

Immunoprecipitation assays were performed as we described previously (Ma and Liu et al., 2012). The HEK293 cells were cotransfected with 1.5 μg pcDNA3.1-3Flag-Bcl2 and 1.5 μg pcDNA3.1-Beclin-1-V5 for 36 h by using Lipofectamine 3000 (Invitrogen) according to manufacturer's instructions. Cell lysates were centrifuged at 12,000 *g* for 15 min