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Biochemical and Biophysical Research Communications 300 (2003) 172–177
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Acetylcholine promotes the proliferation and collagen gene expression of myofibroblastic hepatic stellate cells

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Received 1 November 2002

Abstract

The mechanisms that initiate and perpetuate the fibrogenic response, during liver injury, are unclear. Animal studies, however, strongly support a role for the autonomic nervous system (ANS) in wound healing. Therefore, the ANS may also mediate the development of cirrhosis. Hepatic stellate cells (HSC), the liver's major matrix-producing cells, are activated by injury to become proliferative, fibrogenic myofibroblasts. HSC respond to sympathetic neurotransmitters by changing phenotype, suggesting that HSC may be the cellular effectors of ANS signals that modulate hepatic fibrogenesis during recovery from liver damage. We show here that the parasympathetic neurotransmitter acetylcholine markedly stimulates the proliferation of myofibroblastic HSC and induces HSC collagen gene expression in these cells. By extending evidence that HSC are direct targets of the ANS, these results support the proposed neuroglial role of HSC in the liver and suggest that interrupting ANS signalling may be useful in constraining the fibrogenic response to liver injury.

Keywords: Liver injury; Fibrosis; Autonomic; Hepatic stellate cells; Myofibroblasts; Collagen; Acetylcholine

The mechanisms that initiate and perpetuate the fibrogenic response in the injured liver are not well understood. It is clear, however, that after liver injury, hepatic stellate cells (HSC) are activated, moving from a quiescent phenotype to become proliferative and fibrogenic myofibroblasts [1]. These activated myofibroblastic cells constitute the major matrix-producing cells in the injured liver [1,2]. Moreover, these cells are also contractile and may therefore contribute to portal hypertension, a major complication of hepatic fibrosis [3].

Compelling, albeit indirect, experimental evidence links overactivity of the sympathetic branch of the autonomic nervous system (ANS) with the pathogenesis of cirrhosis and its complications [4–8]. The pro-fibrogenic and portal hypertensive effects of the sympathetic nervous system are presumed to be mediated via the vasculature. However, we recently showed that the sympathetic neurotransmitters, norepinephrine (NE), and neuropeptide Y (NPY), are directly mitogenic for cultured HSC, and that NE also stimulates HSC collagen gene expression [9]. These findings support the view that HSC are an effector arm of the ANS during hepatic fibrogenesis. Given this, it is conceivable that parasympathetic nerve fibres may also influence HSC. At present, however, it is not known what effects, if any, the parasympathetic neurotransmitter acetylcholine (ACh) has on HSC.

The role of the parasympathetic branch of the ANS and its neurotransmitters in controlling the viability and function of other types of cells is, however, well established. For example, in bile duct-ligated rats, the parasympathetic nervous system regulates reconstitution of the damaged biliary tree, because vagotomy abolishes proliferation and induces apoptosis in cholangiocytes [10]. ACh, therefore, is a survival factor for cholangiocytes. During skin wound healing, nicotine and epibatidine, a nicotinic acetylcholine receptor (nAChR) agonist, are known to promote angiogenesis and collagen production [11]. Together, these data suggest that the

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PII: S0006-291X(02)02773-0

parasympathetic nervous system plays an important role in epithelial wound healing.

Whether or not epithelial and endothelial cells are the sole cellular targets of parasympathetic neurotransmitters during tissue repair remains uncertain. Because HSC express glial fibrillary acidic protein (GFAP), nestin, neural cell adhesion molecule (NCAM), synaptophysin, and neurotrophins [3,12–15] and receive innervation by autonomic fibres [16,17], it has been suggested that these cells may be derived from the neural crest and function as liver resident neuroendocrine cells [15]. As such, HSC may also be immediate targets for ANS signals, responding to parasympathetic neurotransmitters by phenotypic changes that potentiate healing of the injured liver. Our recent data that HSC respond directly to NE and NPY [9] and the other studies cited above support this concept. Therefore, the aim of this study was to evaluate the hypothesis that ACh directly regulates HSC, by determining if ACh regulates the proliferation and/or gene expression of cultured HSC.

Materials and methods

Materials

Animals. C57BL-6 mice 10–18 weeks old, were from Jackson Laboratory (Bar Harbor, ME). All experiments satisfied the Guidelines of our Animal Care Committee and the National Institutes of Health.

Stellate cell extraction. Collagenase (type IV), pronase, deoxyribonuclease (DNase), bovine serum albumin (BSA) were from Sigma (St. Louis, MO). Optiprep was purchased from Accurate (Westbury, New York). Intravenous catheters were from Johnson and Johnson (Arlington, Texas).

Tissue culture. RPMI 1640 medium, phosphate-buffered saline (PBS), 2-mercaptoethanol (2-ME), Hanks' buffered salt solution (HBSS), trypsin–EDTA, penicillin–streptomycin were purchased from Invitrogen (Carlsbad, CA). Foetal bovine serum (FBS) was from Intergen (Purchase NY). Tissue culture ware was from Falcon, Becton–Dickinson (Franklin Lakes, NJ).

Immunocytochemistry. Methanol and acetone were from Baker (Phillipsburg, NJ). Anti-α-smooth muscle actin (anti-ASMA) unconjugated-mouse monoclonal antibody and isotype-matched control antibody, and goat anti-mouse IgG-fluorescein isothiocyanate conjugate were from Sigma. Goat polyclonal anti-glial fibrillary acidic protein (GFAP) and anti-goat IgG-Texas red conjugate were from Santa Cruz Biotech (Santa Cruz, CA). Pro-block was from Scytek (Logan, Utah). Hoechst 33342 nuclear stain was from Molecular Probes (Eugene, Oregon).

Cell proliferation assay. Cell counting kit (CCK-8, WST-8) was from Dojindo Molecular Technologies (Gaithersburg, MD). Optical densities were read with an E_{max} precision microplate reader (Molecular Devices, Sunnyvale, CA).

RT-PCR. Rneasy RNA extraction kit was from Qiagen (Valencia, CA). Superscript one-step RT-PCR with platinum Taq kit was from Invitrogen. Collagen *col1α2* primers were made in-house and classic II *18s* Internal standard kit was from Ambion (Austin, Texas).

Drugs. Acetylcholine, mecamylamine, and rat platelet derived growth factor (PDGF) were from Sigma. Methoxyflurane was from Schering-Plough (Union, NJ).

Methods

Stellate cells extraction. Mice were anaesthetized with methoxyflurane. As described [18], livers were perfused in situ with calcium and magnesium free-HBSS containing heparin and then with HBSS (with Ca^{2+} and Mg^{2+}) containing collagenase, pronase, and DNase until the liver lost its firm texture. The liver was excised, placed in HBSS (with Ca^{2+} and Mg^{2+}) with collagenase, pronase and DNase, homogenized, and placed in a shaking water bath at 37 °C for 10 min.

The homogenate was filtered, centrifuged for 2 min to pellet remaining hepatocytes, and then centrifuged, at 400*g* for 10 min, 4 °C. The pellet was resuspended in HBSS (Ca^{2+} and Mg^{2+} free) with BSA and DNase, and recentrifuged. This pellet was resuspended in Optiprep, according to manufacturer's instructions. After centrifuging at 1400*g*, the cell fraction at the HBSS and 11.5% Optiprep interface was gently aspirated, mixed with HBSS, and centrifuged at 400*g* for 10 min, 4 °C. After another wash, the final pellet was resuspended in RPMI medium containing penicillin and streptomycin, 20% FBS, and 2-ME [19].

Cell number and viability were assessed by trypan blue exclusion. In each experiment cells from 4 to 5 mice were pooled. The yield was about 3 million HSC/mouse. Cells were plated at a density of 5×10^4 cm^2 in RPMI on uncoated plastic flasks. After 24 h, medium and non-adherent cells were replaced with fresh medium. At this stage culture purity as assessed by autofluorescence and Sudan black staining was upward of 95% [20]. Culture medium was changed every 48 h thereafter.

At subconfluence (on about day 7), cells were washed with HBSS (Ca^{2+} and Mg^{2+} free) and removed from the flasks by light trypsinization. They were resuspended, washed, and finally resuspended in RPMI (serum and 2-ME free) for the proliferation assays or in normal growth medium for immunocytochemistry.

Immunocytochemistry. Immunocytochemistry was done as described [21] except that the blocking solution was 0.5% BSA or pro-block. Final antibody dilutions were anti-ASMA 1:400, anti-GFAP 1:100, and secondary antibodies 1:200. Slides were examined with a Zeiss 410 confocal microscope or a Nikon E800 fluorescent microscope.

Immunoblot. Immunoblots were performed as described [22]. Final antibody dilutions were: anti-α-smooth muscle actin 1:3000, and anti-GFAP 1:200.

Cell proliferation assay. Test drugs were added to medium containing 10% serum. Antagonist drugs were added 3 h before agonists. The proliferation assay was performed as per manufacturer's instructions [23]. Briefly, cells were incubated with WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt) for 4 h. In viable cells, WST-8 was metabolized to produce a colorimetric dye that is detected at 450 nm. To verify the WST-8 assay results, at the end of some experiments, cultures were photographed after staining with Hoechst 33342.

RT-PCR. RNA was then extracted from duplicate treatment wells with Rneasy kits. Concentration and purity were assessed by absorbance at 260/280 nm. One-step RT-PCR was performed with Superscript one-step RT-PCR with platinum Taq kits. Products were separated by electrophoresis on a 1.5% agarose gel. Bands were quantified with Imagequant software (Molecular Dynamics, Sunnyvale, CA). *Collagen 1α-2* (*col1α2*) transcripts were normalized to 18S RNA expression in the same sample. Primer sequences for *col1α2* were:

Sense	5′-GAA CGG TCC ACG ATT GCA TG-3′
Anti-sense	5′-GGC ATG TTG CTA GGC ACG AAG-3′
Product size	167 bp
GenBank Accession No.	BC007158

Statistics. Statistical analysis, unpaired *t* test, was performed with Graphpad Prism software (San Diego, CA).

Results

Using the HSC isolation and culture conditions that were employed in the present study, others have reported that freshly isolated HSC strongly express GFAP, but little, if any, ASMA. During culture, the HSC become activated and express increasing amounts of ASMA, while GFAP expression declines significantly [24]. To confirm the cellular identity of our cultures, HSC were analysed for expression of ASMA and GFAP at various time points after isolation. At subconfluence (day 7 in culture), cells expressing ASMA are abundant (Fig. 1). In contrast, freshly isolated HSC strongly express GFAP at early time points in culture (day 0–4) (Figs. 2 and 3). Changes in the relative expressions of ASMA and GFAP are demonstrated by immunoblot (Fig. 3). Consistent with the work of others, we show differential regulation of ASMA and GFAP expression during HSC culture.

Before testing the effects of SNS neurotransmitters on HSC proliferation, we evaluated the utility of WST-8, a commercially available reagent, in detecting viable HSC. Increasing numbers of HSC were plated and 48 h later WST-8 counting reagent was added and cell number was evaluated using optical density as described in Methods. The results confirmed the absolute linearity of optical density (OD) with increasing cell number ($p < 0.0001$, $r^2 = 0.98$) [9]. Therefore, in subsequent studies where equal numbers of HSC were added to various wells, it is reasonable to conclude that treatment-related increases in OD reflect increases in HSC number, i.e., proliferation.

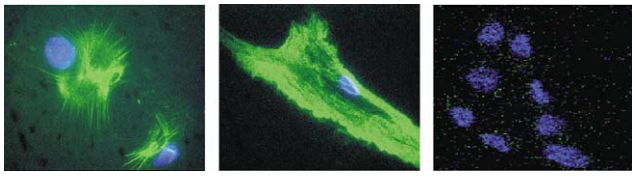

Fig. 1. α-Smooth muscle actin expression by HSC in culture. Murine HSC were grown to subconfluence and then analysed for ASMA expression on day 7. Cell nuclei are demonstrated by Hoechst's stain (blue) and ASMA appears green (left and middle panels). Without primary antibody, binding of the secondary antibody was negligible (right). Final magnification 400×.

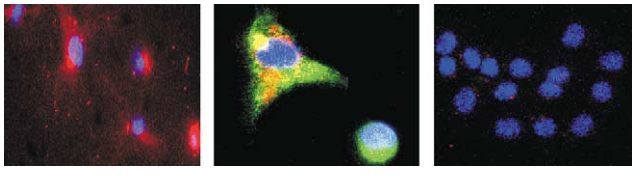

Fig. 2. GFAP and ASMA co-expression in cultured HSC. GFAP and ASMA expression were evaluated in HSC after 4 days in culture. Cell nuclei are demonstrated by Hoechst's stain (blue). GFAP appears red (left and middle panels). ASMA stains green and co-localization of ASMA and GFAP appear yellow (middle panel). Without primary antibodies binding of the secondary antibodies was negligible (right panel). Final magnification 400×.

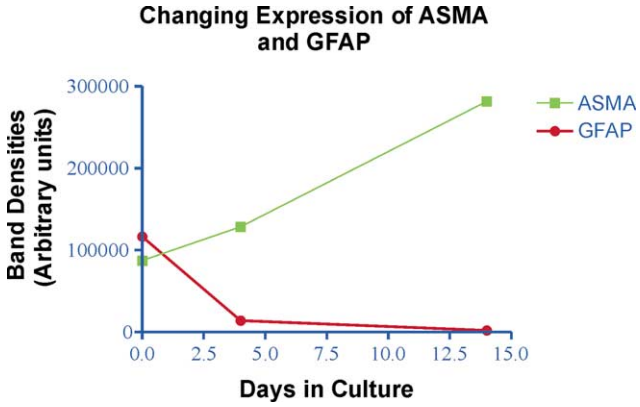

Fig. 3. Immunoblot analysis of ASMA and GFAP expression by HSC at various time points in culture. At the time of plating (day 0) or after 4 or 14 days in culture, ASMA and GFAP expression were assessed by immunoblot (10 μg protein/lane) and the resulting bands were quantified. Results from a representative experiment are shown below. Similar results have been obtained on at least one other occasion.

To determine if the parasympathetic neurotransmitter, ACh, influence HSC proliferation, HSC were cultured for 48 h in either serum-free medium or medium with 10% serum plus PDGF, a known HSC mitogen, or various concentrations of ACh. As expected, serum and PDGF stimulated HSC proliferation. ACh from 0.1 μM to 1 mM caused a dose-dependent increase in proliferation with maximal effect at 100 μM (Figs. 4A and B). The effect of ACh on HSC proliferation was mediated, at least in part via HSC acetylcholine nicotinic receptors, because the ACh-induced proliferation was blocked by mecamylamine, a nicotinic acetylcholine receptor antagonist (Fig. 5).

We next investigated the effect of ACh on collagen gene expression. As shown, similar to PDGF, ACh also increased the expression of *collagen 1α2* by HSC (Fig. 6). This effect was observed at ACh concentrations above 10 μM and was maximal at 1 mM.

Discussion

In this study we have shown that the parasympathetic neurotransmitter, ACh, markedly stimulates proliferation and collagen gene expression by myofibroblastic HSC after their activation in culture. The cells used here are judged to be myofibroblastic HSC by the culture-induced changes in their morphological appearance, α-SMA expression and GFAP expression [24]. Consistent with this interpretation, these cells proliferate, as expected, in response to stimulation by serum and PDGF [25].

The effect of ACh on HSC proliferation is mediated, at least in part, through its direct action on nicotinic

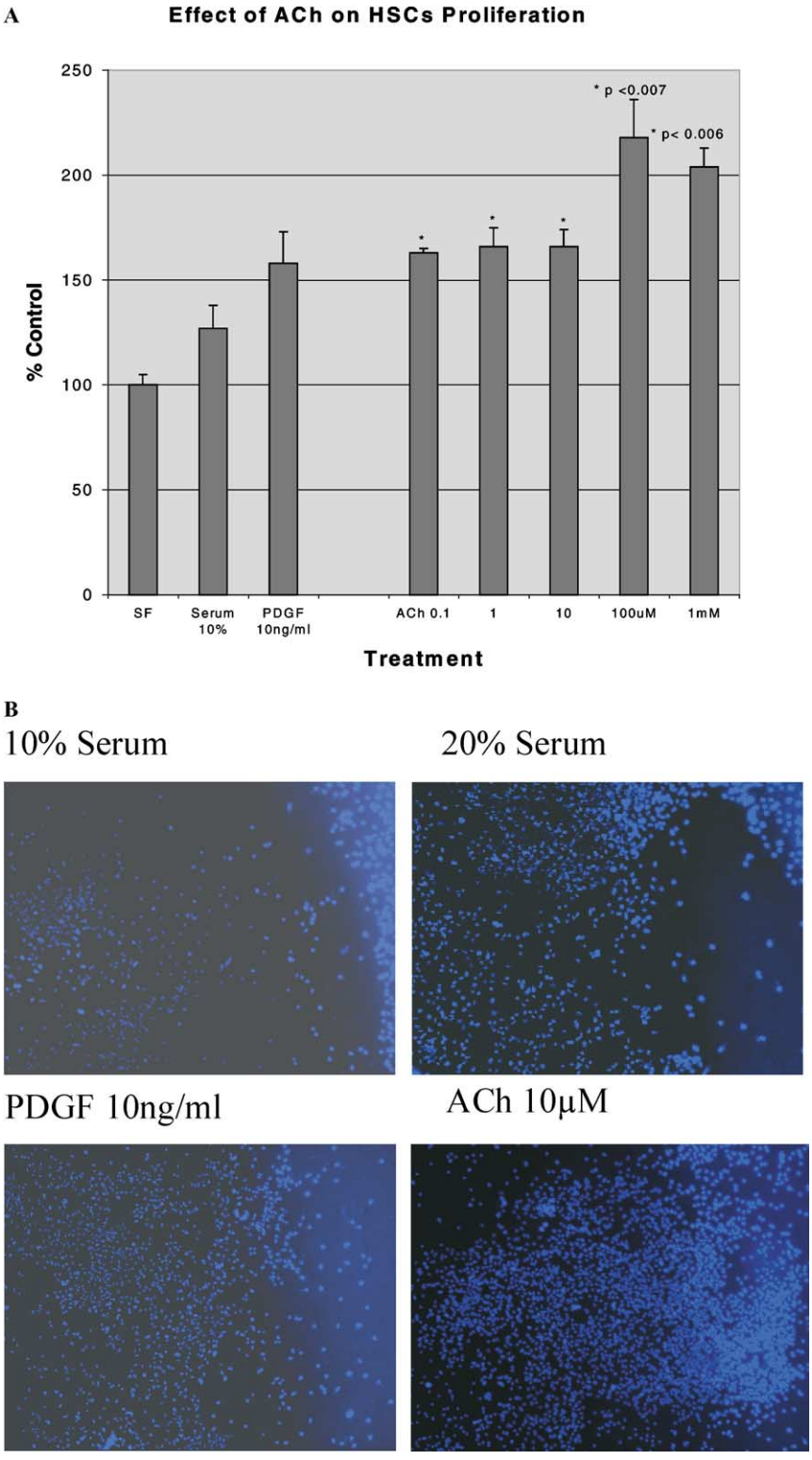

Fig. 4. (A) Effects of ACh on HSC proliferation. HSC were cultured in different concentrations of Ach for 48 h. Cellular proliferation was evaluated using the WST-8 reagent. Results (mean OD ± standard deviation of triplicate responses in one typical experiment) are expressed as a percentage of the response in serum free control wells. Similar results have been obtained on at least one other occasion. SF, serum free. *$p < 0.05$, unpaired t test, unless otherwise specified. (B) Immunocytochemical demonstration of mitogenic of effects on HSC. At the end of treatment, representative cultures were stained with Hoechst and examined in situ (100× magnification) to verify the results of the WST-8 proliferation assays. Each shot was taken to show a view as close as possible to the center of each well.

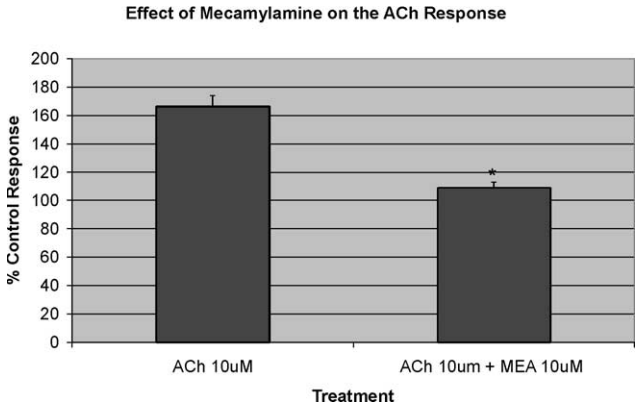

Fig. 5. Antagonism of ACh Effect by mecamylamine. HSC were cultured for 48 h in medium containing Ach with or without the nAChR antagonist mecamylamine. Results (mean OD, ± standard deviation of triplicate responses in one typical experiment) are expressed as a percentage of the response in serum free control wells. Similar results have been obtained on at least one other occasion. $*p < 0.05$, unpaired t test, for MEA plus Ach response versus ACh.

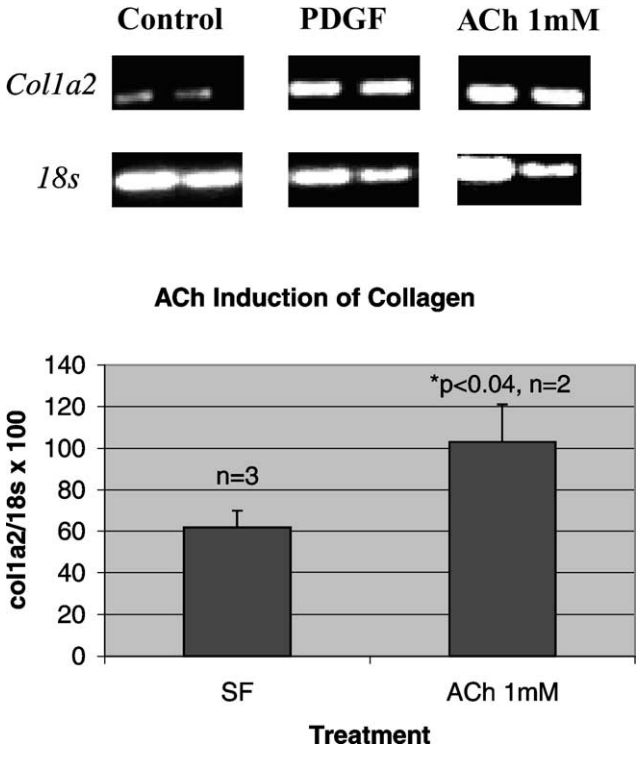

Fig. 6. Induction of collagen gene expression by acetylcholine. HSC were cultured for 48 h in medium containing either PDGF or ACh. Control wells had no drugs added. The bands are from one typical experiment. The graph, however, summarizes treatment effects on *collagen 1α2* gene transcripts, with band densities normalized against the corresponding 18S densities, in multiple experiments as shown (mean ± SEM). Cells from 4 to 5 mice were pooled in each experiment and used at first subconfluence. Similar collagen gene inducing effects of ACh have also been seen at 10 μM doses. $*p < 0.05$ for ACh 1 mM response compared to control.

acetylcholine receptors (nAChRs) because the ACh effect was completely abrogated by pre-treatment of the cells with the nAChRs antagonist, mecamylamine [26]. Although HSC possess adrenoceptors and receive autonomic fibres which terminate directly on them, before our study, they were not known to have nAChRs [16,27] and ACh was not considered to be an HSC mitogen. While evidence that ACh promotes HSC proliferation is new, this finding is completely consistent with reports that other mesenchymal cells that possess nAChRs, such as vascular smooth muscle cells, proliferate in response to nicotine [28]. Similarly, although our study is the first, to our knowledge, to demonstrate that a parasympathetic neurotransmitter directly stimulates collagen gene expression by myofibroblastic HSC, others have shown that nAChR agonists promote collagen production during skin wound healing [11]. This suggests that regardless of their tissue localization, activated myofibroblastic cells, including HSC, share a common phenotype. Nevertheless, the identification of physiologically relevant mitogenic and fibrogenic stimuli for HSC has novel clinical implications, because the recovery of patients with various types of acute or chronic liver injury requires the activation, proliferation, and fibrogenic response of HSC [1].

In this regard, it is important to emphasize that the concentrations of ACh that stimulate HSC proliferation and collagen gene expression are within a physiologically relevant range, because the amount of neurotransmitter in the synaptic cleft reaches millimolar concentrations [27]. Thus, our hypothesis that, via its neurotransmitters, the parasympathetic nervous system directly influences proliferation and collagen gene expression by HSC, is proven. This likely has pathophysiological significance, although further work is necessary to validate our suspicion that ACh–HSC interactions play a role in the pathogenesis of cirrhosis. On the other hand, one report already demonstrates that nicotine and epibatidine, parasympathetic neurotransmitter agonists that act via nAChRs, promote fibrosis and angiogenesis in the skin [11]. Moreover, given dogma that smoking exacerbates fibrostenosis in Crohns disease [29], pathological fibrogenic responses to nAChR activation appear to occur in various parts of the gastrointestinal tract that contain myofibroblastic cells [30].

Finally, our observation that a parasympathetic neurotransmitter directly regulates the phenotype of myofibroblastic HSC extends recent evidence that sympathetic neurotransmitters modulate proliferation and matrix production by mesenchymal cells [31–34], including HSC [9]. Therefore, there is no longer any doubt that both branches of the ANS directly influence stromal cells that maintain the architecture of healthy livers and remodel damaged livers after injury. Given this, further research to delineate the sympathetic and parasympathetic regulation of HSC during liver injury seems

justified. It is conceivable, for example, that targeted interruption of autonomic signalling in HSC may help to constrain the fibrogenic response to liver injury and prevent the development of cirrhosis.

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