



## The sex steroid precursor dehydroepiandrosterone prevents nonalcoholic steatohepatitis by activating the AMPK pathway mediated by GPR30

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### ABSTRACT

The prevalence of nonalcoholic steatohepatitis (NASH) caused by estrogen deficiency increased sharply in recent decades and has become a major threat to liver health in postmenopausal women. There is no effective strategy to control the incidence and development of NASH. Dehydroepiandrosterone (DHEA) is the most abundant circulating steroid with immune and metabolic regulatory properties, and its level markedly declines with increasing age in humans. Importantly, DHEA can convert into active sex hormones depending on the local needs of target tissues with little diffusion, which serves to avoid systemic side-effects from other tissues' exposure to estrogen. Here, we found that DHEA prevented the incidence and development of NASH, which is characterized by the reduction of hepatic steatosis, fibrosis, and inflammation in female mice fed with high-fat/high-cholesterol diets and effectively attenuated lipid accumulation, inflammatory response, and oxidative stress in palmitic acid-challenged hepatocytes. Mechanistically, *in vitro* and *in vivo* studies showed that the anti-NASH function of DHEA depended on its biotransformation into estrogen rather than androgen, and which up-regulates the expression of G protein-coupled estrogen receptor (GPR30), a non-classical estrogen receptor. The activation of GPR30-mediated AMP-activated protein kinase signaling is a necessary prerequisite for the alleviative effects of DHEA on NASH. Collectively, our data show the mechanisms of DHEA treatment and its effects on NASH that were previously overlooked; the data also show that GPR30 can be used as a target for treating lipid metabolism disorders and related diseases, such as NASH. Furthermore, these findings have the potential to help researchers develop new strategies for preventing NASH in postmenopausal women.

### 1. Introduction

With the continuous improvement of living standards and the change in diet, the incidence of nonalcoholic fatty liver disease (NAFLD) has increased, ranging from simple fatty liver (SFL) to nonalcoholic steatohepatitis (NASH), and became the most common and complex chronic liver disease, affecting 25% of adults globally [1]. In general, NASH, which is characterized by hepatic steatosis, chronic inflammation, oxidative stress, and fibrosis in hepatocytes and is caused by the uncontrolled development of SFL, and it is a precursor of numerous serious liver diseases, including hepatocellular carcinoma and cirrhosis [2]. Interestingly, there is a significant gender distinction in the severity and morbidity of NASH. Men are more likely to develop severe

fibrosis in NASH than premenopausal women. The risks of developing liver fibrosis from NASH in postmenopausal women and men are similar [3,4]. More importantly, the incidence of NASH increased markedly with advanced age in women but not in men, due to insufficient estrogen secretion in postmenopausal women. The loss of estrogen in ovariectomized (OVX) or aromatase-deficient animal models can induce fatty liver and insulin resistance [5], which can be improved by supplementing estrogen [6,7]. Clinical trials have found that the incidence and development of NAFLD/NASH is the main side effect of tamoxifen, a selective estrogen receptor modifier, in the treatment of breast cancer [8]. These data suggest that maintaining a certain level of endogenous hormones, especially estrogens, can prevent NASH and related metabolic diseases in postmenopausal women.

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Currently, there is no effective clinical measure for the prevention of NAFLD/NASH; this seriously affects the physical and mental health of postmenopausal women. In clinical trials, improving estrogen deficiency may be a key approach to protecting liver health in postmenopausal women. In the previous studies, researchers mainly focused on the effects and potential mechanisms of exogenous estrogen that help prevent NAFLD/NASH in postmenopausal women; however, the exogenous estrogen will inevitably cause endocrine disorder and increase the incidence of gynecologic cancer [9]. Therefore, we must quickly find and implement a new strategy for maintaining the balance of estrogens in postmenopausal women.

Dehydroepiandrosterone (DHEA) is the most abundant circulating steroid hormone in humans. It is mainly secreted in the adrenal glands, and it can be rapidly converted into active steroid hormones (androgens and estrogens) in target tissues, such as the liver, brain, and ovaries under the catalysis of a related enzymatic system. Widely known as a multidirectional “hormone buffer”, DHEA plays an irreplaceable role in maintaining steroid hormone balance in the body [10,11]. Previous studies have reported the beneficial effects of DHEA in preventing numerous metabolic diseases, such as obesity [12] and diabetes mellitus [13], and those findings have led researchers to focus on the relationship between DHEA levels and human metabolic diseases. At present, DHEA is an over-the-counter drug and dietary supplement approved by the FDA to prevent and treat diseases related to metabolism in the United States. Although epidemiological researchers have found that the decrease of DHEA levels is strongly linked to NAFLD/NASH [14] and type 2 diabetes [15], there is no direct evidence in support of the effects of DHEA in the prevention of NAFLD/NASH in animal models or clinical trials.

The positive role of DHEA in regulating lipid metabolism has been confirmed, and our recent study has shown that DHEA protects against high-fat diet-induced hepatic glycolipid metabolic disorder and insulin resistance via the activation of AMPK-PGC-1 $\alpha$ -NRF-1 and the IRS1-AKT-GLUT2 signaling pathways [16]. To date, the incidence and development of NAFLD/NASH have been summarized using the “two-hit hypothesis”; lipid metabolism disorder plays a key role in the formation of NAFLD/NASH as the first hit [17]. Therefore, we speculated that DHEA can prevent the incidence and development of NAFLD. DHEA levels decline markedly with advanced age and lead to a significant decrease in sex hormones, accompanied by an increase in the prevalence of coronary heart disease and type 2 diabetes [18]. DHEA undergoes biotransformation in target tissues, and based on this characteristic, most researchers’ proposed indirect mechanisms for DHEA in which it exert beneficial biological effects [11]. Our previous study found that DHEA reduced lipid droplet accumulation mainly through conversion into downstream active sex hormones in primary hepatocytes [19], which suggests that the lipid-lowering effect of DHEA may also be closely linked to its biotransformation. Furthermore, the biotransformation of DHEA depends on the local needs of the target tissues; the synthesized active steroids hormones exert their effects on the target cells with little diffusion, and this mechanism serves to prevent the risk of unnecessary side effects for other tissues’ exposure to androgens or estrogens in the body [20]. Thus, we hypothesized that DHEA can be used as an effective agent in preventing the incidence and development of NAFLD/NASH caused by the decrease of estrogen levels, and the beneficial regulatory effects of DHEA may have played a key role in its conversion into downstream active steroid hormones in humans, especially in postmenopausal women.

Besides the well-established role in the activation of classical androgen receptors or estrogen receptors [11,21], DHEA can also exert its effects by binding to the recently identified non-classical steroid receptors G protein-coupled receptors (GPR30 or GPER) [22]. In recent years, GPR30 was discovered to have been expressed in a wide variety of tissues and cell types, and lipid lowering functions mediated by GPR30 have been reported [23], which suggests that GPR30 could be a potential therapeutic target in diseases related to lipid metabolism. However,

the relationship between GPR30 and NAFLD/NASH is unclear, and whether GPR30 plays a critical role in the regulatory effect of DHEA on the incidence and development of NAFLD/NASH still needs further exploration.

In this study, we aimed to evaluate the mechanisms and protective effects of DHEA on lipid accumulation, oxidative stress, and inflammatory response in female mice with NASH induced by high-fat/high-cholesterol (HFHC) diets, or in palmitic acid (PA)-induced BRL-3A and HepG2 cells. The data clearly showed that DHEA prevented the incidence and development of NASH, presented as a decrease in the incidence of hepatic steatosis, fibrosis, and inflammation in female mice fed with an HFHC diet and effectively attenuated the lipid accumulation, inflammatory response, and oxidative stress in PA-challenged hepatocytes. In addition, *in vivo* and *in vitro* studies strongly suggested that DHEA converts into estradiol rather than testosterone and is a prerequisite for DHEA-mediated prevention of NASH. Furthermore, *in vivo* and *in vitro* studies have indicated that DHEA protects against the incidence and development of NASH through activating the AMPK pathway mediated by GPR30. Our study first revealed that DHEA is an effective agent in preventing the incidence and development of NASH, and suggested that GPR30 can be used as a promising therapeutic target for diseases related to lipid metabolism disorder, such as NASH, but more importantly, a new strategy for preventing NAFLD/NASH in postmenopausal women could be developed based on these findings.

## 2. Materials and Methods

### Antibodies and Reagents.

### Animals.

### Cell lines.

### Western blot.

### Real-time quantitative PCR.

### Calculation of organ indexes.

### Transfection of HepG2 cells.

### Sex hormone analyses.

### Cyclic AMP measurement.

### Cytokine measurement.

### Oil Red O staining.

### Nile Red staining.

Intracellular reactive oxygen species (ROS) and mitochondrial ROS assays.

### Intracellular lipid analysis.

### Hepatic lipids analysis.

### Serum assays.

### Transmission electron microscopy.

### Immunofluorescence staining.

### Immunohistochemistry.

### Histopathologic analysis.

### Statistical analysis.

All information regarding Materials and Methods are provided in Supplementary Material.

## 3. Results and discussion

### 3.1. DHEA ameliorates NASH induced by HFHC diets in female mice

Previous studies have clarified that the incidence and development of NAFLD/NASH are linked to obesity, hepatic steatosis, chronic inflammation, and oxidative stress [24], which suggests that alleviating abnormal lipid accumulation, oxidative stress, and inflammatory response are effective strategies for preventing NAFLD/NASH. Despite DHEA’s ability to prevent obesity, reduced lipid accumulation, reduce oxidative stress, and reduce inflammation, as demonstrated in different animal models *in vivo* and *in vitro*, reported studies on the effects of DHEA on NAFLD/NASH are few, especially estrogen deficiency-induced NAFLD/NASH in postmenopausal women.

On the basis that DHEA plays a positive role in regulating lipid metabolism and plays a key role in the incidence and development of NALFD, NASH, and lipid metabolism disorders, the current study first investigated the potential benefits of DHEA treatment on NASH, which is induced by an HFHC diet administered by oral gavage to female mice for 16 weeks [25]. The dose gradient of DHEA was selected as 10 mg/kg, 20 mg/kg, and 40 mg/kg every 2 days, which were referenced together from previous reports on mice [26] or humans [12] (Fig. S1A), and the representative mice in different treatment groups were illustrated in Fig. S1B. Treatment with DHEA at doses of 20 mg/kg and 40 mg/kg every 2 days markedly decreased the body weight, liver weight, and liver index in female mice fed with HFHC diets, while no significant differences were observed in the above parameters in normal chow (NC)-fed female mice by treatment with DHEA at a dose of 40 mg/kg every 2 days (Fig. 1A). Furthermore, an HFHC-induced increase in hepatic lipid (TG, TC, and NEFA) contents were alleviated by treatment with DHEA at 20 mg/kg and 40 mg/kg every 2 days (Fig. 1B). Meanwhile, treatment with DHEA at 20 mg/kg and 40 mg/kg every 2 days dramatically lowered serum lipid (TG, TC, and LDL-C) contents and white adipose mass in female mice fed with HFHC diets, whereas the treatment markedly increased the HDL-C content (Figs. S1C and D). Treatment with DHEA at 20 mg/kg and 40 mg/kg every 2 days mitigated the pathological features of NASH, including hepatic steatosis, liver fibrosis (marked with arrow), circulating inflammatory response, and inflammatory cell infiltration into the liver (marked with arrow) in mice fed with HFHC diets, which is consistent with the above results (Fig. 1C and D; Fig. S1E). RT-qPCR assays showed that DHEA at 20 mg/kg and 40 mg/kg every 2 days of treatment downregulated the mRNA expression levels involved in pro-inflammatory cytokines (*TNF- $\alpha$* , *IL-1 $\beta$* , *IL-6*, and *iNOS*) and fibrosis markers ( *$\alpha$ -SMA*, *TGF- $\beta$ 1*, *Collagen I*, and *Collagen III*) in the livers of female mice fed with HFHC diets (Fig. 1E and F). HFHC-induced adverse changes of proteins involved in the inhibition of AMPK signaling, fatty acid oxidation, and activation of fatty acid synthesis, MAPK signaling, NF- $\kappa$ B signaling, and pro-inflammatory cytokines were markedly attenuated by treatment with DHEA at 20 mg/kg and 40 mg/kg every 2 days in the livers of female mice fed with HFHC diets (Fig. 1G; Fig. S1F). Meanwhile, immunofluorescence results further confirmed that treatment with DHEA at 20 mg/kg and 40 mg/kg every 2 days alleviated the inhibition of AMPK activity in female mice fed with HFHC diets (Fig. S1G). In accordance with the above results, the increase of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) levels induced by HFHC diets were markedly reduced by treatment with DHEA at 20 mg/kg and 40 mg/kg every 2 days (Fig. 1H). These findings indicate that DHEA alleviates NASH-associated steatosis, fibrosis, and inflammatory response in female mice fed with an HFHC diets.

We further evaluated the possibility of toxicity of DHEA in female mice. Notably, no significant differences were observed in the food and energy intake between the HFHC-Vehicle group and the HFHC-DHEA group (Fig. S1I). Treatment with DHEA illustrated a negligible effect on the histological features and function of major organs (heart, lungs, and kidneys) after female mice were fed with an HFHC diet for 16 weeks, as evidenced by organ indexes, marker enzyme activity, and H&E staining (Fig. S1H; Table S2). Interestingly, we found that the content of estradiol (E2) exhibited a clearer tendency to drop in female mice fed with an HFHC diets than the content in the group fed with NC diets, while DHEA at doses of 20 mg/kg and 40 mg/kg every 2 days increased serum E2 content rather than testosterone (T) (Fig. S1J). Therefore, administration of DHEA at 20 mg/kg and 40 mg/kg every 2 days is safe and effective when used to protect against NASH in female mice fed with an HFHC diets. Together, the above data strongly suggest that DHEA treatment is capable of reducing the major pathological features of NASH, including steatosis, inflammation, and fibrosis in female mice fed with HFHC diets.

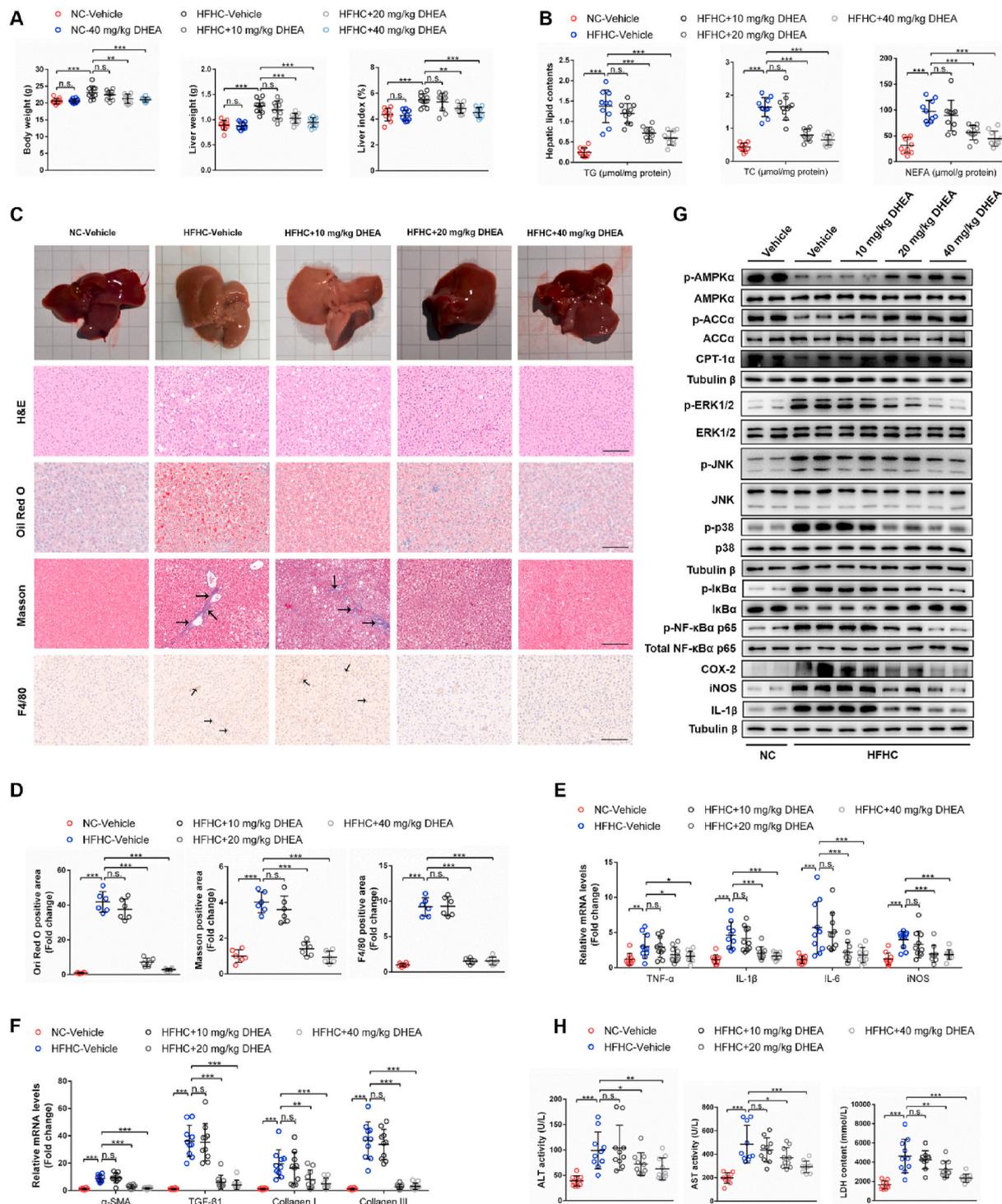
### 3.2. DHEA prevents lipid accumulation, oxidative stress, and inflammation in PA-challenged hepatocytes

A previous study reported that numerous saturated and unsaturated fatty acids, including palmitic acid and oleic acid, can induce lipid metabolism disorder, increase oxidative stress, and increase inflammation in hepatocytes [25,27]. To explore the molecular mechanisms of the anti-NASH effects of DHEA, we first established a model of PA-challenged abnormal lipid droplet accumulation, inflammatory response, and oxidative stress in BRL-3A and HepG2 cells. Nile Red and Oil Red O staining, and the transmission electron microscope (TEM) all showed that both BRL-3A and HepG2 cells treated with PA increased lipid droplet accumulation (Figs. S2A and C). In line with these results, PA treatment also induced oxidative stress in BRL-3A and HepG2 cells, as evidenced by the staining of DCFH-DA and MitoSOX Red probes (Figs. S2B and D). Furthermore, PA-induced adverse changes of protein were involved in the inhibition of AMPK signaling and fatty acid oxidation, and activation of fatty acid synthesis, MAPK signaling, NF- $\kappa$ B signaling, and pro-inflammatory cytokines in both BRL-3A and HepG2 cells (Fig. S2E). These data indicated that PA induced lipid accumulation, inflammation, and oxidative stress in hepatocytes.

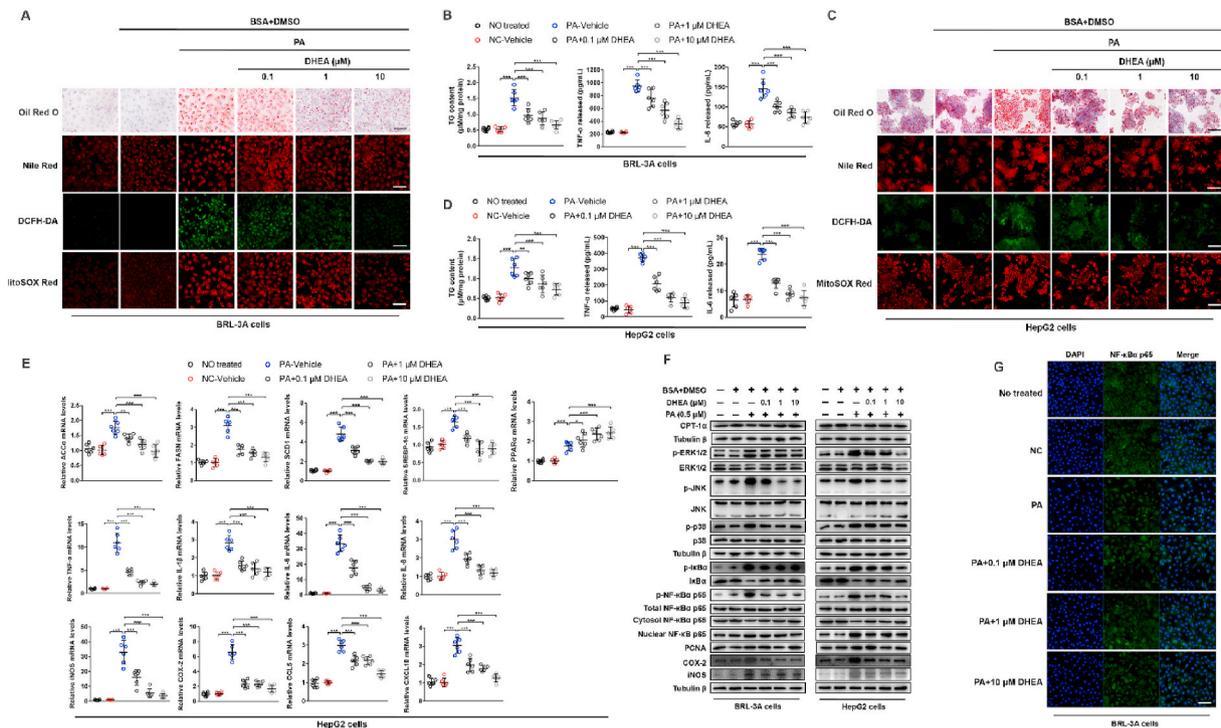
Using the model of PA-challenged hepatocytes, we evaluated the protective effects of DHEA against lipid accumulation, inflammation, and oxidative stress *in vitro*. The result of the experiment *in vitro* corresponded to the results of the experiment *in vivo*, and 0.1–10  $\mu$ M DHEA treatment dose-dependently alleviated PA-induced lipid accumulation, oxidative stress, and inflammatory response in hepatocytes (Fig. 2A–D). As expected, the upregulation of genes involved in fatty acid synthesis genes (*ACCA*, *FASN*, *SCD1*, and *SREBP-1c*) and inflammatory response (*TNF- $\alpha$* , *IL-1 $\beta$* , *IL-6*, *IL-8*, *iNOS*, *COX-2*, *CCL5*, and *CXCL10*), and the downregulation of gene related to fatty acid oxidation (*PPAR $\alpha$* ) caused by PA were markedly diminished by DHEA treatment in HepG2 cells (Fig. 2E). Furthermore, western blotting further confirmed that PA-induced adverse changes of proteins involved in the inhibition of AMPK signaling and fatty acid oxidation, and activation of fatty acid synthesis, MAPK signaling, NF- $\kappa$ B signaling, and pro-inflammatory cytokines were obviously reversed by treatment with DHEA in BRL-3A and HepG2 cells (Fig. 2F; Fig. S3). Immunofluorescence analysis also confirmed that DHEA blocked nuclear translocation of NF- $\kappa$ B in PA-stimulated BRL-3A cells (Fig. 2G). These data strongly suggest that DHEA effectively alleviates lipid accumulation, oxidative stress, and inflammation in PA-challenged hepatocytes.

### 3.3. DHEA relieves NASH, depending on its biotransformation

Both estrogens and androgens are widely known to alleviate NAFLD/NASH [4]. Given the obvious increase of serum E2 levels caused by DHEA in female mice fed with HFHC diets, we inferred that the protective effect of DHEA against NASH may have been associated with its biotransformation. In this study, DHEA significantly increased the contents of T and E2 in PA-stimulated HepG2 cells in a dose-dependent manner (Fig. S4), which suggests that DHEA can be converted into active steroid hormones in hepatocytes. The results are consistent with our recent report, which shows that DHEA regulates lipid metabolism through conversion into downstream active sex hormones in chicken hepatocytes [19]. In addition, a previous study demonstrated that the biotransformation of DHEA to estrogen exhibited a beneficial effect on skin wound healing [11]. As the precursor of sex steroids, DHEA can be rapidly metabolized into androstenedione under the catalysis of 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD) in peripheral target tissues such as tissues in the liver, gonads, and brain; this is the initial rate-limiting step of DHEA's metabolism [28–30]. To explore the beneficial actions of DHEA against NASH, whether they are associated with the biotransformation of DHEA to downstream metabolites, we pretreated BRL-3A cells with the 3 $\beta$ -HSD inhibitor trilostane. We found that the alleviating actions of DHEA on PA-induced lipid metabolism,



**Fig. 1.** DHEA prevents the incidence and development of NASH in female mice fed with HFHC diets (A) Body weight, liver weight, and liver index. n = 10 mice per group. (B) Hepatic lipid (TG, TC, and NEFA) contents. n = 10 mice per group. (C) Representative images of liver overall morphology and liver sections stained with H&E, Oil Red O, Masson, and F4/80. n = 6 mice per group. Scale bars, 100 μm. (D) Quantitative results of Oil Red O, Masson, and F4/80 staining illustrated in (C). n = 6 mice per group. (E) RT-qPCR analysis the transcript levels of liver inflammatory-related genes (*TNF-α*, *IL-1β*, *IL-6*, and *iNOS*). n = 10 mice per group. (F) RT-qPCR analysis of the transcript levels of liver fibrosis-related genes (*α-SMA*, *TGF-β1*, *Collagen I*, and *Collagen III*). n = 10 mice per group. (G) Western blotting analyzed the protein levels involved in AMPK signaling (p-AMPKα and AMPKα), lipid metabolism (p-ACCα, ACCα, and CPT-1α), MAPK signaling (p-ERK1/2, ERK1/2, p-JNK, JNK, p-p38, and p38), NF-κB signaling (p-NF-κBα p65, NF-κBα p65, p-IκBα, and IκBα), and inflammatory factors (COX-2, iNOS, and IL-1β). Tubulin β served as a loading control. (H) Serum ALT, AST, and LDH levels. n = 10 mice per group. Data were presented as the means ± SD. \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001; n.s., no significance, p ≥ 0.05 (one-way ANOVA). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



**Fig. 2.** DHEA alleviates the lipid accumulation, oxidative stress, and inflammation in PA-induced hepatocytes

(A) Oil Red O staining (scale bars, 50  $\mu\text{m}$ ) and Nile Red staining (scale bars, 100  $\mu\text{m}$ ) analysis the accumulation of lipid droplets; ROS (DCFH-DA probe) staining (scale bars, 100  $\mu\text{m}$ ) illustrating ROS levels; and mtROS (MitoSOX Red probe) staining (scale bars, 100  $\mu\text{m}$ ) illustrating mtROS levels in BRL-3A cells treated with DMSO or DHEA (0.1–10  $\mu\text{M}$ ) prior to BSA or 0.5 mM PA-stimulated.

(B) The contents of TG, TNF- $\alpha$ , and IL-6 in BRL-3A cells. n = 6 per group.

(C) Oil Red O staining (scale bars, 50  $\mu\text{m}$ ) and Nile Red staining (scale bars, 200  $\mu\text{m}$ ) analysis the accumulation of lipid droplets; ROS (DCFH-DA probe) staining (scale bars, 100  $\mu\text{m}$ ) illustrating ROS levels; and mtROS (MitoSOX Red probe) staining (scale bars, 100  $\mu\text{m}$ ) illustrating mtROS levels in HepG2 cells treated with DMSO or DHEA (0.1–10  $\mu\text{M}$ ) prior to BSA or 0.5 mM PA-stimulated.

(D) The contents of TG, TNF- $\alpha$ , and IL-6 in HepG2 cells. n = 6 per group.

(E) RT-qPCR analysis of the mRNA levels of lipid metabolism-related factors (*ACC $\alpha$* , *FASN*, *SCD1*, *SREBP-1c*, and *PPAR $\alpha$* ) and inflammatory factors (*TNF- $\alpha$* , *IL-1 $\beta$* , *IL-6*, *iNOS*, *COX-2*, *CCL5*, and *CXCL10*) in HepG2 cells. n = 6 per group.

(F) Western blotting analyzed the protein levels that were involved in lipid metabolism (CPT-1 $\alpha$ ), MAPK signaling (p-ERK1/2, ERK1/2, p-JNK, JNK, p-p38, and p38), NF- $\kappa$ B signaling (p-NF- $\kappa$ B p65, NF- $\kappa$ B p65, cytosol NF- $\kappa$ B p65, nuclear NF- $\kappa$ B p65, p-I $\kappa$ B $\alpha$ , and I $\kappa$ B $\alpha$ ), and inflammatory factors (COX-2 and iNOS) in BRL-3A cells (left) or HepG2 cells (right). PCNA or Tubulin  $\beta$  served as the loading controls.

(G) Immunofluorescence analysis of the nuclear translocation of NF- $\kappa$ B p65 in BRL-3A cells. Scale bars, 100  $\mu\text{m}$ .

Data were presented as the means  $\pm$  SD. \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001 (one-way ANOVA). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

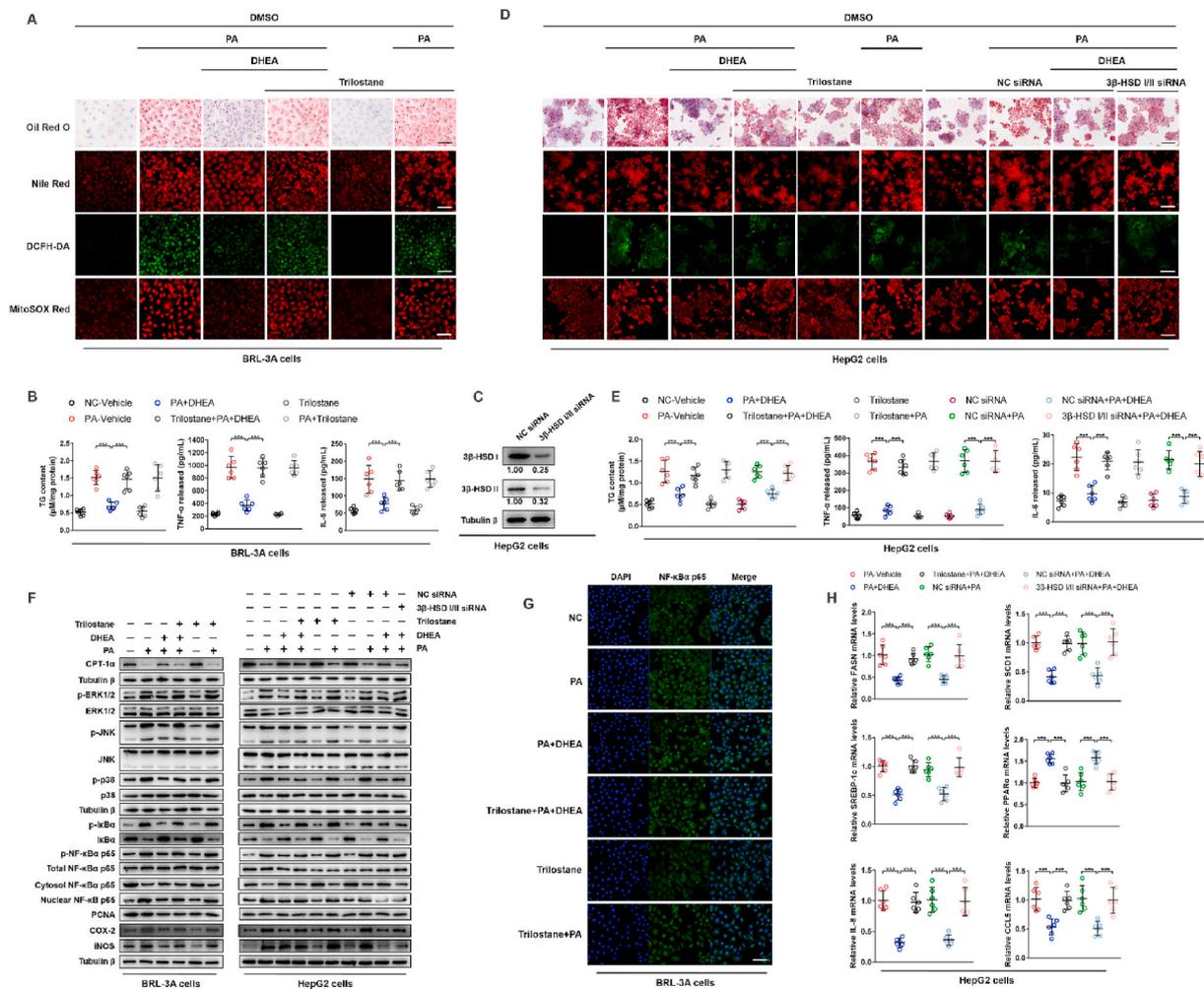
oxidative stress, and inflammatory response were completely reversed by pretreatment with trilostane, which is attributed to DEHEA's biotransformation in BRL-3A cells (Fig. 3A and B).

To confirm these results, we used the short interfering RNA (siRNA) of  $\beta$ -HSD (3 $\beta$ -HSD I/II siRNA) to inhibit the  $\beta$ -HSD protein expression in HepG2 cells (Fig. 3C). We observed that both trilostane and  $\beta$ -HSD I/II siRNA treatment abolished the protective effects of DHEA on PA-induced lipid metabolism, oxidative stress, and inflammatory response in HepG2 cells (Fig. 3D and E). Furthermore, the beneficial effects of DHEA on PA-stimulated expression of protein related to lipid metabolism and inflammation were completely blocked in hepatocytes by pretreatment with trilostane or  $\beta$ -HSD I/II siRNA (Fig. 3F; Fig. S5). Immunofluorescence analysis further confirmed that the down-regulation of DHEA on the transport of NF- $\kappa$ B from the cytoplasm to the nucleus depends on its biotransformation in BRL-3A cells (Fig. 3G). In line with the above results, DHEA-induced downregulation of genes involved in fatty acid synthesis and inflammatory response, and upregulation of fatty acid oxidation gene were completely reversed by pretreatment with trilostane and  $\beta$ -HSD I/II siRNA in PA-stimulated HepG2 cells (Fig. 3H). These results indicate that the conversion of DHEA into active steroid hormone exhibits a prerequisite for its inhibitive effects on the lipid accumulation, inflammatory response, and

oxidative stress in PA-stimulated hepatocytes.

As a metabolic intermediate of DHEA, androstenedione can rapidly convert into T in the presence of 17 $\beta$ -HSD, and which is metabolized into E2 under the catalysis of aromatase. To further certificate the anti-NASH effect of DHEA is related to androgens or estrogens, the aromatase inhibitor anastrozole was used to block the metabolism from T to E2. We found that the alleviating effects of DHEA on PA-induced lipid accumulation and oxidative stress were completely abolished by pretreatment with anastrozole (Figs. S6A–D). The reversing effect of anastrozole on DHEA-repression of lipid accumulation and oxidative stress was eliminated when the PA-stimulated hepatocytes were additionally treated with E2 simultaneously rather than T (Figs. S6A–D). Similar results also appeared in the gene expression closely related to lipid metabolism (Figs. S6E–J). These results strongly demonstrated that the suppressive effects of DHEA on PA-induced lipid accumulation, inflammation, and oxidative stress were acquired by its conversion into E2, rather than T in hepatocytes.

To further validate the findings *in vitro*, the female mice fed with HFHC diets were pretreated with anastrozole one week before gavage administration of DHEA or E2 (Fig. S7A). We found that anastrozole pretreatment obviously reversed the increasing effect of DHEA on E2 content in female mice fed with HFHC diets, whereas no significant



**Fig. 3.** DHEA alleviates the lipid accumulation, oxidative stress, and inflammatory response through conversion into downstream metabolites in PA-stimulated hepatocytes

(A) Oil Red O staining (scale bars, 50 μm) and Nile Red staining (scale bars, 100 μm) analysis the accumulation of lipid droplets, ROS (DCFH-DA probe) staining (scale bars, 100 μm) illustrating ROS levels, and mtROS (MitoSOX Red probe) staining (scale bars, 100 μm) illustrating mtROS levels in BRL-3A cells treated with DMSO or 10 μM DHEA prior to BSA or 0.5 mM PA-stimulated in the presence or absence of 5 μM trilostane (3β-HSD inhibitor).

(B) The contents of TG, TNF-α, and IL-6 in BRL-3A cells. n = 6 per group.

(C) Western blotting analysis 3β-HSD I and 3β-HSD II protein levels in HepG2 cells treated with NC siRNA or 3β-HSD I/II siRNA.

(D) Oil Red O staining (scale bars, 50 μm) and Nile Red staining (scale bars, 200 μm) analysis of the accumulation of lipid droplets; ROS (DCFH-DA probe) staining (scale bars, 100 μm) illustrating ROS levels; and mtROS (MitoSOX Red probe) staining (scale bars, 100 μm) illustrating mtROS levels in HepG2 cells treated with DMSO or 10 μM DHEA prior to BSA or 0.5 mM PA-stimulated in the presence or absence of 5 μM trilostane, NC siRNA, or 3β-HSD I/II siRNA.

(E) The contents of TG, TNF-α, and IL-6 in HepG2 cells.

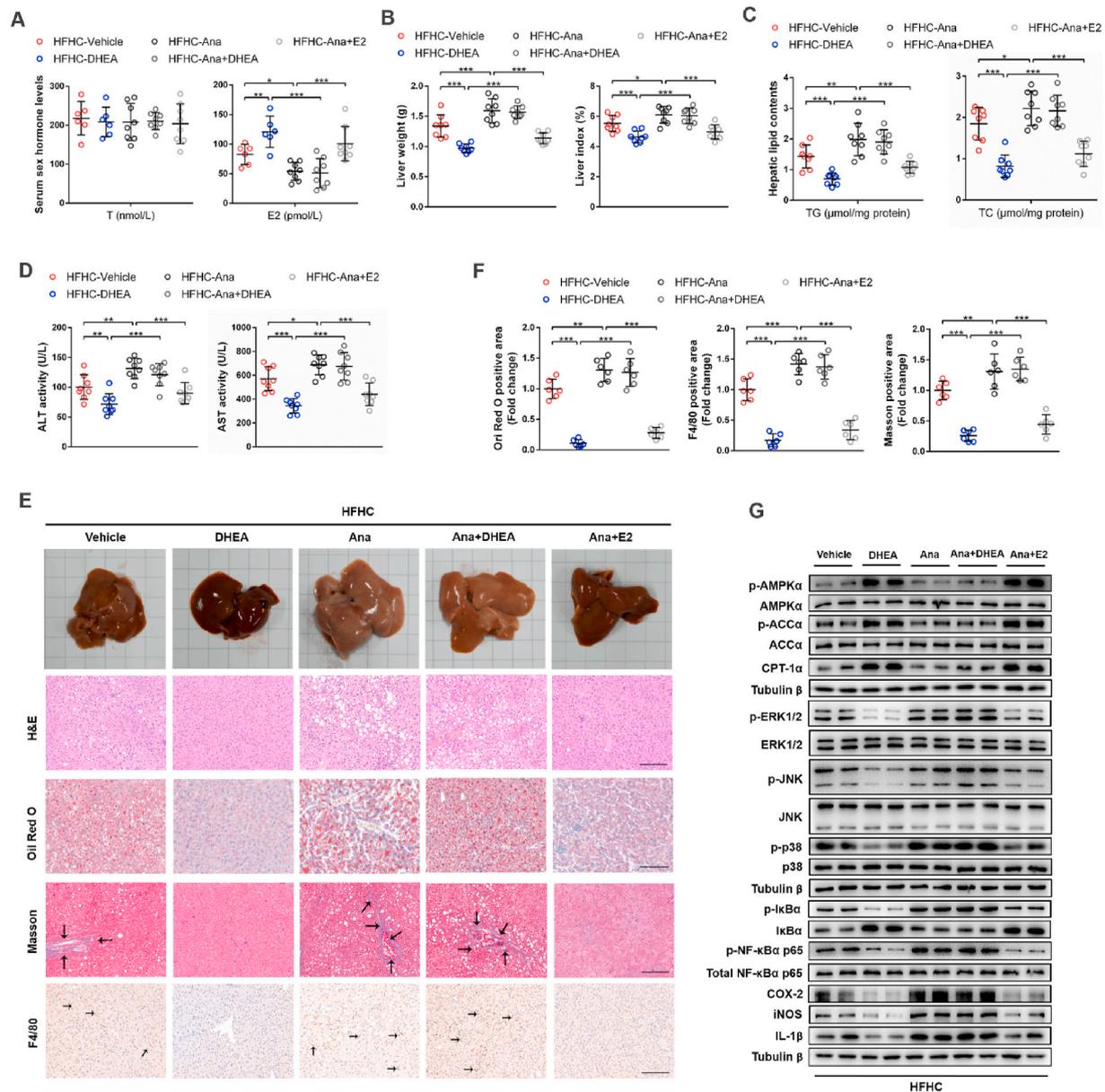
(F) Western blotting analyzed the protein levels that were involved in lipid metabolism, MAPK signaling, NF-κB signaling, and inflammatory factors in BRL-3A cells (left) or HepG2 cells (right). PCNA or Tubulin β served as the loading controls.

(G) Immunofluorescence analysis of the NF-κB p65 nuclear translocation in BRL-3A cells. Scale bars, 100 μm (H) RT-qPCR analysis of the mRNA levels of lipid metabolism-related factors (*FASN*, *SCD1*, *SREBP-1c*, and *PPARα*) and inflammatory factors (*IL-8* and *CCL5*) in HepG2 cells.

Data were presented as the means ± SD. \*\*\*p < 0.001 (one-way ANOVA). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

effects were observed in the T content after DHEA or anastrozole treatment (Fig. 4A), which suggests that the biotransformation of DHEA into E2 was effectively blocked in the body. Furthermore, pretreatment with anastrozole markedly abolished the protective effect of DHEA against NASH, including hepatic steatosis, liver fibrosis, and inflammatory response in female mice fed with HFHC diets (Fig. 4B–G; Fig. S7B). Interestingly, the reduction of E2 levels by anastrozole (an aromatase inhibitor) markedly aggravated the progression of NASH-associated steatosis, fibrosis, and inflammation while the artificial recovery of E2 levels (equal to levels in DHEA-treated female mice) observably alleviated the development of hepatic steatosis, liver fibrosis, and inflammation in female mice fed with HFHC diets when

administered with anastrozole (Fig. 4B–G; Fig. S7B). In addition, the decrease of white adipose mass caused by DHEA was dispelled in female mice fed with HFHC diets and pretreated with anastrozole (Fig. S7C). These data were consistent with previous studies that tamoxifen (a selective estrogen receptor modifier) induces the incidence and development of NAFLD/NASH [8], and which NAFLD/NASH were improved by supplementation of estrogen [6,7]. These results indicate that DHEA suppresses lipid accumulation, liver fibrosis, and inflammation through conversion into E2 in female mice fed with HFHC diets. Collectively, *in vivo* and *in vitro* data suggest that DHEA converts into E2 rather than T; this conversion is a necessary prerequisite for preventing the incidence and development of NASH.



**Fig. 4.** The anti-NASH effects of DHEA depend on its biotransformation into estradiol in female mice fed with HFHC diets

(A) Serum sex hormone (T and E2) levels.  $n = 8$  mice per group.

(B) Liver weight and liver index.  $n = 8$  mice per group.

(C) Hepatic lipid (TG and TC) contents.  $n = 8$  mice per group.

(D) Activity of serum ALT and AST.  $n = 8$  mice per group.

(E) Representative images of liver overall morphology and liver sections stained with H&E, Oil Red O, Masson, and F4/80.  $n = 6$  mice per group. Scale bars, 100  $\mu$ m.

(F) Quantitative results of Oil Red O, Masson, and F4/80 staining illustrated in (E).  $n = 6$  mice per group.

(G) Western blotting analysis of the proteins levels involved in AMPK signaling, lipid metabolism, MAPK signaling, NF- $\kappa$ B signaling, and inflammatory factors. Tubulin  $\beta$  served as a loading control.

Data were presented as the means  $\pm$  SD. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  (one-way ANOVA). Abbreviations: T, testosterone; E2, estradiol. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

### 3.4. The mitigative effect of DHEA on NASH is presented in a GPR30-dependent manner

Estrogens (mainly E2) exert a series of biological effects by binding with classical estrogen receptors (ER, including ER $\alpha$  and ER $\beta$ ), which trigger the transcription of downstream target genes [31]. In addition, studies have shown that E2 can regulate rapid intracellular responses mediated by non-classical receptors, such as G protein-coupled estrogen receptor (GPR30), which play a crucial role in health and disease [23, 32]. To investigate the anti-NASH effects of DHEA and confirm whether

the hormone is mediated by classical or non-classical ER, we first analyzed the ER expression in PA-challenged BRL-3A and HepG2 cells. As shown in Figs. S8A–E, DHEA treatment markedly increased GPR30 protein levels, while no differences were found in the ER $\alpha$  and ER $\beta$  protein levels; the changes in GPR30 protein levels induced by DHEA were blocked in PA-challenged hepatocytes by pretreatment with trilostane, 3 $\beta$ -HSD I/II siRNA, or anastrozole. Furthermore, no effect was observed in the cells treated with T, whereas E2 treatment improved the GPR30 protein level even in the PA-stimulated hepatocytes pretreatment by anastrozole (Figs. S8D and E). These data imply that GPR30

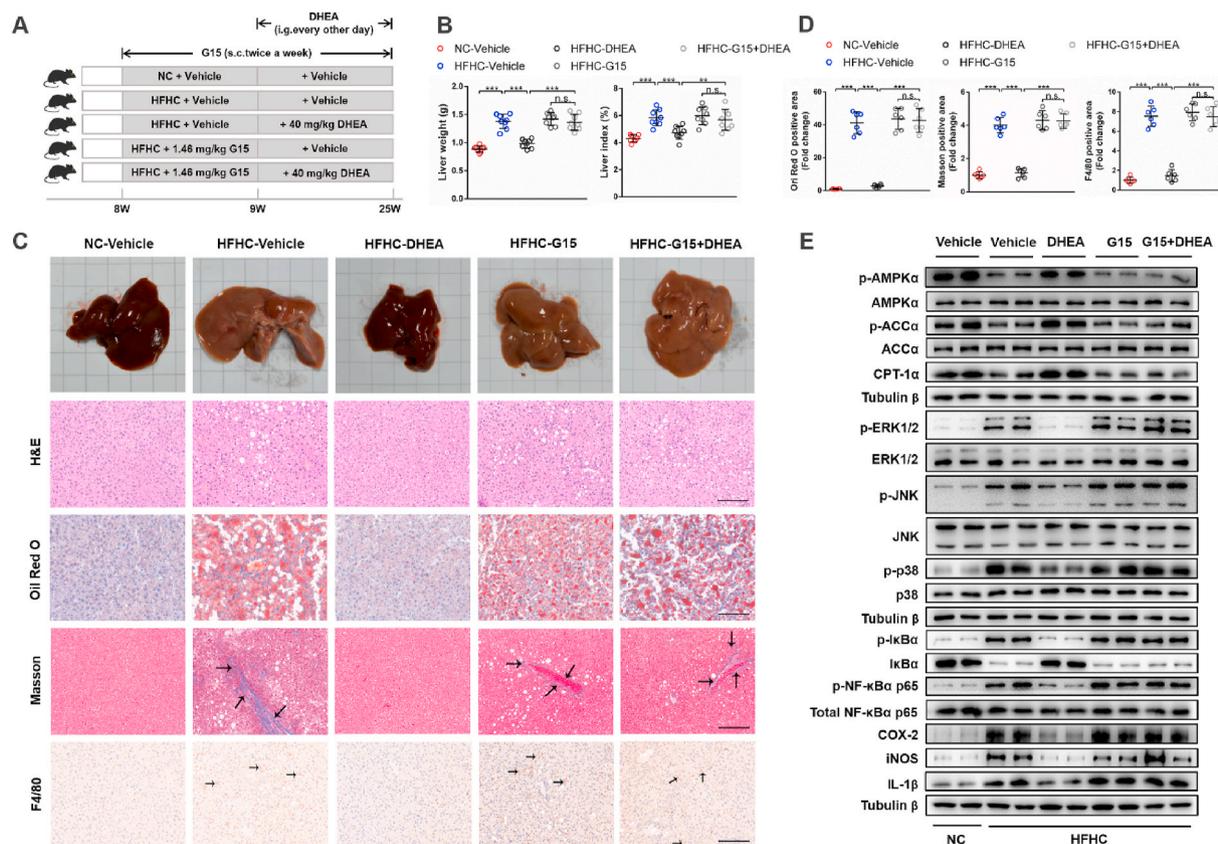
may play a crucial role in the anti-NASH effects of DHEA. Previous studies showed that GPR30 is not a direct target of E2 *in vivo* and *in vitro* [33–35], whereas E2 usually bind to and activates the classical ERs, including ER $\alpha$  and ER $\beta$  [31]. Thus, the conversion of DHEA into E2 may indirectly enhanced the expression level of GPR30 by binding another target, such as the classical ERs. In fact, identification of the direct target of E2 in inducing GPR30 expression can be a worthy study in the future.

A recent study showed that GPR30 selective agonist G1 can alleviate OVX- or diet-induced glucose and lipid metabolism disorders and reduce insulin resistance in female or male mice; this suggest that the activation of GPR30 may be a viable therapeutic approach for treating diabetes, obesity, and related diseases in multiple preclinical male and female models [23]. The incidence and development of NASH are closely related to obesity, diabetes, and related metabolic diseases [36]. Previous studies have shown that DHEA is a ligand of GPR30 and increases GPR30 mRNA and protein expression levels in HepG2 cells [22]. Therefore, we investigated the mediating role of GPR30 in the anti-NASH effects of DHEA both *in vivo* and *in vitro*. BRL-3A or HepG2 cells were pretreated with the antagonist, activator, or siRNA of GPR30; we found that both of the DHEA or the GPR30 activator G1 markedly alleviated the PA-induced lipid accumulation and oxidative stress, which was abolished by pretreatment with GPR30 antagonist G15 (Fig. S9). Similarly, GPR30 siRNA pretreatment also blocked the suppression of DHEA on lipid accumulation and oxidative stress in

PA-stimulated HepG2 cells (Figs. S9D and E). We then verified these findings *in vivo* and observed that the pretreatment of mice with G15 one week before gavage administration of DHEA (Fig. 5A) completely eliminated the attenuating effects of DHEA on major pathological features of NASH, including hepatic steatosis, liver fibrosis, and inflammation in HFHC-induced female mice (Fig. 5B–E; Fig. S10). Together, these data *in vivo* and *in vitro* imply that DHEA prevents the incidence and development of NASH in a GPR30-dependent manner.

### 3.5. DHEA exerts its anti-NASH effects by activating the GPR30-mediated AMPK signaling pathway

DHEA treatment increases intracellular cAMP levels in endothelial cells [37] and our previous study has also found that the lipid-reducing effects of DHEA were mediated by the increase of cAMP levels in chicken hepatocytes [19]. In this study, DHEA remarkably enhanced the cAMP levels in BRL-3A and HepG2 cells (Fig. 6A and B). Furthermore, DHEA dramatically induced the activation of AMPK, which is similar to the inducement of adenylyl cyclase (AC) activator forskolin, and was blocked in PA-stimulated hepatocytes by pretreatment with AC inhibitor MDL-12330A (Fig. 6C; Fig. S11A). These results suggests the possibility that the increased cAMP levels from DHEA were responsible for the activation of the AMPK pathway. cAMP can bind two effectors in most cells, namely cAMP-regulated guanine nucleotide exchange factors



**Fig. 5.** The mitigation effect of DHEA on NASH is exhibited in a GPR30-dependent manner in female mice fed with HFHC diets (A) Scheme for the experimental strategy on NC- or HFHC-diet-fed mice pretreated with 1.46 mg/kg of G15 one week prior to gavage administration of 40 mg/kg of DHEA.

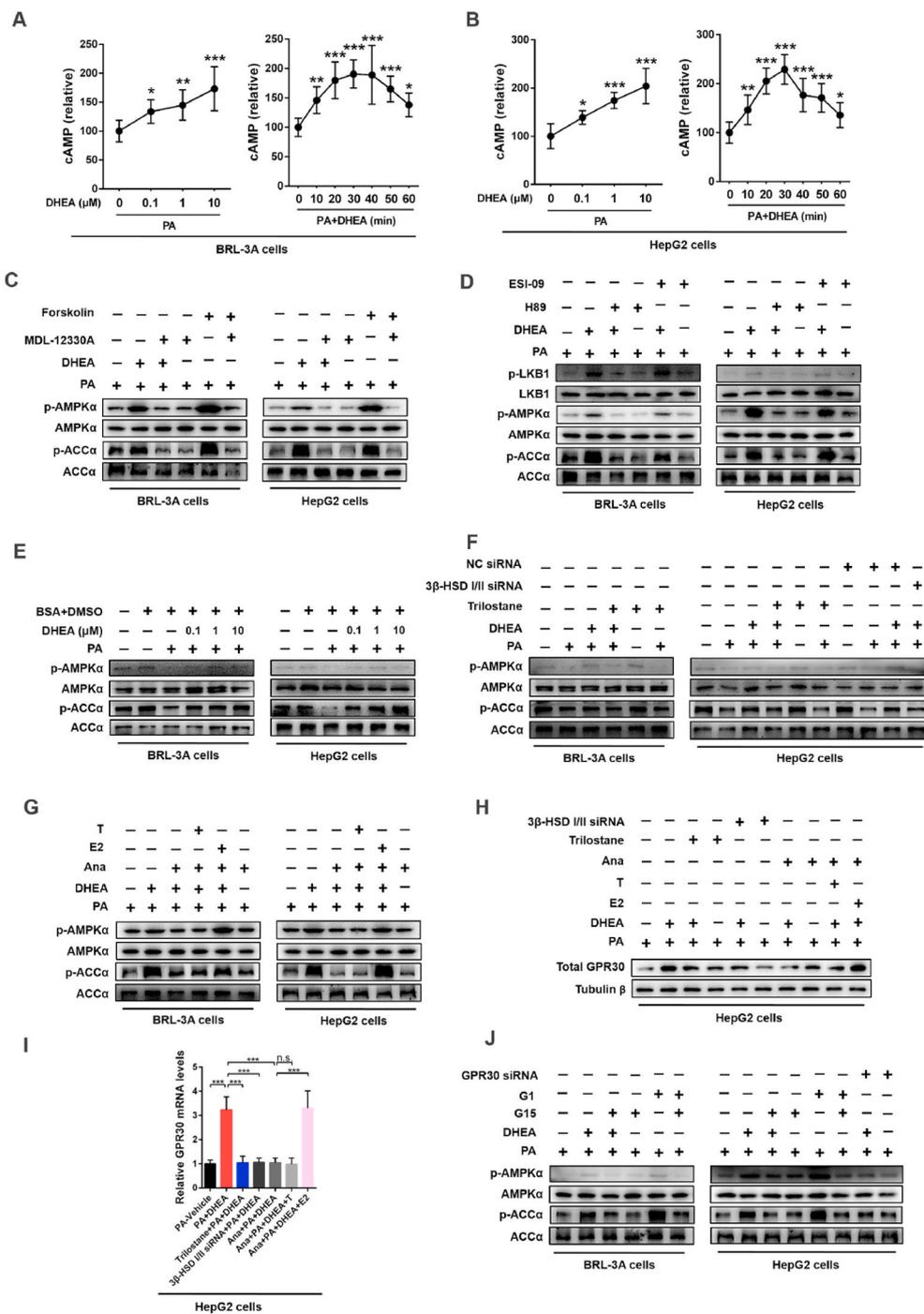
(B) Liver weight and liver index.  $n = 8$  mice per group.

(C) Representative images of liver overall morphology and liver sections stained with H&E, Oil Red O, Masson, and F4/80.  $n = 6$  mice per group. Scale bars, 100  $\mu$ m.

(D) Quantitative results of Oil Red O, Masson, and F4/80 staining illustrated in (C).  $n = 6$  mice per group.

(E) Western blotting analysis of the protein levels involved in AMPK signaling, lipid metabolism, MAPK signaling, NF- $\kappa$ B signaling, and inflammatory factors. Tubulin  $\beta$  served as a loading control.

Data were presented as the means  $\pm$  SD.  $**p < 0.01$ ;  $***p < 0.001$ ; n.s., no significance,  $p \geq 0.05$  (one-way ANOVA). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



**Fig. 6.** DHEA activates the cAMP/PKA-AMPK signaling pathways mediated by GPR30 in PA-stimulated hepatocytes

(A) Cyclic AMP levels (cAMP; left) in BRL-3A cells treated with DMSO or DHEA (0.1–10  $\mu$ M) prior to BSA or 0.5 mM PA-stimulated; in addition, the cAMP levels (right) were analyzed in BRL-3A cells treated with DMSO or 10  $\mu$ M DHEA prior to BSA or 0.5 mM PA-stimulated for the indicated times. n = 6 per group.

(B) cAMP levels (left) in HepG2 cells treated with DMSO or DHEA (0.1–10  $\mu$ M) prior to BSA or 0.5 mM PA-stimulated; in addition, the cAMP levels (right) were analyzed in HepG2 cells treated with DMSO or 10  $\mu$ M DHEA prior to BSA or 0.5 mM PA-stimulated for the indicated times. n = 6 per group.

(C) The phosphorylation protein levels of AMPK (T172) and AMPK substrate ACC (S79) in BRL-3A cells (left) or HepG2 cells (right) treated with DMSO, 20  $\mu$ M forskolin (AC activator) or 10  $\mu$ M DHEA prior to BSA or 0.5 mM PA-stimulated in the presence or absence of 20  $\mu$ M MDL-12330A (AC inhibitor).

(D) The phosphorylation protein level of LKB1, AMPK (T172), and AMPK substrate ACC (S79) in BRL-3A cells (left) or HepG2 cells (right) treated with DMSO or 10  $\mu$ M DHEA prior to BSA or 0.5 mM PA-stimulated in the presence or absence of 10  $\mu$ M ESI-09 (EPAC inhibitor) or 10  $\mu$ M H89 (PKA inhibitor).

(E) The phosphorylation protein level of AMPK (T172) and AMPK substrate ACC (S79) in BRL-3A cells (left) or HepG2 cells (right) treated with DMSO or DHEA (0.1–10  $\mu$ M) prior to BSA or 0.5 mM PA-stimulated.

(F) The phosphorylation protein of AMPK (T172) and AMPK substrate ACC (S79) in BRL-3A cells (left) or HepG2 cells (right) treated with DMSO or 10  $\mu$ M DHEA prior to BSA or 0.5 mM PA-stimulated in the presence or absence of 5  $\mu$ M trilostane or 3 $\beta$ -HSD I/II siRNA.

(G) The phosphorylation protein level of AMPK (T172) and AMPK substrate ACC (S79) in BRL-3A cells (left) or HepG2 cells (right) treated with DMSO, 9 nM testosterone (T), 2 nM estradiol (E2) or 10  $\mu$ M DHEA prior to BSA or 0.5 mM PA-stimulated in the presence or absence of 1  $\mu$ M anastrozole (Ana, aromatase inhibitor).

(H) Western blotting analyzed the GPR30 protein level in HepG2 cells treated with DMSO, 9 nM testosterone (T), 2 nM estradiol (E2) or 10  $\mu$ M DHEA prior to BSA or 0.5 mM PA-stimulated in the presence or absence of 5  $\mu$ M trilostane (3 $\beta$ -HSD inhibitor), 1  $\mu$ M Ana or 3 $\beta$ -HSD I/II siRNA. Tubulin  $\beta$  served

as a loading control.

(I) RT-qPCR analysis of the GPR30 mRNA level in HepG2 cells treated with DMSO, 9 nM testosterone (T), 2 nM estradiol (E2) or 10  $\mu$ M DHEA prior to BSA or 0.5 mM PA-stimulated in the presence or absence of 5  $\mu$ M trilostane (3 $\beta$ -HSD inhibitor), 1  $\mu$ M Ana or 3 $\beta$ -HSD I/II siRNA.

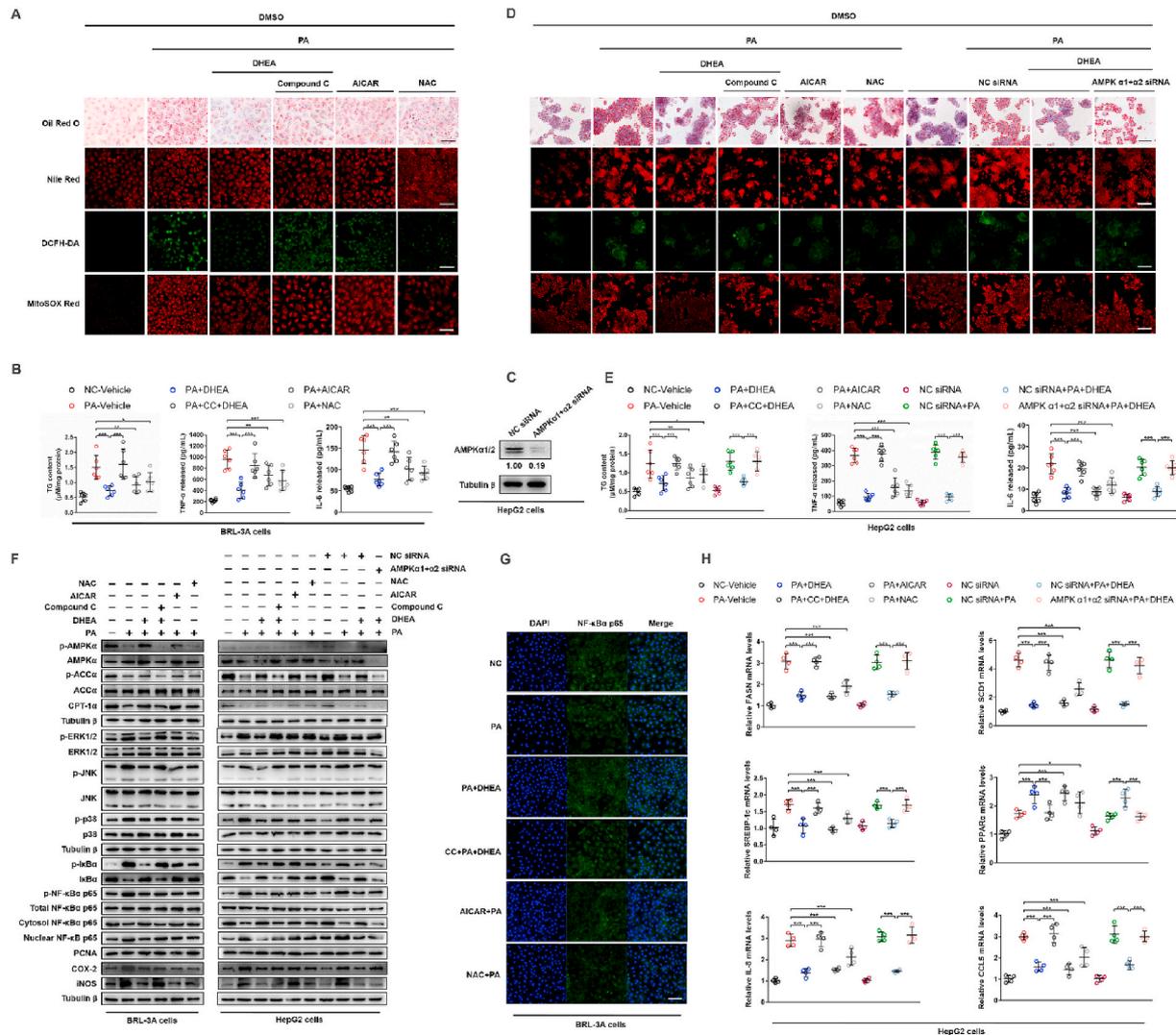
(J) The phosphorylation protein level of AMPK (T172) and AMPK substrate ACC (S79) in BRL-3A cells (left) or HepG2 cells (right) treated with DMSO, 10  $\mu$ M G1 (GPR30 agonists) or 10  $\mu$ M DHEA prior to BSA or 0.5 mM PA-stimulated in the presence or absence of 1  $\mu$ M G15 (GPR30 antagonist) or GPR30 siRNA.

Data were presented as the means  $\pm$  SD. \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001; n.s., no significance, p  $\geq$  0.05 (one-way ANOVA, two-tailed Student's t-test).

(Epac1 and Epac2) or protein kinase A (PKA) [38,39]. To find the effector that participates in the activation of the AMPK pathway caused by DHEA, we pretreated hepatocytes with the PKA inhibitor H89 or the Epac inhibitor ESI-09, respectively. As shown in Fig. 6D and Fig. S11B, DHEA improved the phosphorylation protein levels of LKB1, AMPK $\alpha$ , and ACC $\alpha$  in PA-stimulated hepatocytes under the presence of ESI-09 or absence of H89. These results indicate that PKA rather than Epac contributes to the DHEA-mediated activation of the AMPK pathway.

In this study, we verified that the anti-NASH effect of DHEA depends on its conversion into E2. We further investigated the AMPK activation

to determine whether it is also associated with its biotransformation in PA-challenged hepatocytes. The results showed that DHEA treatment markedly activated the AMPK signaling in PA-stimulated hepatocytes (Fig. 6E; Fig. S11C). However, the activated AMPK signaling pathway mediated by DHEA was completely blocked when the PA-stimulated hepatocytes were pretreated with either trilostane, 3 $\beta$ -HSD I/II siRNA, or anastrozole; treatment with E2 rather than T exerted an activating effect on the AMPK signaling pathway in PA-stimulated hepatocytes under pretreatment with anastrozole (Fig. 6F and G; Figs. S11D and E). Together, these findings first showed that DHEA activates the cAMP/



**Fig. 7.** The AMPK is essential for the suppressive effects of DHEA on lipid accumulation, oxidative stress, and inflammatory response in PA-stimulated hepatocytes (A) Oil Red O staining (scale bars, 50  $\mu$ m) and Nile Red staining (scale bars, 100  $\mu$ m) analyzed the accumulation of lipid droplets; ROS (DCFH-DA probe) staining (scale bars, 100  $\mu$ m) illustrating ROS levels; and mtROS (MitoSOX Red probe) staining (scale bars, 100  $\mu$ m) illustrating mtROS levels in BRL-3A cells treated with DMSO, 500  $\mu$ M AICAR (AMPK activator), 5 mM NAC (ROS scavenger) or 10  $\mu$ M DHEA prior to BSA or 0.5 mM PA-stimulated in the presence or absence of 10  $\mu$ M compound C (CC, AMPK inhibitor). (B) The contents of TG, TNF- $\alpha$ , and IL-6 in BRL-3A cells. n = 6 per group. (C) Western blotting analyzed the AMPK $\alpha$ 1/2 protein in HepG2 cells treated with NC siRNA or AMPK $\alpha$ 1+ $\alpha$ 2 siRNA. (D) Oil Red O staining (scale bars, 50  $\mu$ m) and Nile Red staining (scale bars, 200  $\mu$ m); ROS (DCFH-DA probe) (scale bars, 100  $\mu$ m) and mtROS (MitoSOX Red probe) staining (scale bars, 100  $\mu$ m) in HepG2 cells treated with DMSO, 500  $\mu$ M AICAR (AMPK activator), 5 mM NAC (ROS scavenger), or 10  $\mu$ M DHEA prior to BSA or 0.5 mM PA-stimulated in the presence or absence of 10  $\mu$ M compound C (AMPK inhibitor) or AMPK  $\alpha$ 1+ $\alpha$ 2 siRNA. (E) The contents of TG, TNF- $\alpha$ , and IL-6 in HepG2 cells. n = 6 per group. (F) Western blotting analysis of the protein levels involved in AMPK signaling, lipid metabolism, MAPK signaling, NF- $\kappa$ B signaling, and inflammatory factors in BRL-3A cells (left) or HepG2 cells (right). PCNA or Tubulin  $\beta$  served as the loading controls. (G) Immunofluorescence analysis the nuclear translocation of NF- $\kappa$ B p65 in BRL-3A cells. Scale bars, 100  $\mu$ m (H) RT-qPCR analysis the mRNA levels of lipid metabolism-related factors (*FASN*, *SCD1*, *SREBP-1c*, and *PPAR $\alpha$* ) and inflammatory factors (*IL-8* and *CCL5*) in HepG2 cells. n = 4 per group. Data were presented as the means  $\pm$  SD. \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001 (one-way ANOVA). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

PKA-AMPK signaling pathway, which depends on its biotransformation to E2 rather than to T or DHEA itself.

A previous study reported that the activated GPR30 can improve the production of cyclic AMP (cAMP) in estradiol-17 $\beta$ -D-glucuronide-mediated cholestasis [40]. In addition, estrogens have been reported to alleviate obesity and diabetes, and reduce lipid content in the liver through activation of GPR30 [41]. Based on the findings for anti-NASH effects of DHEA by conversion into E2 and regulating GPR30, the activation of AMPK signaling pathway whether mediated by GPR30 was further analyzed in PA-challenged BRL-3A cells and HepG2 cells. The increase of GPR30 protein and mRNA levels caused by DHEA was largely blocked in PA-stimulated hepatocytes pretreatment with trilostane, 3 $\beta$ -HSD I/II siRNA, and anastrozole, but E2 markedly enhanced the levels of GPR30 protein and mRNA in PA-stimulated hepatocytes pretreated with anastrozole (Fig. 6H and I; Fig. S11F). The changes in the level of GPR30 show a pattern of DHEA-induced AMPK signaling activation in PA-stimulated hepatocytes, which is significant (Fig. 6F). Meanwhile, the activation of AMPK signaling induced by DHEA or the GPR30 activator G1 was dramatically abrogated in PA-stimulated hepatocytes pretreated with GPR30 antagonist G15 and GPR30 siRNA (Fig. 6J; Fig. S11G). These results indicate that GPR30 plays a crucial role in DHEA-induced activation of the AMPK signaling pathway. In brief, the above results showed that the conversion of DHEA to estradiol and then induce the up-regulation of GPR30 is a necessary precursor for inducing the activation of the cAMP-PKA-AMPK pathway.

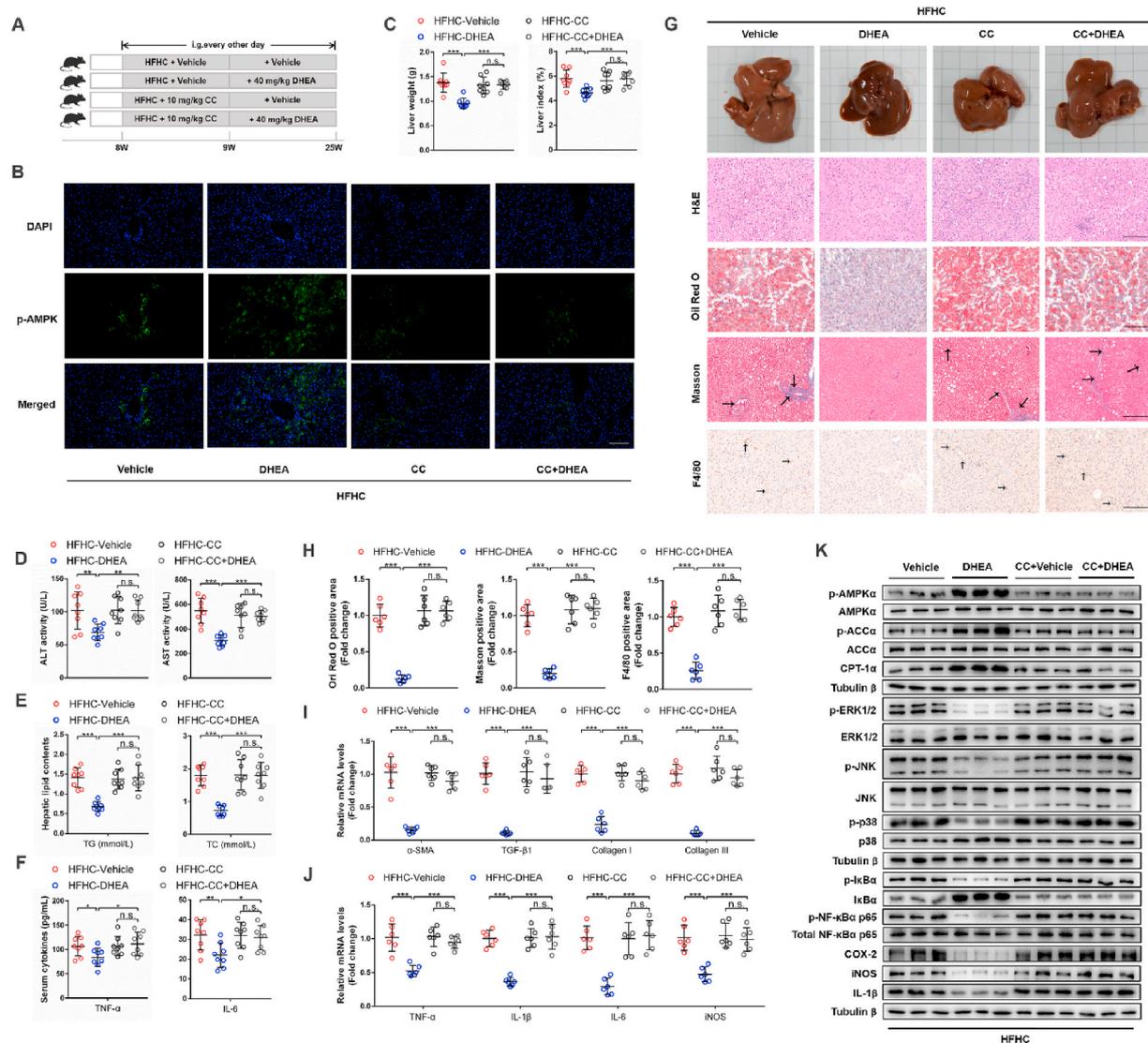
### 3.6. An activated AMPK pathway is required for enabling the protective effects of DHEA against NASH

The AMPK pathway is an important energy sensor and regulator, and its activation has been recognized as a potential target for the prevention and treatment of NASH and its related metabolic diseases through the modulation of lipid metabolic homeostasis, which contributes to the alleviation of oxidative stress, liver fibrosis, and inflammatory responses [42]. In this study, the PA-challenged BRL-3A and HepG2 cells were pretreated with AMPK inhibitor compound C or AMPK siRNA; we aimed to evaluate the suppressive effects of DHEA on PA-challenged lipid accumulation, oxidative stress, and inflammation and determine whether the effects depend on the activation of the AMPK pathway. DHEA reduced the lipid accumulation, oxidative stress, and inflammatory response, which is similar to the reduction by the AMPK activator AICAR or ROS scavenger NAC; however, these effects were completely eliminated in PA-induced BRL-3A cells by pretreated with compound C (Fig. 7A and B). To further verify these results, we synthesized an AMPK  $\alpha$ 1+ $\alpha$ 2 siRNA to inhibit the expression of AMPK $\alpha$  (Fig. 7C), and found that PA-stimulated HepG2 cells pretreated with AMPK siRNA or compound C presented a complete reversal in DHEA-mediated inhibition on lipid accumulation, oxidative stress, and inflammation (Fig. 7D and E). The Western blot assay showed that DHEA, similar to AICAR or NAC, markedly induced the activation of proteins related to the AMPK signaling pathway and fatty acid oxidation but lead to the inhibition of protein involved in MAPK signaling, NF- $\kappa$ B signaling and inflammatory response in PA-stimulated hepatocytes, and these effects were also dramatically reversed in PA-stimulated hepatocytes pretreatment with compound C or AMPK siRNA (Fig. 7F; Fig. S12). Immunofluorescence analysis also confirmed that DHEA treatment, similar to AICAR or NAC treatment, markedly blocked the nuclear translocation of NF- $\kappa$ B, which was largely eliminated in PA-stimulated BRL-3A cells by pretreatment with compound C (Fig. 7G). In line with these observations in hepatocytes, DHEA, AICAR or NAC treatment observably improved the factors mRNA levels related to fatty acid synthesis, fatty acid oxidation, and inflammatory response, and these effects were also remarkably diminished in PA-stimulated HepG2 cells by pretreatment with compound C or AMPK siRNA (Fig. 7H). These results strongly suggest that an activated AMPK pathway is essential for the preventing effects of DHEA on lipid accumulation, oxidative stress, and inflammation in PA-challenged

hepatocytes.

At present, the AMPK has become a key target of NASH prevention research due to its central role in metabolic control [43]. Our recent study reported that DHEA alleviated obesity through the activation of the AMPK signaling pathway in rats that were given a high-fat diet [16]. Based on the *in vitro* results, the female mice were pretreated with compound C for one week and then co-treated with DHEA for another 16 weeks (Fig. 8A) to evaluate whether the AMPK is necessary for enabling the anti-NASH effects of DHEA *in vivo*. We found that pretreatment with compound C before DHEA administration effectively blocked the activation of the AMPK in the liver (Fig. 8B) and markedly abrogated the relieving actions of DHEA on the development of hepatic steatosis, fibrosis, and inflammation in female mice fed with HFHC diets (Fig. 8C-J). Furthermore, the decreasing effect of DHEA on serum lipid contents and white adipose mass were completely abolished in female mice fed with HFHC diets by pretreatment with compound C (Fig. S13 A and B), and western blotting analysis also showed that the beneficial effects of DHEA on the protein factors involved in lipid metabolism and inflammatory response were effectively blocked in female mice fed with HFHC diets by pretreatment with compound C (Fig. 8K; Fig. S13C). These data suggest that an activated AMPK pathway is required for the alleviative effects of DHEA on hepatic steatosis, fibrosis, and inflammation in female mice fed with HFHC diets. Taken together, the evidence *in vivo* and *in vitro* strongly suggests that the AMPK pathway activation, with its downstream signaling effect, is necessary for the DHEA-mediated protection against NASH. This result is consistent with multitudinous studies, which show the potential benefits of DHEA in activating AMPK [16,44].

Although the beneficial effects of DHEA on the health of elderly men and women have been reported [45], the hormone's molecular mechanism remains unknown. This is the first report to systematically elucidate the protective effects and mechanisms of DHEA against NASH, and we have revealed that DHEA protects against the incidence and development of NASH depending on its biotransformation into estradiol rather than testosterone, which improves the expression levels of non-classical estrogen receptor GPR30; the activation of GPR30-mediated AMPK is required for the alleviative effects of DHEA on the major pathological features of NASH, including hepatic steatosis, liver fibrosis, and inflammation. In this study, normal female mice were used according to previous literature [5,7]; certainly, some studies investigated the effects of estrogen deficiency or exogenous estrogen supplementation on the disorder of glycolipid metabolism in postmenopausal women using older female mice to avoid the interference of circulating estrogen [46]. Thus, the mice's age may be considered in future investigations of menopause-related diseases, such as NASH. At present, there is no effective measure for preventing NAFLD/NASH in the clinic, which seriously affect the physical and mental health of patients, especially in postmenopausal women. To date, researchers mainly focused on the protective effect and potential mechanisms of exogenous estrogen when used against NAFLD/NASH in postmenopausal women [47]; however, the exogenous estrogen will inevitably cause endocrine disorder and increase the incidence of gynecologic cancer [9]. The conversion of DHEA into estradiol depends on the local needs of target tissues with little diffusion, which is a mechanism that serves to avoid the risk of unnecessary side effects for other tissues' exposure to estrogen in the body [20]. Therefore, DHEA is expected to be a key substitute for preventing NASH in "estrogen replacement therapy" and the unpleasant side-effects of estrogen can also be avoided. Collectively, these findings are the first to verify that DHEA is an effective anti-NASH agent, and GPR30 can be used as a target factor in preventing lipid metabolism disorder-related diseases, such as NASH; these findings may contribute to the development of a new strategies for the prevention of NAFLD/NASH in postmenopausal women.



**Fig. 8.** DHEA exerts anti-NASH functions that require the activation of the AMPK pathway in female mice fed with HFHC diets

(A) Scheme for the experimental strategy on HFHC-diet-fed female mice pretreated with compound C (CC, AMPK inhibitor) one week prior to gavage administration of 40 mg/kg of DHEA every other day.

(B) Immunofluorescence analyses the p-AMPK protein level. Scale bars, 100  $\mu$ m.

(C) Liver weight and liver index.  $n = 8$  mice per group.

(D) Activity of serum ALT and AST.  $n = 8$  mice per group.

(E) Hepatic lipid (TG and TC) contents.  $n = 8$  mice per group.

(F) Serum cytokines (TNF- $\alpha$  and IL-6) levels.  $n = 8$  mice per group.

(G) Representative images of liver overall morphology and liver sections stained with H&E, Oil Red O, Masson, and F4/80.  $n = 6$  mice per group. Scale bars, 100  $\mu$ m.

(H) Quantitative results of Oil Red O, Masson, and F4/80 staining illustrated in (G).  $n = 6$  mice per group. Scale bars, 100  $\mu$ m.

(I) RT-qPCR analysis the transcript levels of liver fibrosis-related genes ( $\alpha$ -SMA, TGF- $\beta$ 1, Collagen I, and Collagen III).  $n = 6$  mice per group.

(J) RT-qPCR analysis of the transcript levels of liver inflammatory-related genes (TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and iNOS).  $n = 6$  mice per group.

(K) Western blotting analyzed the protein levels involved in AMPK signaling, lipid metabolism, MAPK signaling, NF- $\kappa$ B signaling, and inflammatory factors. Tubulin  $\beta$  served as a loading control.

Data were presented as the means  $\pm$  SD. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; n.s., no significance,  $p \geq 0.05$  (one-way ANOVA). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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## Author contribution

H.M. and L.L. conceived and designed the experiments, analyzed data, and wrote the manuscript. L.L., H.W., Y.Y. and J.C. performed animal and histopathologic experiments and analyzed data. L.L., Z.J., W. Y., X.C., Q.L. and M.L. performed molecular experiments and analyzed data. H.M. designed the project, edited the manuscript, and supervised the study.

## Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled, "The sex steroid precursor dehydroepiandrosterone prevents nonalcoholic steatohepatitis by activating the AMPK pathway mediated by GPR30".

## Abbreviations

$\alpha$ -SMA	$\alpha$ -smooth muscle actin
ACC	acetyl-CoA carboxylase
ALT	alanine aminotransferase
AMPK	AMP-activated protein kinase
Ana	anastrozole
AST	aspartate aminotransferase
BSA	bovine serum albumin
cAMP	cyclic AMP
CC	compound C
CCL5	C-C motif chemokine ligand 5
COX-2	cyclooxygenase-2
CPT-1 $\alpha$	carnitine palmitoyl transferase 1 $\alpha$
CXCL10	CXC chemokine ligand 10
DHEA	dehydroepiandrosterone
E2	estradiol
ER	estrogen receptor
FASN	fatty acid synthase
GPR30	G-protein-coupled estrogen receptor
HDL-C	high-density lipoprotein cholesterol
HFHC	high-fat/high-cholesterol
IL-1 $\beta$	interleukin-1 $\beta$
IL-6	interleukin-6
IL-8	interleukin-8
iNOS	inducible nitric oxide synthase
LDL-C	low-density lipoprotein cholesterol
mtROS	mitochondrial reactive oxygen species
NAFLD	nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
NEFA	non-esterified fatty acid
NC	normal chow
PA	palmitic acid
PPAR $\alpha$	peroxisome proliferator-activated receptor $\alpha$
ROS	reactive oxygen species
SCD1	stearoyl-CoA desaturase 1
SFL	simple fatty liver
SREBP-1	sterol regulatory element-binding protein-1
T	testosterone
TC	total cholesterol
TG	triglyceride;
TGF- $\beta$ 1	transforming growth factor- $\beta$ 1
TNF- $\alpha$	tumor necrosis factor- $\alpha$ ;
3 $\beta$ -HSD	3 $\beta$ -hydroxysteroid dehydrogenase
17 $\beta$ -HSD	17 $\beta$ -hydroxysteroid dehydrogenase

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.redox.2021.102187>.

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