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Aspirin augments the expression of Adenomatous Polyposis Coli protein by suppression of IKKβ

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ABSTRACT

Aspirin has been widely used as analgesic, antipyretic and anti-inflammatory medicine for long. In addition to these traditional effects, clinical studies suggest that aspirin can protect against cancer, but its mechanism has not been explored. To unveil it, we identified the proteins up- or down-regulated after incubation with aspirin by using proteomics analysis with Nano-flow LC/MALDI-TOF system. Interestingly, the analysis identified the protein of Adenomatous Polyposis Coli (APC) as one of the most up-regulated protein. APC regulates cell proliferation or angiogenesis, and is widely known as a tumor-suppressing gene which can cause colorectal cancer when it is mutated. Western blots confirmed this result, and real-time PCR indicated it is transcriptionally regulated. We further tried to elucidate the molecular mechanism with focusing on IKKβ. IKKβ is the essential kinase in activation of nuclear factor-kappa B (NF-κB), major transcriptional factors that regulate genes responsible for inflammation or immune response. Previous reports indicated that aspirin specifically inhibits IKKβ activity, and constitutively active form of IKKβ accelerates APC loss. We found that aspirin suppressed the expression of IKKβ, and the deletion of IKKβ by siRNA increases the expression of APC in HEK294 cells. Finally, we observed similar effects of aspirin in human umbilical vein endothelial cells. Taken together, these results reveal that aspirin up-regulates the expression of APC via the suppression of IKKβ. This can be a mechanism how aspirin prevents cancer at least in part, and a novel link between inflammatory NF-κB signaling and cancer.

1. Introduction

Aspirin has been widely used as analgesic, antipyretic and anti-inflammatory medicine for more than a century. In addition to these traditional effects, recent clinical studies revealed that aspirin has protecting effect against colorectal cancer [1] and several common cancers [2]. Although considerable heterogeneity exists within and between studies, the magnitude of the effect is remarkably consistent, despite differing dosing regimens, duration of use, study populations and geographical locations.

The pharmacological mechanism of aspirin has been intensively explored by researchers. Aspirin inhibits the cyclooxygenase (COX) enzymes COX-1 and COX-2, which synthesize inflammatory mediators like prostaglandins and thromboxanes [3]. Aspirin can also inhibit the transcriptional factors NF-κB pathway involved in the pathogenesis of the inflammatory response [4,5]. Yin et al. have reported that aspirin is the specific inhibitor of IKKβ, which is the essential kinase for NF-κB activation [4]. These reports can account for the classical analgesic effect of aspirin, but the mechanism for its pleiotropic effects of preventing cancer has not been fully explored.

In this study, we identified proteins that are up- or down-regulated by incubation with aspirin using proteomics analysis. Interestingly, treatment with aspirin remarkably increased the expression of APC protein. APC is tumor-suppressing gene and can cause colorectal cancer when it is mutated [6,7]. We further tried to explore the mechanism how aspirin regulates the expression of APC protein, and found IKKβ is the molecule bridging between aspirin and APC. These results could be a new mechanistic insight how aspirin prevents cancer, and reveal a new link between inflammatory NF-κB signaling and tumorigenesis.

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2. Materials and methods

2.1. Antibodies and reagents

The antibodies that were used included anti-IKKβ (Millipore, Billerica, MA, USA), anti-APC (Millipore), anti-APC (Abnova, Taipei City, Taiwan), a series of antibodies to anti-GAPDH, anti-mouse IgG HRP-linked antibody and anti-rabbit IgG HRP-linked antibody (Cell Signaling Technology, Boston, MA, USA (CST)); The reagents that were used acetylsalicylic acid (Aspirin), and cycloheximide (Sigma–Aldrich, St. Louis, MO, USA (Sigma)). Real-time reagents, TaqMan Gene Expression Master Mix and TaqMan Gene Expression Assay were purchased from Applied Biosystems (Foster City, CA, USA (ABI)). Small interfering RNA (siRNA) expression constructs for silencing IKKβ (pKD-IKKb-v3) and control siRNA (pKD-NegCon-v1) were purchased from Upstate (NY, USA). LIVE/DEAD viability/cytotoxicity kit (Thermo Scientific, Rockford, IL, USA).

2.2. Cell culture

The human embryonic kidney 293T cell line (HEK293T) was maintained in DMEM (Wako Pure Chemical Industries, Ltd., Osaka, Japan (Wako)), containing 10% FBS (Gibco, Foster City, CA, USA), 1% penicillin G/streptomycin (Sigma) at 37 °C under 5% CO_2. Human umbilical vein endothelial cells (HUVECs) were maintained in Endothelial cell Basal Medium-2 (EBM-2) (Lonza, Basel, Switzerland) at 37 °C under 5% CO_2.

2.3. Proteomics analysis

HEK293T cells were incubated with 10 mM aspirin for 48 h, and cell culture medium was subjected for analysis after freeze-dried. Sample was reduced with 45 mM of DTT (Wako), alkylated with 100 mM of iodoacetamide (Sigma), and digested with 2000 ng of trypsin (Promega, Madison, WI, USA). One-dimensional peptide fractionation was performed with a DiNa Direct Nano-flow LC/MALDI-TOF system (KYA Technologies, Japan) using a reverse-phase (RP) trap column (HiQ Sil C18-3, 0.8-mm i.d. × 3 mm) and an RP analytical column (HiQ Sil C18-3 Gradient, 0.15-mm i.d. × 50 mm). Peptides was subjected to the trap column and sequentially to the analytical column using a gradient of 0–50% solvent B in solvent A over 65 min [solvent A: 0.1% trifluoroacetic acid (TFA), 2% acetonitrile; solvent B: 0.1% TFA, 70% acetonitrile] and 50–100% solvent B for 15 min at a flow rate 200 nL/min. The RP column eluent was spotted onto a MALDI sample plate using a DiNa Direct Nano-flow LC/MALDI-TOF system (KYA tech.) and analyzed using a 4800 mass spectrometer (ABI). The peptides were fragmented under collision-induced dissociation conditions to give fragment ions that produce sequence information for the peptide. The software packages used for data acquisition and analysis were GPS explorer (ABI) and Mascot (Matrix science, Boston, MA, USA), respectively. Parameters of tolerance for the searches were set to 100 ppm for the MS and 0.2 Da for the MS/MS analyses, respectively.

2.4. Western blotting

Cells were cultured in the 6 cm-dishes and treated with aspirin or indomethacin for 24, 48, 72 h. Cells were scraped into PBS at 4 °C and pelleted (250 g for 5 min), and lysed by addition of the 1× Cell Lyses Buffer (CST) including 1 mM phenylmethylsulfonyl fluoride. After 20 min on ice, lysates were separated (13,000 g for 15 min), and protein content was determined using Dc Protein Assay Kit (Bio-Rad, Hercules, CA, USA). The protein in reducing sample buffer was heated at 95 °C for 5 min, and resolved in 4–20% mini-PROTEAN TGX Gel (Bio-Rad) for 35 min at 200 V. Then, the protein was transferred onto nitrocellulose membrane (Bio-Rad) at 100 V for 90 min. The membrane was pre-blotted in 5% milk-TBST buffer for 30 min, and blotted with first antibody for overnight and species-specific HRP-conjugated secondary antibody (CST) for 60 min. Detection of the bound antibody was performed using ECL reagent (GE Healthcare, Uppsala, Sweden).

To detect multiple signals from one membrane, the membrane was treated with a stripping buffer (Thermo Scientific) for 20 min at RT to remove the bound antibody. All the experiments were conducted for three or more times. Intensity of the immunoblot signal is analyzed quantitatively using ImageJ software.

2.5. Real-time PCR

Cells were scraped into PBS at 4 °C and pelleted (425 g for 3 min). Total RNA was extracted from cells using RNAqueous (Ambion, Foster City, CA, USA) according to the manufacturer's instructions. RNA (~2 μg) was converted to cDNA with the High Capacity RNA-to-cDNA kit (ABI). For quantitative real-time PCR analysis of APC, IKKβ and 18S rRNA reactions were performed using primer-probe sets (ABI). Using an ABI 7300 Real Time PCR System, PCR products were generated in cycle threshold (Ct) between the gene of interest and 18S rRNA using the equation $RQ = 2^{-\Delta\Delta Ct}$ and analyzed using SDS software version 1.4 (ABI).

3. Results

3.1. Proteomics analysis identified APC protein as one of the most up-regulated proteins by incubation with aspirin

To identify proteins affected by treatment with aspirin, HEK293T cells were incubated with aspirin and cell culture medium were subjected to proteomics analysis utilizing Nano-flow LC/MALDI-TOF system (Fig. 1A). Considering that all clinical reports indicated long-term use of high-dose aspirin is required for the anti-cancer effect, 48 h of incubation with high dose of 10 mM aspirin was applied. It was confirmed the treatment has no toxic effect on cells (Fig. 1B). Result indicated that 101 proteins were up-regulated after aspirin incubation, and APC protein was identified as one of the most up-regulated proteins.

3.2. Treatment with aspirin transcriptionally augments the expression of APC protein

To confirm the augmented expression of APC protein by aspirin, we checked the APC protein in HEK293T cells after incubation with aspirin. Cells were incubated with various concentrations of aspirin for 24 and 48 h, and cell lysate was subjected to Western blot. Interestingly, 24-h incubation with aspirin didn't increase the expression of APC protein, but 48-h incubation dramatically increased its expression in the dose of 10 mM (Fig. 2A), reproducing the result of proteomics analysis.

We also examined whether the change of its expression is transcriptionally or post-transcriptionally regulated. As shown in Fig. 2B, treatment with cycloheximide, inhibitor of protein synthesis, blocked the up-regulation of APC protein even after 48-h incubation of aspirin. Real-time PCR results indicated that treatment with aspirin increased mRNA for APC protein in a dose-dependent manner (Fig. 2C). These results indicate that aspirin transcriptionally up-regulates expression of APC.

3.3. Deletion of IKKβ augments the expression of APC protein

We tried to explore the molecular mechanism how aspirin augments the expression of APC protein. Yin et al. have reported

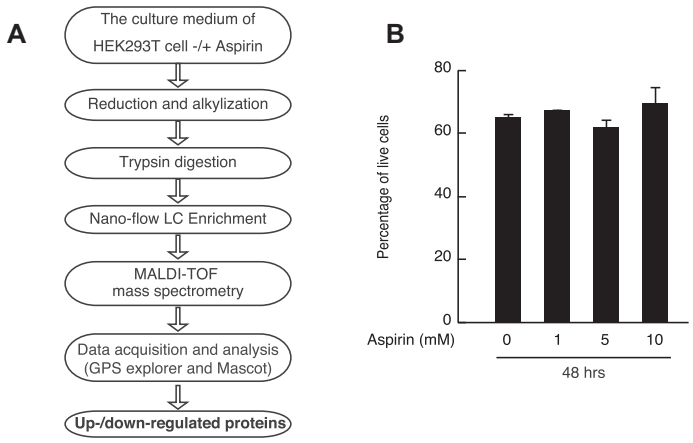

Fig. 1. Identification of up-/down-upregulated proteins by incubation with aspirin using proteomics analysis. (A) Proteomics analysis procedure. (B) The percentage of live HEK293T cells after incubated with various concentrations of aspirin for 48 was determined by LIVE/DEAD viability/cytotoxicity kit.

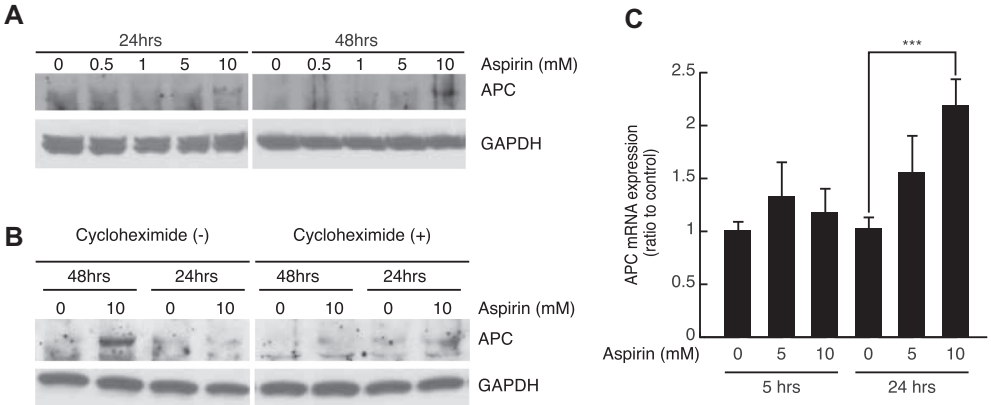

Fig. 2. Long-term incubation with aspirin transcriptionally increases the expression of APC protein. (A) APC immunoblots of HEK293T cells incubated with various concentrations of aspirin for 24 and 48 h. (B) APC immunoblots of HEK293T cells were incubated with 10 mM aspirin with or without cycloheximide (10 μg/mL) for 24 and 48 h. (C) Real-time PCR analysis of APC mRNA expression in HEK293T cells incubated with various concentrations of aspirin for 5 and 24 h. The data shown are from one representative experiment of the three that were performed. Error bar: ±SD. $^{***}P < 0.005$ in a two-sided, Student's *t*-test.

that aspirin inhibits the activity of IKKβ, which is the essential kinase in the NF-κB activation pathway [4]. Interestingly, Shaked et al. have reported that over-expression of constitutively active form of IKKβ accelerates APC loss [8]. Based on these reports, we checked the involvement of IKKβ in the up-regulation of APC protein by aspirin. We found that the deletion of IKKβ by siRNA dramatically increased the expression of APC protein (Fig. 3A) and aspirin suppresses the expression of IKKβ (Fig. 3B). These results implicated that the augmented expression of APC induced by aspirin is due to its inhibitory effect on IKKβ at least in part.

3.4. Aspirin increased the expression of APC and suppressed that of IKKβ in Human Umbilical Vein Endothelial Cells

Next we examined whether those phenomena can be observed in Human Umbilical Vein Endothelial Cells (HUVECs). As shown Fig. 4, aspirin increased the expression of APC protein and suppressed the expression of IKKβ in HUVEC, similarly in HEK293T cells.

4. Discussion

Several lines of clinical studies have shown that aspirin has pleiotropic effect of preventing cancer, but its mechanism has

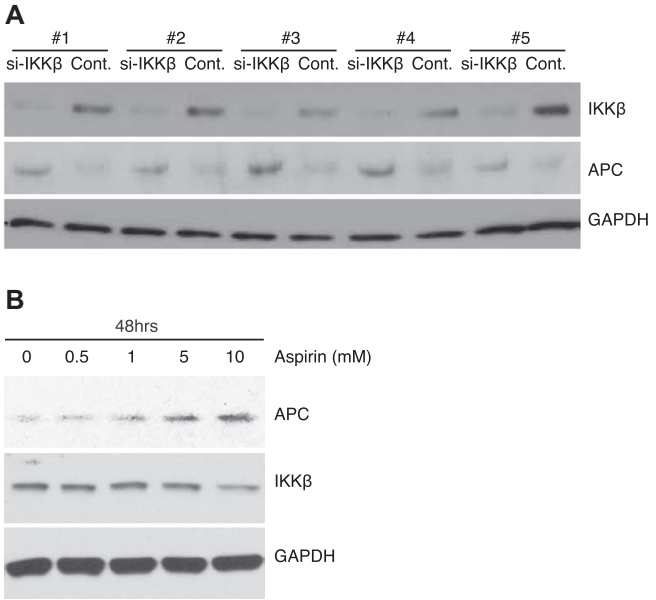

Fig. 3. Deletion of IKKβ increased the expression of APC protein. (A) Immunoblots of APC and IKKβ of HEK293T cells transfected with IKKβ siRNA (si-IKKβ or Control siRNA (Cont.). Five independent experiments are shown. (B) Immunoblots of APC and IKKβ of HEK293T cells incubated with various concentrations of aspirin for 48 h. The data shown are from one representative experiment of the three that were performed.

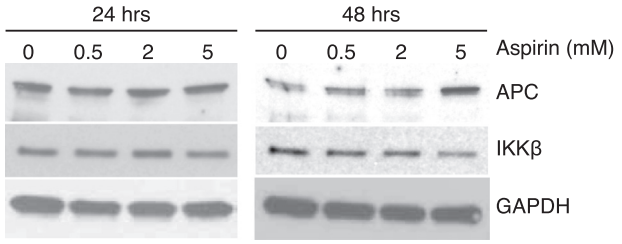

Fig. 4. Aspirin increased the expression of APC and suppressed that of IKKβ in HUVECs. Immunoblots of APC and IKKβ of HUVECs incubated with various concentrations of aspirin for 24 and 48 h. The data shown are from one representative experiment of the three that were performed.

not been explored yet. In this study, we found that the expression of tumor-suppressing APC protein is augmented by incubation with aspirin, possibly through suppression of IKKβ, an essential kinase in NF-κB signaling. Multiple clinical studies have reported that aspirin has preventing effect against cancer, but the effect is seen only when high dose of aspirin is used for long years [9]. It is good coincide with our results that the high dose and long incubation was required for the effect of aspirin.

The APC gene encodes a 310-kDa tumor suppressor protein which has multiple functional domains [10]. Clinical studies indicated that germ-line mutations in one allele of APC give rise to the intestinal polyp disorder, familial adenomatous polyposis (FAP), and mutation of both APC alleles occurs in tumors of FAP patients and the majority of sporadic colorectal cancers and is an early event in tumorigenesis [11]. The APC protein is expressed in most tissues and its expression in the colorectal epithelium contributes to normal growth and differentiation [12,13]. In particular, cells of the gut epithelium undergo cycles of proliferation, adhesion and migration and are sensitive to perturbations in these processes [14]. Functionally, the APC gene product modulates the oncogenic Wnt signal transduction cascade through its effects on cellular levels of β-catenin. In addition, dependent or independent of its ability to titrate β-catenin, APC affects diverse physiologic processes from cell growth to apoptosis in a number of cell types and organisms [15].

NF-κB is transcription factor playing pivotal role in inflammatory response. It generally exist in the cytosol bound to one of three inhibitory, IκB, subunits [16]. In response to a wide variety of inflammatory stimuli, NF-κB is classically activated through serine phosphorylation and degradation of IκB via the ubiquitin pathway, followed by translocation of NF-κB to the nucleus where it activates transcription. Serine phosphorylation of IκB is mediated by a large multi-unit complex containing two catalytic subunits (IKKα and IKKβ) [17,18], as well as the regulatory subunit IKKγ or NEMO, which has no kinase domain [19]. While IKKα plays an important role in skin development independent of its kinase activity [20] as well as a specialized role in the alternative pathway of NF-κB activation that induces specific genes in B cells via NF-κB2 and RelB [21], IKKβ appears to be the primary kinase mediating phosphorylation of IκB in most cell types [22].

Activation of IKKβ-NF-κB signaling in cancer has been reported by multiple groups. For example, Hu et al. have indicated that the expression of IKKβ correlates with poor survival of breast cancer [23]. In terms of its mechanism, the interesting work by Vlantis et al. demonstrates that constitutive IKKβ activity results in activation of the Wnt/β-catenin pathway [24]. However, it remained an open question whether IKKβ directly activates Wnt/β-catenin or indirectly activates Wnt/β-catenin via NF-κB or NF-κB target genes. The observation in this study that IKKβ has inhibitory effect on tumor-suppressing APC protein can be a missing piece to fit in.

However, it is still unclear how the loss of IKKβ up-regulated the expression of APC protein. Shaked et al. have shown that the over-expression of constitutively active of IKKβ accelerates APC loss through iNOS up-regulation [8]. Besides that, our proteomics analysis for identifying binding protein with IKKβ indicated that Cbl, and E3 ligase, binds with the constitutively active of IKKβ (data not shown) and Choi et al. have shown that APC protein is down-regulated by Ubiquitin-Proteinase Pathway by E3 ligase [25]. The further exploration of the mechanism would be of great interest.

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