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**Globular Adiponectin Inhibits Ethanol-Induced ROS Production through Modulation
of NADPH Oxidase in Macrophages: Involvement of LKB1/AMPK pathway**

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Abbreviations:

AICAR: 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside, ALD: Alcoholic liver Disease, AM: Alveolar macrophages, CM-H₂DCFDA: 5-Chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, CYP2E1: Cytochrome P4502E1, DPI: Diphenyleneiodonium chloride, ERK1/2: Extracellular signal regulated kinase 1/2, gAcrp: Globular adiponectin, fAcrp: full-length adiponectin, LKB1: Liver kinase B1, NADPH oxidase: Nicotinamide adenine dinucleotide phosphate-oxidase, NF- κ B: Nuclear factor- κ B, ROS; Reactive oxygen species.

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Abstract

Adiponectin, an adipokine predominantly secreted from adipocytes, has been shown to play protective roles against chronic alcohol consumption. While excessive ROS production in macrophages is considered one of the critical events for ethanol-induced damage in various target tissues, the effect of adiponectin on ethanol-induced ROS production is not clearly understood. In the present study, we investigated the effect of globular adiponectin (gAcrp) on ethanol-induced ROS production and the potential mechanisms underlying these effects of gAcrp in macrophages. Here, we demonstrated that gAcrp prevented ethanol-induced ROS production both in RAW 264.7 macrophages and primary murine peritoneal macrophages. Globular adiponectin also inhibited ethanol-induced activation of NADPH oxidase. In addition, gAcrp suppressed ethanol-induced increase in expression of NADPH oxidase subunits, including Nox2 and p22^{phox}, via modulation of NF- κ B pathway. Furthermore, pretreatment with Compound C, a selective inhibitor of AMPK, or knockdown of AMPK by small interfering RNA (siRNA) restored suppression of ethanol-induced ROS production and Nox2 expression by gAcrp. Finally, we found that gAcrp treatment induced phosphorylation of liver kinase B1 (LKB1), an upstream signaling molecule mediating AMPK activation. Knockdown of LKB1 restored gAcrp-suppressed Nox2 expression, suggesting that LKB1/AMPK pathway plays a critical role in the suppression of ethanol-induced ROS production and activation of NADPH oxidase by gAcrp. Taken together, these results demonstrate that globular adiponectin prevents ethanol-induced ROS production, at least in part, via modulation of NADPH oxidase in macrophages. Further, LKB1/AMPK axis plays an important role in the suppression of ethanol-induced NADPH oxidase activation by gAcrp in

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Introduction

Reactive oxygen species (ROS), highly reactive oxygen-containing molecules, can react with various cellular molecules. Excessive ROS production results in oxidative stress and causes damage in many organs. Ethanol consumption promotes ROS generation, which causes alterations of various signaling pathways and contributes to the development of damages in the target organs. Therefore, ROS production has long been considered as a critical event in the pathogenesis caused by ethanol exposure.

Ethanol-induced ROS generation is mediated via a variety of cellular systems. For example, CYP2E1 plays a critical role in ROS production and oxidative stress in hepatocytes (Gao and Bataller, 2011), while NADPH oxidase, composed of multi-component enzyme complex, including membrane-bound proteins (Nox2 and p22^{phox}) and regulatory cytosolic components (p40^{phox}, p47^{phox}, p67^{phox}, and Rac1/2), is an important source for ROS production in macrophages (Lam et al., 2010). Activation of macrophages and subsequent excessive inflammatory response is one of the key events leading to the pathogenic impact of ethanol on various target organs, including alcoholic liver disease (ALD) (Gao and Bataller, 2011) and impaired lung function (Brown et al., 2009; D'Souza El-Guindy et al., 2007). Previous studies have shown that p47^{phox} knockout mice exhibit reduced ROS production, lower production of inflammatory cytokines from Kupffer cells, the resident macrophages in liver, and are protected from chronic ethanol-induced liver injury (Kono et al., 2000). In addition, recent studies have reported that NADPH oxidase is a main source of ethanol-induced ROS production in alveolar macrophages (AM), which is

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closely associated with dysfunction of AM (Yeligar et al., 2012), and is a critical mediator for LPS-stimulated ROS production in Kupffer cells after chronic ethanol feeding (Thakur et al., 2006b). We have also shown that ethanol treatment increases ROS production in murine peritoneal macrophages via activation of NADPH oxidase (Kim et al., 2013). Collectively, these results indicate that NADPH oxidase plays a critical role in the activation of macrophages and development of various pathological conditions by ethanol consumption.

Adiponectin predominantly secreted from adipocytes plays a role in the control of various physiological and pathophysiological processes. For example, adiponectin plays a crucial role in glucose and lipid metabolism (Mao et al., 2006), insulin sensitizing actions (Kadowaki et al., 2006) and also possesses anti-inflammatory properties (Ouchi and Walsh, 2007). Adiponectin has been also shown to prevent pathological conditions from chronic ethanol administration, particularly in liver (Rogers et al., 2008) and stomach (Yamamoto et al., 2012). Growing evidence suggests that adiponectin generates various biological responses through modulation of oxidative stress (Ouedraogo et al., 2006; Tao et al., 2007). While emerging evidence suggests that adiponectin negatively regulates oxidative stress, the effect of adiponectin on ethanol-induced ROS production and its potential mechanisms have not been explored.

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AMP-dependent protein kinase (AMPK), acting as a molecular sensor in the cellular energy homeostasis, has been considered as a key molecule mediating adiponectin-induced various beneficial effects (Lim et al., 2010). In addition to the critical role in the glucose and lipid metabolism, recent studies have also highlighted that AMPK activation alleviated ROS production in macrophages and endothelial cells (Jeong et al., 2009; Ouedraogo et al., 2006), which, in turn, leads to the negative regulation of NF- κ B signaling and inflammatory responses (Salminen et al., 2011; Yang et al., 2010). While it is well recognized that AMPK signaling plays a critical role in the modulation of ROS production and inflammatory response, its role in macrophages treated with ethanol and/or adiponectin has not been reported.

Liver kinase B1 (LKB1, also known as STK11), originally identified as a tumor suppressor gene, exhibits an important role in energy metabolism mainly via association with AMPK signaling. For example, LKB1 deficiency leads to inactivation of AMPK, and LKB1-induced tumor suppression and modulation of cellular energy homeostasis are mediated through activation of AMPK (Alessi et al., 2006; Shaw et al., 2004). Furthermore, LKB1/AMPK axis is utilized for adiponectin-induced various biological responses (Miller et al., 2011; Vu et al., 2013). However, the effect of adiponectin on LKB1 signaling and its role in the modulation of ROS production in macrophages have not been reported yet.

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Adiponectin plays a protective role in various organs from chronic ethanol treatment. However, the underlying molecular mechanisms are not clearly understood. Herein, we investigated the effect of globular adiponectin on ethanol-induced ROS production and potential mechanisms of gAcrp action in macrophages. In the present study, we demonstrated for the first time that adiponectin prevents ethanol-induced ROS generation through modulation of NADPH oxidase and, furthermore, LKB1/AMPK signaling plays a pivotal role in the suppression of ROS production and NADPH oxidase activation.

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Materials and Methods

Materials

All the cell culture reagents were obtained from Hyclone Laboratories (South Logan, Utah). Recombinant human globular adiponectin (gAcrp) was purchased from peprotech Inc. (Rocky Hill, NJ). Absolute ethanol was purchased from Merck Chemical (Whitehouse Station, NJ). 5-Chloromethyl-2, 7-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) was purchased from Molecular Probes (Eugene, OR). Lucigenin, NADPH, and Diphenyleneiodonium chloride (DPI) were obtained from Enzo Life Sciences (Farmingdale, NY). Bay11-7082, a pharmacological inhibitor of NF- κ B, was obtained from Sigma Aldrich (St. Louis, MO) and 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR), an activator of AMPK, was obtained from Calbiochem (San Diego, CA). Luciferase assay kit and all related products were purchased from Promega (Madison, WI). Polyclonal antibodies against phosphorylated and total forms of AMPK α and LKB1 were obtained from Cell Signaling Technology Inc. (Beverly, MA). Antibody against Nox2 was purchased from Santa Cruz (Delaware, CA).

Isolation and culture of murine peritoneal macrophages

Murine peritoneal macrophages were isolated essentially as described previously (Kim et al., 2013). Briefly, 8 to 10 week of mice were I.P. injected with 1ml of 4 % Brewer thioglycollate medium. After 3 days of injection, peritoneal cells were washed with 10 ml of ice-cold Hank's balanced salt solution (HBSS) (calcium and magnesium free). Media

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prepared from peritoneum were centrifuged at 12,000 rpm for 5 min. The pellet was resuspended in RBC lysis buffer (BioLegend, CA) and then centrifuged at 12,000 rpm for 5 min. After washing by 20 ml of HBSS, cells were seeded on 4-well culture slides (5×10^5 cells/well) or 96-well plate (1×10^5 cells/well). These cells were allowed to adhere for 2 h and then washed out with 1X PBS to remove non-adherent cells. After 24 h of culture, adherent cells were used in the experiments.

Cell culture

The RAW 264.7 macrophage cell line was purchased from the Korean cell line bank (Seoul, Korea) and routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % (v/v) fetal bovine serum (FBS) and 1 % (v/v) penicillin-streptomycin at 37°C in an incubator with a humidified atmosphere of 5 % CO₂.

Total RNA isolation, reverse transcription and quantitative PCR (qPCR)

To measure the mRNA levels of the target genes, total RNAs were extracted using Qiagen lysis solution (Qiagen, Hilden, Germany) according to the manufacturer's instructions and reverse-transcribed into cDNA using the GoScript Reverse Transcription system (Promega). Quantitative Real-time PCR was then performed with LightCycler 1.5 (Roche Diagnostics, Mannheim, Germany) using QPCR SYBR Green Capillary Mix (ABgene, Surrey, UK) at 95°C for 15 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec

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and 72°C for 30 sec. The primer sequences used for amplification of the target genes are listed in Table 1. The amount of target mRNA was assessed via the comparative threshold (C_t) method after normalizing target mRNA C_t values to those for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, ΔC_t) as a housekeeping gene.

Measurement of total ROS generation

Intracellular ROS production was assessed by measurement of the changes in the fluorescence of 5-chloromethyl-2, 7-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA). In brief, RAW 264.7 macrophages were seeded at a density of 2×10^5 cells/well in 4-well culture slides. Cells were pretreated with 0.1 μ g/ml of globular adiponectin (gAcrp) for 18 h followed by treatment with 100 mM ethanol for additional 24 h. Cells were treated with 10 μ M CM-H₂DCFDA in Hank's balanced salt solution (HBSS) in the dark for 30 min and then washed with HBSS to remove excess dye. For the measurement of ROS production, the change in fluorescence intensity was immediately observed by fluorescence microscopy (Nikon, Tokyo, Japan). The fluorescence intensities were analyzed by quantitative digital analysis using Image Inside from FOCUS (Seoul, South Korea).

Determination of NADPH oxidase activity

NADPH oxidase-dependent ROS formation was determined by measuring lucigenin-derived chemiluminescence using FLUOstar OPTIMA luminometer (BMG Labtech,

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Ortenberg, Germany). Briefly, peritoneal macrophages and RAW 264.7 macrophages were seeded onto 96-well white culture plates at a density of $0.5-1 \times 10^5$ cells/well. After overnight culture, cells were treated with gAcrp and ethanol as indicated in figure legends and then lysed with Hank's balanced salt solution (HBSS) containing 0.1 % Triton X-100 and 0.1 N NaOH. Cellular lysates were incubated with NADPH (200 μ M) and lucigenin (100 μ M) in HBSS for 30 min at 37°C in the dark. Chemiluminescence was measured in relative light units every 5 min over a period of 60 min.

Transient transfection and luciferase assay

Transcriptional activity of NF- κ B was determined using luciferase reporter assay kit (Promega) according to the manufacturer's instructions. Briefly, cells were initially seeded in 24-well plates at a density of 5×10^5 cells/well. After overnight culture, the cells were co-transfected with control (pRL-TK) and expression vectors (pGL4/NF- κ B) using Fugene HD (Promega). After 6 h incubation, media were replaced with DMEM containing 10 % FBS and cells were cultured for additional 18 h. Thereafter, the cells were pretreated with 0.1 μ g/ml gAcrp followed by 100 mM ethanol for additional 24 h and extracted with 0.1 ml of passive lysis buffer (Promega). *Firefly* and *Renilla* luciferase activities were measured by the Dual Luciferase Reporter Assay System (Promega). Statistical analyses for luciferase expression were carried out on the ratios of relative luciferase activity to *Renilla* luciferase.

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Transient transfection with small interfering RNA (siRNA)

RAW 264.7 macrophages were initially seeded on 35-mm dishes at a density of 7×10^5 cells/well. After overnight incubation, cells were transfected with scrambled control siRNA or siRNA targeting AMPK α 1 (25 nM) or LKB1 (50 nM) using Hiperfect transfection reagent (Qiagen) according to the manufacturer's instructions. Gene silencing efficiency was assessed by qRT-PCR after 24 h or 48 h of transfection. The ON-TARGET plus SMART of murine specific siRNA targeting AMPK α 1 and control siRNA were purchased from Dharmacon (Lafayette, CO). Targeting siRNA of LKB1 was obtained from Bioneer (Daejeon, South Korea). The sequences of the siRNA used are listed in (Table 2).

Preparation of cellular extracts and Western blot analysis

RAW 264.7 macrophages were seeded in 35-mm dishes at a density of 1×10^6 cells per well. After overnight incubation, cells were treated with gAcrp and/or ethanol as indicated in figure legends. Total proteins were then isolated using RIPA lysis buffer containing Halt Protease and Phosphatase Inhibitor Single-Use Cocktail (Thermo scientific, Rockford). For immunoblot analysis, 15 μ g of protein were loaded by 10 % SDS-PAGE. The proteins were then transferred to PVDF membranes, blocked with 5 % skim milk in PBS/ Tween20 for 1 h and incubated with the designated primary antibodies overnight at 4°C. Subsequently, the membrane was washed and incubated with secondary HRP-labeled antibody. The images of the blots were captured using Fujifilm LAS-4000 mini (Fujifilm,

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Tokyo, Japan). The membranes were then stripped and re-probed with β -actin or total form of AMPK α or LKB1 antibody as the loading control.

Statistical analysis

Values were presented as mean \pm SEM derived from at least three separate experiments.

Data were assessed by one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests using GraphPad prism software version 5.01 (California, USA).

Differences between groups were considered to be significant at $P < 0.05$.

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Results

Globular adiponectin inhibits ethanol-induced ROS production via modulation of NADPH oxidase in RAW 264.7 macrophages and peritoneal macrophages isolated from mice

Excessive ROS production in macrophages is a critical event for the development of pathological condition by chronic ethanol treatment. We investigated if adiponectin inhibits ethanol-induced ROS production in macrophages. As shown in Fig. 1A, ethanol treatment enhanced ROS production consistent with previous results, but pretreatment with gAcrp significantly suppressed ethanol-induced ROS production in murine macrophages (RAW 264.7 macrophages). Similar effects were also observed in the primary peritoneal macrophages isolated from mice (Fig. 1B), indicating that adiponectin prevents ethanol-induced ROS production in different types of macrophages. We and other previous reports have shown that NADPH oxidase system is the major source for ethanol-induced ROS production in macrophages (Kim et al., 2013; Yeligar et al., 2012). To elucidate the potential mechanisms implicated, we next examined the effect of gAcrp on ethanol-induced activation of NADPH oxidase using lucigenin-based assay. As shown in Fig. 1C and 1D, ethanol-induced increase in ROS production by NADPH oxidase was restored by pretreatment with gAcrp in both RAW 264.7 macrophages (Fig. 1C) and primary murine peritoneal macrophages (Fig. 1D). All these data indicate that gAcrp inhibits ethanol-induced ROS production in macrophages via modulation of NADPH oxidase-dependent ROS production.

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Globular adiponectin suppresses ethanol-induced Nox2 and p22^{phox} expression in RAW

264.7 macrophages

NADPH oxidase is composed of multiple components localized in cytosol and plasma membrane. To identify the subunits responsible for regulation of NADPH oxidase by gAcrp, we examined the effect of gAcrp on the expression of Nox2 and p22^{phox}, since Nox2 and p22^{phox} are critical subunits for ethanol-induced NADPH oxidase activation and subsequent increase in ROS production (Kim et al., 2013). As shown in Fig. 2A, gAcrp treatment decreased the expression of Nox2 mRNA level. Nox2 protein expression was also significantly decreased by treatment with gAcrp, which showed a pattern similar to the regulation of mRNA expression (Fig. 2B). Globular adiponectin also significantly decreased p22^{phox} mRNA expression in a time-dependent manner (Fig. 2C). We also examined the effect of adiponectin on the expression of other NADPH oxidase subunits, such as p40^{phox}, p47^{phox} and p67^{phox}. But, treatment with gAcrp did not significantly affect expression of other subunits of NADPH oxidase (data not shown), suggesting that gAcrp affects expression of NADPH oxidase subunits in a selective manner. In addition, ethanol-induced increase in Nox2 expression was restored by pretreatment with gAcrp at both mRNA (Fig. 2D) and protein (Fig. 2E) level, as expected. Pretreatment with gAcrp also significantly suppressed ethanol-induced increase in p22^{phox} expression (Fig. 2F) similar to the pattern for the modulation of Nox2 expression. Taken together, these data suggest that gAcrp modulates ethanol-induced activation of NADPH oxidase probably via down-regulation of Nox2 and p22^{phox} expression.

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Globular adiponectin suppresses ethanol-induced Nox2 and p22^{phox} expression through modulation of NF- κ B signaling pathway in RAW 264.7 macrophages

To further elucidate signaling mechanisms underlying suppression of ethanol-induced Nox2 and p22^{phox} by gAcrp, we examined whether gAcrp affects ethanol-induced activation of p38 MAPK and NF- κ B pathway, since p38 MAPK/NF- κ B signaling pathway plays a critical role in ethanol-induced Nox2 and p22^{phox} expression and further ROS production in macrophages (Kim et al., 2013). For this, we first confirmed involvement of p38 MAPK and NF- κ B signaling in ethanol-induced Nox2 and p22^{phox} expression in our experimental condition. As indicated in Fig. 3A and 3B, pretreatment with Bay 11-7082, a pharmacological inhibitor of IKK, significantly inhibited ethanol-induced mRNA expression of Nox2 and p22^{phox}. In addition, pretreatment with p38 MAPK inhibitor (SB203580) also significantly suppressed ethanol-induced Nox2 and p22^{phox} expression (Fig. 3C and 3D) consistent with previous observations, confirming the critical role of p38 MAPK/NF- κ B signaling in ethanol-induced Nox2 and p22^{phox} expression in macrophages. We next examined the effect of gAcrp on ethanol-induced activation of NF- κ B signaling. As indicated in Fig. 3E, pretreatment with gAcrp significantly suppressed ethanol-induced NF- κ B activation assessed by luciferase reporter gene assay. All these results imply that gAcrp suppresses ethanol-induced Nox2 and p22^{phox} expression via modulation of NF- κ B signaling pathway.

AMPK signaling is implicated in the suppression of ethanol-induced Nox2 expression by gAcrp in RAW 264.7 macrophages

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AMPK is well known as a mediator of adiponectin-induced various biological responses and modulator of NF- κ B signaling (Salminen et al., 2011). To investigate the underlying molecular mechanisms, we investigated the role of AMPK signaling in the suppression of ethanol-induced Nox2 expression by gAcrp. For this, we first examined the effect of ethanol on AMPK activity and further its role in Nox2 expression in RAW 264.7 macrophages. As shown in Fig. 4A, ethanol treatment decreased the phosphorylation of AMPK α in a time-dependent manner. Furthermore, restoration of AMPK activity by pretreatment with AICAR, a pharmacological activator of AMPK, attenuated ethanol-induced Nox2 mRNA expression (Fig.4B), and AICAR treatment also significantly decreased ethanol-induced NF- κ B activation (Fig. 4C), which plays a critical role in ethanol-induced Nox2 expression. In these experiments, we confirmed increased phosphorylation level of AMPK by treatment with AICAR in RAW 264.7 macrophages, as expected (Supplementary Fig. 2). All these results suggest that ethanol treatment causes down-regulation of AMPK signaling, which in turn leads to the activation of NF- κ B and Nox2 expression. We next examined the potential role of AMPK signaling in prevention of ethanol-induced Nox2 expression by gAcrp. Treatment with gAcrp induces phosphorylation of AMPK up to 24 h in our experimental condition (Fig. 4D), consistent with previous reports. Furthermore, pretreatment with gAcrp restored ethanol-suppressed AMPK phosphorylation in RAW 264.7 macrophages (Fig. 4E). Finally, we dissected the role of AMPK signaling in the suppression of ethanol-induced Nox2 expression by gAcrp. Pretreatment of the cells with compound C, an inhibitor of AMPK, abolished suppression of Nox2 expression by gAcrp in ethanol-treated cells (Fig. 4F). Moreover, it also abrogated the suppression of ethanol-induced NF- κ B activation by gAcrp (Fig. 4G). The role of AMPK activation by gAcrp in suppression of ethanol-induced NF- κ B activation was

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confirmed by gene silencing of AMPK α 1. As depicted in Fig. 4H, knocking down AMPK α 1 by siRNA reverted inhibition of ethanol-induced NF- κ B activation by gAcrp similar to the results induced by treatment with compound C. Taken together, these data suggest that gAcrp-induced AMPK activation plays an important role in suppression of ethanol-induced expression of Nox2 via modulation of NF- κ B signaling in RAW 264.7 macrophages.

AMPK signaling is involved in the suppression of ethanol-induced ROS production by gAcrp in RAW 264.7 macrophages

NADPH oxidase is a main source for ethanol-induced ROS production in macrophages and we found that gAcrp inhibited ethanol-induced NADPH oxidase activation via activation of AMPK signaling (Fig. 4). Next, we finally examined whether AMPK signaling is implicated in modulation of ROS production by gAcrp. As shown in Fig. 5A, while gAcrp treatment significantly suppressed ethanol-induced intracellular ROS production, pretreatment with compound C reversed suppression of ROS production by gAcrp, as assessed by conversion of CM-H₂DCF-DA to the fluorescent DCF. Moreover, gene silencing of AMPK α 1 reversed inhibitory effect of gAcrp on ethanol-induced ROS production, while no significant effect was observed by scrambled siRNA (Fig. 5B). Taken together, these data confirm that the AMPK signaling also plays a critical role in the prevention of ethanol-induced ROS production by gAcrp.

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LKB1 signaling is implicated in the suppression of ethanol-induced Nox2 expression by gAcrp in RAW 264.7 macrophages

Liver kinase B1 (LKB1) is widely known as an upstream signaling molecule of AMPK. To further elucidate upstream signaling mechanisms leading to the AMPK activation and suppression of ROS production by gAcrp in ethanol-treated macrophages, we examined if LKB1 signaling plays a role in the suppression of Nox2 expression by gAcrp. For this, we first investigated the effect of gAcrp on LKB1. As indicated in Fig. 6A, gAcrp treatment rapidly increased phosphorylation level of LKB1, showing a maximal level at 30 min. In addition, suppression of LKB1 phosphorylation by ethanol was restored by pretreatment with gAcrp in RAW 264.7 macrophages (Fig. 6B), which is similar to the pattern for the modulation of AMPK phosphorylation. Furthermore, gene silencing of LKB1 by transfection with siRNA targeting LKB1 significantly restored suppression of Nox2 mRNA expression by gAcrp (Fig. 6C) and it also reverted gAcrp-suppressed NF- κ B activity in ethanol-treated cells (Fig. 6D) without significant effect by transfection with scrambled siRNA. We also observed that depletion of LKB1 gene prevented gAcrp-induced AMPK phosphorylation (data not shown) consistent with previous reports. All these data suggest that LKB1 signaling plays an important role in the suppression of ethanol-induced Nox2 expression and NF- κ B signaling via acting as an upstream molecule leading to the AMPK activation. LKB1 signaling is regulated by a number of upstream signaling pathways. Particularly, ERK1/2 MAPK has been shown to activate LKB1 signaling in various experimental models (Sapkota et al., 2001). Thus, to identify signaling molecules responsible for gAcrp-induced LKB1 activation, we examined the involvement of MAPKs. As depicted in Fig. 6E, pretreatment of cells with

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U0126, a selective inhibitor of ERK1/2, suppressed gAcrp-induced LKB1 phosphorylation, whereas inhibitors of JNK (SP600125) and p38 MAPK (SB203580) did not significantly affect gAcrp-induced LKB1 activation, indicating a critical role of ERK1/2 signaling in gAcrp-induced LKB1 activation.

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Discussion

Reactive oxygen species (ROS) has been shown to induce diverse pathological events such as DNA mutation, carcinogenesis, aging, etc. (Cederbaum et al., 2009), although it is required for normal physiological processes as well. Ethanol treatment caused significant increase in ROS production, which is a critical event for the development of pathophysiological condition in various target tissues. Macrophages are main cellular source responsible for ethanol-induced ROS production and excessive ROS production by macrophages leads to the damage in target tissues, such as alcoholic liver injury and impairment of lung function. Adiponectin, the most abundant adipokine in plasma, is implicated in a number of beneficial processes. It plays a potent protective role against chronic alcohol consumption (Nepal et al., 2012; Rogers et al., 2008) and prevents ROS production in response to various stimuli (Ouedraogo et al., 2006; Thakur et al., 2006a). Although, it is widely accepted that ROS production in macrophages is a critical step leading to the various pathological conditions by ethanol exposure and adiponectin plays a protective role against chronic ethanol consumption, effect of adiponectin on ethanol-induced ROS production in macrophages are not clearly understood. In the present study, we examined the effect of adiponectin on ethanol-induced ROS production in macrophages and its potential mechanisms. Herein, we provided the first evidence that globular adiponectin (gAcrp) inhibited ethanol-induced ROS production. Additional mechanistic studies have demonstrated that suppression of ethanol-induced ROS production by gAcrp is mediated via modulation of NADPH oxidase and, further, LKB1/AMPK pathway plays an important role in the suppression of NADPH oxidase and ROS production by gAcrp (Fig. 7).

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Ethanol consumption promotes the production of ROS via multiple mechanisms in a cell type specific manner. Accumulated line of evidence demonstrated a critical role of NADPH oxidase in ethanol-induced ROS production in macrophages. For example, ethanol treatment increases ROS production via enhancing expression of Nox2 and p22^{phox} in RAW 264.7 macrophages (Kim et al., 2013), induces oxidative stress through up regulation of NADPH oxidases in alveolar macrophages (Yeligar et al., 2012) and enhances LPS-stimulated TNF- α production via modulation of NADPH oxidase-dependent ROS production (Thakur et al., 2006b), collectively indicating that NADPH oxidase would be a main source for ethanol-induced ROS production in macrophages and therefore, it would be a promising target for modulation of ethanol-induced pathogenic conditions.

Adiponectin has been shown to possess anti-oxidative and anti-inflammatory properties in various experimental conditions, which are compatible with the present study. Herein, we demonstrated that gAcrp prevented ethanol-induced ROS production via modulation of NADPH oxidase in both RAW 264.7 macrophages and murine peritoneal macrophages (Fig. 1), providing another evidence for the anti-oxidative effect of adiponectin. While a number of studies have supported notion that adiponectin acts as an inhibitor of anti-oxidative stress, other studies have also suggested that adiponectin induces ROS production. Akifusa and colleagues reported that adiponectin treatment increased ROS/RNS production, which causes Bcl-2 modulation and induction of apoptosis in RAW 264 macrophages (Akifusa et al., 2009; Akifusa et al., 2008). It seems differential effects of adiponectin on ROS production would be

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derived from experimental conditions. For example, in the current study, pretreatment with 0.1 $\mu\text{g/ml}$ of gAcrp for 24 h reduced ethanol-stimulated ROS production. However, in previous studies, treatment with higher concentration of gAcrp alone (10 $\mu\text{g/ml}$) leads to the ROS formation. To further investigate the effect of adiponectin on ROS production in macrophages, we examined the effect of gAcrp on ROS production at various dose and time points. In these experiments, relatively higher concentration (1 and 10 $\mu\text{g/ml}$) of gAcrp increased ROS production in a time-dependent manner consistent with previous reports, but no significant effect was observed by treatment with 0.1 $\mu\text{g/ml}$ of gAcrp (Supplementary Fig. 1). These results suggest that treatment with lower concentration of gAcrp does not significantly affect ROS production, but has preventive effect on ethanol-induced ROS production in RAW 264.7 macrophages. We could not investigate the effect of higher concentration of gAcrp on ethanol-induced ROS production, since treatment of RAW 264.7 macrophages with ethanol and higher concentration of gAcrp (1 or 10 $\mu\text{g/ml}$) caused severe cytotoxicity. All these results indicate that lower concentration of gAcrp prevents ethanol-induced ROS production in macrophages, while higher concentration of gAcrp further accelerates ethanol-induced cell death in macrophages. Although, at this stage, we cannot identify detailed mechanisms underlying opposite responses by adiponectin, it is possible that diverse responses to treatment condition of adiponectin are attributable to the different responses by adiponectin.

AMP-dependent protein kinase (AMPK) is well known as a key mediator of adiponectin-induced various biological responses (Nepal and Park, 2013; Zhou et al., 2009). In the present

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study, we observed that pretreatment with AMPK inhibitor or targeted knockdown of AMPK α 1 efficiently reversed inhibitory effect of gAcrp on ethanol-induced Nox2 expression and ROS production (Fig. 4), providing another evidence that AMPK mediates adiponectin-induced beneficial effects. With regards to the regulation of AMPK signaling, ethanol consumption has been shown to dysregulate AMPK activity in cultured hepatic cell lines, as well as in vivo model (Garcia-Villafranca et al., 2008; Shen et al., 2010; You et al., 2004). Based on previous reports regarding the regulation of AMPK by ethanol and adiponectin, we hypothesized that ethanol-induced ROS production may be mediated via dysregulation of AMPK signaling and gAcrp prevents ROS production through restoration of AMPK activity. In the current study, we demonstrated that treatment with AMPK activator inhibited ethanol-induced Nox2 expression and NF- κ B activation (Fig. 4B and C). Furthermore, inhibition of AMPK signaling restored suppression of ethanol-induced activation of NF- κ B signaling, Nox2 expression (Fig. 4F-H) and finally ROS production (Fig. 5A and 5B) by gAcrp, clearly demonstrating that AMPK plays a critical role in regulation of ROS production by ethanol and adiponectin in macrophages.

Liver Kinase B1 (LKB1) plays important roles in various biological responses through regulation of multiple downstream kinases. Growing evidences have demonstrated LKB1 efficiently phosphorylates the catalytic α -subunit of AMPK at Thr172 upon binding with Ste20-related adaptor protein (STRAD) and mouse protein 25 (MO25), regulatory proteins of LKB1, and that a number of LKB1-induced biological responses are mediated through AMPK dependent manner (Alessi et al., 2006; Shackelford and Shaw, 2009; Shaw et al., 2004).

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Recent studies have also shown that LKB1/AMPK pathway is implicated in adiponectin-induced various biological responses. For example, adiponectin-induced glucose uptake and suppression of breast cancer cells migration are mediated via LKB1/AMPK dependent manner (Vu et al., 2013). Although it is well known that LKB1 is implicated in adiponectin-induced biological responses, the role of LKB1 signaling in anti-oxidative effect induced by adiponectin has not been demonstrated. To our knowledge, the present study demonstrates for the first time that LKB1 signaling plays a critical role in the inhibition of ROS production and modulation of NADPH oxidase by adiponectin. In the present study, gAcrp treatment induced increase in phosphorylation of LKB1 (Fig. 6A) and further pretreatment with gAcrp ameliorated suppression of LKB1 phosphorylation by ethanol (Fig. 6B), which exactly correlates to the modulation of AMPK signaling by ethanol and adiponectin. Furthermore, we also observed that depletion of LKB1 gene prevented gAcrp-induced AMPK phosphorylation (data not shown) and blocked the suppressive effects of gAcrp on Nox2 expression (Fig. 6D). Based on previous reports and data presented here, it seems highly likely that LKB1 acts as upstream of AMPK for the prevention of ethanol-induced ROS production, and further LKB1 would be a promising target for protective effects by gAcrp against chronic alcohol consumption.

LKB1 is phosphorylated at different residues (Ser31, Ser325, Thr366, and Ser428) via various kinases, including PKA, ribosomal S6 protein kinase (RSK) and PKC- ζ . Previous studies have shown that PKC- ζ is responsible for phosphorylation of LKB1 at Ser428. In addition, ethanol has been shown to inhibit phosphorylation of PKC- ζ (Guizzetti and Costa, 2002). Furthermore, in the present study, we found that ethanol decreased and gAcrp reversed

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phosphorylation of LKB1 at Ser428 (Fig. 6B), in which phosphorylation is modulated by PKC- ζ . All these previous reports and results from this study suggest ethanol and gAcrp may modulate LKB1 phosphorylation via regulation of PKC- ζ . In the present study, we did not identify the detailed mechanisms underlying LKB1 phosphorylation modulated by ethanol and adiponectin, since this project would be beyond the scope of the current manuscript. It would be interesting to investigate the involvement of PKC- ζ signaling in suppression and restoration of LKB1 activation by ethanol and adiponectin for future study.

In conclusion, the data presented in this study demonstrated that globular adiponectin prevents ethanol-induced ROS production through modulation of NADPH oxidase in macrophages and this inhibitory effect is mediated by LKB1/AMPK axis (Fig.7). Based on these findings, we suggest that inhibition of NADPH oxidase would be a mechanism underlying for the hepatoprotective effect and other beneficial effects by adiponectin. The present study utilizes RAW 264.7 macrophages and primary peritoneal macrophages to understand mechanisms underlying modulation of ROS production by gAcrp. Further studies are now required to validate our findings in murine peritoneal macrophages and macrophage cell line to the appropriate *in vitro* and *in vivo* model.

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Authorship Contribution

Participated in research design: Kim, Nagy and Park

Conducted experiments: Kim and Park

Performed data analysis: Kim and Park

Wrote or contributed to the writing of the manuscript: Kim, Nagy and Park

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Footnotes

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Figure legends

Fig. 1. Effects of globular adiponectin on ethanol-induced ROS production and NADPH oxidase activation in macrophages. (A) RAW 264.7 macrophages cultured in 4-well culture slides were pretreated with gAcrp (0.1 $\mu\text{g/ml}$) for 18 h followed by stimulation with 100 mM ethanol for additional 24 h. Cells were then further stimulated with Hank's Buffered salt solution (HBSS) containing 10 μM of CM-H₂DCF-DA and ROS production was assessed by conversion of CM-H₂DCF-DA to the oxidized fluorescent DCF, which was monitored by fluorescence microscopy as described in materials and methods. Representative images from three independent experiments are shown along with quantification with green signal intensity (lower panel). Values are expressed as mean \pm SEM (n = 3). * $P < 0.05$ compared with cells not treated with ethanol; # $P < 0.05$ compared with cells treated with ethanol. (B) Peritoneal macrophages isolated from mice were incubated with 0.1 $\mu\text{g/ml}$ of gAcrp for 18 h followed by treatment with 100 mM ethanol for additional 24 h. ROS production was assessed as described previously and representative images from three independent experiments are shown along with the quantitative analysis of green fluorescent intensity (lower panel). Values shown are presented as mean \pm SEM (n = 3). * $P < 0.05$ compared with cells not treated with ethanol; # $P < 0.05$ compared with cells treated with ethanol. (C) RAW 264.7 macrophages were treated with 0.1 $\mu\text{g/ml}$ of gAcrp for 18 h followed by incubation with 100 mM ethanol for 24 h. NADPH oxidase activity was determined by lucigenin-based assay as described in the materials and methods. Briefly, cell lysates were incubated with Hank's Buffered salt solution (HBSS) containing 100 μM lucigenin and 200 μM NADPH for 30 min. NADPH oxidase activity was then assessed by chemiluminescence and expressed in relative

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light units. Values represent fold increase in comparison to the cells not treated with ethanol and are expressed as mean \pm SEM (n = 6). * $P < 0.05$ compared with cells not treated with ethanol; # $P < 0.05$ compared with cells treated with ethanol. (D) Primary murine peritoneal macrophages were pretreated with gAcrp (0.1 μ g/ml) for 18 h followed by incubation with ethanol (100 mM) for additional 24 h. NADPH oxidase activity was measured as described previously. Values are presented as fold increase compared to the control cells and are expressed as mean \pm SEM (n = 4). * $P < 0.05$ compared with cells not treated with ethanol; # $P < 0.05$ compared with cells treated with ethanol.

Fig. 2. Effects of globular adiponectin on ethanol-induced Nox2 and p22^{phox} expression in RAW 264.7 macrophages. (A) Cells were incubated with gAcrp (0.1 μ g/ml) for the indicated time periods. Nox2 mRNA expression was analyzed by qRT-PCR as described in material and methods. Values are represented as fold increase relative to control and are expressed as mean \pm SEM (n = 4). * $P < 0.05$ compared with cells not treated with gAcrp. (B) Cells were treated with gAcrp (0.1 μ g/ml) for the indicated time periods. Nox2 protein expression levels were analyzed by Western blot analysis as described in materials and methods. Representative image from four independent experiments is shown along with β -actin for internal loading control. Band intensities were quantified by densitometric analysis and normalized by β -actin. Values are represented as fold changes relative to control and are expressed as mean \pm SEM. (C) Cells were treated with gAcrp (0.1 μ g/ml) for the indicated time periods. p22^{phox} mRNA expression was analyzed by qRT-PCR as described previously. Values shown are represented as mean \pm SEM (n = 3). * $P < 0.05$ compared with cells not treated with ethanol. (D) Cells

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were pretreated with gAcrp (0.1 $\mu\text{g/ml}$) for 18 h followed by 100 mM of ethanol for additional 24 h. Nox2 mRNA expression level was assessed by qRT-PCR as described previously. Values are presented as mean \pm SEM (n = 4). * $P < 0.05$ compared with cells not treated with ethanol; # $P < 0.05$ compared with cells treated with ethanol. (E) Cells were pretreated with 0.1 $\mu\text{g/ml}$ of gAcrp for 18 h followed by ethanol for additional 24 h. Nox2 protein was measured by Western blot analysis as described previously. Images are representative of three separate experiments that showed similar results. Band intensities were quantified by densitometric analysis and normalized by β -actin. Values are represented as fold changes relative to control and are expressed as mean \pm SEM. (F) Cells were treated with gAcrp (0.1 $\mu\text{g/ml}$) for 18 h followed by incubation with 100 mM ethanol (100 mM) for 24 h. p22^{phox} mRNA expression was measured as described previously. Values are presented as mean \pm SEM (n = 4). * $P < 0.05$ compared with control; # $P < 0.05$ compared with cells treated with ethanol.

Fig. 3. Involvement of p38 MAPK/NF- κ B pathway in the suppression of ethanol-induced Nox2 and p22^{phox} expression by gAcrp in RAW 264.7 macrophages. (A) and (B) Cells were pretreated with Bay 11-7082 (5 μM), a selective inhibitor of IKK, for 1 h followed by stimulation with 100 mM ethanol for 24 h. Messenger RNA levels of Nox2 (A) and p22^{phox} (B) were assessed by qRT-PCR as described previously. Values are expressed as mean \pm SEM (n = 8). * $P < 0.05$ compared with control; # $P < 0.05$ compared with cells treated with ethanol. (C) and (D) Cells were pretreated with SB203580 (10 μM), a selective inhibitor of p38 MAPK, for 1 h followed by treatment with 100 mM ethanol for additional 24 h. Nox2 (C)

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and p22^{phox} (D) mRNA levels were measured by qRT-PCR as described previously. Values represent fold change relative to control cells and are expressed as mean \pm SEM (n = 4). * $P < 0.05$ compared with the cells not treated with ethanol; # $P < 0.05$ compared with the cells treated with ethanol. (E) Cells were transiently co-transfected with pNF κ B-*luc* and *Renilla* reporter gene as described in materials and methods. After 24 h of transfection, cells were then pretreated with gAcrp (0.1 μ g/ml) for 18 h followed by treatment with 100 mM ethanol for additional 24 h. Transcriptional activity of NF- κ B was measured by luciferase reporter assay. Values are presented as fold increase compared to the control samples and are expressed as mean \pm SEM (n = 5). * $P < 0.05$ compared with the cells not treated with ethanol; # $P < 0.05$ compared with cells treated with ethanol.

Fig. 4. Role of AMPK signaling in the suppression of ethanol-induced Nox2 expression by gAcrp in RAW 264.7 macrophages. (A) Cells were incubated with 100 mM ethanol for the indicated time periods. The level of phosphorylated AMPK α was measured by Western blot analysis as described previously. Images are representative of three independent experiments that showed similar results. Band intensities were quantified by densitometric analysis and normalized by total AMPK. Values are represented as fold changes relative to control and are expressed as mean \pm SEM (n=3). (B) Cells were pretreated with AICAR (1 mM), an activator of AMPK, for 1 h followed by stimulation with 100 mM ethanol for 18 h. Nox2 mRNA level was measured by qRT-PCR. Values represent fold change relative to the control cells and are expressed as mean \pm SEM (n = 3). * $P < 0.05$ compared with the cells not treated with ethanol; # $P < 0.05$ compared with cells treated with ethanol. (C) Cells were co-transfected

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with pNF κ B-*Luc* plasmid and *Renilla* control reporter gene as described previously. After 24 h of transfection, cells were pre-incubated with AICAR (1 mM) for 1 h followed by treatment with 100 mM ethanol for additional 24 h. Transcriptional activity of NF- κ B was assessed by reporter assay. Values are the results of six separate experiments and expressed as mean \pm SEM (n = 6). * $P < 0.05$ compared with the cells not treated with ethanol; # $P < 0.05$ compared with cells treated with ethanol. (D) Cells were treated with gAcrp (0.1 μ g/ml) for the indicated time periods. The level of phosphorylation of AMPK α was analyzed by Western blot analysis. Band intensities were quantified by densitometric analysis and normalized by total AMPK α . Values are represented as fold changes relative to control and are expressed as mean \pm SEM. (E) Cells were pretreated with 0.1 μ g/ml of gAcrp for 18 h followed by stimulation with 100 mM ethanol for 1 h. The level of AMPK α phosphorylation was measured by Western blot analysis. Representative images from three independent experiments are shown along with β -actin for loading control. Band intensities were quantified by densitometric analysis and normalized by β -actin. Values are represented as fold changes relative to control and are expressed as mean \pm SEM. (F) Cells were pretreated with 0.1 μ g/ml of gAcrp for 18 h in the absence or presence of compound C (1 μ M), an AMPK inhibitor, followed by 100 mM ethanol treatment. Nox2 mRNA was analyzed by qRT-PCR as described previously. Values are the results of four separate experiments and presented as mean \pm SEM (n = 4). * $P < 0.05$ compared with the cells not treated with ethanol; # $P < 0.05$ compared with the cells treated with ethanol. (G) Cells were transiently co-transfected with pNF κ B-*Luc* plasmid and *Renilla* reporter gene as described previously. After 24 h of culture, cells were pretreated with 0.1 μ g/ml of gAcrp for 18 h in the absence or presence of compound C (1 μ M) followed by incubation with 100 mM ethanol for additional 24 h. Transcriptional activity of NF- κ B was

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assessed by luciferase reporter assay as described previously. Values are expressed as fold increase relative to control cells, mean \pm SEM (n = 3). * $P < 0.05$ compared with the cells not treated with ethanol; # $P < 0.05$ compared with cells treated with ethanol; § $P < 0.05$ compared with the cells treated with gAcrp and ethanol. (H) (Upper panel) Cells were transfected with different concentrations of siRNA targeting AMPK α 1 or scrambled siRNA. After overnight incubation, expression level of AMPK α was determined by Western blot analysis. (Lower panel) Cells were transfected with siRNA targeting AMPK α (25 nM) or scrambled control siRNA. After 6 h incubation, cells were then co-transfected with pNF κ B-*Luc* plasmid and *Renilla* reporter gene. After overnight incubation, cells were pretreated with gAcrp (0.1 μ g/ml) for 18 h followed by 100 mM ethanol stimulation for additional 24 h. Transcriptional activity of NF- κ B was determined by luciferase reporter assay. Values are presented as fold change relative to control cells and expressed as mean \pm SEM (n = 3). * $P < 0.05$ compared with the cells not treated with ethanol; # $P < 0.05$ compared with cells treated with ethanol.

Fig. 5. Role of AMPK signaling in suppression of ethanol-induced ROS production by gAcrp in RAW264.7 macrophages. (A) Cells were pretreated with gAcrp in the absence or presence of compound C (1 μ M) for 18 h followed by incubation with 100 mM ethanol. After 24 h incubation, the media were replaced with Hank's balanced salt solution (HBSS) containing 10 μ M CM-H₂DCF-DA and cells were further incubated for 30 min in dark. ROS generation was assessed as described previously and representative images from three independent experiments are shown along with quantitation of green signal intensity. Values are presented as mean \pm SEM (n = 3). * $P < 0.05$ compared with control; # $P < 0.05$ compared with cells

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treated with ethanol; [§] $P < 0.05$: compared with the cells treated with gAcrp and ethanol. (B) Cells were transfected with siRNA targeting AMPK α (25 nM) or scrambled control siRNA. After 6 h incubation, media were replaced with DMEM containing 10 % FBS and then further incubated for additional 22 h. Cells were treated with 0.1 $\mu\text{g/ml}$ of gAcrp for 18 h followed by stimulation with 100 mM ethanol for additional 24 h. ROS production was determined as described previously and representative images from three independent experiments are shown along with quantitation of fluorescence intensity (lower panel). Values are expressed as fold change relative to control cells. * $P < 0.05$ compared with control group; # $P < 0.05$ compared with cells treated with only ethanol; [§] $P < 0.05$ compared with the cells treated with gAcrp and ethanol.

Fig. 6. Role of LKB1 signaling in the suppression of ethanol-induced ROS production by gAcrp in RAW 264.7 macrophages. (A) Cells were incubated with gAcrp (0.1 $\mu\text{g/ml}$) for the indicated time periods. The level of LKB1 Phosphorylation was analyzed by Western blot analysis as described previously. Images are representative of three independent experiments along with total LKB1 for internal loading control. Band intensities were quantified by densitometric analysis and normalized by total LKB1. Values are represented as fold changes relative to control and are expressed as mean \pm SEM. (B) Cells were pretreated with 0.1 $\mu\text{g/ml}$ of gAcrp for 18 h followed by incubation with 100 mM ethanol for 1 h. The level of LKB1 phosphorylation was measured by Western blot analysis. Representative images from three independent experiments are shown along with total LKB1 for loading control. (C) (Upper panel) Cells were transfected with different concentrations of siRNA targeting LKB1 or

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scrambled siRNA. After overnight incubation, expression level of LKB1 was determined by Western blot analysis. (Lower panel) Cells were transfected with siRNA targeting LKB1 (50 nM) or scrambled control siRNA. After 6 h incubation, media were changed with DMEM containing 10 % FBS and cells were further cultured for additional 22 h. Cells were treated with 0.1 $\mu\text{g/ml}$ gAcrp for 18 h followed by stimulation with 100 mM ethanol for additional 24 h. Nox2 mRNA expression levels were assessed by qRT-PCR as described previously. Values are presented as fold increase relative to control group and expressed as mean \pm SEM (n = 5). * $P < 0.05$ compared with control group; # $P < 0.05$ compared with cells treated with only ethanol; § $P < 0.05$ compared with the cells treated with gAcrp and ethanol. (D) Cells were transfected with siRNA targeting LKB1 (50 nM) or scrambled control siRNA. After 6 h incubation, cells were then co-transfected with pNF κ B-*Luc* plasmid and *Renilla* reporter gene. After 6 h of transfection, media were replaced with DMEM containing 10 % FBS and further incubated for 18 h. Finally, cells were pretreated with 0.1 $\mu\text{g/ml}$ of gAcrp for 18 h followed by stimulation with ethanol for additional 24 h and the transcriptional activity of NF- κ B was measured as described previously. Values are expressed as fold change relative to control cells, mean \pm SEM (n = 3). * $P < 0.05$ compared with control group; # $P < 0.05$ compared with cells treated with ethanol; § $P < 0.05$ compared with the cells treated with gAcrp and ethanol. (E) Cells were pretreated with U0126 (5 μM), a selective inhibitor of ERK1/2, SP600125 (10 μM), a selective inhibitor of JNK, or SB203580 (10 μM), a selective inhibitor of p38 MAPK for 1 h followed by stimulation with gAcrp (0.1 $\mu\text{g/ml}$) for additional 30 min. Phosphorylated LKB1 levels were analyzed by Western blot analysis along with total LKB1 for internal control. Band intensities were quantified by densitometric analysis and normalized by total

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LKB1. Values are represented as fold changes relative to control and are expressed as mean \pm SEM.

Fig. 7. Proposed model for the modulation of ethanol-induced Nox2 expression by globular adiponectin and its potential role in the suppression of ethanol-induced ROS production in macrophages. Ethanol treatment induces increase in reactive oxygen species (ROS) production in murine macrophages via NADPH oxidase activation, which is composed of various cytosolic and membrane subunits. Ethanol-induced NADPH oxidase activation is accompanied with Nox2 expression through p38 MAPK/NF- κ B pathway. Globular adiponectin treatment causes prevention of ROS production induced by ethanol treatment. The inhibitory effect of gAcrp on ethanol-induced ROS production is mediated by modulation of NADPH oxidase, particularly via inhibition of Nox2 expression. Abrogation of ethanol-induced Nox2 expression by globular adiponectin is mediated through LKB1/AMPK axis signaling mechanisms. LKB1 and AMPK signaling pathway reverted ethanol-induced Nox2 expression, probably via negative regulation of transcriptional activity of NF- κ B. Detailed mechanisms underlying inhibition of NF- κ B signaling by LKB1/AMPK axis remain to be determined.

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Table 1. Sequences of the primers used for quantitative RT-PCR

Target gene	Primer	Sequence
Nox2	Forward	5' - TTGGGTCAGCACTGGCTCTG -3'
	Reverse	5' - TGGCGGTGTGCAGTGCTATC -3'
p22 ^{phox}	Forward	5' - GTCCACCATGGAGCGATGTG -3'
	Reverse	5' - CAATGGCCAAGCAGACGGTC -3'
GAPDH	Forward	5'-ACCACAGTCCATGCCATCAC-3'
	Reverse	5'-TCCACCACCCTGTTGCTGTA-3'

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Table 2. Sequences of small interfering RNA used in transfection

Target gene	Primer	Nucleotide sequence
LKB1	Forward	5'- CCACCGAGGUAUUCUACCA -3'
	Reverse	5'- UGGUAGAUUACCUCGGUGG -3'
AMPK α 1	Forward	5'- GCAGAAGUUUGUAGAGCAA -3'
		5'- UCUUAUAGUUCAACCAUGA -3'
		5'- ACCAGGAAGUCAUACAAUA -3'
		5'- CGAGUUGACCGGACAUAAA -3'
Scrambled	Forward	5'-UGGUUUACAUGUCGACUAA-3'
		5'-UGGUUUACAUGUUGUGUGA-3'
		5'-UGGUUUACAUGUUUUCUGA-3'
		5'-UGGUUUACAUGUUGUCUUA-3'

Fig. 1

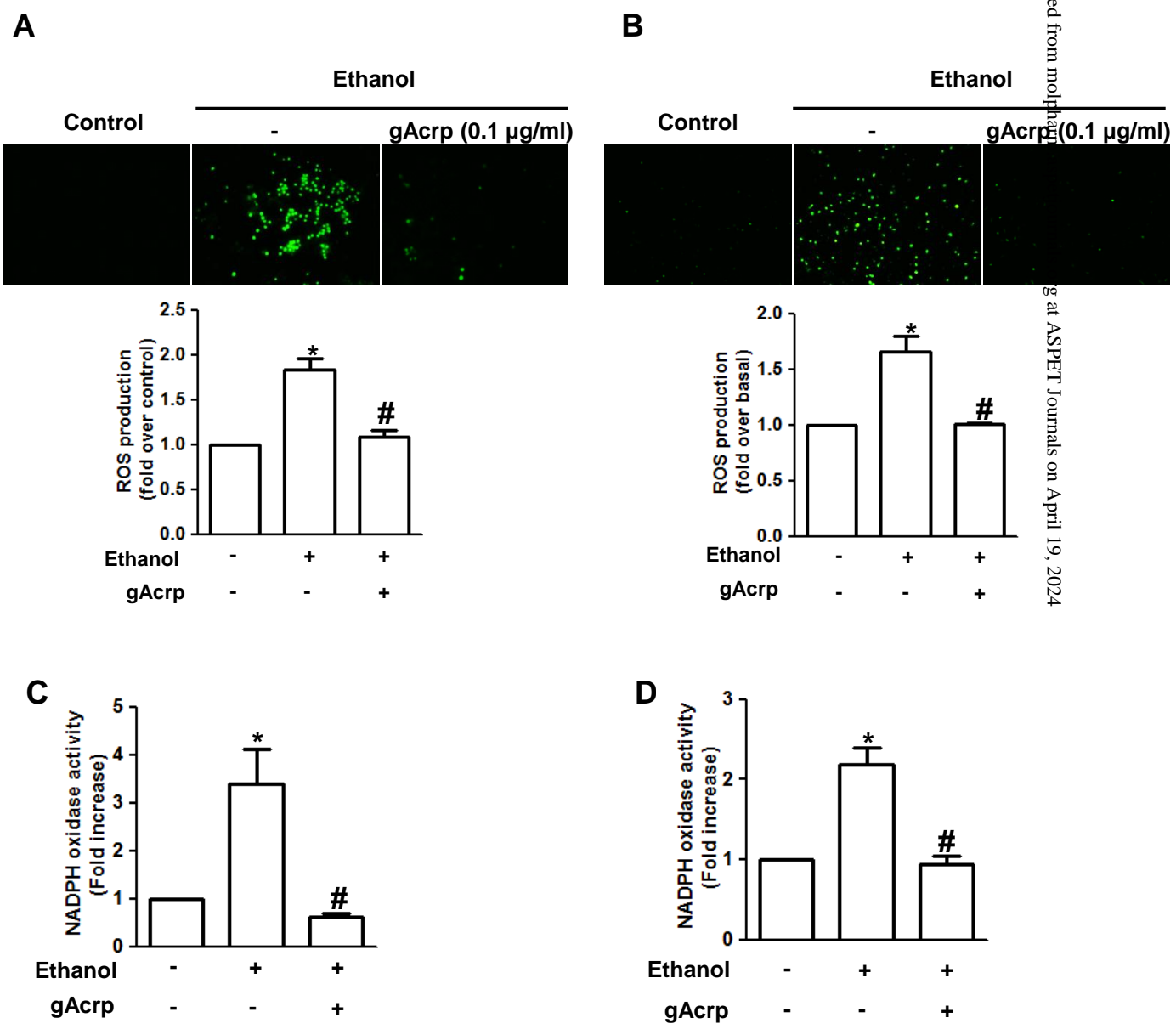


Fig. 2

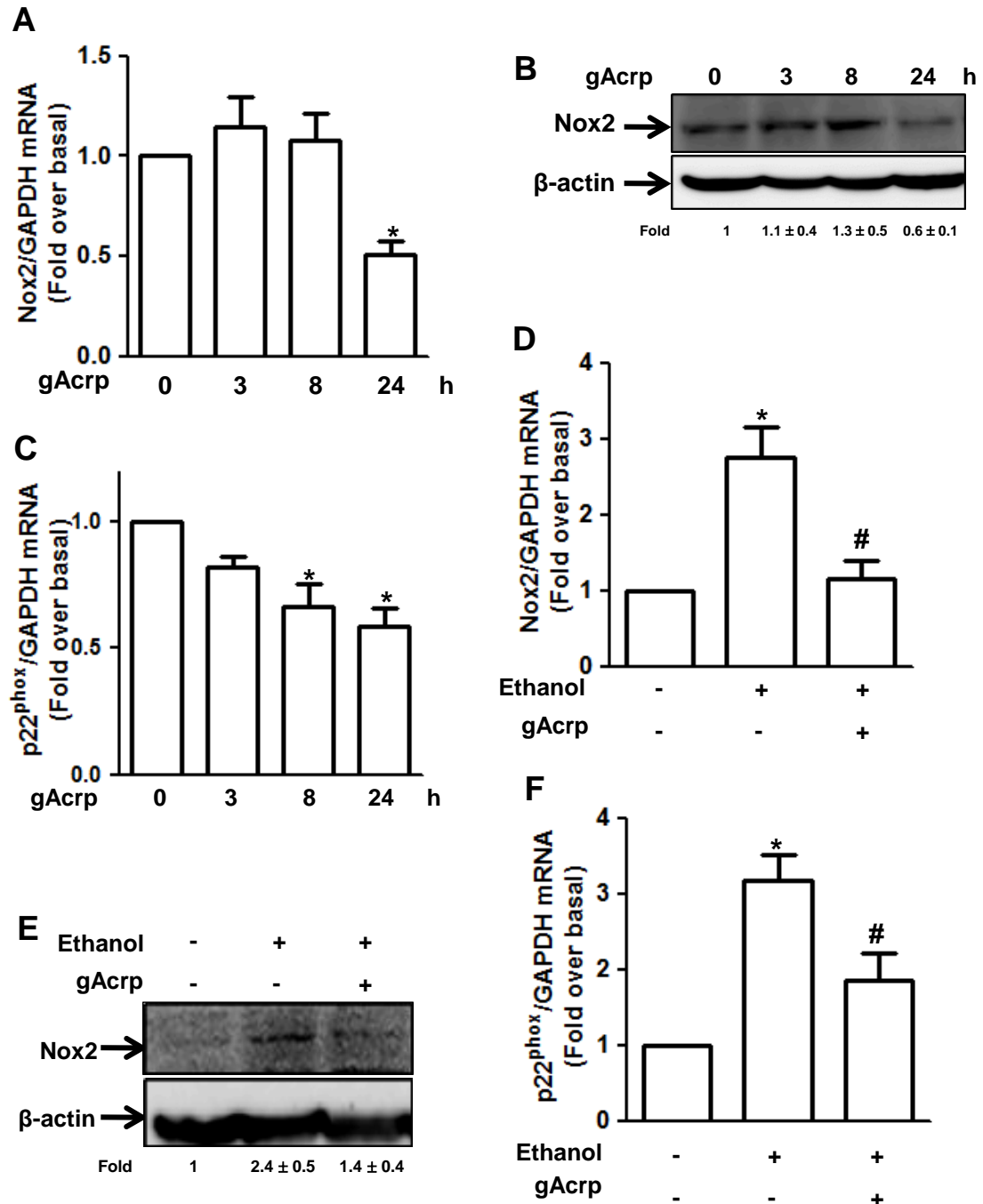


Fig. 3

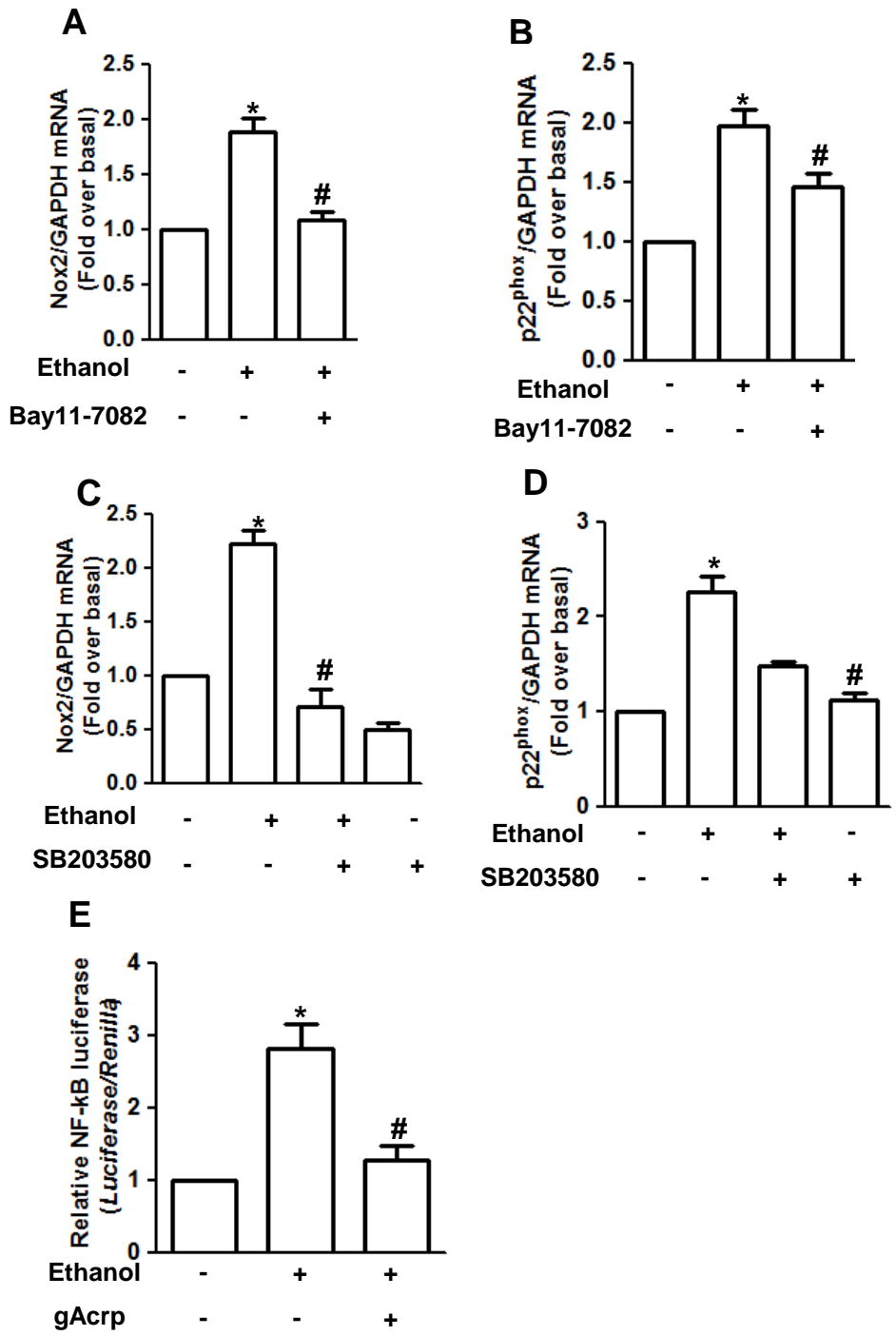
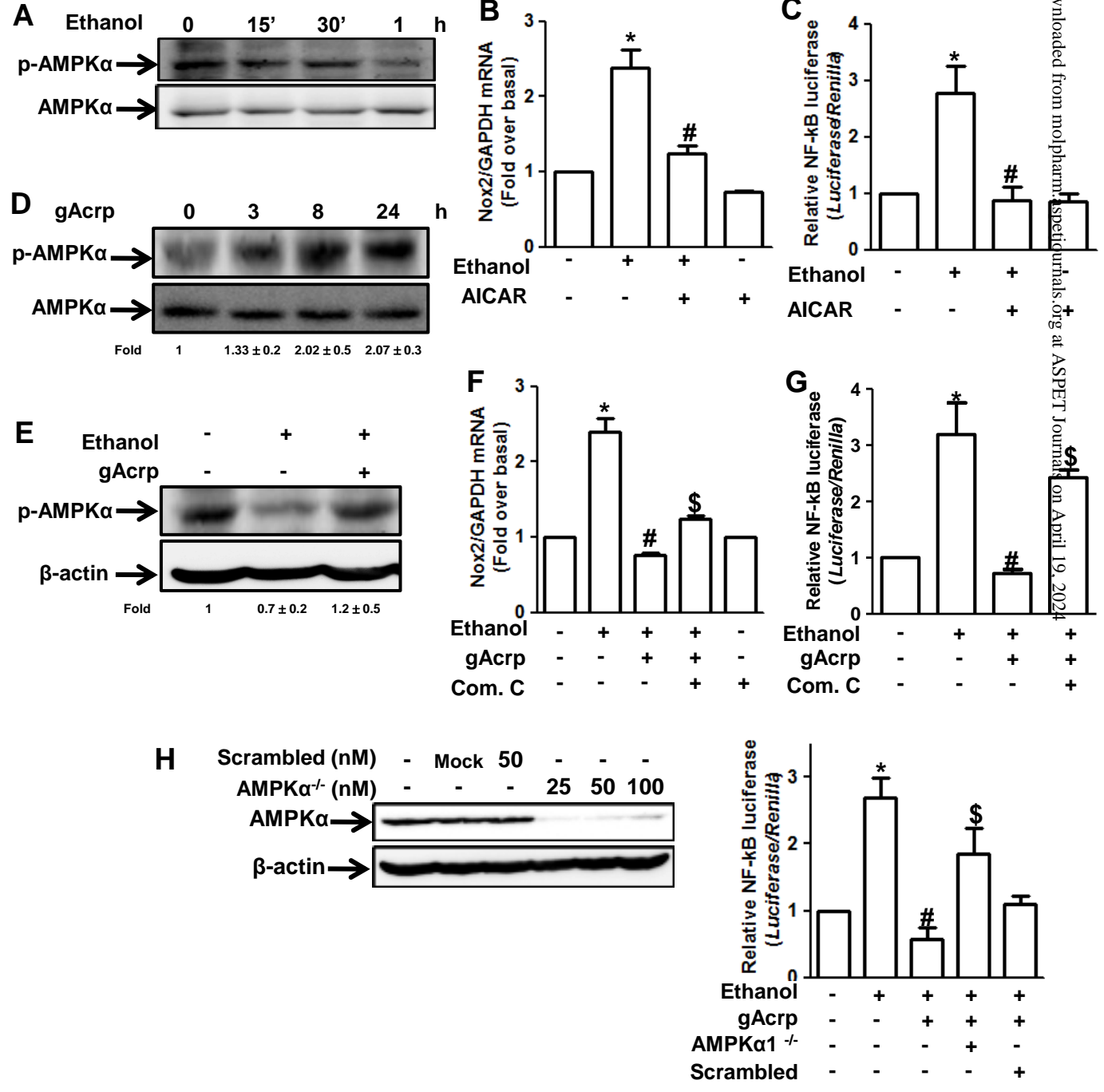
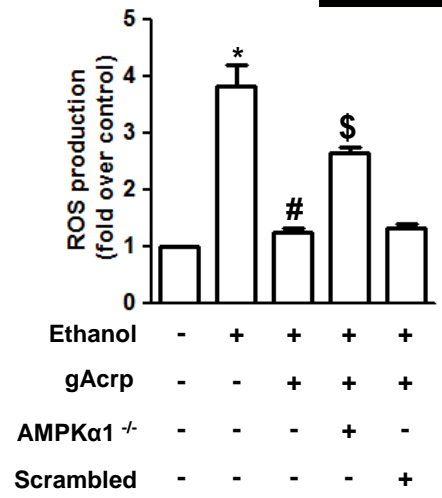
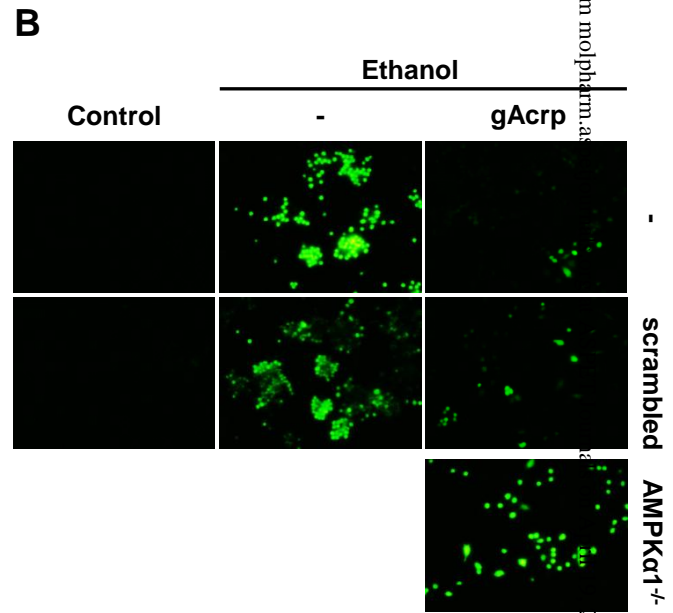
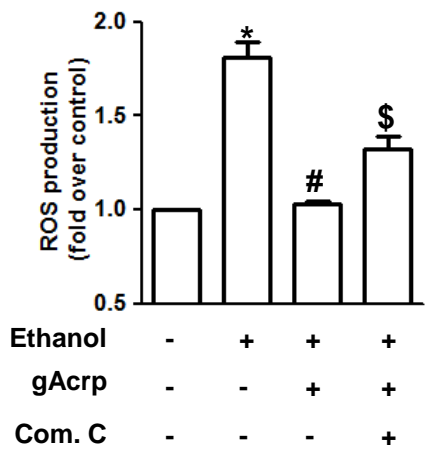
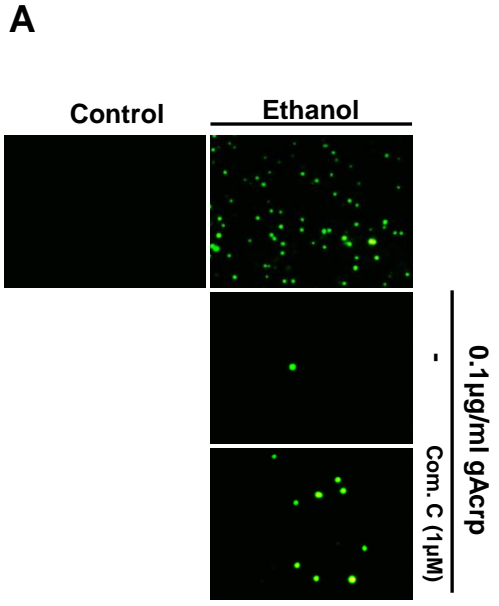


Fig. 4



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Fig. 5



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Fig. 6

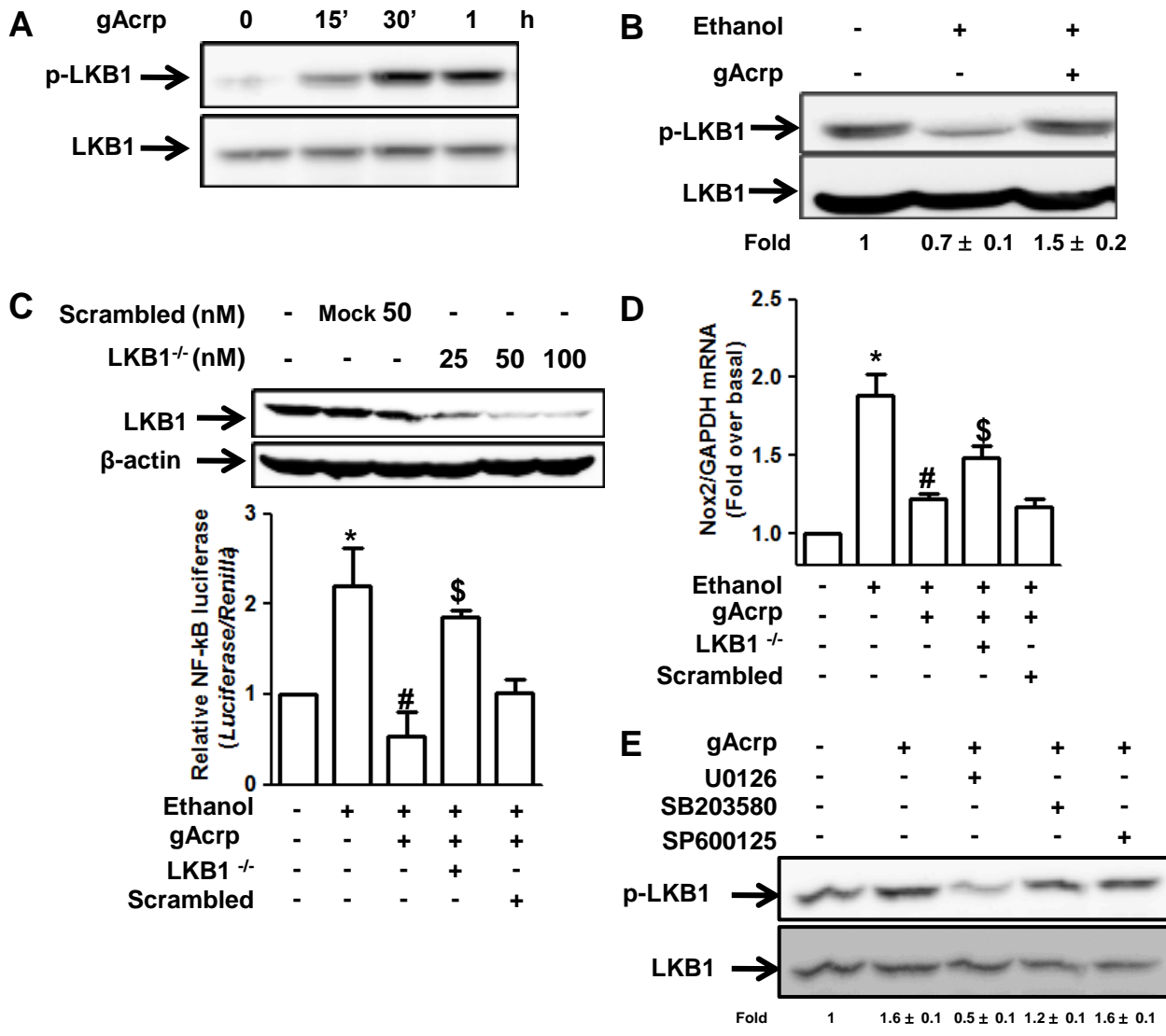


Fig. 7

