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Sex-associated preventive effects of low-dose Aspirin on obesity and NAFLD in mouse offspring with in-utero over-nutrition

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Abstract

Aspirin has been found to diminish hypertriglyceridemia and hyperglycemia in both obese rodents and patients with type 2 diabetes mellitus. We aim to test if and how low dose aspirin is able to prevent obesity and the progression of non-alcoholic fatty liver disease (NAFLD) in the high risk subjects. We used offspring mice with maternal over-nutrition as a high risk model of obesity and NAFLD. The offspring were given postnatal HF-diet and diethylnitrosamine (DEN) to induce obesity and NAFLD, and were treated with or without a low dose of aspirin for 12 weeks (ASP or CTL groups). Aspirin treatment reduced body weight gain, reversed glucose intolerance, and depressed hepatic lipid accumulation in female, but not in male mice. Female mice displayed re-sensitized Insulin/Akt signaling and overactivated AMPK signaling, with enhanced level of hepatic PPAR-γ, Glut4 and Glut2, while male mice only enhanced hepatic PPAR-α and PPAR-γ levels. The female ASP mice had inhibited p44/42 MAPK activity and enhanced *Pten* expression, while male displayed activated p38 MAPK signaling. Furthermore, the female but not the male ASP mice reduced Wnt signaling activity via both the epigenetic regulation of *Apc* expression and the posttranscriptional regulation of β-catenin degradation. In summary, our study demonstrated a

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AUTHOR CONTRIBUTIONS

L.X. and Y. Z. designed the study, L.X., H.P and Y. H. wrote the manuscript, Y. Z., H.P., Z. L. C.J and M. D. performed experiments, K. Z. performed data analysis.

COMPETING FIANCIAL INTERESTS

No potential conflicts of interest relevant to this article were reported.

sex-associated effect of low dose aspirin on obesity and NAFLD prevention in female but not in male mice.

Keywords

aspirin; high-fat diet; non-alcoholic fatty liver disease; insulin signaling

INTRODUCTION

The rapid rise in obesity and associated diseases throughout the world now has a major negative impact on human health and healthcare resources. Regarded as the hepatic manifestation of a metabolic syndrome associated with obesity, hyperinsulinemia and peripheral insulin resistance, the non-alcoholic fatty liver disease (NAFLD) affects 10% to 24% of the general population in various countries and the prevalence has even been up to 75 percent in obese people[1]. NAFLD encompasses a spectrum of diseases from simple steatosis to nonalcoholic steatohepatitis (NASH), which can progress to cirrhosis and hepatocellular carcinoma (HCC). In recent years, with the changes of the diet structure and decrease in physical activity, the population of NAFLD patients is increasing and becoming younger [2].

Recent studies have put more efforts on investigating maternal over-nutrition, which reflects the dietary habits of the Western society and supported that maternal over-nutrition is associated with increased obesity and NAFLD. In human, maternal obesity dramatically influenced the risk of NAFLD in adolescents[3]. It is recently reported that maternal pre-pregnancy obesity and early introduction of supplementary milk is associated with a higher risk for adolescent NAFLD[3]. In mouse model, peri-conceptional obesogenic exposure induces sex-specific programming of fatty liver in offspring [3–8]. In male offspring with maternal high-fat (HF) diet, simple steatosis was presented at the age of 30 weeks with normal chow diet; while NASH was developed as early as week 15 if exposed to postnatal HF diet [4]. These evidence support that the next generation with overnutrition during early-development is a high risk population for NAFLD. Thus, there is an urgent need for an effective early prevention strategy for adolescent fatty liver diseases, for which current recommendations provide no guidelines unfortunately.

Aspirin has been used to prevent the development of atherosclerosis due to its important role of anti-inflammation and inhibiting the enzyme clyclooxygenase[9]. High-dose aspirin treatment diminishes hypertriglyceridemia in both obese rodents[10] and patients with type 2 diabetes mellitus[11]. Similarly, low-dose aspirin ameliorated hyperlipidemia induced by HF-diet in rodent models and the mechanisms involve decreased adhesion molecule and chemokine production in the platelet and lymphocytes[12], and reduced VLDL-triglyceride production[13]. Regardless of the cumulative-dose response or duration-response of aspirin, a large population-based study has shown that aspirin intake is associated with a 41% lower risk of hepatocellular carcinoma[14], for which NASH has been identified as an important risk factor. These all suggest a preventive effect of aspirin on liver injury.

In this study, we aims to evaluate if aspirin is effective to prevent juvenile liver injury in a NAFLD high-risk population and to understand why. We used mouse subjects (offspring) from a dam of maternal HF-diet and the liver injury was induced by postnatal HF-diet and diethylnitrosamine (DEN) for 12 weeks. By using this model, we wish to provide evidence based- data to support a preventive strategy for adolescent fatty liver diseases for those who have high risks due to early exposure to over-nutrition. The effect of low-dose aspirin on preventing the liver injury, the glucose intolerance, and the associated molecular and cellular alterations were investigated.

MATERIALS AND METHODS

Chemicals, Antibodies and Reagents

Diethylnitrosamine (DEN) and aspirin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies against IRS1, phospho- IRS-1(Ser1101), NF-kappaB, phospho-NF-kappaB, AKT, phospho-AKT(Ser473), phosphor-AKT (Thr308), JNK, phospho-SAPK/JNK (Thr183/Tyr185), phospho-p38MARK (Thr180/Tyr182), phospho-p44/42 MARK (Erk1/2) (Thr202/Tyr204), AMPK-α, phospho-AMPK-α(Thr172),ACC, phospho-ACC(Ser79),β-Catenin, phospho-β-Catenin (Ser33/37/Thr41) and GAPDH were purchased from Cell Signaling Technology (USA). Antibodies against Srebp1c, Glut4, Glut2 were from Santa Cruz Biotechnology Inc. (USA). The antibody against PPAR-α was acquired from Millipore Sigma (USA). The antibody against PPAR-γ was obtained from Abcam Co. (USA). The bicinchonic acid (BCA) protein assay kit and the phosphatase inhibitor tablets were purchased from Thermo Fisher Scientific (USA). Concentrations of the primary and secondary antibodies are described in the supplemental table.

Study design

Three-month-old mice of mixed background (B6/129/SvEv) were fed a HF diet (60% fat) for 9 weeks before conception. The breeding pairs were given the HF diet throughout gestation and lactation. The offspring were divided into two groups: the ASP group and the CTL group. After weaning, all offspring were given DEN and the HF diet for 12 weeks, while the ASP group were treated with aspirin at the same time. The REF group, which were fed the control diet and were born to breeding pairs given the control diet, served as the reference. Then, they were euthanized by CO2 with the flow rate 3.1–4 LPM, and cervical dislocations were placed to ensure mice were completely dead. After that, the blood and the liver were collected for additional experiments. Mouse experiments were completed according to a protocol reviewed and approved by the Institutional Animal Care and Use Committee of Texas A&M University, in compliance with the USA Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Diet and treatments

Diet was purchased from Research Diets, LLC (New Brunswick, NJ, USA). The HF diet (cat. no. D12492) had an energy density of 5.157 kcal/g (60% fat energy, 20% carbohydrate energy and 20% protein energy). The reference diet (cat. no. D12450B) had an energy density of 3.771 kcal /g (10% fat energy, 70% carbohydrate energy and 20% protein energy). The fat source is composed of 92% lard and 8% soybean oil. The concentration of vitamins,

minerals and proteins were modified to ensure that these nutrients in the HF diet were equivalent to those in the NF diet on a per kilocalorie basis.

DEN treatments: DEN was injected intraperitoneally (i.p.) with 20–25 μg/g and 50 μg/L in their drink water at 21 days of age.

Aspirin treatments: Aspirin (30mg/l in drinking water, pH 6.4) was treated in their drinking water, which was replaced with fresh water every other day. Considering that each animal drinks in average 3 to 4 mL of water per day, this would be equal to 90μg to 120μg aspirin per day for a mouse of 30g weight.

Intraperitoneal injected glucose tolerance test (IPGTT)

At the end of week 12, offspring mice from each experimental group fasted overnight and were subsequently subjected to IPGTT early the next morning. Glucose tolerance tests were conducted with 20% D-glucose in 0.9% saline.The final concentration of the administered dose was 2.0 g/kg body weight.Tail vein blood glucose level was measured with an automated glucometer (Bayer, Elkhart, IN) at baseline and 30, 60, and 120 min after the injection.

Analysis of serum alanine aminotransferase (ALT) activity and insulin concentration

Serums were collected and stored in deep freeze −80°C until measured. Serum ALT activity was measured using an ALT Activity Assay kit (MAK052, Sigma-Aldrich,USA) according to the manufacturer's instructions. The plasma concentrations of insulin were determined using an insulin enzyme-linked immunosorbent assay (ELISA) commercial kits (RayBiotech, USA) according to the manufacturer's instructions.

Histological analysis

Liver tissues were fixed in 4% paraformaldehyde overnight at 4°C, dehydrated with FLEX 100 (70%, 80%, 90%, 95% and 100%), and embedded in paraffin. Specimens were sliced into sections of 5μm thickness using a Leica RM2235 microtome. The hematoxylin and eosin (H&E) staining was performed according to standard method. All HE slides were blindly examined according to the Kleiner scoring system.

Protein extraction and western blot

100mg Frozen tissue was homogenized in 1mL of lysis buffer (RIPA10ml-Add 1 tablet of cOmplete ULTRA Tablet, 1 tablet of Phosphatase inhibitor Tablet, and 200mM PMSF 100ul). The lysate was then centrifuged at 12000rmp for 20 min, and the supernatant fraction was collected. The protein concentration was determined using a BCA protein assay. Aliquots of 150ug were diluted in SDS sample buffer, boiled and run immediately on 7% acrylamide SDS–PAGE gels. Proteins were transferred electrophoretically to nitrocellulose membranes, and the membranes were blocked in 5% Bovine Serum Albumin(BSA)/Tris-buffered saline and reacted with primary antibody to each specific protein overnight in Tris-buffered saline plus 5% BSA. After washing three times with Tris-buffered saline containing 0.1% Tween- 20, the membranes were treated with peroxidase-

conjugated secondary antibodies and visualized using ECL Ultra western HRP Substrate(Millipore Sigma,USA).

RNA extraction and RT-PCR

Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, USA), and concentration was determined in triplicate using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific,USA). Total mRNA (1ug) was amplified and reversed transcribed using ReadyScript®cDNA Synthesis Mix (Sigma-Aldrich, USA), and qPCR was performed using CFX384™ Real-Time System (BIO-RAD, USA). Each reaction consisted of 0.2μl amplified cDNA, 2 μl primers, 5 μl All-in-One qPCR Mix (Gene Copoeia Co.,USA), and nuclease-free water (final volume of 10 μl). All reactions were performed in triplicate on a Bio-Rad real-time PCR machine with the CFX Manager 3.1 software. Primers used for RT-PCR analysis are shown in Table 1.

DNA extraction, Bisulfite conversion and Methylation-specific PCR (MSP)

To extract genomic DNA, 20mg frozen liver tissue was used and followed the work flow of solid tissues, as instructed by the Quick-DNA™ Universal Kit (Zymo Research,USA). One microgram of genomic DNA was bisulfite converted using EZ DNA Methylation-Gold™ Kit (Zymo Research, USA) according to the manufacture's instruction. Bisulfite converts unmethylated cytosine into uracil, whereas methylated cytosine is left unchanged. The sequences of the *Apc* promoter region of mouse was retrieved from (http://genome.ucsc.edu). The tested GC-rich regions is located 185 bp upstream of the *Apc* start codon, and appropriate primers were designed in the website http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi (shown in Table 2). MSPs were performed using CFX384™ Real-Time System (BIO-RAD, USA). All methylation-specific PCRs (MSPs) were performed under the following conditions: 95°C for 10 minutes, then 40 cycles of 95°C for 10 seconds, 58°C for 20 seconds, and 72°C for 15 seconds. The CFX Manager 3.1software (Bio-Rad) was used to measure threshold cycle (Ct) values.

Statistical analysis

Differences among the control and ASP groups across different genders were analyzed by one-way ANOVA. For the longitudinal data such as body weight and food consumption, a linear mixed model was used for the analysis of repeated measures with each individual mouse as a random effect. All analyses were carried out by using SAS JMP software (SAS Institute Inc., Cary, NC, USA) and R statistical programming language. $P<0.05$ is considered significant different.

RESULTS

Aspirin treatment prevented the excess body weight gain and the glucose intolerance in female but not in the male mice.

We monitored the body weight gain during the 12-week experimental period after weaning. Both the male and female mice were weaned at similar body weight and significantly gained body weight by HF-diet through the experimental period. However, the low-dose aspirin treatment reduced the amount of body weight gain in female mice, but not in the male mice

(Figs.1A and 1B). At the end of week 12, the female mice treated with aspirin weighed significantly less than the control female (Fig.1A).

IPGTT was measured at the end of week 12. The fasting glucose level of the CTL group and the ASP group was similar as the REF mice (offspring from the normal fed dam and with postnatal chow diet), regardless of male or female (Figs.1C and 1D). Both the male and female offspring from over-nutrient dam displayed glucose intolerance after 12-week exposure to postnatal HF-diet and DEN (Figs.1C-F, P=0.03 vs. REF for female; P=0.035 vs. REF for male). However, aspirin treatment prevented glucose intolerance in the female ASP group, while the male ASP mice remained glucose intolerant (Figs.1C-F, P=0.793 vs. REF for female; P=0.044 vs. REF for male). In addition, we measured the fasting insulin level at the end of week 12. The result showed that the ASP male mice had significantly higher serum insulin concentration versus either the REF or the CTL mice (Fig.1H), while the female mice insulin level was not different among REF, CTL and ASP groups (Fig.1G).

Aspirin treatment reduced fatty acid accumulation in female hepatocytes, but did not ameliorate the NASH in male mice

Consistent with previous reports[15–17], we observed significant different histological and pathological changes in the female and the male liver at the end of week 12. Clearly, the male mice displayed NASH with severe hepatic steatosis and liver injury featured with balloon-like hepatocytes (Fig.2B), while the female mice only had simple fatty acid accumulation in hepatocytes (Fig.2A). The ASP treatment reduced the amount of fatty acid accumulation in female hepatocytes (Fig.2A), however, the liver injury and hepatosteatosis in ASP male mice seemed not ameliorated compared to the CTL male mice (Fig.2B). We further measured the serum ALT level, which was unchanged under ASP treatment in either female or male offspring comparing to CTL group (Figs.2C and D).

Aspirin treatment re-sensitized hepatic Insulin-Akt signaling in female but not male mice.

The reversed glucose intolerance in ASP female offspring drove us to measure the insulin signaling activity in liver. In female liver, IRS-1 level was unchanged in the ASP group versus the CTL group (Figs.3A and C). However, the phosphorylation at Ser1101 of IRS-1, one of the well-known negative phosphor-regulatory sites, was depressed in the ASP group (Figs.3A and C), suggesting re-sensitizing of insulin signaling by aspirin treatment. Unlike the female mice, activity of insulin signaling in male liver was not altered by aspirin treatment (Figs.3B and D).

We next examined if insulin-dependent Akt signaling also displayed a higher basal activity by aspirin treatment. Consistently, the ASP female group had hyperphosphorylation at Thr308 of the Akt protein (Figs.3E and G), suggesting a higher basal level of Akt signaling. However, Akt activation was not observed in male liver treated with aspirin although the expression of Akt protein was higher (Figs.3F and H).

We further measured the expression of Glut4 and Glut2 to better understanding if aspirin promoted the capacity of glucose transport in liver. Glut4 expression was significantly higher in liver of ASP female but not ASP male (Figs.3A-D). Glut2 is the major hepatic

glucose transporter, but is not insulin-responsive. ASP treatment remarkably increased the level of Glut2 in female liver but not in male liver (Figs.3A-D).

Aspirin treatment activated the AMPK signaling in female, but not male liver.

We examined the AMPK pathway activity to address how aspirin intervention promoted the cellular lipid homeostasis in liver of the female and male mice. The expression of AMPK-α and ACC was not different in both groups regardless from male or female mouse (Figs.4A-C). However, the p-AMPK-α level was significantly higher in ASP group than in the CTL group of the female mice, suggesting an overactivation of AMPK signaling. Consistently, the p-ACC/ACC was also remarkably increased in the ASP group versus the CTL group of the female mice. These data overall suggested enhanced β-oxidation in the female hepatocytes (Figs.4A and C). Unfortunately, the p-AMPK-α and the p-ACC/ACC levels were unchanged in ASP male comparing to the CTL male mice (Figs.4B and D).

Next, we measured the expression level of three key transcription factors for lipid metabolism, Srebp-1c, PPAR-γ and PPAR-α. Srebp-1c expression was unchanged in ASP hepatocytes, regardless of male or female. Unlike the significantly higher expression of hepatic PPAR-γ but not PPAR-α in ASP female (Figs.4E and G); the male mice significantly enhanced PPAR-α and PPAR-γ expression in liver (Figs.4F and H).

Aspirin treatment enhanced activity of JNK signaling in male liver.

According to the “two hits” hypothesis, hepatic inflammation triggers the “second hit” for NAFLD progression[18]. Consistent with the histological changes in female liver that hepatic inflammation did not exist, the aspirin treatment had no effect on either the JNK or the NF-κB signaling in female liver (Figs.5A and C). Neutrophil infiltration was observed in the CTL and ASP male livers, thus we measured if aspirin activated JNK signaling and NF-κB signaling in liver. The results showed that aspirin treatment significantly activated JNK signaling by enhanced expression of JNK protein and hyperphosphorylation of p54 JNK in male liver (Figs.5B and D). The ASP male mice also increased expression of hepatic NF-κB although the phosphorylation of NF-κB was similar as the CTL male mice (Figs.5B and D). We further detected the expression of some inflammatory-related genes including *Il-6, Il-1β, Il-10, Tnf-α, Ccl1, Ccl2* and *Il-4* by realtime-PCR. However, ASP treatment did not change the expression of any of these genes (Fig.5E).

Aspirin treatment depressed hepatic p44/42 MAPK signaling in female and enhanced p38 MAPK signaling in male.

DEN has been widely used as a chemical carcinogen to cause liver HCC. A previous study reported that DEN leads to carcinogenesis ,promotion and progression at doses above 25–30 mg/kg of body weight [19]. Although exposure to DEN at a dose of 20–25 mg/kg of body weight for 12 weeks might not be toxic enough to cause HCC, the liver injury was obvious. Therefore, we measured at the molecular level the activity of Mitogen-activated protein kinase (MAPK) pathways in liver, aims to understand the status of the machinery that controls fundamental cellular processes such as proliferation, differentiation, and apoptosis.

In female, aspirin treatment resulted in hypophosphorylation of p44/42 MAPK in liver exposed to HF diet and DEN for 12 weeks (Figs.6A and C). Unlike the changes in female, the ASP male group had a hyperphosphorylation of p38 MAPK (Figs.6B and D).

Several important cell cycle genes, including *Atm, Cdk2, Cdk4, Cdk6, Chk1, Pal, Pten, P21, CycB* and *CycD2*, were measured for its expression level in liver. The aspirin treatment enhanced hepatic expression of *Pten* in female liver (Fig.6E), while expression levels of these genes in male liver were unchanged (Fig.6F).

Aspirin treatment inhibited Wnt-signaling via both epigenetic regulation of *Apc* promoter region and posttranscriptional phosphorylation of β-catenin

Wnt/β-catenin signaling is known to play an important role in liver development and liver regeneration via one of its main roles in regulating cell cycle progression. We thus investigated whether aspirin treatment could affect the Wnt signaling via downregulation or upregulation the gene expression of key modulator genes. In female ASP mice versus the CTL mice, there was a higher expression of hepatic *Apc* (Figure 7A); however none of the examined Wnt modulator genes were found upregulated or downregulated in male ASP mice (Fig.7B). We hypothesized that *Apc* upregulation was due to hypomethylation at its CpG islands, therefore we utilized methylation-specific PCR to answer this question. As expected, hypomethylation of CpG island located 185 bp upstream of the *Apc* start codon was found in ASP female offspring liver (Figs.7C). In contrast, methylation status was unchanged at this site in ASP male versus the CTL male mice (Figs.7D).

We wonder if aspirin also effects on the Wnt-signaling via post-transcriptional regulation of β-catenin, therefore the protein level and the phosphorylation of β-catenin were measured. We showed significant decrease in β-catenin expression in the ASP liver compared to the CTL liver (Figs.7E and F), suggesting a potential increased degradation of this protein. Consistently, the ASP liver had increased phosphorylation at Ser33/37 and Thr41 of β-catenin (Figs.7E and F), the major phosphorylation sites whose activation is associated with its disability.

DISCUSSION

It is probably due to widely reported gender differences of the hepatic phenotype in mouse models of postweaning diet-induced obesity (DIO) [16, 20–28], low dose of aspirin has been previously studied only in male rodents with HF-diet and is reported to reduce the serum lipid content and inhibited the hepatic inflammation [12, 13]. There is unfortunately zero information about aspirin on modulating the lipid metabolism in female mice. We, for the first time, reported that the effects of aspirin to prevent obesity and its related complications is different in male versus in female: a low-dose aspirin intake not only reduced the body weight gain but also reversed the impaired glucose tolerance in female mice; while such effects were not observed in male mice; instead, an impaired insulin sensitivity might occur in male mice evidenced by a higher fasting serum insulin level. This sex-associated effect is also manifested by different metabolic response in liver: the female ASP mice displayed less fatty acid accumulation, while the hepatic steatosis and liver injury remained severe in male ASP mice. Thus, one hypothesis to explain the aspirin effect disparity between male and

female is that the different preventive effect was actually attributable to the more advanced NASH in male than in female, rather than the sex-different. Following this hypothesis, low-dose of aspirin only prevented the progression of NAFLD in the early rather than the later stage. However, this hypothesis needs to be tested in different stages of NAFLD in animal models of the same sex. Interestingly, we are not the first study to report the differing effects of aspirin in male and female, which has been widely reported in both human and animal models and especially in atherothrombotic benefit [29–34]. Although most studies about the sex-related difference of aspirin effects are observational, the data have suggested the contribution of the interaction of aspirin and sex hormones, especially the level of testosterone and estrogen [35–40]. As the major organ to metabolite the sex hormones, the dysfunctional liver can greatly affect the levels and the normal function of sex hormones (reviewed in [41]). Thus, another hypothesis could be the interactive effect of aspirin, sex hormones and fatty liver, which needs to be systematically tested in the future study. Nonetheless, our data suggested to us that a low dose of aspirin might be efficient to prevent excessive body weight gain, glucose intolerance and an early progression of NAFLD.

According to the “two-hit” hypothesis, the “first hit” initiates the hepatic lipid accumulation. During the “first hit”, diminished insulin signaling by serine phosphorylation of IRS-1 and IRS-2 contributes to fat accumulation[42–46]. To be noted, previous human data are not consistent about the effect of aspirin in insulin sensitivity. Earlier studies completed in diabetic patients support that aspirin is responsible to insulin sensitization[47–49], while some recent studies in healthy subjects found a detrimental effect [50–52]. This difference is attributable to a higher dose and a longer duration in the earlier studies than in the recent study (6–9g/d vs. <3g/d and 1–3 weeks vs. a few days). In our study, we aimed to investigate the preventive effect of aspirin, thus a very low dose of aspirin (3–4mg/kg/d) but a long-term treatment (12 weeks) was given to ASP mice daily. We demonstrated that a long-term and low-dose of aspirin improved insulin sensitivity via activation of Insulin-Akt signaling via modulating glucose and lipid metabolism. As an evidence, the liver expressed increased hepatic expression of Glut2 and PPAR-γ. These results are consistent with more recent studies in human[11] and rodent’s models[10] to support the important role of aspirin in maintaining glucose homeostasis, although a higher dose and a shorter-term of aspirin was applied. We also provide evidence to show that aspirin also contributed to maintain lipid homeostasis via promoting lipid catabolism to fight against DEN and HFD, through activating AMPK signaling and enhancing PPAR-γ which transcriptionally regulate β-oxidation[53].

Previous study has shown that aspirin is able to treat or prevent a variety of cancers including colorectal cancer[54–56], squamous cell esophageal cancer[57–59] and prostate cancer[60–63], suggesting that aspirin plays a positive role in anti-hyperproliferation during tumorigenesis. In our study with liver injury induced by DEN and HFD, a low dose of aspirin depressed p42/44 MAPK signaling in female. The female mice also had enhanced expression of hepatic *Pten*, a well-known tumor suppressor and a well-established negative regulator of cell-cycle progression[64]. One mechanism to explain the effect of aspirin on cancer prevention is through its cyclooxygenase-independent effects of aspirin/Wnt interactions. Previous report has shown that aspirin treatment results in the increased ubiquination of β-catenin though increased phosphorylation of protein phosphatase 2A

(PP2A), which further leads to a reduction in enzymatic PP2A activated Wnt-signaling[65]. Here we demonstrated the novel role of aspirin to epigenetically regulate the Wnt-signaling via decreased DNA methylation and identified the methylation site within its transcriptional regulation region. Apc protein is known to inhibit Wnt signaling by interacting with β-catenin and Axin to form an Apc/Axin/β-catenin complex. We showed the phosphorylation at Ser33/37 and Thr41 site of β-catenin further leads to its degradation, which is consistent with previous reports [66]. Our results suggested a novel mechanisms how aspirin plays a cyclooxygenase-independent role in regulating Wnt-signaling. Although *Apc* knockout is associated with HCC [67], it is not clear if *Apc* overexpression is able to inhibit the DEN-induced hyperproliferation. Here we were unable to detect transcriptional changes of known proliferation related Wnt-pathway downstream targets responsible to aspirin treatment, but we also could not exclude expression changes of unknown Wnt-pathway targets contributing to anti-hyperproliferation. Nevertheless, our data confirmed a potential role of aspirin to prevent the progression of NAFLD via down-regulating Wnt-signaling to block DEN-triggered hyperproliferation.

Our data does not support that aspirin plays a positive effect on preventing obesity, insulin resistance and NASH in male offspring with high risks. Although we showed an overexpression of hepatic PPAR-α and PPAR-γ, we also showed glucose intolerance and increased fasting insulin in male mice, suggesting the increments might be attributable to a compensatory effect in responsible to hyperglycemia and hyperinsulinemia. Previous studies have shown that a high dose of aspirin activates IKKβ signaling[10, 68] and p38 MAPK signaling[69], suggesting the activated IKKβ/NFκB and p38 MAPK mediated anti-inflammation and anti-insulin resistance during liver injury. In our study, we did observe activated p38 MAPK, NFκB signaling and JNK signaling in male ASP offspring mice. However, expression of well-known inflammatory cytokine genes in liver were irresponsible to aspirin-treatment in our study. Thus, the role of Aspirin in mediating insulin sensitivity and hepatic fat accumulation-induced injury might be complicated and needs more intensive studies.

In summary, we adapted a mouse model with high risks of NAFLD to examine the preventive effect of low dose aspirin. The preventive effect of low dose aspirin on obesity and NAFLD is different in male and female mice. It prevented body weight gain, glucose intolerance and lipid accumulation in female via sensitizing the Insulin/Akt signaling, activating AMPK signaling, inhibiting the MAPK signaling and Wnt-signaling through both epigenetic regulation and the post-transcriptional modification (Figure 8). However, these effects of low dose aspirin were not obvious in male mice with NASH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCE

[1]. Miele L, Forgione A, Hernandez AP, Gabrieli ML, Vero V, Di Rocco P, Greco AV, Gasbarrini G, Gasbarrini A, Grieco A: The natural history and risk factors for progression of non-alcoholic fatty liver disease and steatohepatitis. Eur Rev Med Pharmacol Sci 2005, 9:273–7. [PubMed: 16231589]

[2]. Charlton M: Nonalcoholic fatty liver disease: a review of current understanding and future impact. Clin Gastroenterol Hepatol 2004, 2:1048–58. [PubMed: 15625647]

[3]. Ayonrinde OT, Oddy WH, Adams LA, Mori TA, Beilin LJ, de Klerk N, Olynyk JK: Infant nutrition and maternal obesity influence the risk of non-alcoholic fatty liver disease in adolescents. J Hepatol 2017.

[4]. Bruce KD, Cagampang FR, Argenton M, Zhang J, Ethirajan PL, Burdge GC, Bateman AC, Clough GF, Poston L, Hanson MA, McConnell JM, Byrne CD: Maternal high-fat feeding primes steatohepatitis in adult mice offspring, involving mitochondrial dysfunction and altered lipogenesis gene expression. Hepatology 2009, 50:1796–808. [PubMed: 19816994]

[5]. Ashino NG, Saito KN, Souza FD, Nakutz FS, Roman EA, Velloso LA, Torsoni AS, Torsoni MA: Maternal high-fat feeding through pregnancy and lactation predisposes mouse offspring to molecular insulin resistance and fatty liver. J Nutr Biochem 2012, 23:341–8. [PubMed: 21543214]

[6]. Dahlhoff M, Pfister S, Blutke A, Rozman J, Klingenspor M, Deutsch MJ, Rathkolb B, Fink B, Gimpfl M, Hrabe de Angelis M, Roscher AA, Wolf E, Ensenauer R: Peri-conceptional obesogenic exposure induces sex-specific programming of disease susceptibilities in adult mouse offspring. Biochim Biophys Acta 2014, 1842:304–17. [PubMed: 24275555]

[7]. Kruse M, Seki Y, Vuguin PM, Du XQ, Fiallo A, Glenn AS, Singer S, Breuhahn K, Katz EB, Charron MJ: High-fat intake during pregnancy and lactation exacerbates high-fat diet-induced complications in male offspring in mice. Endocrinology 2013, 154:3565–76. [PubMed: 23861375]

[8]. Li J, Huang J, Li JS, Chen H, Huang K, Zheng L: Accumulation of endoplasmic reticulum stress and lipogenesis in the liver through generational effects of high fat diets. J Hepatol 2012, 56:900–7. [PubMed: 22173165]

[9]. Maranhao RC, Leite AC, Jr., Development of anti-atherosclerosis therapy based on the inflammatory and proliferative aspects of the disease. Curr Pharm Des 2015, 21:1196–204. [PubMed: 25312729]

[10]. Yuan M, Konstantopoulos N, Lee J, Hansen L, Li ZW, Karin M, Shoelson SE: Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. Science 2001, 293:1673–7. [PubMed: 11533494]

[11]. Hundal RS, Petersen KF, Mayerson AB, Randhawa PS, Inzucchi S, Shoelson SE, Shulman GI: Mechanism by which high-dose aspirin improves glucose metabolism in type 2 diabetes. J Clin Invest 2002, 109:1321–6. [PubMed: 12021247]

[12]. Lin HL, Yen HW, Hsieh SL, An LM, Shen KP: Low-dose aspirin ameliorated hyperlipidemia, adhesion molecule, and chemokine production induced by high-fat diet in Sprague-Dawley rats. Drug Dev Res 2014, 75:97–106. [PubMed: 24648214]

[13]. van Diepen JA, Vroegrijk IO, Berbee JF, Shoelson SE, Romijn JA, Havekes LM, Rensen PC, Voshol PJ: Aspirin reduces hypertriglyceridemia by lowering VLDL-triglyceride production in mice fed a high-fat diet. Am J Physiol Endocrinol Metab 2011, 301:E1099–107. [PubMed: 21862721]

[14]. Sahasrabuddhe VV, Gunja MZ, Graubard BI, Trabert B, Schwartz LM, Park Y, Hollenbeck AR, Freedman ND, McGlynn KA: Nonsteroidal anti-inflammatory drug use, chronic liver disease, and hepatocellular carcinoma. J Natl Cancer Inst 2012, 104:1808–14. [PubMed: 23197492]

[15]. Burcelin R, Dolci W, Thorens B: Glucose sensing by the hepatoportal sensor is GLUT2-dependent: in vivo analysis in GLUT2-null mice. Diabetes 2000, 49:1643–8. [PubMed: 11016447]

[16]. Hwang LL, Wang CH, Li TL, Chang SD, Lin LC, Chen CP, Chen CT, Liang KC, Ho IK, Yang WS, Chiou LC: Sex differences in high-fat diet-induced obesity, metabolic alterations and learning, and synaptic plasticity deficits in mice. Obesity 2010, 18:463–9. [PubMed: 19730425]

[17]. Fu Q, Olson P, Rasmussen D, Keith B, Williamson M, Zhang KK, Xie L: A short-term transition from a high-fat diet to a normal-fat diet before pregnancy exacerbates female mouse offspring obesity. Int J Obes (Lond) 2016, 40:564–72. [PubMed: 26607040]

[18]. Byrne CD, Olufadi R, Bruce KD, Cagampang FR, Ahmed MH: Metabolic disturbances in non-alcoholic fatty liver disease. Clin Sci (Lond) 2009, 116:539–64. [PubMed: 19243311]

[19]. Verna L, Whysner J, Williams GM: N-nitrosodiethylamine mechanistic data and risk assessment: bioactivation, DNA-adduct formation, mutagenicity, and tumor initiation. Pharmacol Ther 1996, 71:57–81. [PubMed: 8910949]

[20]. Cecconello AL, Trapp M, Hoefel AL, Marques CV, Arbo BD, Osterkamp G, Kucharski LC, Ribeiro MF: Sex-related differences in the effects of high-fat diets on DHEA-treated rats. Endocrine 2014.

[21]. Bellisario V, Berry A, Capoccia S, Raggi C, Panetta P, Branchi I, Piccaro G, Giorgio M, Pelicci PG, Cirulli F: Gender-dependent resiliency to stressful and metabolic challenges following prenatal exposure to high-fat diet in the p66(Shc−/−) mouse. Front Behav Neurosci 2014, 8:285. [PubMed: 25202246]

[22]. Hariri N, Thibault L: High-fat diet-induced obesity in animal models. Nutrition research reviews 2010, 23:270–99. [PubMed: 20977819]

[23]. Deeb SS, Zambon A, Carr MC, Ayyobi AF, Brunzell JD: Hepatic lipase and dyslipidemia: interactions among genetic variants, obesity, gender, and diet. Journal of lipid research 2003, 44:1279–86. [PubMed: 12639974]

[24]. Mischke M, Pruis MG, Boekschoten MV, Groen AK, Fitri AR, van de Heijning BJ, Verkade HJ, Muller M, Plosch T, Steegenga WT: Maternal Western-style high fat diet induces sex-specific physiological and molecular changes in two-week-old mouse offspring. PLoS One 2013, 8:e78623. [PubMed: 24223833]

[25]. Cecconello AL, Trapp M, Hoefel AL, Marques CV, Arbo BD, Osterkamp G, Kucharski LC, Ribeiro MF: Sex-related differences in the effects of high-fat diets on DHEA-treated rats. Endocrine 2015, 48:985–94. [PubMed: 25300783]

[26]. Deeb SS, Zambon A, Carr MC, Ayyobi AF, Brunzell JD: Hepatic lipase and dyslipidemia: interactions among genetic variants, obesity, gender, and diet. J Lipid Res 2003, 44:1279–86. [PubMed: 12639974]

[27]. Hariri N, Thibault L: High-fat diet-induced obesity in animal models. Nutr Res Rev 2010, 23:270–99. [PubMed: 20977819]

[28]. Hwang LL, Wang CH, Li TL, Chang SD, Lin LC, Chen CP, Chen CT, Liang KC, Ho IK, Yang WS, Chiou LC: Sex differences in high-fat diet-induced obesity, metabolic alterations and learning, and synaptic plasticity deficits in mice. Obesity (Silver Spring) 2010, 18:463–9. [PubMed: 19730425]

[29]. Adelman EE, Lisabeth L, Brown DL: Gender differences in the primary prevention of stroke with aspirin. Womens Health (Lond) 2011, 7:341–52; quiz 52–3. [PubMed: 21612354]

[30]. Amateau SK, Alt JJ, Stamps CL, McCarthy MM: Brain estradiol content in newborn rats: sex differences, regional heterogeneity, and possible de novo synthesis by the female telencephalon. Endocrinology 2004, 145:2906–17. [PubMed: 14988386]

[31]. Ayala DE, Hermida RC: Sex differences in the administration-time-dependent effects of low-dose aspirin on ambulatory blood pressure in hypertensive subjects. Chronobiol Int 2010, 27:345–62. [PubMed: 20370474]

[32]. Kelton JG, Hirsh J, Carter CJ, Buchanan MR: Sex differences in the antithrombotic effects of aspirin. Blood 1978, 52:1073–6. [PubMed: 698392]

[33]. Morikawa M, Kojima T, Inoue M, Tsuboi M: Sex difference in the inhibitory effect of aspirin on prostacyclin production of rat aortae. Jpn J Pharmacol 1984, 35:1–7. [PubMed: 6433093]

[34]. Menguy R, Desbaillets L, Masters YF, Okabe S: Evidence for a sex-linked difference in aspirin metabolism. Nature 1972, 239:102–3. [PubMed: 4562107]

[35]. Albert O, Desdoits-Lethimonier C, Lesne L, Legrand A, Guille F, Bensalah K, Dejucq-Rainsford N, Jegou B: Paracetamol, aspirin and indomethacin display endocrine disrupting properties in the adult human testis in vitro. Hum Reprod 2013, 28:1890–8. [PubMed: 23670170]

[36]. Bauer SR, Fortner RT, Gates MA, Eliassen AH, Hankinson SE, Tworoger SS: Analgesic use in relation to sex hormone and prolactin concentrations in premenopausal women. Cancer Causes Control 2013, 24:1087–97. [PubMed: 23515936]

[37]. Didolkar AK, Gurjar A, Joshi UM, Sheth AR, Roychowdhury D: Effects of aspirin on blood plasma levels of testosterone, LH and FSH in maturing male rats. Int J Androl 1980, 3:312–8. [PubMed: 6773893]

[38]. Duggan C, Wang CY, Xiao L, McTiernan A: Aspirin and serum estrogens in postmenopausal women: a randomized controlled clinical trial. Cancer Prev Res (Phila) 2014, 7:906–12. [PubMed: 24924486]

[39]. Gates MA, Tworoger SS, Eliassen AH, Missmer SA, Hankinson SE: Analgesic use and sex steroid hormone concentrations in postmenopausal women. Cancer Epidemiol Biomarkers Prev 2010, 19:1033–41. [PubMed: 20332258]

[40]. Kristensen DM, Lesne L, Le Fol V, Desdoits-Lethimonier C, Dejucq-Rainsford N, Leffers H, Jegou B: Paracetamol (acetaminophen), aspirin (acetylsalicylic acid) and indomethacin are anti-androgenic in the rat foetal testis. Int J Androl 2012, 35:377–84. [PubMed: 22612476]

[41]. Gawrieh S: Sex Hormones, Sex Hormone-Binding Globulin, and Liver Fat: Which Came First, the Chicken or the Egg? Clin Gastroenterol Hepatol 2015, 13:1694–6. [PubMed: 25956841]

[42]. Kodama Y, Brenner DA: c-Jun N-terminal kinase signaling in the pathogenesis of nonalcoholic fatty liver disease: Multiple roles in multiple steps. Hepatology 2009, 49:6–8. [PubMed: 19111006]

[43]. Tarantino G, Caputi A: JNKs, insulin resistance and inflammation: A possible link between NAFLD and coronary artery disease. World J Gastroenterol 2011, 17:3785–94. [PubMed: 21987620]

[44]. Singh R, Wang Y, Xiang Y, Tanaka KE, Gaarde WA, Czaja MJ: Differential effects of JNK1 and JNK2 inhibition on murine steatohepatitis and insulin resistance. Hepatology 2009, 49:87–96. [PubMed: 19053047]

[45]. Solinas G, Vilcu C, Neels JG, Bandyopadhyay GK, Luo JL, Naugler W, Grivennikov S, Wynshaw-Boris A, Scadeng M, Olefsky JM, Karin M: JNK1 in hematopoietically derived cells contributes to diet-induced inflammation and insulin resistance without affecting obesity. Cell Metab 2007, 6:386–97. [PubMed: 17983584]

[46]. Kamata H, Honda S, Maeda S, Chang L, Hirata H, Karin M: Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. Cell 2005, 120:649–61. [PubMed: 15766528]

[47]. Reid J, Lightbody TD: The insulin equivalence of salicylate. Br Med J 1959, 1:897–900. [PubMed: 13629156]

[48]. Reid J, Macdougall AI, Andrews MM: Aspirin and diabetes mellitus. Br Med J 1957, 2:1071–4. [PubMed: 13472052]

[49]. Williamson RT: On the Treatment of Glycosuria and Diabetes Mellitus with Sodium Salicylate. Br Med J 1901, 1:760–2. [PubMed: 20759517]

[50]. Bratusch-Marrain PR, Vierhapper H, Komjati M, Waldhausl WK: Acetyl-salicylic acid impairs insulin-mediated glucose utilization and reduces insulin clearance in healthy and non-insulin-dependent diabetic man. Diabetologia 1985, 28:671–6. [PubMed: 3905478]

[51]. Giugliano D, Sacca L, Scognamiglio G, Ungaro B, Torella R: Influence of acetylsalicylic acid on glucose turnover in normal man. Diabete Metab 1982, 8:279–82. [PubMed: 6819169]

[52]. Newman WP, Brodows RG: Aspirin causes tissue insensitivity to insulin in normal man. J Clin Endocrinol Metab 1983, 57:1102–6. [PubMed: 6355137]

[53]. Varga T, Czimmerer Z, Nagy L: PPARs are a unique set of fatty acid regulated transcription factors controlling both lipid metabolism and inflammation. Biochim Biophys Acta 2011, 1812:1007–22. [PubMed: 21382489]

[54]. Cook NR, Lee IM, Zhang SM, Moorthy MV, Buring JE: Alternate-day, low-dose aspirin and cancer risk: long-term observational follow-up of a randomized trial. Ann Intern Med 2013, 159:77–85. [PubMed: 23856681]

[55]. Friis S, Riis AH, Erichsen R, Baron JA, Sorensen HT: Low-Dose Aspirin or Nonsteroidal Anti-inflammatory Drug Use and Colorectal Cancer Risk: A Population-Based, Case-Control Study. Ann Intern Med 2015, 163:347–55. [PubMed: 26302241]

[56]. Rothwell PM, Wilson M, Elwin CE, Norrving B, Algra A, Warlow CP, Meade TW: Long-term effect of aspirin on colorectal cancer incidence and mortality: 20-year follow-up of five randomised trials. Lancet 2010, 376:1741–50. [PubMed: 20970847]

[57]. Corley DA, Kerlikowske K, Verma R, Buffler P: Protective association of aspirin/NSAIDs and esophageal cancer: a systematic review and meta-analysis. Gastroenterology 2003, 124:47–56. [PubMed: 12512029]

[58]. Malik S, Ullah S, Afzal M, Lal K, Haque S: Clinical and descriptive genetic study of polydactyly: a Pakistani experience of 313 cases. Clin Genet 2014, 85:482–6. [PubMed: 23772746]

[59]. Zhang L, Wu YD, Li P, Tu J, Niu YL, Xu CM, Zhang ST: Effects of cyclooxygenase-2 on human esophageal squamous cell carcinoma. World J Gastroenterol 2011, 17:4572–80. [PubMed: 22147962]

[60]. Bosetti C, Rosato V, Gallus S, La Vecchia C: Aspirin and prostate cancer prevention. Recent Results Cancer Res 2014, 202:93–100. [PubMed: 24531782]

[61]. Moyad MA, Vogelzang NJ: Heart healthy equals prostate healthy and statins, aspirin, and/or metformin (S.A.M.) are the ideal recommendations for prostate cancer prevention. Asian J Androl 2015, 17:783–91. [PubMed: 25657084]

[62]. Vidal AC, Freedland SJ: Aspirin and prostate cancer prevention. Aging (Albany NY) 2015, 7:292–3. [PubMed: 26022347]

[63]. Vidal AC, Howard LE, Moreira DM, Castro-Santamaria R, Andriole GL, Freedland SJ: Aspirin, NSAIDs, and risk of prostate cancer: results from the REDUCE study. Clin Cancer Res 2015, 21:756–62. [PubMed: 25520389]

[64]. Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, Ruland J, Penninger JM, Siderovski DP, Mak TW: Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. Cell 1998, 95:29–39. [PubMed: 9778245]

[65]. Bos CL, Kodach LL, van den Brink GR, Diks SH, van Santen MM, Richel DJ, Peppelenbosch MP, Hardwick JC: Effect of aspirin on the Wnt/beta-catenin pathway is mediated via protein phosphatase 2A. Oncogene 2006, 25:6447–56. [PubMed: 16878161]

[66]. Yost C, Torres M, Miller JR, Huang E, Kimelman D, Moon RT: The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in Xenopus embryos by glycogen synthase kinase 3. Genes Dev 1996, 10:1443–54. [PubMed: 8666229]

[67]. Colnot S, Decaens T, Niwa-Kawakita M, Godard C, Hamard G, Kahn A, Giovannini M, Perret C: Liver-targeted disruption of Apc in mice activates beta-catenin signaling and leads to hepatocellular carcinomas. Proc Natl Acad Sci U S A 2004, 101:17216–21. [PubMed: 15563600]

[68]. Yin MJ, Yamamoto Y, Gaynor RB: The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. Nature 1998, 396:77–80. [PubMed: 9817203]

[69]. Oshima T, Miwa H, Joh T: Aspirin induces gastric epithelial barrier dysfunction by activating p38 MAPK via claudin-7. Am J Physiol Cell Physiol 2008, 295:C800–6. [PubMed: 18667601]

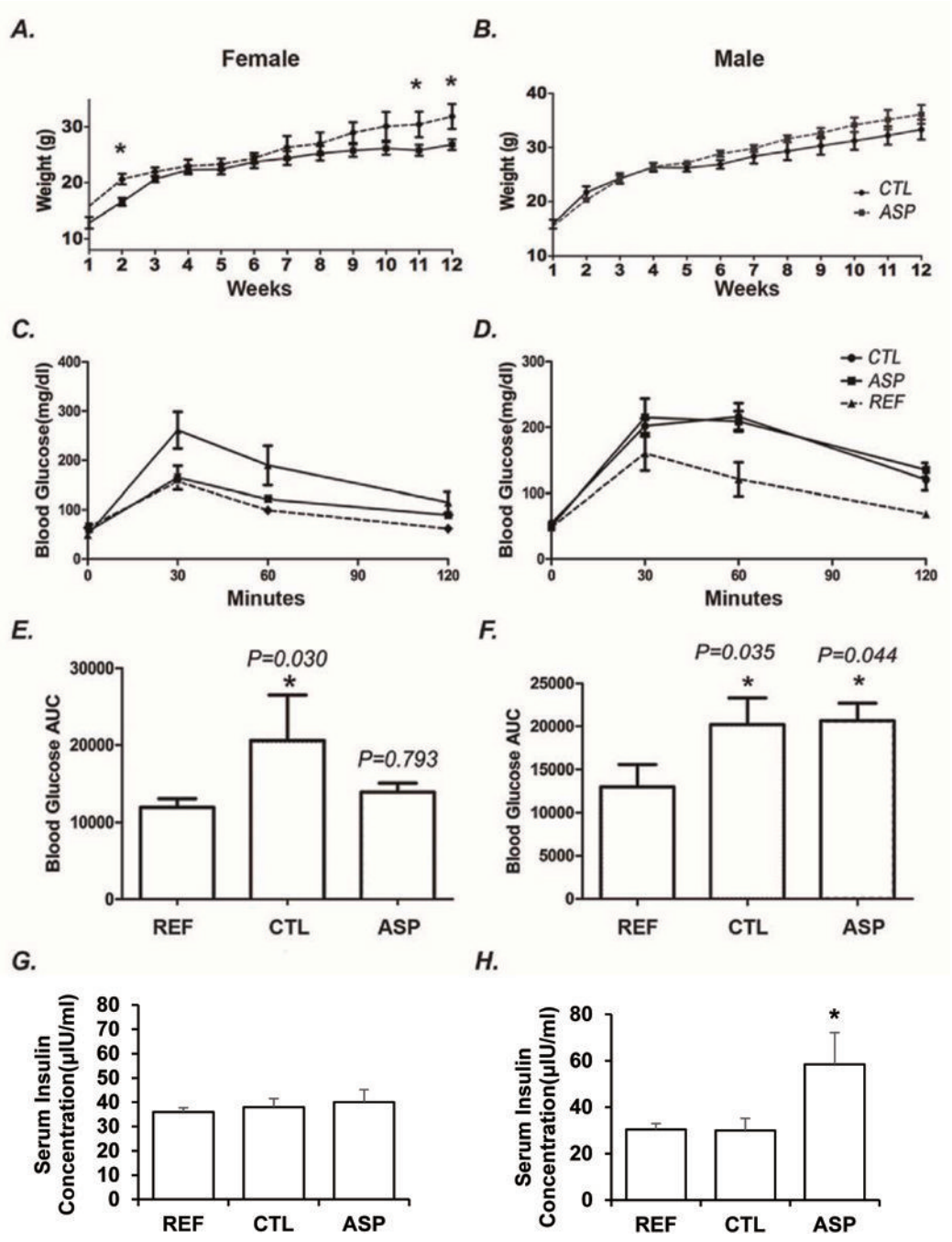

Figure 1. Aspirin treatment prevented the excess body weight gain and the glucose intolerance in female but not in the male mice.

1A-B. Body weight of the female (A) and male (B) offspring were recorded weekly since weaning for 12 weeks.

1C-D. IPGTT was measured at the end of week 12 in female (C) and male mice (D).

1E-F. Area under the curve (AUC) was calculated for the results of IPGTT in female (E) and male mice (F).

1G-H. The fasting insulin level was measured at the end of week 12 in female (G) and male mice (H).

Data is presented as Mean ± SD, n=4–6. Significance (P<0.05) is presented between two groups.

* P<0.05 vs. CTL/REF group.

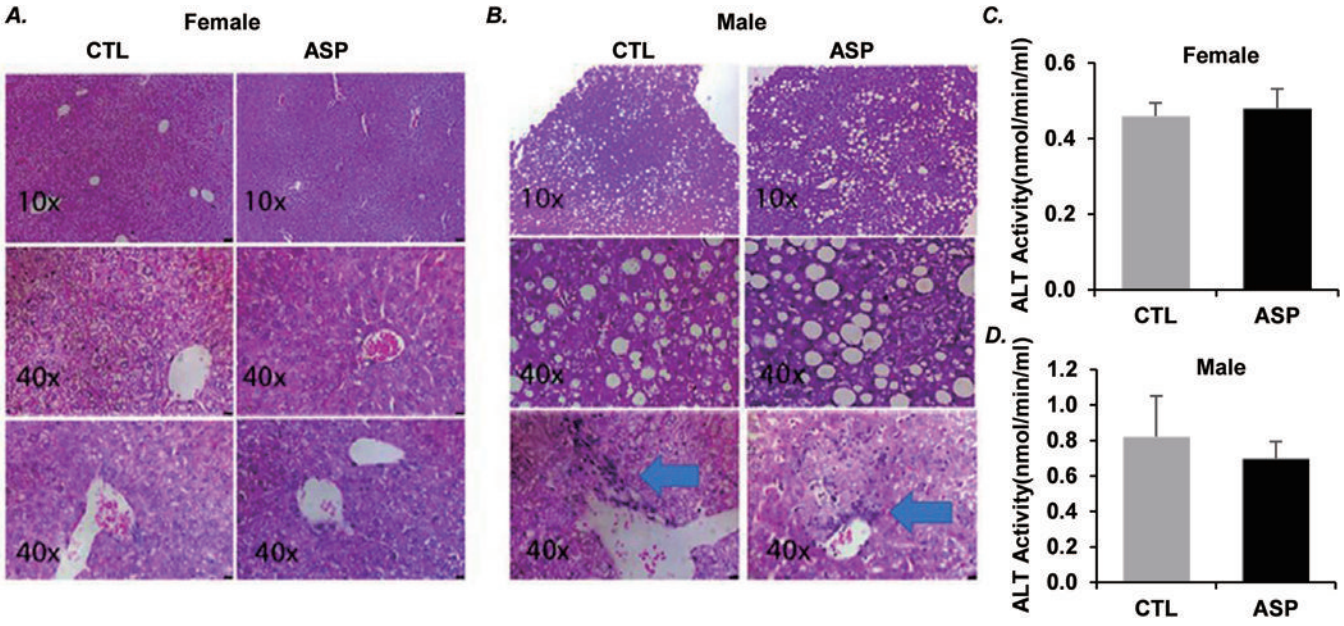

Figure 2. Aspirin treatment reduced fatty acid accumulation in female hepatocytes, but did not ameliorated the NASH in male.

2A. By HE staining, the ASP treatment reduced the amount of fatty acid accumulation in female hepatocytes.

2B. By HE staining, the liver injury and hepatosteatosis in ASP male mice were not ameliorated comparing to the CTL male mice. (The blue arrow indicates inflammatory cells infiltration)

2C-D. The serum ALT level were not altered by ASP treatment in either female or male offspring comparing to CTL group in female (C) and male mice (D).

Data is presented as Mean ± SD, n=4–6. Significance (P<0.05) is presented between two groups.

* P<0.05 vs. CTL group.

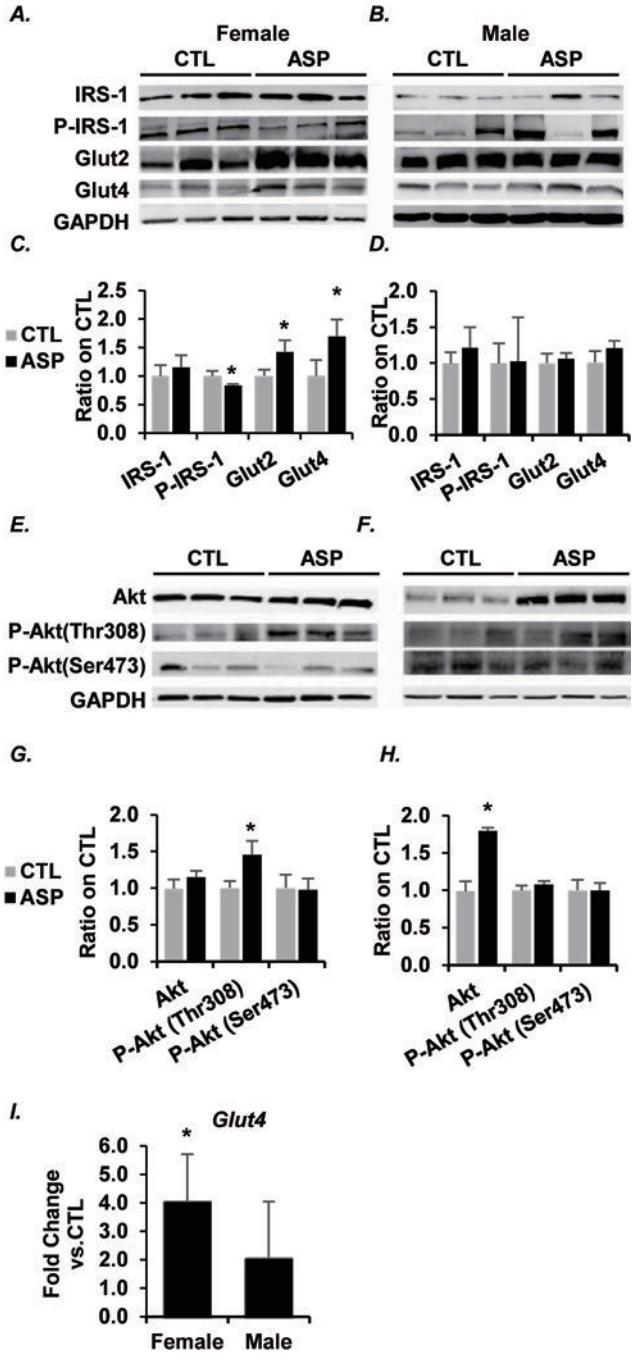

Figure 3. Aspirin treatment re-sensitized hepatic insulin-AKT signaling in female but not male mice.

3A-B. IRS-1, phosphorylation of IRS-1 at Ser1101, Glut2 and Glut4 in liver tissue were detected by western blots in female (A) and male mice (B).

3C-D. Relative amounts were expressed as the ratios of IRS1/GAPDH, Phospho-IRS-1-Ser^{1101}/GAPDH, Glut2/ GAPDH and Glut4/ GAPDH in female (C) and male mice (D)..

3E-F. Akt, phosphorylation of Akt at Thr308, phosphorylation of Akt at Ser473, in liver tissue were detected by western blots in female (E) and male mice(F).

3G-H. Relative amounts were expressed as the ratios of Akt/GAPDH, Phospho-Akt (Thr308)/GAPDH, Phospho-Akt (Ser473)/GAPDH in female (G) and male mice (H).
3I. *Glut4* were measured for their expression level in liver by realtime-PCR in female and male.
Data is presented as Mean ± SD, n=4–6. Significance ($P<0.05$) is presented between two groups.
* $P<0.05$ vs. CTL group.

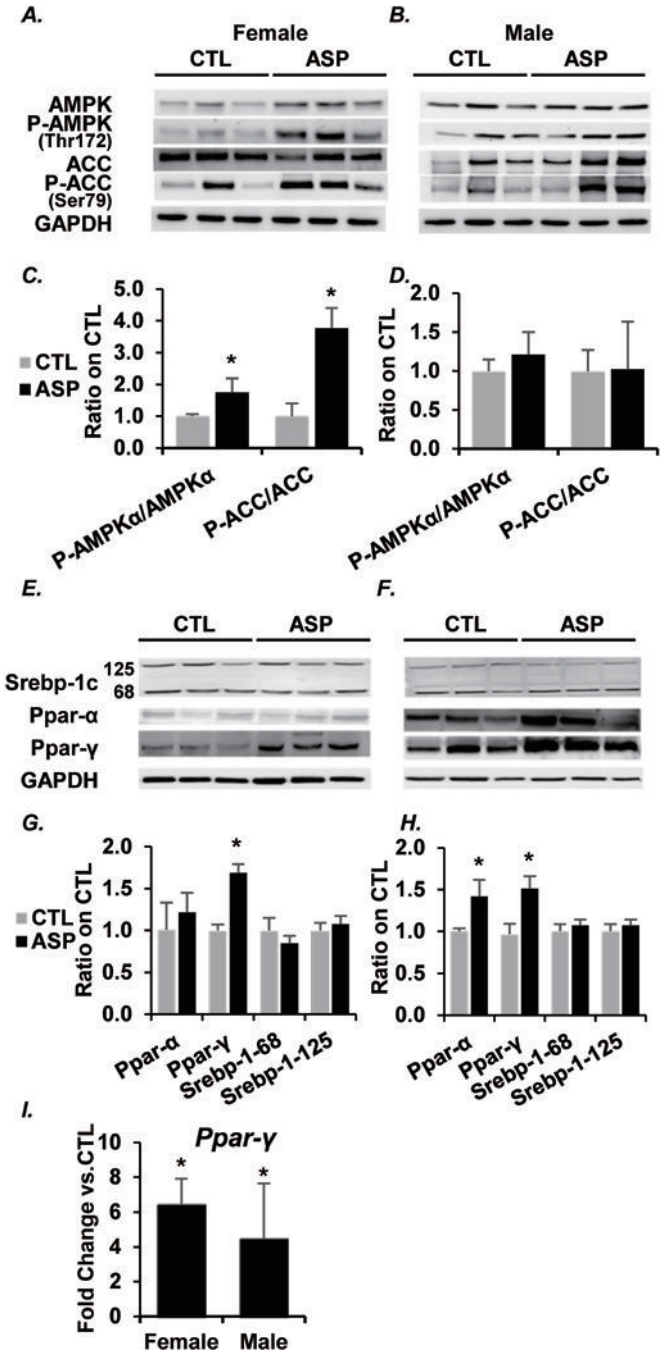

Figure 4. Aspirin treatment activated the AMPK signaling in female, but not male liver.

4A-B. AMPK-α, phosphorylation of AMPK-α at Thr172, ACC,phosphorylation of ACC at Ser79 in liver tissue were detected by western blots in female (A) and male mice (B).

4C-D. Relative amounts were expressed as the ratios of Phospho- AMPK-α (Thr172)/ AMPK-α /GAPDH, Phospho- ACC (Ser79)/ACC/GAPDH in female (C) and male mice (D).

4E-F. Srebp1–1c, PPAR-α and PPAR-γ in liver tissue were detected by western blots in female (E) and male mice(F).

4G-H. Relative amounts were expressed as the ratios of Srebp1–1c/GAPDH, PPAR-α/GAPDH and PPAR-γ/GAPDH in female (G) and male mice (H).

4I. *PPAR-γ* were measured for their expression level in liver by realtime-PCR in female and male. Data is presented as Mean ± SD, n=4–6. Significance ($P<0.05$) is presented between two groups. * $P<0.05$ vs. CTL group.

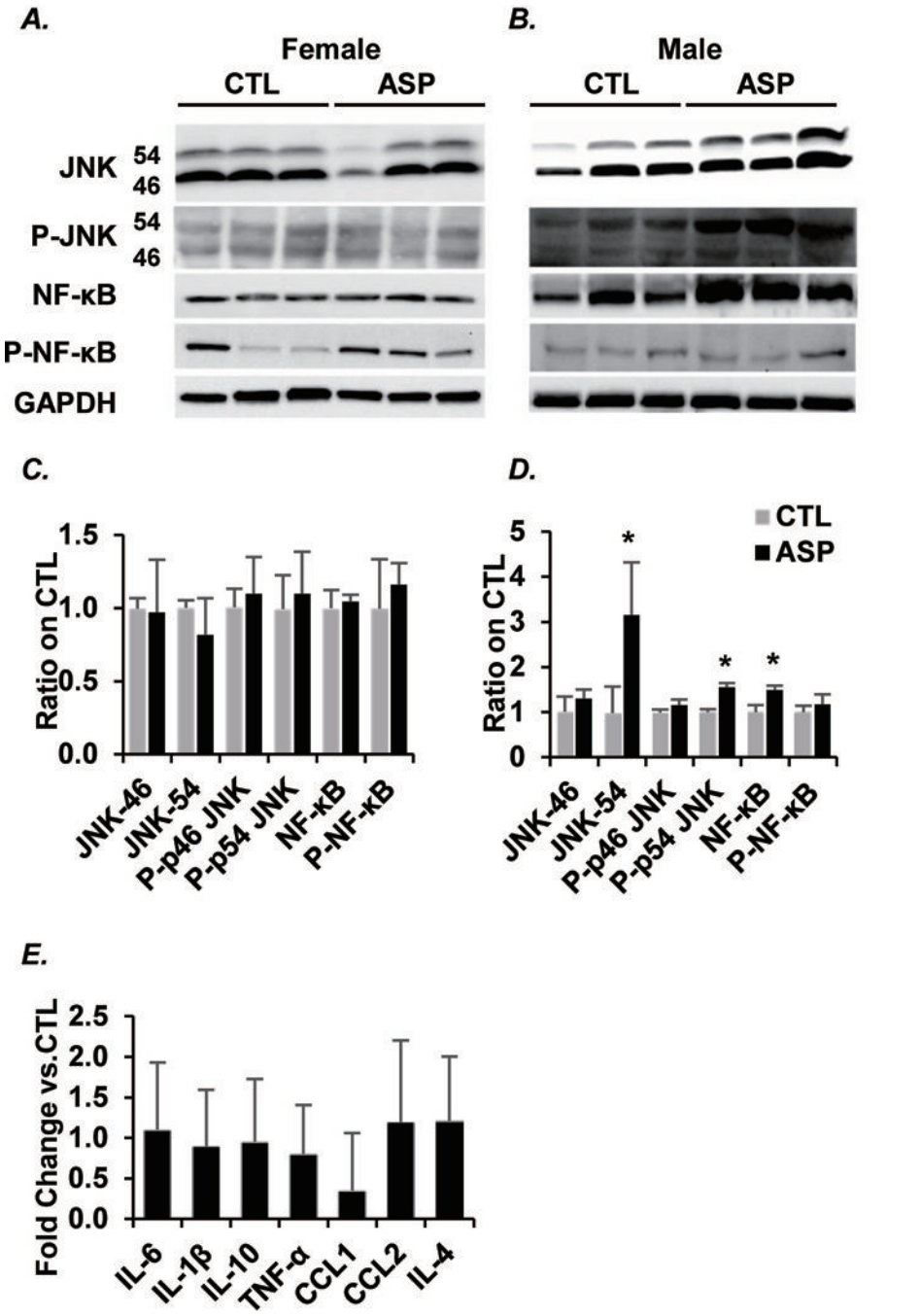

Figure 5. Aspirin treatment enhanced activity of JNK signaling in male liver.

6A-B. JNK, phosphorylation of JNK, NF-κB, and phosphorylation of NF-κB in liver tissue were detected by western blots in female (A) and male mice (B).

6C-D. Relative amounts were expressed as the ratios of JNK /GAPDH, Phospho-JNK / GAPDH, NF-κB /GAPDH, Phospho-NF-κB /GAPDH in female (C) and male mice (D).

6E. *IL-6*, *IL-1β*, *IL-10*, *TNF-α*, *CCL-1*, *CCL-2* and *IL-4* were measured for their expression level in liver by realtime-PCR in male (E).

Data is presented as Mean ± SD n=4–6. Significance ($P<0.05$) is presented between two groups.

* $P<0.05$ vs. CTL group.

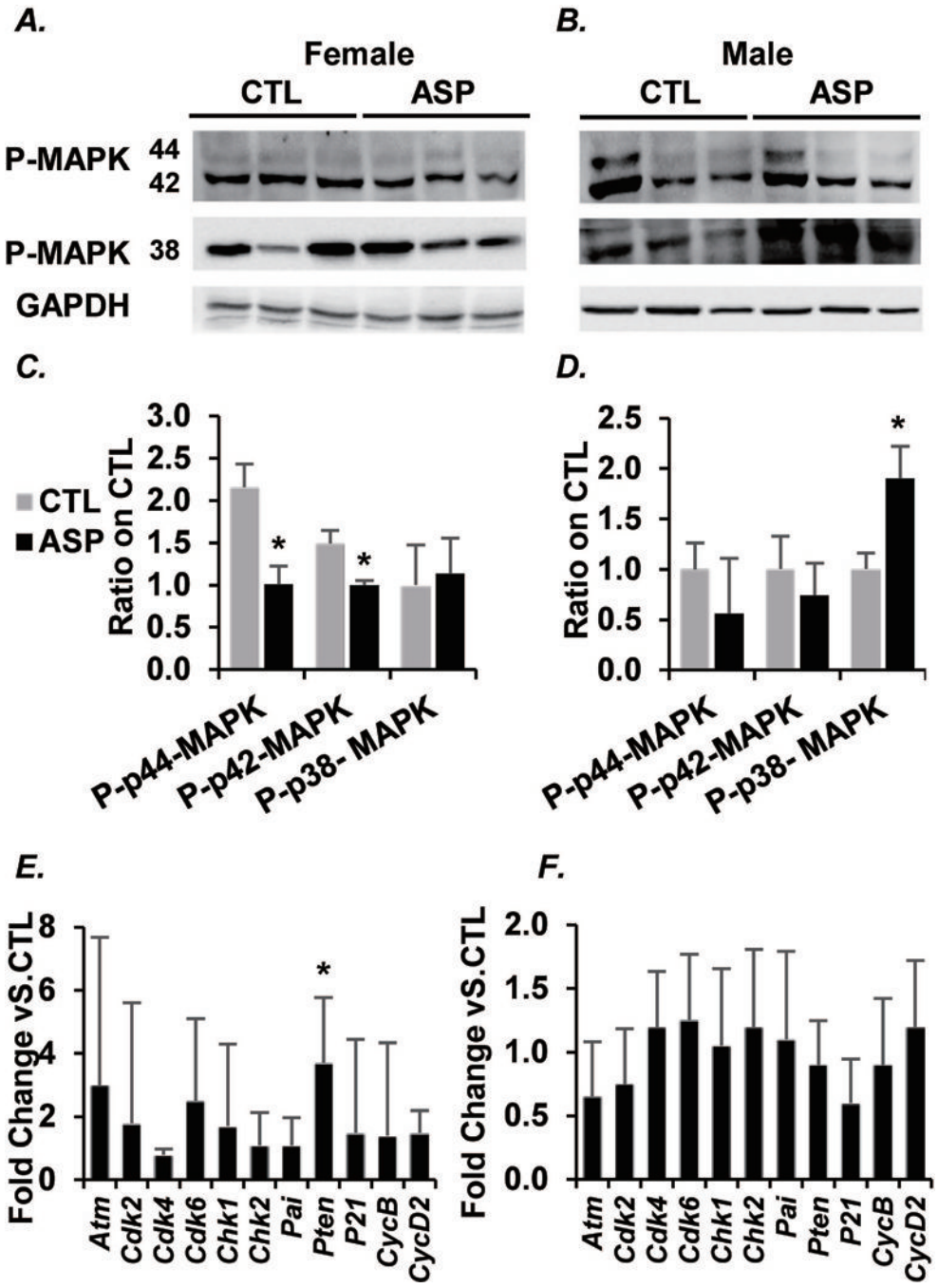

Figure 6. Aspirin treatment depressed hepatic p44/42 MAPK signaling in female and enhanced p38 MAPK signaling in male.

6A-B. Phosphorylation of p44/42 MAPK and phosphorylation of p38 MAPK in liver tissue were detected by western blots in female (A) and male mice (B).

6C-D. Relative amounts were expressed as the ratios of Phospho-p44/42 MAPK /GAPDH and Phospho-p38 /GAPDH in female (C) and male mice (D).

6E-F. *Atm, Cdk2, Cdk4, Cdk6, Chk1, Pal, Pten, P21, CycB* and *CycD2* were measured for their expression level in liver by realtime-PCR in female (E) and male mice (F).

Data is presented as Mean ± SD, n=4–6. Significance (P<0.05) is presented between two groups.

* P<0.05 vs. CTL group.

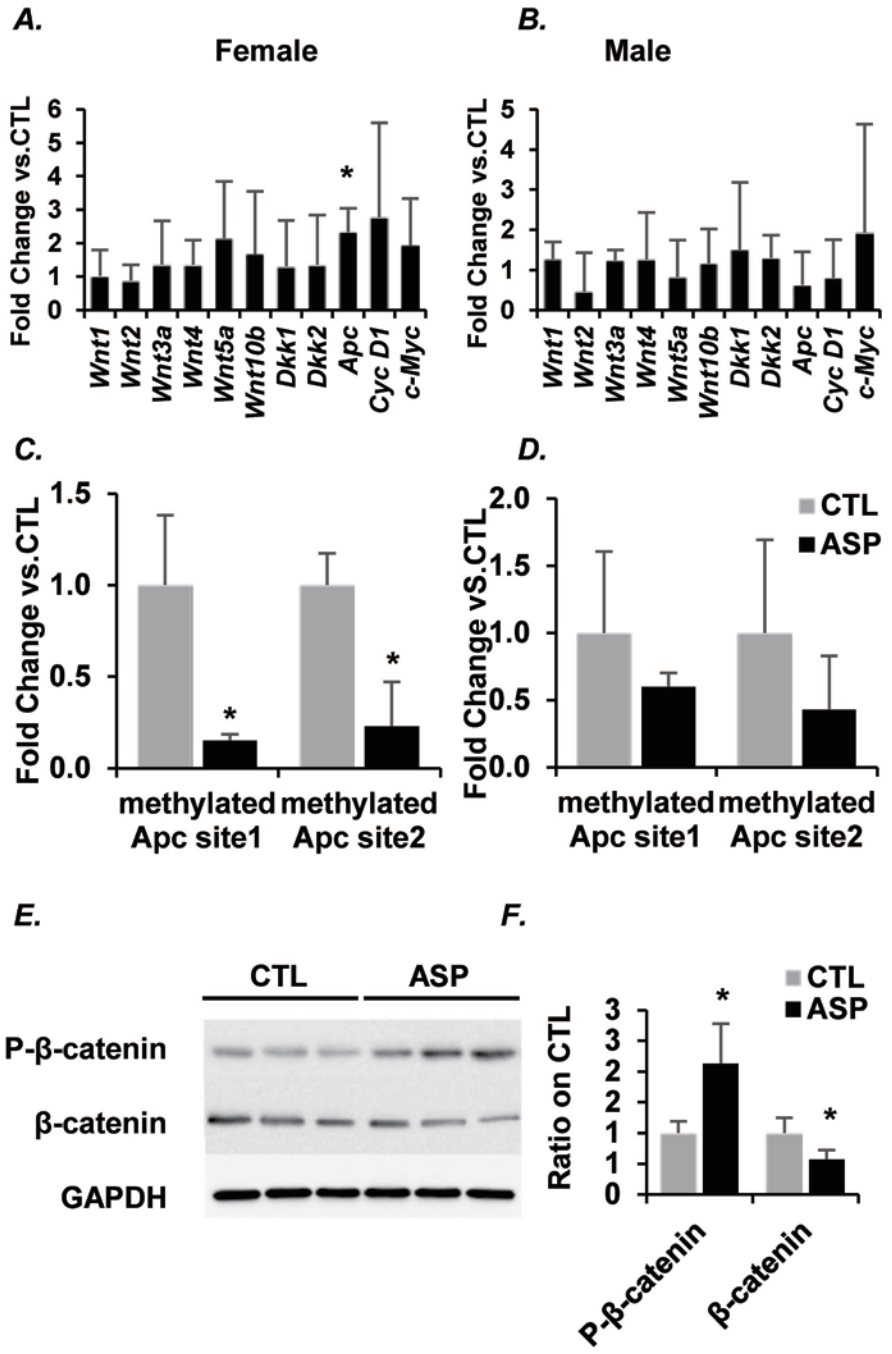

Figure 7. Aspirin treatment enhanced Apc expression in female liver via decreased DNA methylation at the CpG site of Apc promoter region

7A-B.Hepatic expressions of Wnt-signaling related genes were measured by real-time PCR in female (A) and male mice (B).

7C-D. Methylation status of the two CpG sites within the regulatory region of the *Apc* gene was measured by MSP in female (C) and male mice (D).

7E-F.β-Catenin, phospho -β-Catenin (Ser33/37/Thr41) in liver tissue were detected by western blots in female (E). Relative amounts were expressed as the ratios of β-Catenin / GAPDH, phospho -β-Catenin (Ser33/37/Thr41) in female (F).

Data is presented as Mean ± SD, n=4–6. Significance (P<0.05) is presented between two groups.

* P<0.05 vs. CTL group.

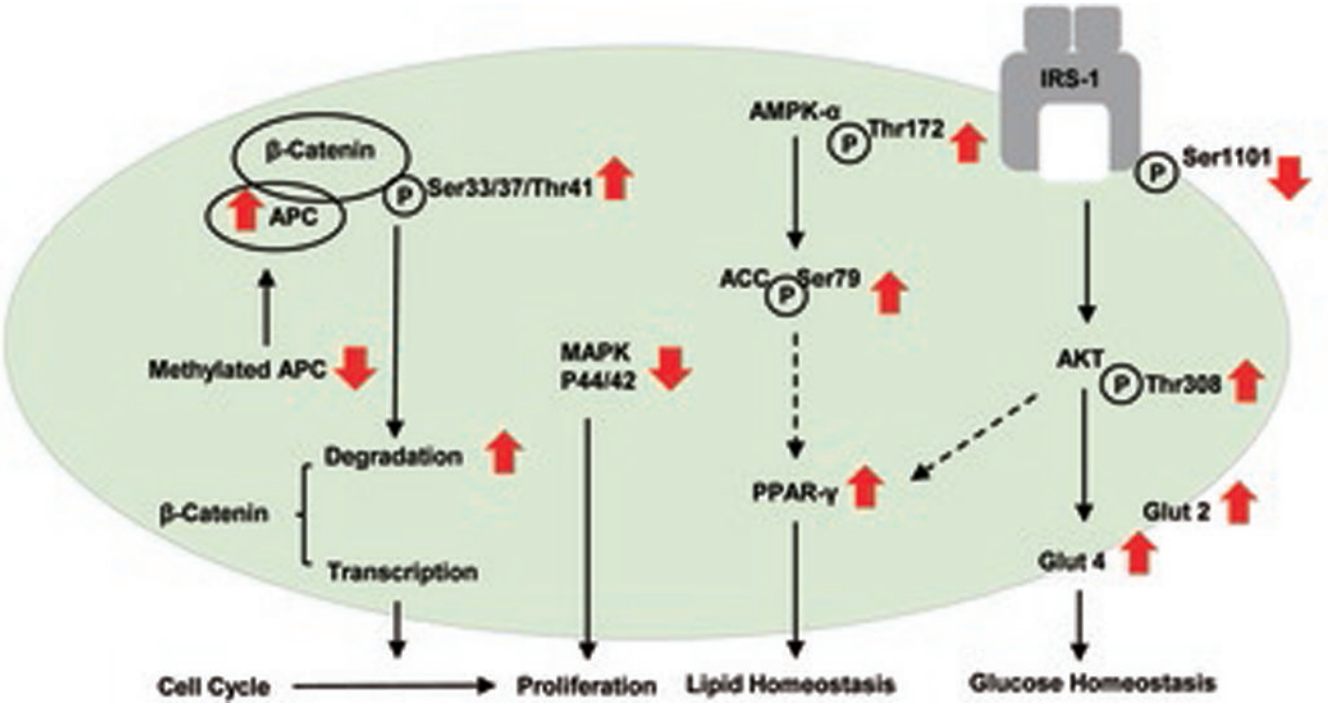

Figure 8. Schematic mechanisms how low-dose aspirin prevented NAFLD progression.

A long-term and low-dose of aspirin treatment in female mice re-sensitized the Insulin/Akt signaling via reducing phosphorylation at Ser1101 of IRS-1 and hyperphosphorylation at Thr308 of Akt, and increased the glucose homeostasis via enhanced expression of hepatic Glut4, Glut2, and hepatic PPAR-γ. In female, aspirin contributed to maintain lipid homeostasis via promoting lipid catabolism through activating AMPK signaling and enhancing PPAR-γ. Aspirin treatment suppressed the cell proliferation by inhibiting the MAPK signaling via hypophosphorylation of p44/42 MAPK in liver and upregulating the *Apc* gene expression via hypomethylation of CpG island located 185 bp upstream of the *Apc* start codon. Overexpression of Apc further inhibited Wnt signaling by hyperphosphorylating β-catenin at Ser33/37/Thr41 to form an Apc/Axin/β-catenin complex, which leads to β-catenin degradation.

Table 1

Primer sets used for PT-PCR

	Forward(5'-3')	Reverse(5'-3')
Glut4	GTGACTGGAACACTGGTCCTA	CCAGCCACGTTGCATTGTAG
PPAR-γ	CCTGAAGCTCCAAGAATACCAAA	AGAGTTTTTCAGAATAATAAGG
IL-6	TGCCTTCTTGGGACTGAT-GC	TGGGAGTGGTATCCTCTG-TGA
IL-1β	AAATACCTGTGGCCTTGGGC	CTTGGGATCCACACTCTCCAG
IL-10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
TNF-α	TAGCCAGGAGGGAGAACAGA	TTTTCTGGAGGGAGATGTGG
CCL1	GGCTGCCGTGTGGATACAG	AGGTGATTTTGAACCCAC-GTTT
CCL2	TTAAAAACCTGGATCGGA-ACCAA	GCATTAGCTTCAGATTTA-CGGGT
IL-4	CGCAGCTCTAGGAGCATGTG	TGCCTGCTCTTACTGACT-GG
Atm	CAGGCACAACCCTTGGTCC	TGCAGGTGTGAATCAACA-TGAC
Cdk2	CCTGCTTATCAATGCAGAGGG	CCTGCTTATCAATGCAGAGGG
Cdk4	ATGGCTGCCACTCGATATGAA	TCCTCCATTAGGAACTCTCACAC
Cdk6	GGCGTACCCACAGAAACCATA	AGGTAAGGGCCATCTGAAAACT
Chk1	GTTAAGCCACGAGAATGTAGTGA	GATACTGGATATGGCCTTCCCT
Chk2	TGACAGTGCTTCCTGTTCACA	GAGCTGGACGAACCCTGATA
Pai	TTCAGCCCTTGCTTGCCTC	ACACTTTTACTCCGAAGTCGGT
Pten	TTTGCTAGTGAGTGGAATCCTCT	TGTGACAAAAGTGACACAGATCA
P21	CCTGGTGATGTCCGACCTG	CCATGAGCGCATCGCAATC
CycB	AAGGTGCCTGTGTGTGAACC	GTCAGCCCCATCATCTGCG
CycD2	CGCACAGAGCGATGAAGGT	CGCACAGAGCGATGAAGGT
Wnt2	CTGATGTAGACGCAAGGGGG	CCTGTAGCTCTCATGTACCACC
Wnt3a	TCTGGTGGTCCTTGGCTGTG	GGGCATGATCTCCACGTAGTT
Wnt4	AGACGTGCGAGAAACTCAAAG	GGAACTGGTATTGGCACTCCT
Wnt5a	CAACTGGCAGGACTTTCTCAA	CATCTCCGATGCCGGAACT
Wnt10b	GAAGGGTAGTGGTGAGCAAGA	GGTTACAGCCACCCCATTCC
Dkk1	CCAACGCGATCAAGAACCTG	GGTAGGGCTGGTAGTTGTCA
Dkk2	CTGATGCGGGTCAAGGATTCA	CTCCCCTCCTAGAGAGGACTT
c-Myc	GGTGTCTGTGGAGAAGAGGC	TTGTGCTGGTGAGTGGAGAC
Apc	TCCGAGTCCTTCATCTTTTGGA	CTTGTGGCCCAGTTAAAATCTGA
CycD1	GCGTACCCTGACACCAATCTC	CTCCTCTTCGCACTTCTGCTC
B2M	TTCTGGTGCTTGTCTCAC-TGA	CAGTATGTTCGGCTTCCC-ATTC

Table 2

Primer sets used for methylation-specific PCR (MSP)

	Forward(5'-3')	**Reverse(5'-3')**
Apc-m1	GTAGAGGTAGGGTATAGGTTGTTGC	CCATCTTATTAAAAACGAAAAACGA
Apc-u1	TAGAGGTAGGGTATAGGTTGTTGTG	CATCTTATTAAAAACAAAAAACAAA
Apc-m2	GAGTGTGGTTGTCGGAAATTC	CAAAAAAACGTACATAAAAAACGCT
Apc-u2	TGTGGTTGTTGGAAATTTGG	CAAAAAAACATACATAAAAAACACT

Definition of abbreviations: m=methylated; u=unmethylated.