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Nitric Oxide Suppresses Transforming Growth Factor-β1–Induced Epithelial-to-Mesenchymal Transition and Apoptosis in Mouse Hepatocytes

Xinchao Pan, Xunde Wang, Weiwei Lei, Lihua Min, Yanan Yang, Xin Wang, and Jianguo Song

Nitric oxide (NO) is a multifunctional regulator that is implicated in various physiological and pathological processes. Here we report that administration of NO donor S-nitroso-N-acetylpenicillamine (SNAP) inhibited transforming growth factor-β1 (TGF-β1)-induced epithelial-to-mesenchymal transition (EMT) and apoptosis in mouse hepatocytes. Overexpression of inducible NO synthase (iNOS) by transfection of the iNOS-expressing vector, which increased NO production, also inhibited the TGF-β1-induced EMT and apoptosis in these cells. Treatment of cells with proinflammatory mediators, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and interferon (IFN)-γ, which increased the endogenous NO production, produced the same inhibitory effect. Furthermore, exogenous NO donor SNAP treatment caused a decrease in the intracellular adenosine triphosphate (ATP) levels. Consistently, depletion of intracellular ATP by mitochondrial uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) inhibited the TGF-β1-induced EMT and apoptosis, suggesting that an NO-induced decrease of ATP involved in the NO-mediated inhibition of TGF-β1-induced EMT and apoptosis. NO and FCCP also inhibited TGF-β1-induced STAT3 activation, suggesting that signal transducer and activator of transcription 3 inactivation is involved in the NO-induced effects on TGF-β1-induced EMT and apoptosis. *Conclusion:* Our study indicates that NO plays an important role in the inhibition of TGF-β1-induced EMT and apoptosis in mouse hepatocytes through the downregulation of intracellular ATP levels. The data provide an insight into the *in vivo* mechanisms on the function of NO during the processes of both EMT and apoptosis. (HEPATOLOGY 2009;50:1577-1587.)

Epithelial-to-mesenchymal transition (EMT) is a phenomenon whereby epithelial cells lose their epithelial cell characteristics and acquire mesenchymal phenotypic traits. EMT is considered to play an important role in embryogenesis in the formation of many different tissues and organs; it is also involved in other physiological and pathological events, including tissue remodeling, wound healing, tumor progression, and some types of sclerosis.[1,2] Fibrosis is a common scarring response to chronic liver injury, which can lead to cirrhosis, liver cancer, and other liver dysfunction. It has been well documented that activated fibroblasts are key contributors to liver fibrosis. Although stellate cells are considered the principal source of liver fibroblasts,[3,4] some evidence suggests that adult hepatocytes also play a role by way of EMT in the accumulation of activated fibroblasts.[5,6] Hepatocyte apoptosis has also been shown to contribute to liver inflammation and fibrosis.[7,8] Thus, exploring the mechanisms of EMT and apoptosis is of importance in developing new and efficacious therapies for treatment of cirrhosis, portal hypertension, liver cancer, and some other liver diseases.

Nitric oxide (NO) has been characterized as a critical modulator in liver inflammation, cirrhosis, portal hypertension, and liver injury, and the roles of NO in liver are

Abbreviations: ATP, adenosine triphosphate; cGMP, cyclic guanosine monophosphate; EMT, epithelial-to-mesenchymal transition; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; IFN-γ, interferon-γ; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; SNAP, S-nitroso-N-acetylpenicillamine; STAT3, signal transducer and activator of transcription 3; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.

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diverse and complex.[9,10] Endogenous NO is synthesized from L-arginine by a family of NO synthase (NOS) enzymes, which includes three classes in mammalian cells: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS).[11] iNOS is a major class of NOS in liver, which is not expressed constitutively in healthy liver. Liver injury or pathogen stimulation can cause the liver to secrete proinflammatory mediators, including interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6, which can subsequently lead to the secretion of high concentrations of NO, derived from iNOS, in most liver cell types, including hepatocytes, Kupffer cells, endothelial cells, and hepatic stellate cells.[12] Some evidence suggests that NOS-induced NO have a protective effect on liver injury and fibrogenesis.[13,14]

The multifunctional cytokine transforming growth factor-β (TGF-β) has been implicated as a mediator of fibrosis in many liver diseases.[15] In addition to apoptosis, TGF-β1 also induces the EMT of hepatocytes. Because TGF-β-induced EMT has been implicated in many liver diseases, the TGF-β signaling pathway is regarded as a promising therapeutic target in hepatic fibrosis.[15] TGF-β and NO typically have opposite effects in regulating cell behavior. TGF-β is an antiinflammatory regulator that can inhibit NO-related inflammatory responses by inhibition of iNOS messenger RNA (mRNA) transcription activity, leading to the inhibition of iNOS protein expression and NO production.[16,17] Thus, investigating the regulatory effect of NO on TGF-β-mediated biological events in hepatocytes is also important for understanding the mechanisms of some liver diseases.

In this study we examined the potential mechanism of TGF-β1-induced EMT and apoptosis in mouse hepatocytes. We found that NO can block TGF-β1-induced EMT and apoptosis, and this effect of NO is accompanied with the inhibition of signal transducer and activator of transcription 3 (STAT3). Moreover, our data indicate that NO-mediated decrease of intracellular adenosine triphosphate (ATP) levels played an important role in inhibiting EMT and apoptosis.

Materials and Methods

Materials. SNAP, Ac-DEVD-pNA and 8-Bromo-cGMP (cyclic guanosine monophosphate) were from Calbiochem (La Jolla, CA). Nitrocellulose membranes were from Amersham Pharmacia Biotech (Buckinghamshire, UK). TNF-α, IL-1β, and IFN-γ were purchased from R&D Systems (Minneapolis, MN). The mouse monoclonal antibody against E-cadherin and the rabbit polyclonal antibody against iNOS were from BD Pharmingen (San Diego, CA). Rabbit polyclonal antibodies against ZO-1 and STAT3, a goat polyclonal antibody against β-actin and horseradish peroxidase (HRP)-conjugated antirabbit, antimouse, and antigoat secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit polyclonal antibody against fibronectin was from Sigma (St. Louis, MO). The mouse monoclonal antibody against p-STAT3 (Tyr705) and rabbit polyclonal antibody against caspase-3 were from Cell Signaling Technology (Beverly, MA). Other reagents were from Sigma unless otherwise indicated.

Mouse Hepatocyte Isolation and Cell Culture. Mouse hepatocytes were isolated by a two-step in situ collagenase perfusion procedure as described previously, with minor modifications.[5,18] Briefly, 6-week-old C57BL/6 mice were purchased from the Shanghai Experimental Animal Center (Chinese Academy of Sciences). Livers were perfused *in situ* through the portal vein with calcium- and magnesium-free Earle's Basic Salt Solution (EBSS) for 3 minutes at a rate of 5 mL/min, followed by EBSS for another 3 minutes at 5 mL/min. Then the liver was digested by 0.5 mg/mL of type IV collagenase in EBSS for 10 minutes at 37°C. The hepatocytes were collected by centrifuging and seeded in Dulbecco's Modified Eagle's Medium (DMEM), containing 10% fetal bovine serum (FBS), and media was changed after 6 hours. Primary culture of mouse hepatocytes were performed at 37°C in a humidified atmosphere of 5% CO_2 in DMEM with 10% FBS, 100 units/mL penicillin, and 100 g/mL streptomycin. The cell medium was changed every 2 days. AML12 hepatocytes (American Type Culture Collection, Manassas, VA) were cultured as described.[19]

Morphological Examination. Morphological changes in the cells were observed under an inverted phase-contrast microscope (Olympus, Japan). The photographs were taken at 200× magnification using a digital camera.

DNA Fragmentation Assay. After being treated with TGF-β1 and/or other reagents, cells were rinsed twice with phosphate-buffered saline (PBS) and lysed on ice for 30 minutes in 10 mM Tris-HCl, pH 8.0, 25 mM ethlyene diamine tetraacetic acid (EDTA), pH 8.0, and 0.25% Triton X-100. Then the cells were scraped and centrifuged (13,800*g*, 15 minutes). The supernatant was incubated with RNase A at 37°C for 60 minutes and then with proteinase K at 56°C overnight. The contents were extracted sequentially with phenol, phenol:chloroform (1:1), and chloroform. The DNA in the aqueous phase was precipitated and analyzed by 1.5% agarose gel electrophoresis. The gel was stained and photographed under transmitted UV light.

Flow Cytometry Analysis. After being treated with TGF-β1 and other reagents, the cell apoptotic rate was determined by flow cytometry analysis and the percentage

of cells with the sub-G_1 DNA content was regarded as the apoptotic rate of the cell population.[20]

Caspase-3 Activity Assay. After being treated with different reagents, cells were collected and caspase-3 activities were detected as described.[20]

Cell Lysate Preparation and Immunoblotting. Cells were lysed in lysis buffer (10 mM Tris, pH 7.4, 1 mM EDTA, 0.5 mM EGTA, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 10 mM $Na_4P_2O_7 \cdot 10\ H_2O$, 5 mg/L aprotinin, 5 mg/L leupeptin, 1 mM phenylmethylsulfonyl fluoride). Proteins (50 μg) were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels and transferred onto nitrocellulose membranes (Hybond, Amersham). After being blocked with 5% skim milk in Tris-buffered saline (TBS), containing 0.1% Tween-20 (TBS-T), the membranes were incubated with appropriate primary antibodies. Then the membranes were washed with TBS-T and incubated with HRP-conjugated secondary antibodies. The immunoreactive bands were detected by enhanced chemiluminescent (ECL) reagents (Amersham).

Immunofluorescence. Cells were seeded on glass slides and treated as indicated. Then the slides were washed with PBS, followed by fixing in 100% methanol at −20°C for 10 minutes. After being blocked with 2.5% bovine serum albumin and 2.5% skim milk, the samples were subjected to probing with appropriate primary antibodies. Then the samples were incubated with goat anti-mouse Cy2-conjugated antibody, goat antirabbit Cy2, or Cy3-conjugated secondary antibodies (Jackson Laboratory, Bar Harbor, ME). The nuclei were stained with DAPI and the fluorescence was visualized under confocal microscopy (Leica).

NO Production Assay. NO production was detected by measuring nitrite, a stable NO metabolite, in the culture medium using a spectrophotometric method, based on the Griess reaction, as described by Kim et al.[21]

ATP Quantification. Intracellular ATP content was determined by the luciferin/luciferase method using the ATP bioluminescent assay kit (Sigma). ATP quantification was performed according to the manufacturer's instruction.

Intracellular cGMP Quantification. After being pretreated with 3-isobutyl-1-methylxanthine (IBMX) for 2 hours, cells were incubated with correspondent reagents. The cGMP content was determined by a cGMP enzyme-linked immunosorbent assay (ELISA) assay kit (R&D Systems).

iNOS Construction and Transfection. The mammalian expression vector pcDNA3.1B-iNOS containing sense-oriented murine iNOS complementary DNA (cDNA) was constructed by polymerase chain reaction (PCR). Total mRNA was extracted from AML12 cells treated with 2 ng/mL of TNF-α, 10 ng/mL of IL-1β, and 2 ng/mL of IFN-γ for 24 hours, and then a cDNA library was prepared by reverse-transcription PCR (RT-PCR) using total mRNA. To clone a full-length iNOS cDNA, three complete sets of clones from separate PCR reactions were sequenced in both forward and reverse directions to reduce the probability of synthesis errors. The three sets of primers are: F1-sense: 5′-gctagcgcttgggtcttgttcactcc-3′, F1-antisense: 5′-gggcccggtactcattctg-3′, F2-sense: 5′-gggcccgtggaggctgcc-3′, F2-antisense: 5′-gggccctcggctgccctc-3′, F3-sense: 5′-gggcccagctacctgcct-3′, F3-antisense: 5′-aagctttcagagcctcgtggctttg-3′. The primers were checked using BLAST to ensure that they are specific for iNOS. These three fragments were spliced together to get a full-length iNOS cDNA. The vectors pcDNA3.1B and pcDNA3.1B-iNOS were transfected into AML12 cells and selected with 800 μg/mL of G418 to get monoclones. The monoclones were maintained in 500 μg/mL of G418.

Data Analysis. Data are expressed as means ± standard error of the mean (SEM) from at least three independent experiments. Statistical analysis was performed using Student's *t* test or analysis of variance (ANOVA). *P* values < 0.05 were deemed statistically significant.

Results

Exogenous NO Inhibited TGF-β1-Induced EMT. Induction of EMT by TGF-β1 was performed according to the method in our previous report.[19,22] TGF-β1-induced EMT in both primary hepatocytes and AML12 cell line was identified by the cell's morphological alterations from an epithelial shape to a fibroblast-like shape. In line with these changes, there were decreased expression levels of ZO-1 and E-cadherin and increased fibronectin levels. Introduction of SNAP, an NO donor, suppressed TGF-β1-induced EMT, as determined by morphological changes (Fig. 1A) and the expression levels of marker proteins of EMT (Fig. 1B). This inhibitory effect of SNAP on EMT is in a dose-dependent manner (Fig. 1C,D), and was confirmed in mouse primary hepatocytes. In mouse primary hepatocytes, SNAP treatment also inhibited TGF-β1-induced cell morphological changes (Supporting Fig. 1A), and abrogated the down-regulation of E-cadherin and ZO-1, and the up-regulation of fibronectin (Fig. 1E,F). TGF-β1-induced increase in cell migration in AML12 cells and mouse primary hepatocytes also can be inhibited by SNAP (Supporting Fig. 1B). These results demonstrate that NO plays an important role in suppressing TGF-β1-induced EMT and in maintaining the epithelial phenotype of mouse hepatocytes.

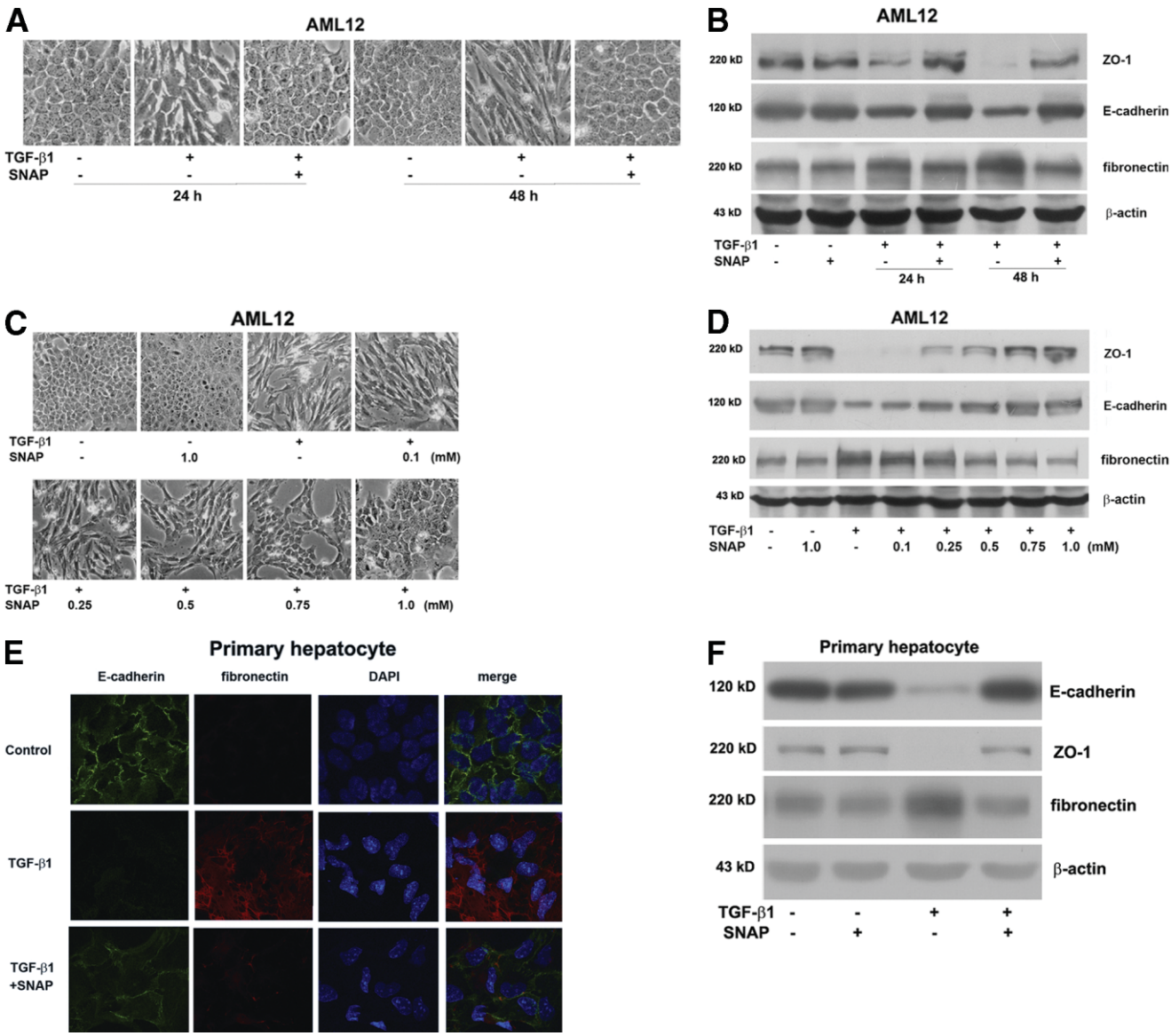

Fig. 1. NO inhibits TGF-β1–induced EMT in mouse hepatocytes. AML12 cells were treated with TGF-β1 (5 ng/mL) and SNAP (1 mM). EMT was examined by (A) observing cell morphological changes or by (B) immunoblotting of cellular levels of ZO-1, E-cadherin, and fibronectin. The dose-effect of SNAP was examined by treating cells with TGF-β1 (5 ng/mL) and different concentrations of SNAP for 48 hours. EMT was examined by (C) cell morphological changes or by (D) immunoblotting of ZO-1, E-cadherin, and fibronectin. Mouse primary hepatocytes were treated with SNAP (1 mM) and TGF-β1 (5 ng/mL) for 48 hours, and EMT was examined by (E) immunofluorescence and (F) immunoblotting.

Exogenous NO Inhibited TGF-β1-Induced Apoptosis. We next examined the effect of NO on TGF-β1-induced apoptosis. TGF-β1-induced apoptosis at both 24 and 48 hours was inhibited by pretreating the cells with SNAP (Fig. 2A). The dose effect of SNAP-mediated inhibition of apoptosis is shown in Fig. 2B. In addition, TGF-β1-induced apoptosis as shown by nuclear condensation, and fragmentation in AML12 cells was inhibited by SNAP (Supporting Fig. 2A,B). Further experiments showed that SNAP treatment blocked TGF-β1-induced caspase-3 cleavage in both AML12 (Fig. 2C) and mouse primary hepatocytes (Fig. 2D). SNAP also abrogated TGF-β1-induced caspase-3 activity (Fig. 2E). These results demonstrate that NO is also important in the control of survival and death in mouse hepatocytes.

NO Generated by iNOS Inhibited TGF-β1-Induced Apoptosis and EMT. To further confirm the effect of NO, we constructed a plasmid containing the mouse iNOS gene and transfected it into AML12 cells. Strikingly, transfection of iNOS, which increased the expression of iNOS (Fig. 3A) and the production of NO (Fig. 3B), suppressed TGF-β1-induced EMT, as assessed by morphological changes (Fig. 3C) and the protein levels of marker proteins (Fig. 3D). Transfection of iNOS also

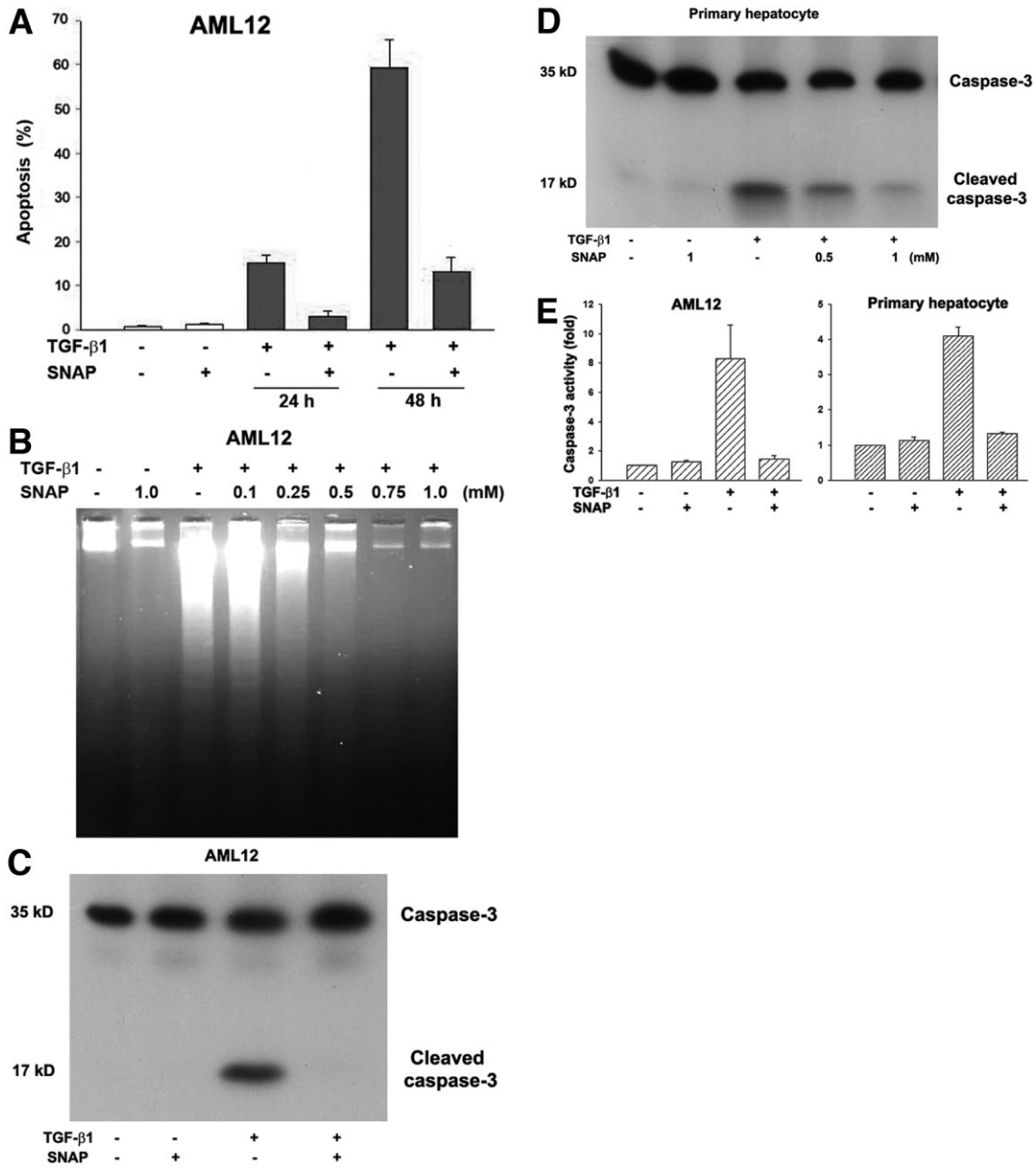

Fig. 2. NO inhibits TGF-β1–induced apoptosis in mouse hepatocytes. AML12 cells were treated with TGF-β1 (5 ng/mL) and SNAP (1 mM) for 24 hours or the indicated time, and apoptosis was detected by using (A) FACS analysis, (B) DNA fragmentation, and (C) immunoblotting assays. Mouse primary hepatocytes were treated with TGF-β1 (5 ng/mL) in the presence or absence of SNAP at different concentrations for 48 hours, and apoptosis was detemined by examining the caspase-3 cleavage using (D) immunoblotting and (E) detecting caspase-3 activity.

abrogated TGF-β1-induced apoptosis (Fig. 3E). These results confirm that NO is a potent inhibitor of TGF-β1-induced apoptosis and EMT.

The administration of TNF-α, IL-1β, and IFN-γ in combination has generally been used to induce iNOS expression and NO generation, to mimic an inflammatory-like situation in hepatocytes. L-NAME is an iNOS inhibitor that can inhibit the generation of NO, but not iNOS expression. To investigate the biological effect of NO, we also treated cells with L-NAME and then examined TGF-β1-induced EMT and apoptosis in the presence of TNF-α, IL-1β, and IFN-γ. In AML12 cells and mouse primary hepatocytes, basal levels of iNOS and NO production were very low (undetectable). Adding TNF-α, IL-1β, and IFN-γ induced iNOS expression and NO production, and the administration of L-NAME inhibited NO production (Fig. 4A), but the iNOS expression level was not influenced (Fig. 4B). On the other hand, adding TGF-β1 did not affect NO production as detected. To determine the effect of endogenous NO production on TGF-β1-induced EMT and apoptosis, we pretreated cells with TNF-α/IL-1β/IFN-γ and/or

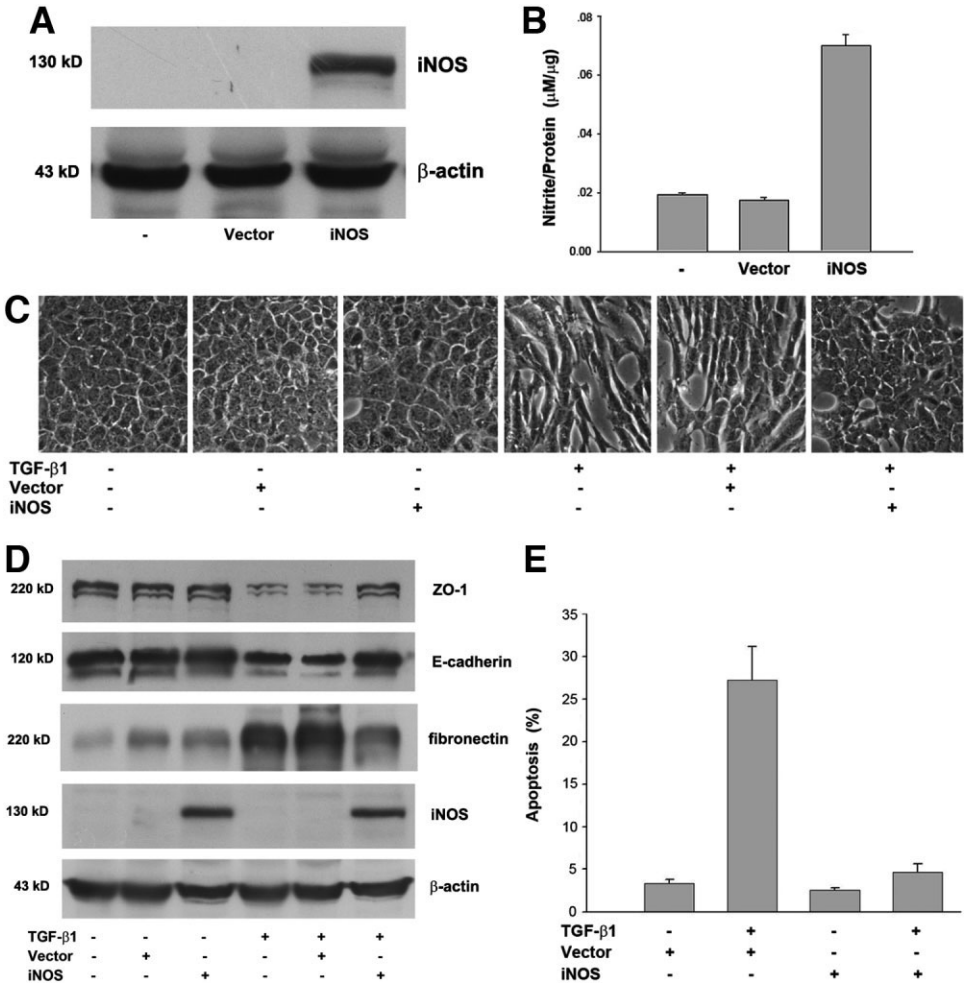

Fig. 3. Overexpression of iNOS inhibits apoptosis and EMT. AML12 cells were stably transfected with iNOS. (A) The expression of iNOS and β-actin was determined by immunoblotting. (B) The cells were cultured for 24 hours, and NO generation was determined by examining the nitrite accumulated in the culture media. The cells were treated with TGF-β1 (2 ng/mL) for 48 hours, and EMT was examined by (C) cell morphological changes and (D) immunoblotting. (E) At 24 hours after the TGF-β1 (5 ng/mL) treatment, the cell apoptosis was determined by FACS.

L-NAME for 24 hours and then stimulated with TGF-β1 for a further 24 or 48 hours. TNF-α/IL-1β/IFN-γ-induced iNOS expression and NO production inhibited TGF-β1-induced EMT (Fig. 4C-E) and apoptosis (Fig. 4F). These results further support a role for NO in the suppression of TGF-β1-induced apoptosis and EMT.

Decreased Intracellular ATP Is Implicated in the Control of Apoptosis and EMT. As cGMP signaling is a classic pathway implicated in certain NO-mediated biological events,[23] we examined whether the alterations of intracellular cGMP levels are associated with NO's effect on apoptosis and EMT. We found that SNAP only induced a transient weak increase of the intracellular cGMP level (Fig. 5A) in AML12 cells. Furthermore, treatment of cells with the exogenous cGMP analog, 8-Bromo-cGMP, generated no effect on TGF-β1-induced apoptosis, as examined by fluorescence-activated cell sorting (FACS) and caspase-3 cleavage (Fig. 5B,C). The effect of 8-Bromo-cGMP on EMT was also examined and no effect was observed (Fig. 5D,E). These results indicate that the increased intracellular cGMP level may not be a critical factor for NO-mediated suppression of TGF-β1-induced apoptosis and EMT.

Mitochondrial components have been shown to be involved in NO-mediated signaling and biological events, and some respiratory chain complexes have been shown to be the targets of NO.[24,25] Thus, alterations in the ATP levels may be implicated in the NO-mediated cellular responses. It has been shown that apoptosis is an energy-requiring process and a relatively high concentration of intracellular ATP is important for completion of apoptotic processes. Loss of ATP can switch cells from apoptosis to necrosis, in response to certain insults, such as NO stimulation.[26,27]

We observed in this study that treatment of AML12 cells with SNAP caused a decrease in intracellular ATP levels (Fig. 6A). Interestingly, TNF-α/IL-1β/IFN-γ treatment also decreased intracellular ATP levels (Fig. 6A). To determine whether NO-mediated reduction in intracellular ATP level was involved in the inhibitory function of NO on TGF-β1–induced apoptosis and EMT, we treated cells with the FCCP, a mitochondrial

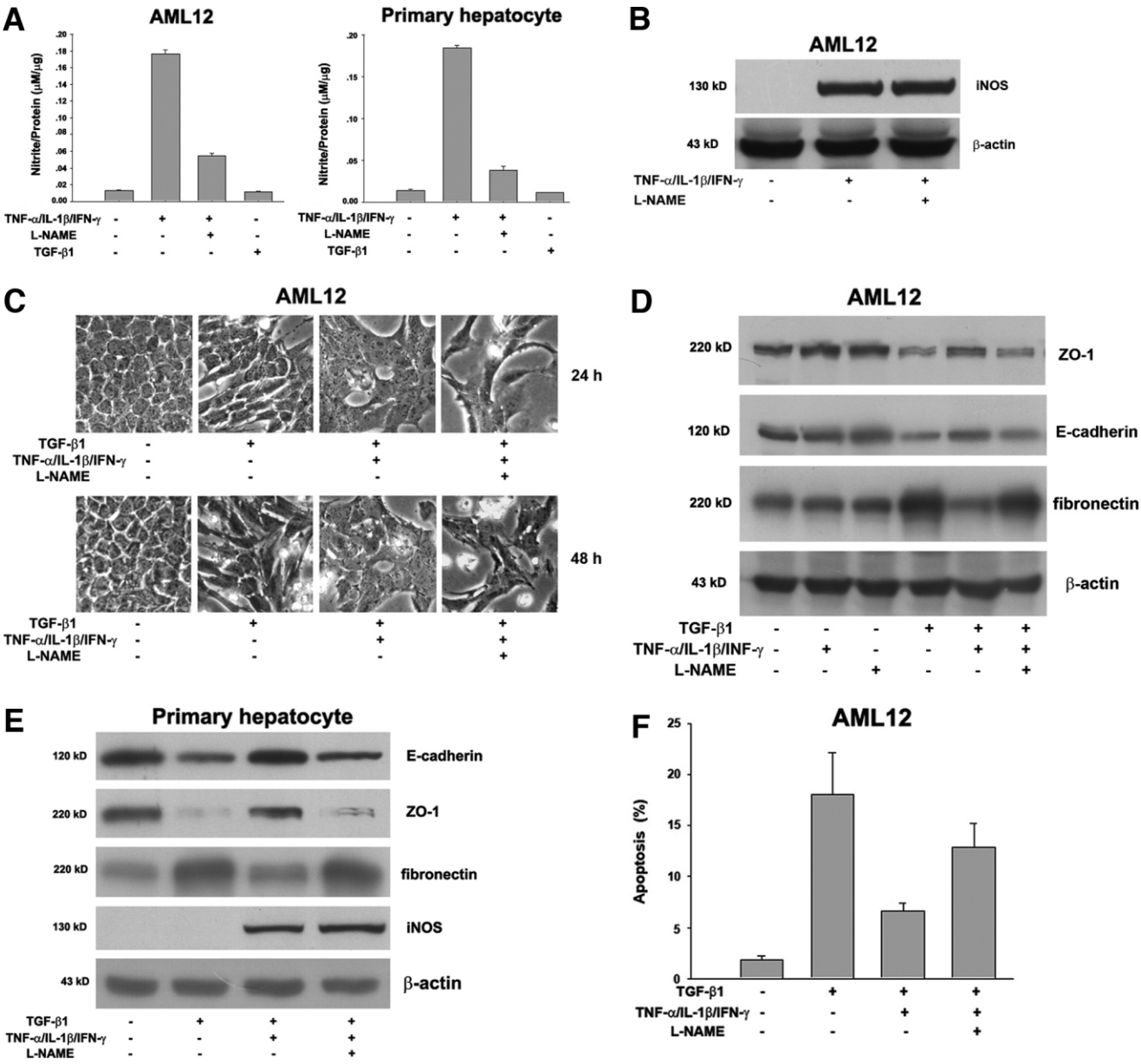

Fig. 4. Induction of NO inhibits TGF-β1-induced EMT and apoptosis. Cells were treated with the combination of TNF-α (2 ng/mL), IL-1β (10 ng/mL), IFN-γ (2 ng/mL), and/or L-NAME (0.2 mM) for 24 hours. (A) The induction of NO generation was examined by determining nitrite accumulation in the culture media, and (B) iNOS expression was detected by immunoblotting using anti-iNOS antibody. Cells were further incubated with TGF-β1 (2 ng/mL) for another 24 hours or indicated times. EMT was detected by (C) morphological change, or by (D) immunoblotting. Mouse primary hepatocytes were also pretreated with TNF-α (2 ng/mL), IL-1β (10 ng/mL), IFN-γ (2 ng/mL), and L-NAME (0.2 mM) for 24 hours, then treated with TGF-β1 (2 ng/mL) for another 48 hours. (E) The protein levels were determined by immunoblotting. (F) The apoptosis of AML12 cells were determined by FACS after being treated with TGF-β1 for 24 hours.

uncoupler of oxidative phosphorylation. As shown in Fig. 6A, 4 μM of FCCP treatment caused a decrease of intracellular ATP levels. In addition, FCCP treatment blocked TGF-β1–induced EMT, as shown by cell morphology (Fig. 6B) and the expression levels of E-cadherin, ZO-1, and fibronectin (Fig. 6C,D). FCCP treatment also blocked TGF-β1–induced apoptosis (Fig. 6E). These results indicate that the inhibitory effect of NO and FCCP on TGF-β1–induced apoptosis and EMT may be mediated by the inhibition of mitochondrial ATP generation.

NO and FCCP Inhibited the TGF-β1–Induced STAT3 Phosphorylation. The observations that both NO and STAT3 have been involved in the regulation of TGF-β1–induced apoptosis and EMT suggest a possibility that NO can regulate STAT3. Thus, we examined whether NO mediates TGF-β1-induced apoptosis and

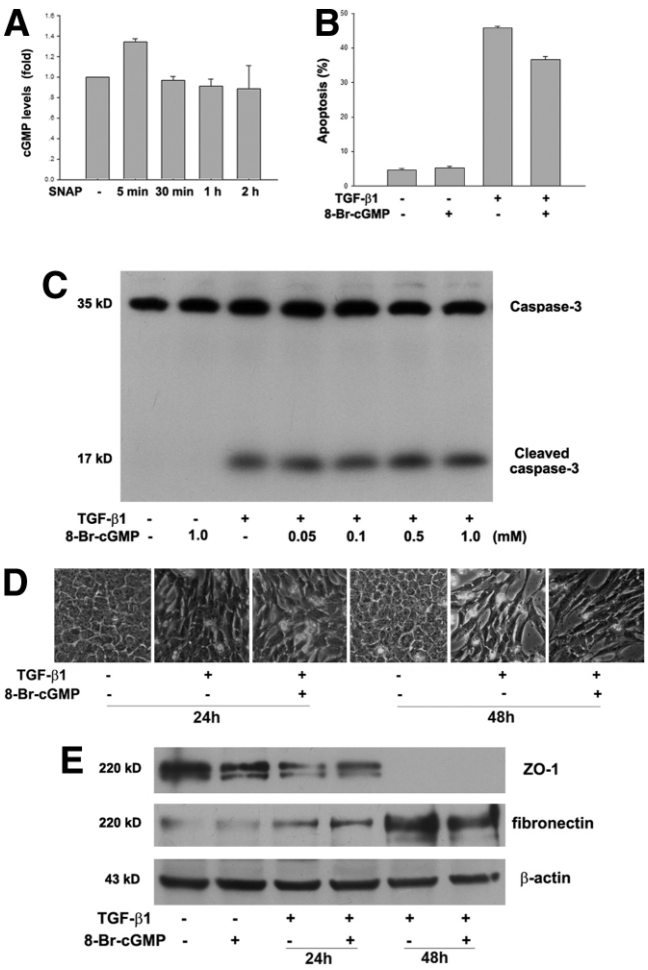

Fig. 5. The effect of cGMP on EMT and apoptosis. (A) AML12 cells were treated with SNAP, and intracellular cGMP levels were detected. AML12 cells were treated with TGF-β1 (5 ng/mL) and 8-Br-cGMP (1 mM) for 36 hours, and apoptosis was determined by (B) FACS analysis and (C) detecting caspase-3 cleavage by immunoblotting. AML12 cells were treated with TGF-β1 (5 ng/mL) and 8-Br-cGMP (1 mM) for 48 hours, and EMT was examined by (D) cell morphological changes and (E) immunoblotting of ZO-1 and fibronectin.

EMT by way of mechanisms involving the regulation of STAT3 activation. TGF-β1 stimulated STAT3 phosphorylation in a dose-dependent manner (Fig. 7A). NO donor SNAP potently inhibited TGF-β1–induced STAT3 phosphorylation (Fig. 7B) in a dose-dependent manner (Fig. 7C). NO production induced by TNF-α/IL-1β/IFN-γ and transfected iNOS also abrogated TGF-β1–induced STAT3 activation (Fig. 7D,E). Interestingly, FCCP treatment also inhibited TGF-β1–induced STAT3 activation (Fig. 7F). Further results show that SB431542, an inhibitor of the TGF-β type I receptor, decreased STAT3 phosphorylation (Supporting Fig. 3A) and blocked TGF-β1–induced EMT and apoptosis in AML12 cells (Supporting Fig. 3B-D). These results suggest that regulation of STAT3 by NO or intracellular ATP levels may be involved in the suppression of TGF-β1–induced apoptosis and EMT by NO.

Discussion

It has been demonstrated that liver cells undergo EMT at various stages of liver development.[28,29] Various factors are known to be involved in the regulation of EMT, and TGF-β is the most widely used cytokine for studying EMT *in vitro*.[30] EMT has been increasingly recognized as a process involved in fibrotic diseases, and it plays an important role in the progression of liver cirrhosis.[31] Cirrhosis is a complication of many liver diseases accompanied with abnormal structure and function of the liver. The structural distortion of the intrahepatic microcirculation in cirrhotic liver can increase the hepatic resistance, which accounts for the development of portal hypertension. Because TGF-β is a major inducer of liver fibrosis and cirrhosis, studies on TGF-β-induced EMT and apoptosis in mouse hepatocytes is of great importance for understanding the mechanism of the progress of liver cirrhosis and portal hypertension.

NO has diverse and complex roles in the liver and NO production is often increased in response to acute insult in the liver, which may protect the liver under some circumstances.[32,33] It has been known that NO is an important regulator in cirrhosis and portal hypertension.[9,10] Except for the fact that an enhanced production of NO accounts for portal hypertension, there is also much evidence showing that NO is an important vasodilator that can reduce portal perfusion pressure in normal and cirrhotic livers.[34] Because both apoptosis and EMT in hepatocytes have been implicated in fibrogenesis-related diseases, our new finding that NO inhibits TGF-β1-induced apoptosis and EMT suggests that NO can be potentially used for the treatment or prevention of liver fibrosis, cirrhosis, and portal hypertension.

It has been reported that NO plays dual roles in tumor progression,[35] but very little is known about the mechanism underlying these roles. TGF-β1-induced apoptosis and EMT have been shown to be two independent events that are contingent on the cell cycle phases.[22] The fact that NO can suppress both apoptosis and EMT induced by TGF-β1 suggests that NO may exert effects through regulating EMT and apoptotic events *in vivo.* Because EMT enhances the migration capacity of tumor cells, inhibition of EMT by NO may attenuate cancer metastasis. On the other hand, the suppressive effect of NO on apoptosis may promote cancer progression. Thus, this study may also provide an explanation on the dual roles of NO in tumor pro-

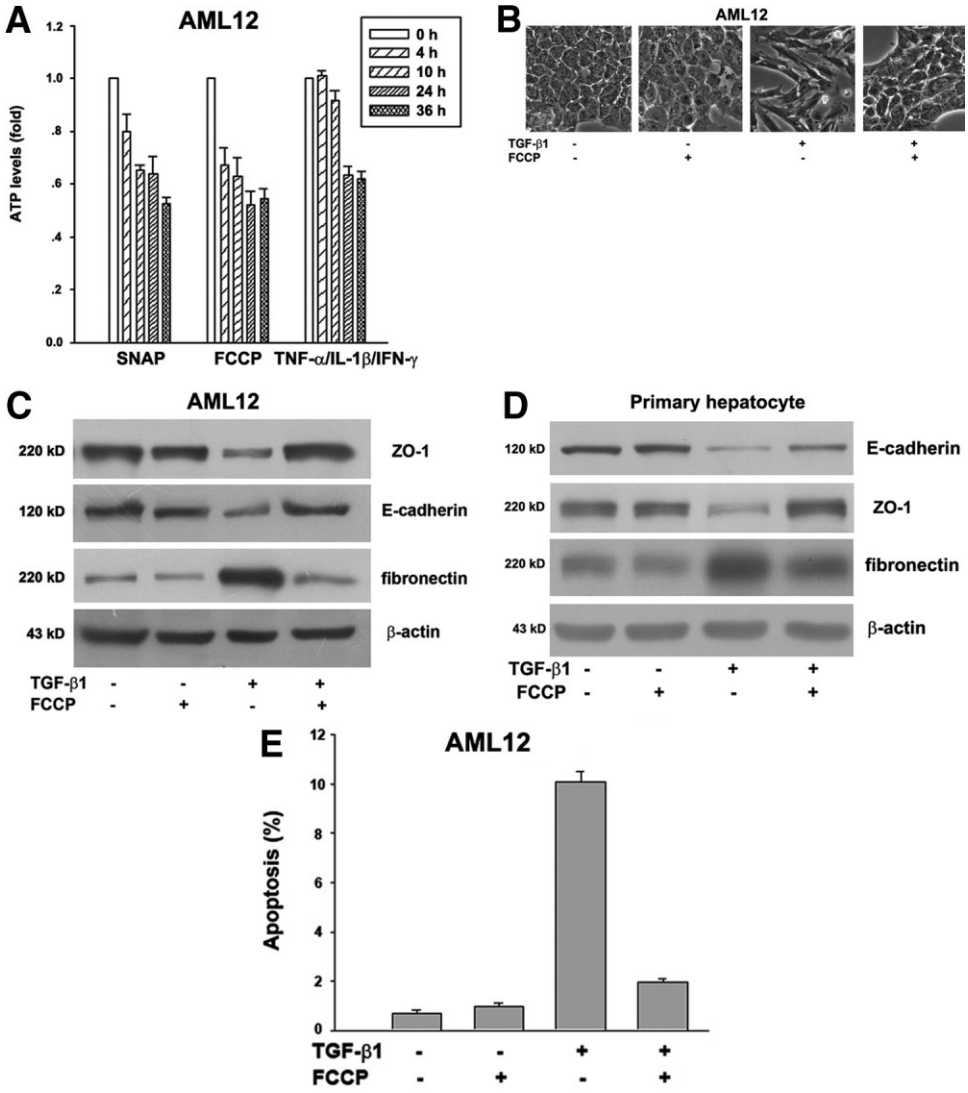

Fig. 6. eduction of intracellular ATP inhibits EMT and apoptosis. (A) AML12 cells were treated with SNAP (1 mM), FCCP (4 μM), or the combination of TNF-α (2 ng/mL), IL-1β (10 ng/mL), and IFN-γ (2 ng/mL) and the intracellular ATP levels were examined. AML12 cells were treated with TGF-β1 (5 ng/mL) and FCCP (4 μM) for 48 hours, and EMT was examined by (B) cell morphological changes or (C) immunoblotting. Mouse primary hepatocytes were treated with TGF-β1 (5 ng/mL) and FCCP (4 μM) for 48 hours. (D) EMT was determined by immunoblotting. AML12 cells were treated with TGF-β1 (5 ng/mL) and FCCP (4 μM) for 24 hours. (E) Apoptosis was determined by FACS analysis.

gression. The specific effect generated by multifunctional NO molecules may depend on the cellular and environmental context.

It has been reported that NO can decrease the activity of some components of the respiratory chain, and inhibition of the respiratory chain by NO will lead to mitochondrial ATP depletion.[24] That NO and the mitochondrial uncoupler reduced the intracellular ATP levels and abrogated TGF-β1-induced EMT suggests that TGF-β1-induced EMT is also an energy-demanding process, like apoptosis. As the normal intracellular ATP level is important for protein phosphorylation, a sufficient intracellular ATP level may be required for TGF-β1-induced STAT3 activation. We observed that the decreased ATP level, by NO and the mitochondrial uncoupler, blocked TGF-β1-induced STAT3 phosphorylation. This evidence indicates that the intracellular ATP level is important in TGF-β1-induced STAT3 phosphorylation.

Interactions between TGF-β1 and NO signaling could be important in modulating biological processes in liver cells. The inhibitory effect of NO on TGF-β1-mediated EMT and apoptosis suggests the possibility that NO may directly or indirectly modify key molecules in the TGF-β1 signaling pathway. Smads are important intracellular mediators of TGF-β-induced responses by regulating the transcription of target genes.[36] It has been reported that Smads are critical regulators in TGF-β1-induced EMT in AML12 cells.[37] We observed that treatment of cells with exogenous NO donor inhibited TGF-β1-induced activation of Smads (Supporting Fig. 4), suggesting that NO's inhibitory effect on EMT and apoptosis is associated with the inhibition of TGF-β-induced Smad2/3 transcriptional activities.

The cGMP-dependent cascade is the best-characterized NO signaling event.[23] Although we did not find the

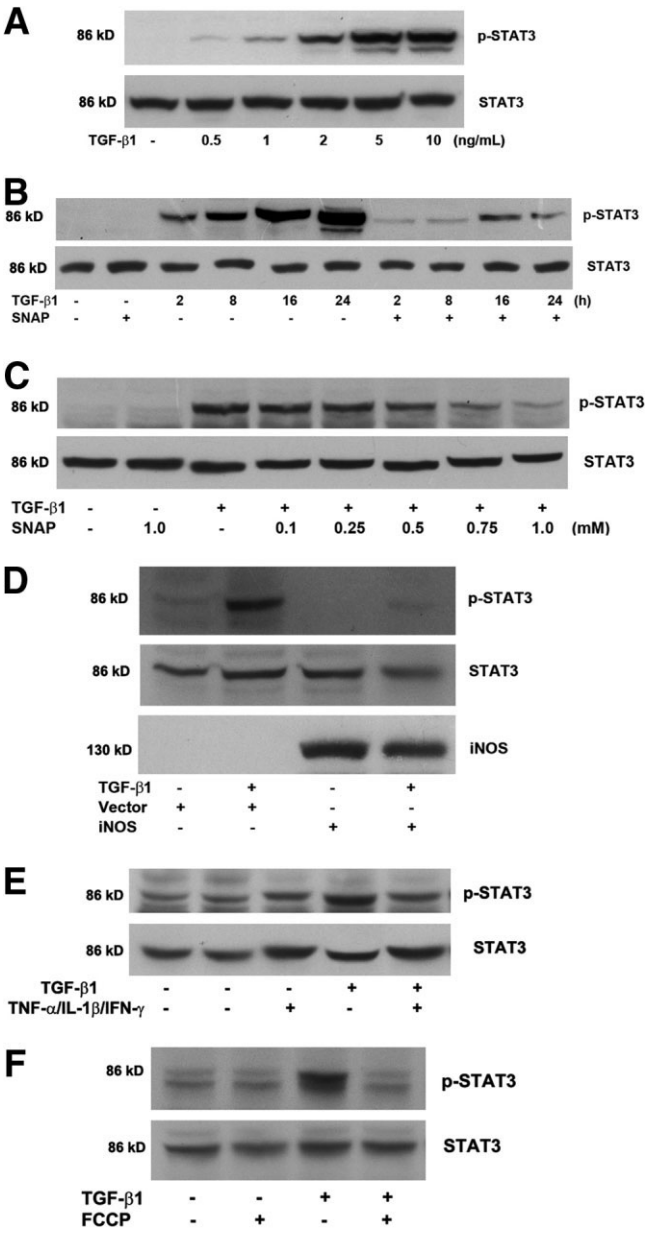

Fig. 7. NO inhibits TGF-β1–induced STAT3 activation. AML12 cells were treated with different concentrations of TGF-β1 for 4 hours, and (A) cell lysates were immunoblotted with anti-STAT3 (Tyr705) and anti-STAT3. (B) AML12 cells were treated with SNAP (1 mM) and TGF-β1 (5 ng/mL), and p-STAT3 (Tyr705) and STAT3 were detected by immunoblotting. (C) AML12 cells were treated with TGF-β1 (5 ng/mL) and different concentrations of SNAP, p-STAT3 (Tyr705), and STAT3 were detected by immunoblotting. (D) AML12 cells transfected with iNOS were treated with TGF-β1 (2 ng/mL) for 4 hours, and cell lysates were immunoblotted with anti-p-STAT3 (Tyr705), anti-STAT3, and anti-iNOS. (E) AML12 cells were pretreated with TNF-α (2 ng/mL), IL-1β (10 ng/mL), and IFN-γ (2 ng/mL) for 24 hours, and then were treated with TGF-β1 (2 ng/mL) for another 4 hours. Cell lysates were immunoblotted with anti-p-STAT3 (Tyr705) and anti-STAT3 antibodies. (F) AML12 cells were treated with FCCP (4 μM) and TGF-β1 (5 ng/mL) for 4 hours, and cell lysates were immunoblotted with anti-p-STAT3 (Tyr705) and anti-STAT3 antibodies.

involvement of intracellular cGMP level in NO-mediated suppression of TGF-β1-induced apoptosis and EMT, we cannot exclude the possibility that basal levels of cGMP signaling are required in these events. NO-mediated protein modifications have been shown in recent years to play an important role in regulating protein activity. For instance, the tyrosine residues of some proteins can be nitrated by NO, causing inactivation of the proteins[38]; the S-nitrosylation of cysteine residues is another kind of modification induced by NO.[39,40] S-nitrosylation affects the stability of targeted proteins. The modification of important proteins in TGF-β1 signaling may be another mechanism underlying the inhibitory effects of NO. Future studies on this possibility may improve our mechanism-based understanding of apoptosis, EMT, and relevant liver diseases.

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