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Nicotine induces fibrogenic changes in human liver via nicotinic acetylcholine receptors expressed on hepatic stellate cells

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ABSTRACT

Background and aims: Cigarette smoke (CS) may cause liver fibrosis but possible involved mechanisms are unclear. Among the many chemicals in CS is nicotine – which affects cells through nicotinic acetylcholine receptors (nAChR). We studied the effects of nicotine, and involved pathways, on human primary hepatic stellate cells (hHSCs), the principal fibrogenic cells in the liver. We then determined possible disease relevance by assaying nAChR in liver samples from human non-alcoholic steatohepatitis (NASH).
Methods: hHSC were isolated from healthy human livers and nAChR expression analyzed – RT-PCR and Western blotting. Nicotine induction of hHSC proliferation, upregulation of collagen1-α2 and the pro-fibrogenic cytokine transforming growth factor beta 1 (TGF-β1) was determined along with involved intracellular signaling pathways. nAChR mRNA expression was finally analyzed in whole liver biopsies obtained from patients diagnosed with non-alcoholic steatohepatitis (NASH).
Results: hHSCs express muscle type (α1, β1, delta and epsilon) and neuronal type (α3, α6, α7, β2 and β4) nAChR subunits at the mRNA level. Among these subunits, α3, α7, β1 and ε were predominantly expressed as confirmed by Western blotting. Nicotine induced hHSC proliferation was attenuated by mecamylamine ($p < 0.05$). Additionally, collagen1-α2 and TGF-β1 mRNA expression were significantly upregulated by nicotine and inhibited by mecamylamine. α1 and α3-nAChR mRNA expression was significantly upregulated in NASH fibrosis compared to normal livers.
Conclusion: Nicotine at levels in smokers' blood is pro-fibrogenic, through actions on hHSCs expressed nAChRs. Therefore, CS, via its nicotine content, may worsen liver fibrosis. Moreover, nicotinic receptor antagonists may have utility as novel anti-fibrotic agents.

1. Introduction

It is accepted that cigarette smoking (CS) increases the risk of cardiovascular and pulmonary diseases as well as carcinogenesis. A growing body of evidence suggests that cigarette smoking may also accelerate the progression of renal, pulmonary, and cardiac fibrosis [1–3]. Moreover, in smokers, with chronic liver diseases such as primary biliary cirrhosis and chronic hepatitis C there is a documented increased severity of hepatic fibrosis [4–6]. More recently, CS has been shown to exacerbate the histological severity of NAFLD in obese Zucker rats [7] and to be associated with advanced liver fibrosis in a large multi-center cohort of NAFLD patients [8]. The mechanisms underlying the fibrogenic effect of CS in NAFLD however, are largely unknown.

Among the many substances contained in cigarette smoke, is nicotine which has an affinity for the nicotinic acetylcholine receptor (nAChR). nAChRs are ligand-gated, pentameric ion channels which are sensitive to acetylcholine and nicotine [9]. To date, 16 nAChR subunits have been identified in mammals, consisting of α1–10, β1–4, δ, γ and ε. Nicotinic receptors are classified into two subtypes: muscle and neuronal. The muscle subtypes comprise of α1, β1, δ, and ε subunits, whereas the neuronal subtypes are various combinations of 12 different nicotinic receptor subunits. The muscle type nAChR is present at the neuromuscular junction and activated by ACh to cause muscular contraction. The neuronal type receptor is generally responsible for synaptic transmission in the brain and autonomic ganglia. Additionally, growing evidence suggests that non-neuronal cells also express the nAChR and that it may play various roles in physiological processes such as inflammation, cell proliferation and survival [10,11].

Abbreviations: CS, cigarette smoking; NASH, Non-alcoholic steatohepatits; nAChR, nicotinic acetylcholine receptors; hHSCs, human hepatic stellate cells.

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Hepatic stellate cells (HSC), the liver's principal fibrogenic cells when stimulated by liver injury, become proliferative fibrogenic myofibroblasts and our group has previously shown that these cells synthesize and respond to cathecholamines via their expressed adrenoceptors [12]. We further showed that acetylcholine induced fibrotic phenotypic changes in murine HSC [13]. The expression by human HSC (hHSCs) of functional nAChR has not described. If hHSCs are proven to express functional nAChR, it would strengthen the emerging argument that patients with NAFLD should be advised not to smoke. Our aim therefore, was to test the hypothesis that hHSCs express functional nAChR and that the fibrogenic effect of CS is at least partially through the action of CS nicotine on these nAChR.

2. Materials and methods

2.1. Chemicals

Nicotine hydrogen tartrate salt, mecamylamine hydrochloride, pertussis toxin (PT), wortmannin (WT), PD98059 (PD), SB202190 (SB), and Ro-32-0432 (RO), D-Tubocurarine (DTC) were purchased from Sigma.

2.2. Isolation and culture of human primary hepatic stellate cells

Human HSCs were isolated from resected human liver tissue with appropriate local Ethics Committee approval and patient consent. Human HSCs were isolated by collagenase (type IV) perfusion, followed by density gradient centrifugation, as described elsewhere [13]. Cell identity was confirmed by autofluorescence, and the expression of two well-accepted HSC markers, alpha smooth muscle actin (ASMA) and glial fibrillary acidic protein (GFAP), was verified by immunocytochemistry. Experiments were performed with human HSCs cultured between days 7 and 20.

2.3. Cell proliferation assay

Cell proliferation was analyzed using CCK-8 Cell counting kit (Dojindo Molecular Technologies (NBS Biologicals, UK) as described [13]. Optical densities were read with an Emax precision microplate reader (Anthos HT III).

2.4. RT-PCR

Total RNA was isolated using TRizol reagent according to the manufacture's protocol. The primers sequences are as shown in Table 1. Semi-quantitative RT-PCR was performed using the one-step PCR kit (Invitrogen) according to the manufacture's instruction. Relative quantification of the mRNA was performed by real-time PCR using Rotorgene RG-3000 instrument (Corbett Research) and SuperScript III platinum SYBR Green One-step qRT-PCR kit. To monitor the specificity, final PCR products were analyzed by melting curves and electrophoresis. The amount of transcript was calculated and expressed as the difference relative to the control gene GAPDH ($2^{-\Delta\Delta Ct}$, where ΔCt represents the difference in threshold cycles between the target and control genes).

2.5. Western blotting

Cultured hHSCs were homogenized in a buffer containing 20 mmol/L Tris–HCl (pH 7.5), 150 mmol/L NaCl, 1% Nonidet P-40, 0.1% SDS, 1% sodium deoxycholate, 1 mmol/L PMSF, and mixed protease inhibitor cocktail (Roche). The homogenates were centrifuged at 12,000 g for 10 min at 4 °C and supernatants collected. Protein concentration was measured with a Bradford protein assay (BioRad). Equal concentrations of protein from liver homogenates were dissolved in sample buffer (Invitrogen), loaded onto 10% polyacrylamide gel and electrophoresed. Proteins were then transferred to a polyvinylidene difluoride membrane (Invitrogen) by electroblotting. Membranes were blocked for 1 h at room temperature with 5% nonfat dried milk in TBS containing 0.1% (vol/vol) Tween-20 (TBS-T), and incubated overnight with primary antibodies (Table 2) diluted in TBS-T containing 5% bovine serum albumin (BSA). After washing in TBS-T three times, the membranes were incubated for 1 h with peroxidase-conjugated goat anti-mouse antibody (Santa Cruz) or goat anti-rabbit antibody (Cell Signaling) diluted in TBS-T containing 5% BSA. After washing in TBS-T, blots were developed by enhanced chemiluminescence (ECL; Amersham) and exposed to X-ray film (Kodack). Positive controls include total human brain or skeletal muscle lysates (Abcam).

Table 1
Primer sequences of genes used for PCR.

Primer	Product size	Sequence
Alpha 1[a]	580/505	CGT TCT GGT GGC AAA GCT CCG CTC TCC ATG AAG TT
Alpha 2[a]	466	CCG GTG GCT TCT GAT GA CAG ATC ATT CCA GCT AGG
Alpha 3[a]	464	CTG GTG AAG GTG GAT GAA GT CTC GCA GCA GTT GTA CTT GA
Alpha 4[a]	444	GGA TGA GAA GAA CCA GAT GA CTC GTA CTT CCT GGT GTT GT
Alpha 5[a]	525	GAT AAT GCA GAT GGA CGT TGA TGG TAT GAT CTC TTC
Alpha 6[a]	372	GTG GCC TCT GGA CAA GAC AA CCT GCA GTT CCA AAT ACA CA
Alpha 7[a]	375	GGA GCT GGT CAA GAA CTA CA CAG CGT ACA TCG ATG TAG CA
Beta 1[a]	479	CTA CGA CAG CTC GGA GGT CA GCA GGT TGA GAA CCA CGA CA
Beta 2[a]	453	CAA TGG CTC TGA GCT GGT GA CCA CTA GGT GTG AAG TCG TCC A
Beta 3[a]	439	TGGAGA GTA CCT GCT GTT CA CGA GCC TGT TAC TGA CAC TA
Beta 4[a]	524	GTG AAT GAG CGA GAG CAG AT GGG ATG ATG AGG TTG ATG GT
Delta[a]	471	CAG ATC TCC TAC TCC TGC AA CCA CTG ATG TCT TCT CAC CA
Gamma[a]	546	CGC CTG CTC TAT CTC AGT CA GGA GAC ATT GAG CAC AAC CA
Epsilon[a]	432	GTA ACC CTG ACG AAT CTC AT GTC GAT GTC GAT CTT GTT GA
Gapdh	320	ACA GTC CAT GCC ATC ACT GCC GCC TGC TTC ACC ACC TTC TTG
Collagen1-α2	236	ATA TTG CAC CTT TGG ACA TC TGC TCT GAT CAA TCC TTC TT
TGFβ1	157	AAC CCA CAA CGA AAT CTA TG GTG CTG CTC CAC TTT TAA CT
18S	488	AM1716 Ambion

[a] From Ref. [17].

Table 2
Primary antibodies used for Western blot analysis.

Antibody	Species	Manufacture	Catalog number	Dilution
α1 nAchR	Rabbit	Santa Cruz	SC-11371	1:500
α3 nAchR	Rabbit	Proteintech	10333-1-AP	1:600
α7 nAchR	Rabbit	Genscript	A0 1420	1:1000
β1 nAchR	Rabbit	Santa Cruz	SC-11371	1:1000
β2 nAchR	Rabbit	Santa Cruz	SC-11372	1:1000
δ nAchR	Rabbit	Abcam	AB26095	1:1000
ε nAchR	Rabbit	Abcam	AB49141	1:800
βactin	Rabbit	Cell Signaling	4967	1:1000

nAchR: nicotinic acethylcholine receptor.

2.6. Expression of nAChR in human livers with confirmed NASH fibrosis

To determine if the observed ex vivo effects are functionally relevant in vivo, we then assayed by RT-PCR the expression of

selected nAChR subtypes in liver biopsy specimens from patients with confirmed severe NASH fibrosis and compared this with expression in control livers.

2.7. Statistical analysis

All data are expressed as mean (±SEM). Statistical analyses were performed using the Mann–Whitney test. Significance was accepted as $p < 0.05$.

3. Results

3.1. Primary hHSCs express nAChRs

Activated hHSCs expressed muscle and neuronal type nAChR subunits: α1, β1, δ, ε (muscle type) and α2-α7, β2, β4 (neuronal type), Fig. 1A and B. nAChR expression was confirmed at the protein level by Western blotting with observed high expression of α1, β1, δ, and ε (muscle type) and α3, α7, β2 (neuronal type), nAChR subunits (Fig. 1C), confirming that hHSCs express muscle and neuronal type nAChRs.

3.2. Nicotine induces mecamylamine inhibitable hHSC proliferation via PI3K and PKC

The effect of increasing nicotine concentrations (1 pM–1 mM) on hHSC proliferation was now evaluated. We observed a biphasic proliferative response of hHSCs following treatment with nicotine. Peak proliferation was induced by nicotine concentrations of 1 nM and 10 μM (Fig. 2A). Mecamylamine, a neuronal nAChR antagonist, inhibited nicotine induced proliferation of hHSCs but DTC a muscle type nAChR antagonist had no effect on nicotine induced hHSC proliferation (Fig. 2B), suggesting that the muscle type nAChR does not mediate hHSC proliferation.

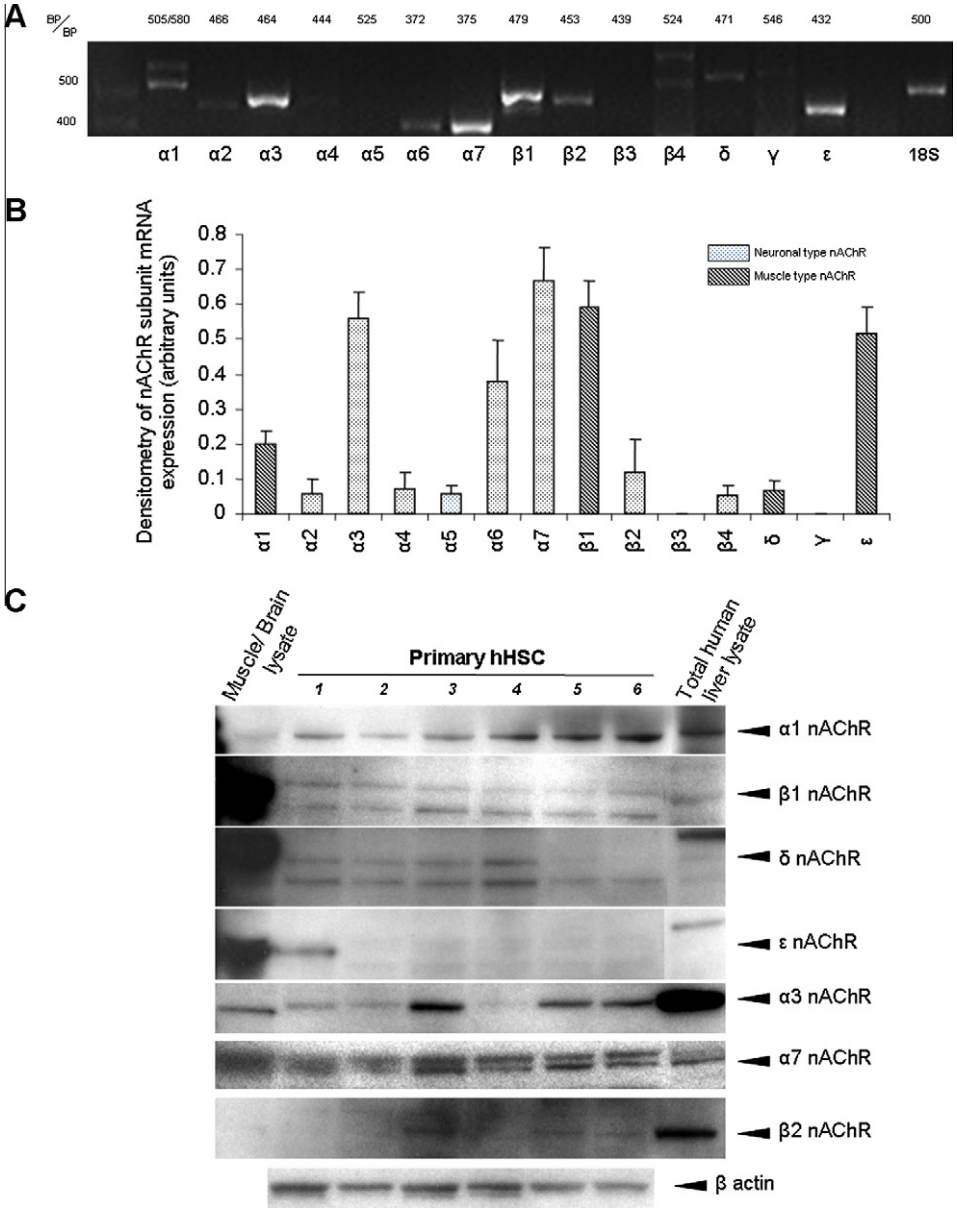

Fig. 1. nAChR subunits expression by hHSC. (A) a representative Rt-PCR analysis showing hHSC expression of α1–7, β1–4, δ and ε nAChR subunits. (B) Relative nAChR subunits mRNA expression in hHSC. Gene expression was determined by Rt-PCR and band densities normalized to 18S. Each bar represents mean ± SEM ($n = 5$). (C) Western blotting analysis confirmation of hHSC nAChR subunit expression at protein level. Arrows point to the immuno-reactive band of the predicted size for each subunit. Whole liver lysates were analyzed as evidence that the observed bands are not an in vitro or ex vivo artifact.

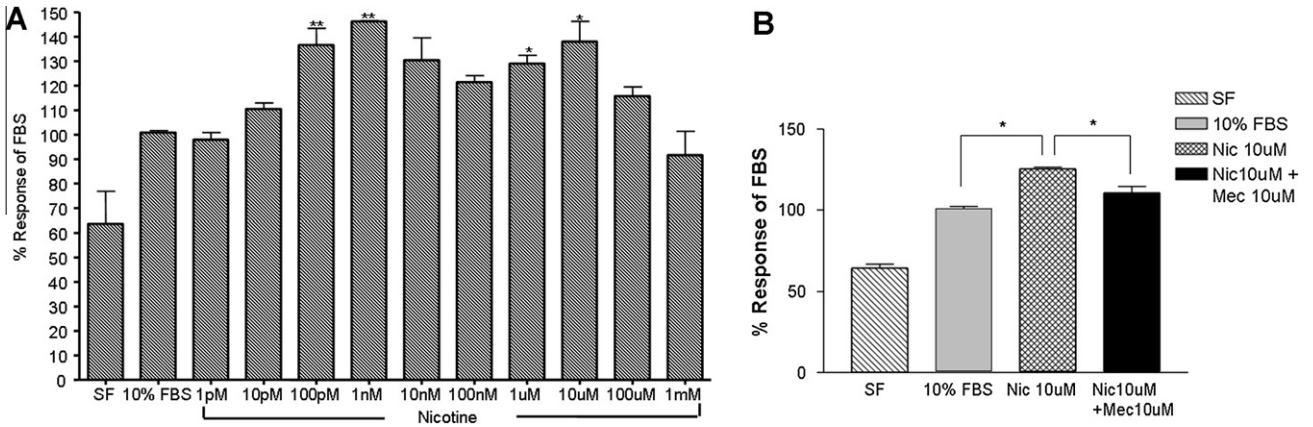

Fig. 2. Nicotine induction of hHSC proliferation and effect of mecamylamine and D-Tubocurarine. (A) nicotine at 10–100 μM induced hHSC proliferation with a biphasic response profile and maximal effects at 1 nM and 10 μM. Responses were normalized to the control response in FBS alone containing media, $^{*}p < 0.05$, $^{**}p < 0.01$. SF (serum free culture media) acted as a negative control. (B) Nicotine induced hHSC proliferation was blocked by 10 uM mecamylamine a nicotinic (neuronal type) antagonist. The muscle type nicotinic antagonist DTC had no effect on nicotine induced hHSC proliferation.

To further investigate the mechanisms underlying nicotine induced proliferation of hHSC, we then examined the effect of pertussis toxin (PT), wortmannin (WT), PD98059 (PD), SB202190 (SB), or Ro-32-0432 (RO), which are specific inhibitors of G-protein, PI3K, MEK, p38 and PKC, respectively. Nicotine induced proliferation was significantly inhibited by treatment with WT and RO (Fig. 3), suggesting that nicotine's proliferative action on hHSCs is mediated through PI3 kinase and PKC activation.

3.3. Nicotine induces TGF-β1 and collagen gene expression

To further investigate the role of nicotine in hHSC fibrogenesis, TGF-β1 and collagen1-α2 gene expression were now assessed. Nicotine significantly induced up-regulation of both genes at a concentration of 10 μM (Fig. 4A and B) an effect inhibited by mecamylamine (Fig. 4C and D).

3.4. nAChR expression is enhanced in human livers with NASH fibrosis

To now study the possible relevance of nAChR in human liver fibrosis in the context of a common liver disease, the expression of the neuronal and muscle type nAChR subtypes whose presence was detected in cultured hHSC, namely α1, β1, ε (muscle type) and α3, α7 (neuronal type), was now assayed by RT-PCR in human livers with biopsy proven NASH fibrosis and compared with their expression in control livers. We observed a marked up-regulation of the α1 (muscle type) and α3 (neuronal type) nAChR subunits in livers with biopsy proven NASH induced severe fibrosis (Brunt–Kleiner stage F3) or cirrhosis (F4), (Fig. 4E and F).

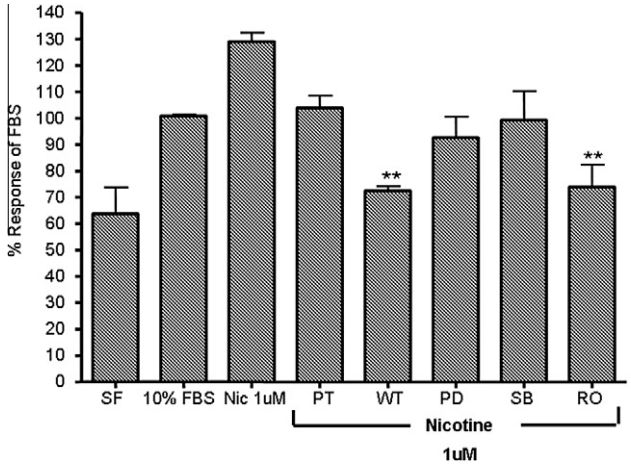

Fig. 3. Intra-cellular pathways involved in nicotine's induction of hHSC proliferation pertussis toxin (PT) an inhibitor of $G_{\alpha i/o}$-protein (100 ng/ml); wortmanin (WT) – inhibitor of PI3-K (100 nm); PD 98059 (PD) – inhibitor of MEK (20 nM); SB 202190 (SB) – inhibitor of p38 MAPK (10 uM) and RO 320432 (RO) – inhibitor of PKC (1 uM) were cultured with hHSC in the presence of nicotine 1 μM. WT and RO significantly inhibited nicotine induced hHSC proliferation.

4. Discussion

Epidemiological studies have shown an association between cigarette smoking and accelerated progression of liver fibrosis in patients with a variety of chronic liver diseases [14]. Numerous mechanisms for this observed effect have been proposed and include tissue hypoxia, iron accumulation, tissue inflammation, oxidative stress and lipid peroxidation [15]. In experimental models Azzalini et al. [7] recently showed that CS causes oxidative stress and worsens the severity of nonalcoholic fatty liver disease in obese rats. Presently, there are few experimental evidence to support the contention that cigarette smoke induces liver fibrosis. Here, we show for the first time that primary hHSCs express functional nAChR and that nicotine, a major constituent of cigarette smoke, directly stimulates hHSCs through these receptors. Additionally, we observed that nicotine induces hHSC proliferation and up-regulation of the fibrogenic markers, TGF-β and collagen.

nAChRs play a critical role in synaptic transmission and thus the primary focus of nicotine and its receptors has been on its physiological effects within the nervous system. However, there is growing evidence suggesting that non-neuronal cells also express nAChRs [10,11]. In our present study, we have shown that culture activated hHSC express nAChR subunits of both the muscle and neuronal type. In support of our findings, renal, pulmonary and cutaneous fibrogenic cells have been shown to express nAChR subunits [3,16–18] and nicotine has been shown to be mitogenic for vascular smooth muscle cells [19]. The expression of nAChRs in non-neuronal cells suggests that they possess functions independent of neurotransmission. We have here now also demonstrated a role for nAChRs in hHSC proliferation and fibrogenesis.

Mecamylamine, a specific nicotinic receptor antagonist, inhibited nicotine induced hHSC proliferation suggesting that hHSC expressed nAChRs are functionally involved in the observed nicotine's enhancement of hHSC proliferation. The latter phenotypic effect requires PI3K and PCK pathways as evidenced by the effects of WT and RO. Besides proliferation, nicotine also directly stimulated

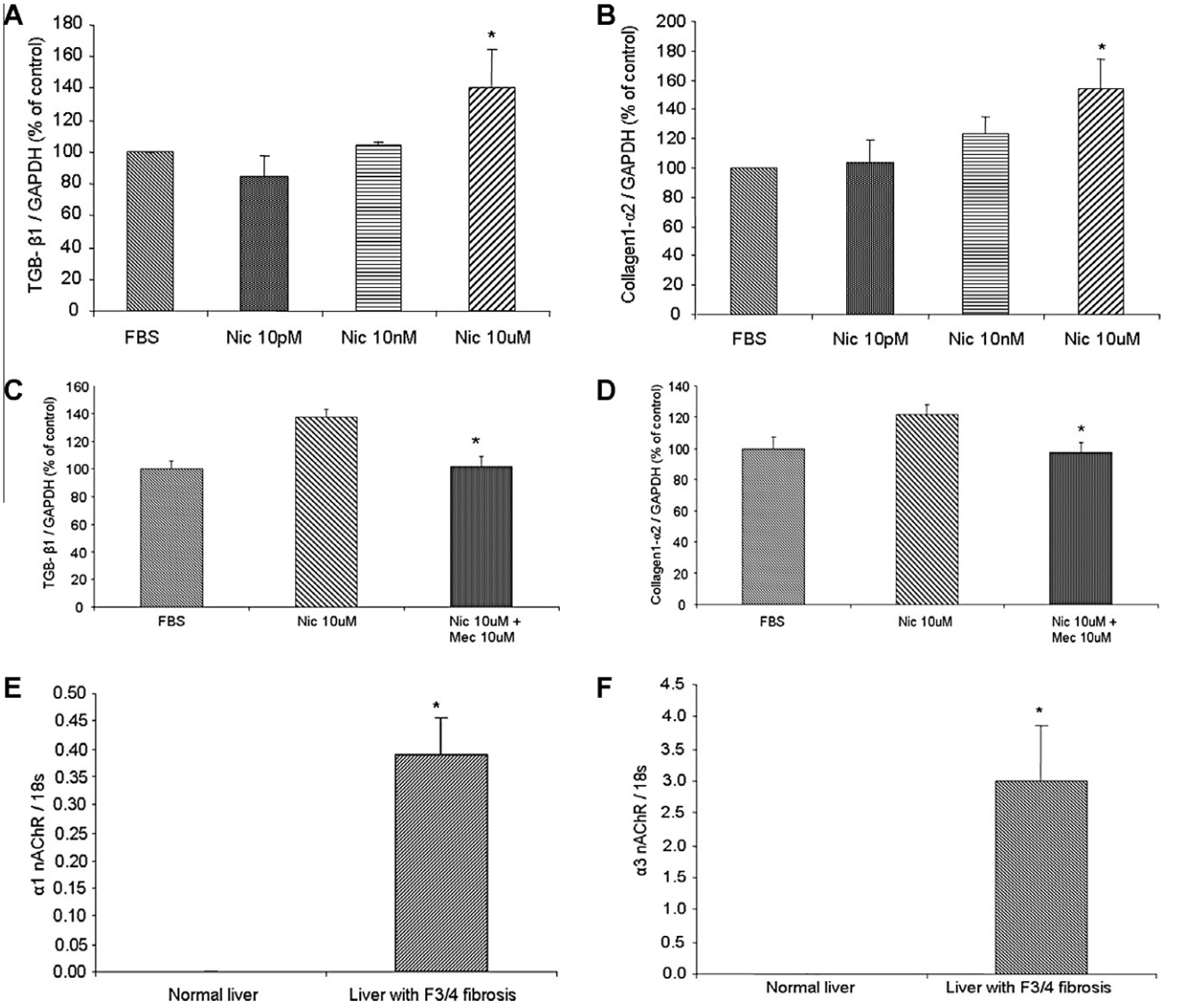

Fig. 4. Nicotine induces TGF-β1 and collagen 1-α2 gene expression in hHSC. (A) Nicotine 10 μM significantly upregulated TGF-β1 expression in hHSC, $^{*}p < 0.05$. (B) Nicotine 10 μM similarly significantly upregulated collagen 1-α2 gene expression in hHSC $^{*}p < 0.05$. Nicotine induction of TGF-β1 and collagen 1-α2 gene expression in hHSC is reversed by mecamylamine. (C) Mecamylamine, 10 μM, significantly inhibited nicotine induced TGF-β1. (D) Mecamylamine, 10 μM, also significantly inhibited nicotine induced collagen 1-α2 gene expression. nAChR subunit expression in livers with NASH fibrosis. Rt-PCR analysis of the 14 nAChR subunits, normalized against 18 s, showed that compared to control livers, α1 (E) and α3 (F) subunits were significantly upregulated in livers with NASH fibrosis of severity F3/F4 ($n = 3$/group).

hHSC TGF-β and collagen gene expression thus confirming its role in hepatic fibrogenesis. Moreover, we have also shown that expression of nAChRs in the livers of patients with severe NASH fibrosis or cirrhosis is enhanced compared to control livers.

Given that we do not presently advise smokers with liver disease to refrain from smoking, our results imply that this approach may be incorrect and that we should in fact be advising patients with liver disease to abandon smoking, not for cardiovascular health alone, but also for fear that it may exacerbate liver fibrogenesis. The blood concentration of nicotine in smokers has been reported to be up to micromolar or higher concentrations [20]. Since we have in our present studies observed pro-fibrogenic effects at nicotine concentrations as low as picomolar concentrations, the observed effects are likely therefore to be of clinical relevance. In vivo, the effects of nicotine may be further augmented by its release of cathecholamines [21,22] which we showed previously to be involved in hepatic fibrogenesis [23].

In conclusion, nicotine a major constituent of CS is pro-fibrogenic via its interaction with nAChRs on hHSCs. These findings suggest that patients with liver disease should be advised not to smoke for fear that CS may worsen hepatic fibrosis and accelerate their trajectory to cirrhosis.

Disclosures

The authors disclose no conflicts.

Writing assistance

None.

Author contributions

All the authors contributed to the work reported here through study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; statistical analysis;

obtained funding; administrative, technical, or material support or study supervision.

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