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Ethanol Augments PDGF-Induced NADPH Oxidase Activity and Proliferation in Rat Pancreatic Stellate Cells

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Key Words
NADPH oxidase · Reactive oxygen species · Proliferation · Platelet-derived growth factor · Ethanol

Abstract
Background/Aims: Activated stellate cells are considered the principal mediators of chronic alcoholic pancreatitis/fibrosis. However the mechanisms of alcohol action on pancreatic stellate cells (PaSCs) are poorly understood. The aims of this study were to determine the presence and role of the NADPH oxidase system in mediating alcohol effects on PaSCs with specific emphasis on proliferation. ***Methods:*** PaSC NADPH oxidase components mRNA and protein were determined by RT-PCR and Western blot. The NADPH oxidase activity was measured by detecting the production of reactive oxygen species using lucigenin-derived chemiluminescence assay. PaSC DNA synthesis, a measure of proliferation, was performed by determining the [^{3}H] thymidine incorporation into DNA. ***Results:*** mRNA for NADPH oxidase components Nox1, gp91^{phox}, Nox4, p22^{phox}, p47^{phox} and p67^{phox} and protein for NADPH oxidase subunits gp91^{phox}, p22^{phox}, p47^{phox} and p67^{phox} are present in PaSCs. Treatment with platelet-derived growth factor (PDGF) significantly increased the NADPH oxidase activity and DNA synthesis in cultured PaSCs. Alcohol treatment markedly augmented both the NADPH oxidase activity and the DNA synthesis caused by PDGF, which was prevented by antioxidant N-acetyl-L-cysteine, ROS scavenger tiron, and the NADPH oxidase inhibitor diphenylene iodium. The effects of PDGF on NADPH oxidase activity and DNA synthesis were prevented in PaSCs isolated from the pancreas of mice with a genetic deficiency of p47^{phox}. ***Conclusions:*** Ethanol causes proliferation of stellate cells by augmenting the activation of the cell's NADPH oxidase system stimulated by PDGF. These results provide new insights into the mechanisms of alcohol-induced fibrosing disorders.

Introduction

Chronic pancreatitis is a chronic disorder characterized by fibrosis, parenchymal tissue loss and chronic inflammation. Alcohol abuse has long been recognized as the most common associated factor leading to chronic pancreatitis [1].

Several lines of evidences indicate that pancreatic stellate cells play a key role in the pathogenesis of chronic pancreatitis. In its normal and quiescent state surrounding the pancreatic acinus, the stellate cell contains lipid droplet stores of vitamin A. With activation triggered by alcohol, cytokines, chemokines and growth factors, stellate cells release the vitamin A stores, start proliferating

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and produce large quantities of extracellular matrix proteins [2]. Histological studies demonstrate that pancreatic stellate cells are activated in the pancreas of patients with chronic pancreatitis [3].

In vitro experiments demonstrate that alcohol alone causes pancreatic stellate cells to produce extracellular matrix proteins, however alcohol alone does not cause proliferation of pancreatic stellate cells in vitro [2].

A recent finding of significance to the fibrosing process is that activation and proliferation of hepatic stellate cells by angiotensin II are mediated by the NADPH oxidase system that produces reactive oxygen species (ROS) [4]. Further, the NADPH oxidase system is a necessary factor for inducing fibrosis in an experimental model of bile duct ligation [4]. Finally, recent data support the notion that NADPH oxidase plays a necessary role in the platelet-derived growth factor (PDGF)-induced proliferation of hepatic stellate cells [5]. These findings indicate that ROS generated through the NADPH oxidase system mediate proliferative and fibrosing responses in hepatic stellate cells.

NADPH oxidase has traditionally been regarded as a host defense system which plays an important role in the killing of bacteria by leukocytes. However, more recently NADPH oxidase has increasingly been recognized as a crucial signaling system mediating mitogenic responses in nonleukocyte cell types [6, 7]. ROS have been found to play a crucial role in cell proliferation in vascular endothelial, smooth muscle, and hepatic stellate cells [5, 6, 8, 9].

Leukocyte NADPH oxidase is composed of several subunits. These subunits include constitutive subunits $p22^{phox}$ and $gp91^{phox}$ (Nox2), which are located on the cell membrane, and regulatory subunits $p47^{phox}$ and $p67^{phox}$, and possibly $p40^{phox}$ stationed in cytosol. In neutrophils activated by stimuli, $p47^{phox}$ and $p67^{phox}$ are phosphorylated and translocate to the cell membrane and form active catalytic complexes with $p22^{phox}$ and $gp91^{phox}$ resulting in the generation of ROS [6].

In the present study, we demonstrated the presence of an NADPH oxidase system in pancreatic stellate cells and determined the role of ROS generated by the NADPH oxidase system in mediating the effects of alcohol and PDGF on pancreatic stellate cell DNA synthesis.

Materials and Methods

Materials

Antibodies for $gp91^{phox}$ (Nox2), $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$ were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). Nycodenz was purchased from Accurate Chemical and Scientific Corp. (Westbury, N.Y., USA). [Methyl-^{3}H] thymidine was purchased from MP Biomedicals Inc. (Irvine, Calif., USA). DMEM/F12 was purchased from Invitrogen (Carlsbad, Calif., USA). Lucigenin was obtained from Molecular Probes (Eugene, Oreg., USA). Matrigel was obtained from BD Biosciences (San Diego, Calif., USA). Rat PDGF BB and hPDGF BB were used for experiments with rat pancreatic stellate cells and mouse pancreatic stellate cells, respectively, and were purchased from Sigma (St. Louis, Mo., USA) and Roche (Chicago, Ill., USA), respectively. All other chemicals were obtained from Sigma-Aldrich Co. (St. Louis, Mo., USA). The $p47^{phox}$-deficient mice and corresponding wild-type mice were obtained from The Jackson Laboratory (Bar Harbor, Me., USA).

Pancreatic Stellate Cell Isolation and Treatment

Pancreatic stellate cells were isolated from the whole pancreas of Sprague-Dawley rats weighing 250–300 g as previously described by Apte et al. [10]. In brief, the pancreas was digested with a mixture of 0.05% (wt/vol) collagenase P, 0.02% pronase and 0.1% deoxyribonuclease in Gey's balanced salt solution. The resulting suspension of cells was centrifuged in a 13.2% Nycodenz gradient at 1,400 *g* for 20 min. Stellate cells separate into a fuzzy band just above the interface of the Nycodenz solution. Isolated stellate cells were washed and resuspended in DMEM/F12 supplemented with penicillin (100 units/ml), streptomycin (0.1 mg/ml) and 15% fetal bovine serum and then incubated at 37°C in a 5% CO_2 humidified atmosphere. The cells become activated when grown on a plastic surface at about 48 h as demonstrated by α-SMA staining with a purity of >98% [11]. The cell culture medium was replaced every 3 days. Once the cells grew to confluence, they were removed from the plate with trypsin treatment and replated. The replated cells were referred to as passage 1 cells. A second replating was referred to as passage 2 cells. Passage 1 or 2 cells were used for all experiments. All incubations were in culture medium described above. The cells were treated with ethanol or PDGF or different inhibitors for indicated times and concentrations.

The isolation of pancreatic stellate cells from mice was performed as described above with modification. The pancreatic tissue from wild-type and $p47^{phox}$-deficient mice was digested with a mixture of 0.0375% (wt/vol) collagenase P, 0.015% pronase and 0.075% deoxyribonuclease at 37°C followed by separation on the gradient system as described above. The preparation was >96% purity as confirmed by desmin and α-SMA staining. The isolated cells were cultured using the same conditions described above for rat pancreatic stellate cells and passage 1 and 2 cells were used for experiments as our preliminary experiments demonstrated that passage 1 and 2 cells showed the greatest viability and there was no significant difference in terms of NADPH oxidase activity between passage 1 and 2 cells. The culture medium was refreshed every 24 h once the treatment with chemical or ethanol started. The culture media were changed every 24 h during treatments in order to diminish the effects of evaporation of ethanol. The pancreatic stellate cells isolated from $p47^{phox}$ knockout mice grown in vitro acquire myofibroblast-like phenotype similar to that isolated from wild-type animals with loss of lipid droplets and positive staining with α-SMA.

Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from pancreatic stellate cells using TRI reagent from Molecular Research Center (Cincinnati, Ohio,

Table 1. Primer sequences for rat NADPH oxidase subunits

mRNA	Forward primer	Reverse primer	GenBank™ accession No.
Nox1	5′-ttccctggaacaagagatgg	5′-taagaaaaccccaccacag	NW_053683
gp91^{phox}	5′-caaagcctgtggctgtgata	5′-cttgagaatggaggcagagg	NW_023965
Nox3	5′-gacccaactggaatgaggaa	5′-aatgaacgcccctaggatct	XM_217848
Nox4	5′-tccagtggtttgcagacttg	5′-ggtccacagcagaaaactcc	NW_047561
p22^{phox}	5′-gccattgccagtgtgatcta	5′-ctcctcttcggcctcactt	NW_047536
p47^{phox}	5′-gcccagcgacagattagaag	5′-ggagtcgatggattgtcctt	NW_047371
p67^{phox}	5′-tctaagaagctggcgctctc	5′-gcgtctgagttttcccttttg	AB002664
β-Actin	5′-ctcttccagccttccttcct	5′-cttctgcatcctgtcagcaa	NM_031144

USA). 5 μg of total RNA was reverse-transcribed using Superscript II Preamplification System from Invitrogen using oligo (dT) as the primer. cDNA derived from 0.5 μg of total RNA was amplified by PCR using primers for different NADPH oxidase subunits listed in table 1. PCR was performed with denaturation for 3 min at 95°C, followed by 30 amplification cycles, each comprising 30 s at 94°C, 45 s at 56°C, and 1 min at 72°C. The PCR products were analyzed by 1.8% agarose electrophoresis gels stained with ethidium bromide.

Western Blotting

Passage 1 pancreatic stellate cells were washed twice with phosphate-buffered saline (pH 7.4) and then suspended in RIPA lysis buffer (50 mM NaCl, 50 mM Tris/HCl, pH 7.2, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS) for at least 30 min at 4°C in a rotator, followed by centrifugation at 15,000 *g* for 20 min. The supernatants were collected for Western blot analysis. For Western blot analysis, 25 μg of protein was separated by 4–20% SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane from Schleicher & Schuell Co. (Keene, N.H., USA). Nonspecific binding was blocked with 5% nonfat dry milk in Tris-buffered saline (4 mM Tris base, 100 mM NaCl, pH 7.5). The membranes were then washed in Tris-buffered saline with 0.05% Tween-20, incubated overnight with primary antibodies (1:1,000) in buffer containing 1% nonfat dry milk in Tween-20, washed and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (1:2,000 dilution). Membranes were developed with Super Substrate Ultra ECL kit from Pierce (Rockford, Ill., USA).

NADPH Oxidase Activity Measurements

NADPH oxidase activity in cell membranes and cytosolic fractions was measured by detecting the production of ROS using lucigenin-derived chemiluminescence assay with NADPH as substrate as we previously described [12]. Briefly, 25 μg of protein prepared from pancreatic stellate cells was diluted in 400 μl of 50 mM phosphate buffer containing 1.0 mM EGTA and 150 mM sucrose. The reaction was carried out at 37°C. Chemiluminescence was measured at 15-second intervals for a total of 75 s in a Tunner 20/20 luminometer (Sunnyvale, Calif., USA) started immediately after adding dark-adapted lucigenin into the reaction mixture. The obtained values were analyzed by Prizm (Stillwater, Okla., USA) and the slope for enzyme reaction was used for NADPH oxidase activity analysis and for statistical calculation. Protein concentration for all assays was measured by Bradford assay (Hercules, Calif., USA). Previous reports indicated that high lucigenin concentrations can by themselves stimulate additional superoxide production [13]. However experiments done by our laboratory in pancreatic cancer cells have demonstrated that lucigenin at a concentration of 5–50 μM does not induce artificial superoxide production [12]. Similar experiments were performed in our pancreatic stellate cell preparation showing that lucigenin at a concentration of 5–75 μM did not produce additional superoxide production. The data presented here were obtained with 25 μM lucigenin.

DNA Synthesis

Pancreatic stellate cell DNA synthesis, a measure of proliferation, was performed by determining the [^{3}H] thymidine incorporation into DNA as described before with modification [10]. In brief, passage 1 or 2 pancreatic stellate cells were seeded onto 12- or 24-well plates. Once the pancreatic stellate cells reached about 70% confluence, they were treated with vehicle, ethanol, PDGF or inhibitors alone or in combination for indicated times at indicated concentrations. For these experiments, [^{3}H] thymidine at a final concentration of 1.0 μCi/ml was added to the cells 24 h prior to measuring the amount of [^{3}H] thymidine incorporated into DNA. The cells were precipitated with trichloroacetic acid (10% wt/vol) twice, then the precipitate was washed with iced phosphate-buffered saline 3 times and air-dried, followed by digesting with 1 N NaOH for 30 min and neutralized with 1 N HCl. DNA-associated [^{3}H] thymidine was measured using standard scintillation counting techniques.

Statistical Analysis

Results were expressed as the means and the standard deviation (SD) of the values of 3–4 independent experiments. ANOVA was used for multiple variable analyses followed by unpaired Student's t test; p values <0.05 were considered statistically significant.

Results

Presence of NADPH Oxidase(s) in Pancreatic Stellate Cells

First we determined the presence of components of the NADPH oxidase system in pancreatic stellate cells using RT-PCR analysis of mRNA from pancreatic stellate cells.

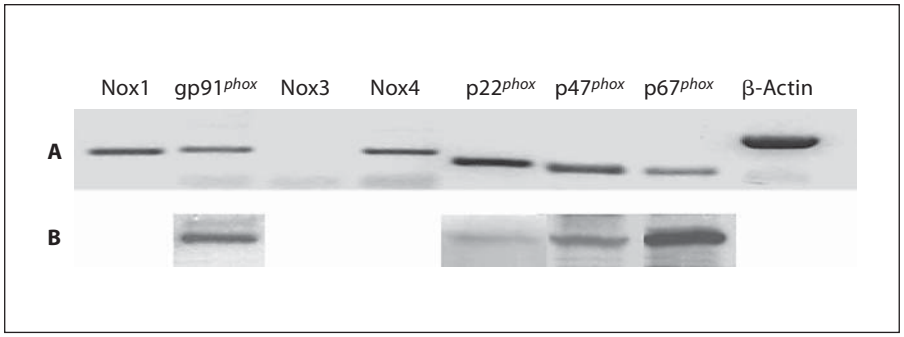


Fig. 1. Pancreatic stellate cells express mRNA and protein for NADPH oxidase components. Pancreatic stellate cells, passage 1 or 2, were used for mRNA extraction for RT-PCR analysis, and protein preparation for Western blot as described in 'Materials and Methods'. **A** RT-PCR of mRNA for NADPH oxidase subunits. **B** Western blot analysis for protein expression of NADPH oxidase subunits. These results are representative of two independent experiments.

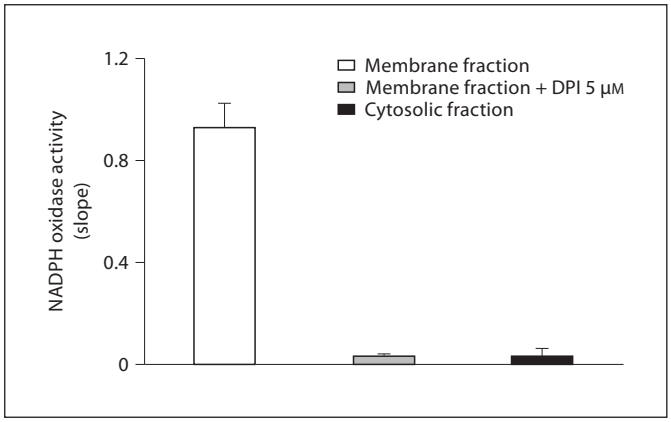


Fig. 2. NADPH oxidase activity located in the membrane fraction of pancreatic stellate cells. Pancreatic stellate cells were lysed in lysis buffer and the membrane and cytosolic fractions were separated by centrifugation. The activity of NADPH oxidase in each fraction was analyzed as described in 'Materials and Methods'. DPI was added to the membrane fraction 10 min prior to activity assay. The results represent the means and SD of the values of three independent experiments.

Table 1 illustrates the primers used for RT-PCR analysis. Figure 1A shows the RT-PCR products from RNA extracted from stellate cells. The results indicated that mRNA for NADPH oxidase system subunits is present in isolated pancreatic stellate cells, including Nox1, gp91^{phox} (Nox2), Nox4, p22^{phox}, p47^{phox}, p67^{phox}. Using Western blot analysis, we found that gp91^{phox}, p22^{phox}, p47^{phox} and p67^{phox} were all present in pancreatic stellate cells at the protein level (fig. 1B). These results indicated that an NADPH oxidase system is present in activated pancreatic stellate cells. Further, the composition of components demonstrated by Western blot indicates that stellate cells have an NADPH oxidase system similar to that of leukocytes.

NADPH Oxidase Activity Is Present in Pancreatic Stellate Cell Membranes

In the neutrophil, the subunits of NADPH oxidase are located in different parts of the cell. That is, gp91^{phox} (Nox2) and p22^{phox} are anchored in the cell membrane, while the regulatory subunits p47^{phox} and p67^{phox} are present in the cytosol of the resting neutrophil. With activation, p47^{phox} and p67^{phox} translocate to the cell membrane to form a complex with the membrane-located subunits to activate the NADPH oxidase resulting in ROS generation. In order to determine the location of active NADPH oxidase activity, we cultured pancreatic stellate cells for 48 h in vitro in the presence of 15% fetal bovine serum. Then the cells were lysed and the membrane and cytosolic portions were separated by centrifugation in order to determine the distribution of enzyme activity. We found that NADPH oxidase activity was predominantly located in the cell membrane fraction (95%) compared to the cytosolic fraction (5%) of the stellate cells (fig. 2). The activity was specific to NADPH, that is, there was activity only with NADPH, but not with NADH added as a substrate (fig. 3). Furthermore, NADPH oxidase activity was blocked by preincubating the membranes with the NADPH oxidase inhibitor diphenylene iodium (DPI).

PDGF Treatment Increases NADPH Oxidase Activity

In order to investigate the effect of growth factors on NADPH oxidase activity, we treated pancreatic stellate cells with PDGF at different concentrations. We found that PDGF dose-dependently increased NADPH oxidase activity, with an EC_{50} at 10 $\pm$ 4 ng/ml and E_{max} at 20–40 ng/ml (fig. 4A). Next, we treated the pancreatic stellate cells with PDGF at a concentration of 20 ng/ml for different time periods. We found that PDGF time-dependently increased NADPH oxidase activity (fig. 4B). The increase in NADPH oxidase activity by PDGF treatment was predominantly located in the membrane fraction of pancreatic stellate cells (fig. 5).

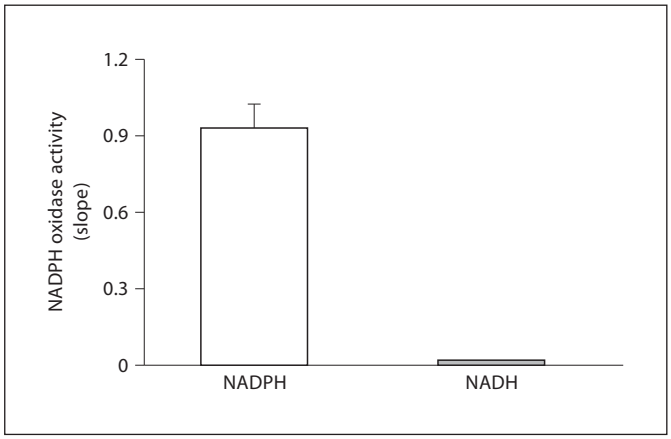
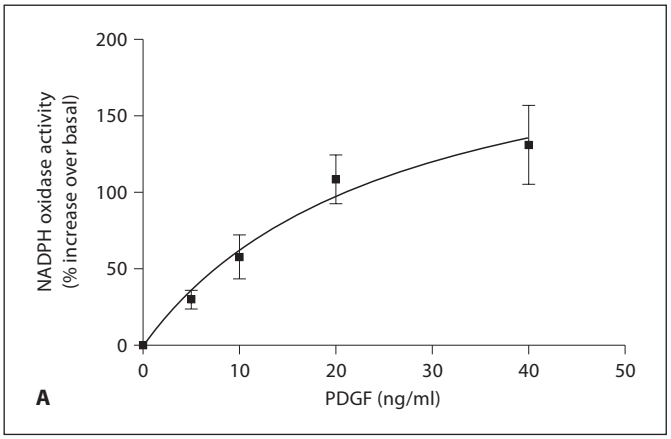


Fig. 3. NADPH oxidase measurement is specific for NADPH. The pancreatic stellate cell membrane fraction was used to examine the chemiluminescence generation with the addition of either NADPH or NADH. The results are the means and SD of the values of three independent experiments.



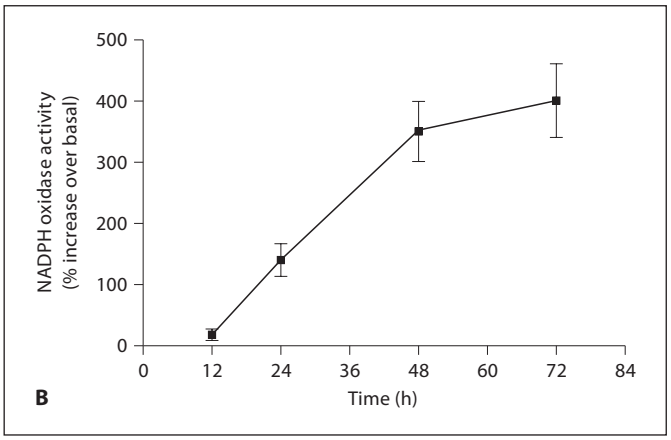


Fig. 4. PDGF treatment increases NADPH oxidase activity in pancreatic stellate cells in a dose- and time-dependent fashion. Pancreatic stellate cells were treated with PDGF at the indicated concentrations for the indicated times. The membrane fraction was used for NADPH oxidase activity assay. **A** Cells were treated with rat PDGF BB at indicated concentrations for 24 h. **B** Cells were treated with PDGF 20 ng/ml for indicated times. The results represent the mean and SD of the values of four independent experiments.

Ethanol Treatment Augments the PDGF-Induced NADPH Oxidase Activity in Pancreatic Stellate Cells

We next investigated the effects of ethanol treatment on NADPH oxidase activity. Preliminary experiments had shown that ethanol at concentrations of 25, 50, and 100 mM and treatment for 24, 48 and 72 h generated no significant effects on NADPH oxidase activity and [^{3}H] thymidine incorporation into DNA. However, treatment with ethanol at concentrations of 50 and 100 mM for 72 h produced a synergistic effect on 10 ng/ml PDGF (EC_{50})-induced NADPH oxidase activity with a robust effect at 100 mM ethanol. Therefore the concentration of 100 mM and the time period of 72 h were selected for further experiments; these conditions are widely used in in vivo and in vitro experiments [14–16]. The pancreatic stellate cells once grown to about 70% confluence were treated with ethanol at a concentration of 100 mM for 72 h, and PDGF was added to the culture for the last 24 h of ethanol treatment. While ethanol treatment itself did not show a significant effect on NADPH oxidase activity, it significantly augmented the effect of PDGF (10 ng/ml) on NADPH oxidase activity (fig. 6). This effect was blocked by the NADPH oxidase inhibitor DPI. However, treatment with DPI alone at the concentration used did not have a significant effect on NADPH oxidase activity in the cell membrane fraction (data not shown).

Ethanol Treatment Potentiated PDGF-Induced DNA Synthesis in Pancreatic Stellate Cells

We investigated the effect of PDGF and ethanol treatment alone and in combination on pancreatic stellate cell DNA synthesis by measuring the [^{3}H] thymidine incorporation into DNA. PDGF treatment at 10 ng/ml for 24 h significantly increased stellate cell DNA synthesis, while ethanol did not show a significant effect on stellate cell DNA synthesis. However, ethanol augmented the effect of PDGF on cell DNA synthesis. These effects were inhibited by the NADPH oxidase inhibitor DPI. Increased DNA synthesis was also prevented by concomitant treatment of

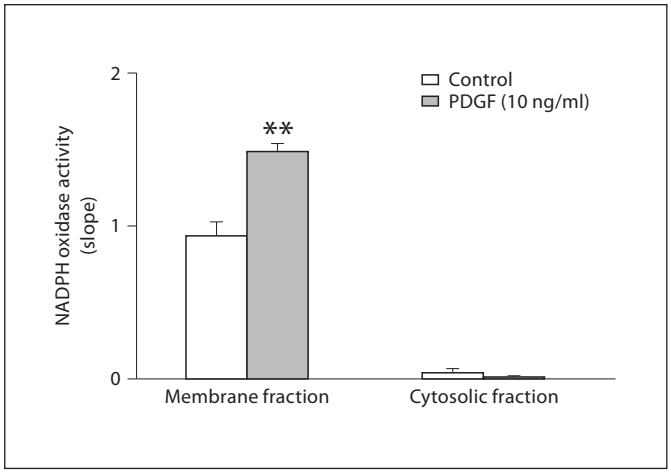


Fig. 5. PDGF treatment increases NADPH oxidase activity in the membrane fraction of pancreatic stellate cells. Pancreatic stellate cells were treated without or with rat PDGF BB 10 ng/ml for 24 h. The membrane and cytosolic fractions were separated as described in 'Materials and Methods'. The results shown are means and SD of the values of three to four independent experiments. ** $p < 0.001$ as compared to control.

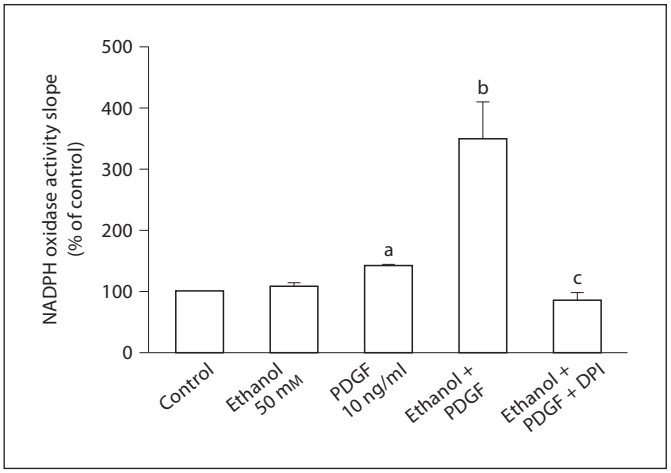


Fig. 6. Ethanol treatment augments PDGF-induced NADPH oxidase activity in pancreatic stellate cells. Activity is inhibited by DPI. Pancreatic stellate cells were treated with or without ethanol (100 mM) for 3 days and rat PDGF BB (10 ng/ml) and DPI (5 μM) for the last 24 h of incubation. The results represent means and SD of the values of three to four independent experiments. [a] $p < 0.0001$ as compared to control; [b] $p < 0.05$ as compared to PDGF alone; [c] $p < 0.01$ as compared to ethanol + PDGF.

the cells with the antioxidant N-acetylcysteine (NAC) or the ROS scavenger tiron (fig. 7). Preliminary experiments demonstrated that treatment with the NADPH oxidase inhibitors alone at the indicated concentration did not have significant effects on DNA synthesis as measured by [^{3}H] thymidine incorporation into DNA.

Effects of Ethanol and PDGF Treatment on NADPH Oxidase Activity and DNA Synthesis in Pancreatic Stellate Cells Grown on Matrigel

In order to more closely mimic in vivo conditions, we next investigated if pancreatic stellate cells grown on Matrigel respond in the same manner as those grown on plastic. Our results show that PDGF treatment increased NADPH oxidase activity (fig. 8) and DNA synthesis (fig. 9) and these effects were augmented by ethanol. Similar to stellate cells grown on plastic, these effects were blocked by DPI, NAC and tiron.

Effect of p47^{phox} Genetic Deletion on PDGF-Mediated NADPH Oxidase Activity and DNA Synthesis in Mouse Pancreatic Stellate Cells

Genetic p47^{phox}-deficient mice were used for investigating the role of the NADPH oxidase system in hepatic pathogenesis and demonstrated decreased expression of p47^{phox} RNA and protein [4]. In the present study, using

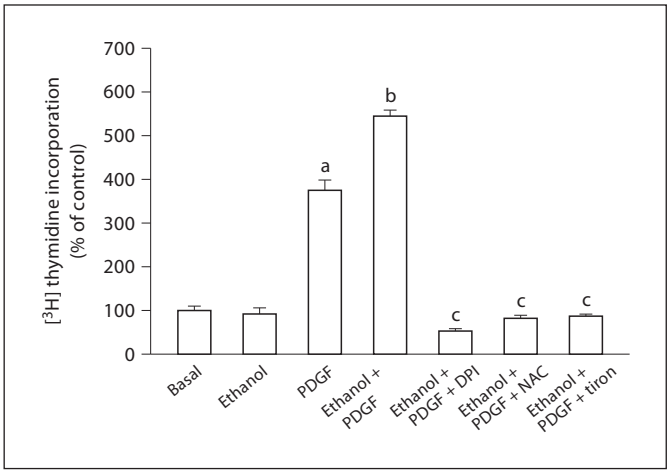


Fig. 7. Ethanol treatment augments the effect of PDGF on DNA synthesis. Activity is inhibited by DPI, NAC and tiron. Pancreatic stellate cells were treated with or without 100 mM ethanol for 72 h and rat PDGF BB (10 ng/ml) or in combination with DPI (5 μM), NAC (10 mM), or tiron (1.0 mM) for 24 h. The results represent means and SD of the values of three to four independent experiments. [a] $p < 0.001$ as compared to basal; [b] $p < 0.001$ as compared to PDGF alone; [c] $p < 0.001$ as compared to ethanol + PDGF.

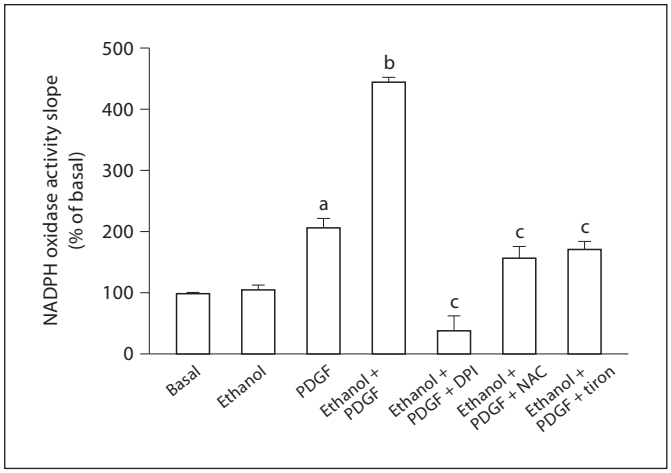


Fig. 8. Ethanol augments the effect of NADPH oxidase activity in pancreatic stellate cells grown on Matrigel. Activity is inhibited by DPI, NAC and tiron. Pancreatic stellate cells grown on Matrigel were treated with ethanol (100 mM) for 72 h and rat PDGF BB (10 ng/ml), DPI (5 μM), NAC (10 mM), or tiron (1.0 mM) for the last 24 h of incubation. The results represent means and SD of the values of three independent experiments. [a] $p < 0.01$ as compared to basal; [b] $p < 0.001$ as compared to PDGF alone; [c] $p < 0.001$ as compared to ethanol + PDGF.

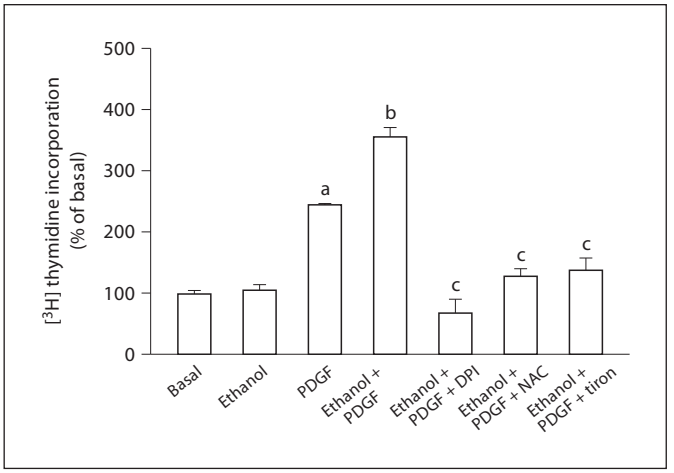


Fig. 9. Ethanol augments the effect of PDGF on DNA synthesis in pancreatic stellate cells grown on Matrigel and inhibition by DPI, NAC and tiron. Pancreatic stellate cells grown on Matrigel were treated with ethanol for 72 h, and rat PDGF BB (10 ng/ml), DPI (5 μM), NAC (10 mM) alone or tiron (1.0 mM) in the presence of 1.0 μCi [3H] thymidine for 24 h. The results represent means and SD of the values of three independent experiments. [a, b] $p < 0.001$ as compared to control and PDGF alone, respectively; [c] $p < 0.01$ as compared to ethanol + PDGF.

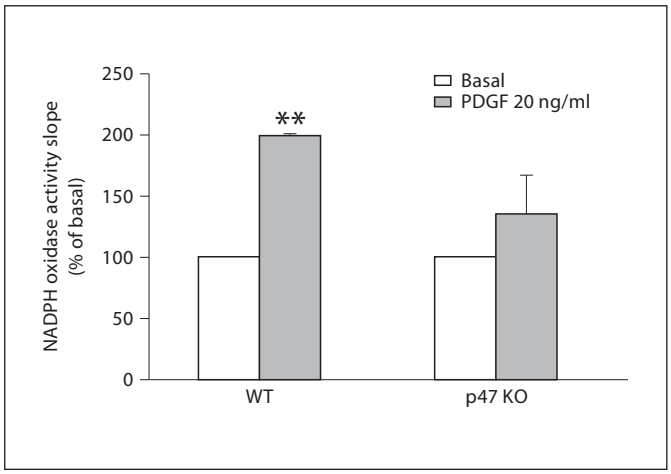


Fig. 10. PDGF activation of NADPH oxidase requires the p47^{phox} subunit of NADPH oxidase in pancreatic stellate cells. Pancreatic stellate cells were isolated from wild-type (WT) and p47^{phox} genetically deficient mice as indicated in 'Materials and Methods'. The cells were treated with hPDGF BB at the indicated concentration for 24 h. The results represent means and SEM of the values of three independent experiments. ** $p < 0.001$ as compared to basal.

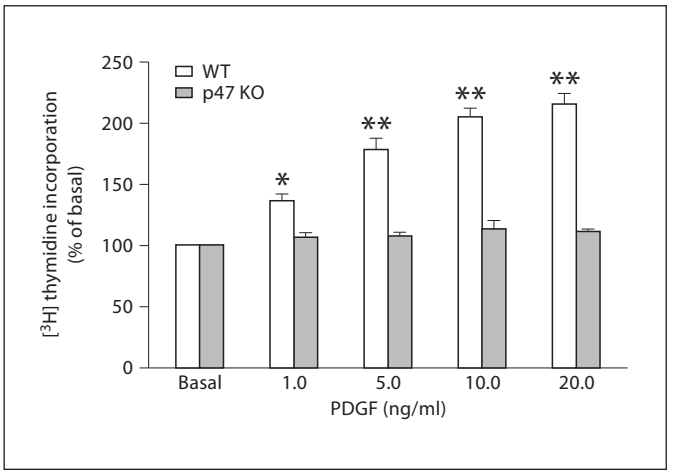


Fig. 11. NADPH oxidase p47^{phox} subunit mediates PDGF-induced DNA synthesis in pancreatic stellate cells. Pancreatic stellate cells isolated from wild-type and p47^{phox} genetically deficient mice were grown as indicated in 'Materials and Methods'. The cells were treated with hPDGF BB at indicated concentrations for 24 h. The results represent means and SEM of the values of three independent experiments. * $p < 0.05$ and ** $p < 0.01$ as compared to basal.

mice with a genetic deletion of p47^{phox}, we further investigated the specific role of this key subunit of NADPH oxidase in mediating PDGF-induced augmentation of NADPH oxidase activity and DNA synthesis. In pancreatic stellate cells isolated from wild-type mice, PDGF significantly increased NADPH oxidase activity, but not in mice with genetic deletion of p47^{phox} (fig. 10). PDGF also dose-dependently increased [^{3}H] thymidine incorporation into DNA in pancreatic stellate cells from wild-type, but not in cells from p47^{phox} knockout mice (fig. 11). These results confirm the necessary role of the NADPH oxidase system in PDGF-induced pancreatic stellate cell proliferation.

Discussion

An ample body of evidence indicates that activated pancreatic stellate cells play key roles in the pancreatic fibrosis of chronic pancreatitis and the desmoplastic reaction of pancreatic cancer [3, 17]. In these diseases the pancreatic stellate cells transdifferentiate from their quiescent state into a myofibroblastic phenotype under stimulation of chemokines, cytokines and growth factors as well as ethanol and its metabolites [18]. Activated pancreatic stellate cells produce collagen and matrix metalloproteinases which provide for matrix deposition and turnover [19]. Pancreatic stellate cells are functionally and morphologically similar to hepatic stellate cells. Previous experiments have demonstrated that NADPH oxidase appears to be a key signal in alcohol-induced liver disease, as mice lacking p47^{phox}, a cytoplasmic component required for enzymatic activation, are protected from the effects of alcohol [20]. Furthermore, recent studies show that the NADPH oxidase system in hepatic stellate cells plays a critical role in hepatic fibrogenesis [4]. That is, an NADPH oxidase system is present in hepatic stellate cells; this system is activated by angiotensin II and it participates in the hepatic fibrosing process in a bile duct ligation experimental animal model. More recently, evidence has been presented that PDGF also activates the NADPH oxidase system and that this system mediates the proliferation of hepatic stellate cells [5].

The presence of an NADPH oxidase system in pancreatic stellate cells has not been previously demonstrated. In the present study we found that the NADPH oxidase system is present in pancreatic stellate cells and contains common subunits of the neutrophil NADPH oxidase system such as gp91^{phox}, p47^{phox}, p67^{phox} and p22^{phox}.

Chronic alcohol abuse is the leading cause of chronic pancreatitis and pancreatic fibrosis, and alcohol use also increases the risk of pancreatic cancer through its effects on pancreatic fibrosis [1]. Histological studies [3] reveal that only activated stellate cells cause pancreatic fibrogenesis. Furthermore, those studies suggest the participation of PDGF receptor in alcohol-induced chronic pancreatic fibrosis.

Our results indicate that ethanol potentiated the effects of PDGF. These findings suggest that alcohol abuse sensitizes or primes the pancreatic stellate cells so that they have a robust response to growth factors such as PDGF with an inflammatory insult to the pancreas.

It has been reported that alcohol directly stimulates pancreatic stellate cells to produce collagen [2]. The mechanisms underlying the effect of ethanol on pancreatic fibrosis are poorly understood although signal transduction systems involving phosphatidylinositol 3-kinase, mitogen-activated protein kinase, and extracellular signal-regulated kinase have been postulated to be involved in the alcohol-mediated pancreatic fibrogenic process [2, 21, 22]. However, the effects of ethanol in these studies on pancreatic stellate cell DNA synthesis and proliferation response have not been addressed. It is well known that pancreatic stellate cell proliferation is widely accepted as the crucial step for the development of pancreatic fibrosis. Our results with the NADPH oxidase inhibitor and mice with genetic deletion of p47^{phox} indicate that the NADPH oxidase system is necessary to mediate stimulated DNA synthesis. These results suggest that ethanol sensitizes or primes the pancreatic stellate cell to augment the pathological proliferation response to growth factors, such as PDGF produced during pancreatic injury by augmenting the NADPH oxidase response.

A growing body of pharmacological and biochemical evidence supports the hypothesis that ROS contribute to the pathology of pancreatitis, pancreatic cancer and pancreatic fibrosis. Evidence from animal studies has shown that antioxidants ameliorate the biochemical and morphological features associated with chronic pancreatitis [23, 24]. In particular, vitamin E treatment likely acting as an antioxidant decreased the number of activated stellate cells in rat pancreatic tissue in a model of chronic pancreatitis [24]. Clinically, antioxidants may prevent recurrent chronic pancreatitis [25–27]. Patients with chronic pancreatitis have evidence of multiple antioxidant deficiencies [25]. However, how ROS mediate the pathological processes of pancreatitis is poorly understood.

In summary, the present study demonstrates that an NADPH oxidase system is present in pancreatic stellate

cells. This system is regulated by the growth factor PDGF. More importantly, ethanol, the most common etiology of chronic pancreatitis/fibrosis, augments the activation of NADPH oxidase by PDGF, resulting in augmentation of the proliferative response of pancreatic stellate cells to the growth factor. These findings provide new insights into the pancreatic fibrosis process and may reveal novel targets for therapy.

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