Hepatic fibrogenesis requires sympathetic neurotransmitters

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Background and aims: Hepatic stellate cells (HSC) are activated by liver injury to become proliferative fibrogenic myofibroblasts. This process may be regulated by the sympathetic nervous system (SNS) but the mechanisms involved are unclear.

Methods: We studied cultured HSC and intact mice with liver injury to test the hypothesis that HSC respond to and produce SNS neurotransmitters to promote fibrogenesis.

Results: HSC expressed adrenoceptors, catecholamine biosynthetic enzymes, released norepinephrine (NE), and were growth inhibited by 2- and 3-adrenoceptor antagonists. HSC from dopamine b-hydroxylase deficient (Dbh−/−) mice, which cannot make NE, grew poorly in culture and were rescued by NE. Inhibitor studies demonstrated that this effect was mediated via G protein coupled adrenoceptors, mitogen activated kinases, and phosphatidylinositol 3-kinase. Injury related fibrogenic responses were inhibited in Dbh−/− mice, as evidenced by reduced hepatic accumulation of α-smooth muscle actin and decreased induction of transforming growth factor β1 (TGF-β1) and collagen. Treatment with isoprenaline rescued HSC activation. HSC were also reduced in leptin deficient ob/ob mice which have reduced NE levels and are resistant to hepatic fibrosis. Treating ob/ob mice with NE induced HSC proliferation, upregulated hepatic TGF-β1 and collagen, and increased liver fibrosis.

Conclusions: HSC are hepatic neuroglia that produce and respond to SNS neurotransmitters to promote hepatic fibrosis.

Liver injury activates hepatic stellate cells (HSC) to move from a quiescent phenotype to a proliferative, fibrogenic, myofibroblastic phenotype.1 These activated myofibroblastic cells are responsible for the progressive accumulation of collagen that occurs as injured livers become cirrhotic. HSC are also contractile and may therefore contribute to portal hypertension.2 The mechanisms that initiate and perpetuate the fibrogenic response in injured livers are not understood.

Sympathetic nervous system (SNS) inhibitors markedly reduce experimentally induced liver fibrosis,3 and the spontaneously hypertensive rat, which has an overactive SNS, develops unusually severe liver fibrosis when given hepatotoxins.1 3 Although these observations suggest that SNS activity promotes hepatic fibrosis, the cellular target of the SNS in mediating these effects, and the mechanisms involved, are not known.

HSC may be targets for SNS regulation because we showed that cultured HSC proliferate and express collagen mRNA in response to SNS neurotransmitters.4 5 Given that HSC also express stereotypical neuronal proteins,2 6 8–11 possess synaptic vesicles,10 and are innervated by autonomic fibres,12 13 we hypothesise that HSC produce SNS neurotransmitters and function as an effector arm of the SNS, automodulating their own activation via SNS neurotransmitters.

METHODS

Drugs
All drugs were from Sigma (St Louis, Missouri, USA), except pertussis toxin, wortmannin, SB202190, PD98059, and RO-32-0432 (all purchased from Calbiochem, San Diego, California, USA).

Animal experiments
Adult (10–18 weeks), male, lean, and ob/ob C57BL/6 mice were from Jackson Laboratory (Bar Harbor, Maine, USA). Similarly aged male Sprague-Dawley rats were from Charles River (Wilmington, Massachusetts, USA). Male dopamine b-hydroxylase deficient (Dbh−/−) and Dbh+/− mice (30–40 weeks) were from our colony.14 Most animals were used to provide HSC for in vitro experiments (see below). However, other experiments were done in intact mice. In those studies, Dbh−/− mice (n = 12) and Dbh+/− littermates (n = 6) were fed methionine restricted choline deficient (MCD) diets (ICN, Aurora, Ohio, USA) for four weeks to induce steatohepatitis and liver fibrosis.15 16 Half of these Dbh−/− mice were given subcutaneous osmotic minipumps (Alzet, Cupertino, California, USA) that infused isoprenaline at 20 mg/kg/day to induce SNS activation.7 Some ob/ob mice (n = 15) were similarly infused with vehicle or norepinephrine (NE 2.5 mg/kg/day). Such NE treatment induces SNS activation in normal mice.8 During in vivo experiments, mice were weighed weekly until sacrifice when livers were harvested, fixed (in buffered formalin or optimal cutting temperature fixative (Sakura, Torrance, California, USA)), or snap frozen and stored at −80°C. All experiments satisfied the guidelines of our Institutional Animal Care Committee and the National Institutes of Health.

Stellate cell isolation and culture
Pronase-collagenase liver digestion was used to isolate HSC from wild type, Dbh+/−, and Dbh−/− mice, or healthy rats. For

Abbreviations: ASMA, alpha smooth muscle actin; Dbh, dopamine b-hydroxylase; DOPAC, 3,4-dihydroxyphenylacetic acid; GFAP, glial acidic fibrillary protein; HPLC, high pressure liquid chromatography; HSC, hepatic stellate cells; HVA, homovanilllic acid; MCD, methionine restricted choline deficient; MMP, matrix metalloproteinase; NE, norepinephrine; 5-HT, 5-hydroxytryptamine (serotonin); RT-PCR, reverse transcription-polymerase chain reaction; SNS, sympathetic nervous system; TGF-β1, transforming growth factor β1; TH, tyrosine hydroxylase; TIMP, tissue inhibitor of metalloproteinase; 5-HIAA, 5-hydroxyindoleacetic acid
each experiment, HSC were pooled from six mice of each genotype. A single rat provided sufficient HSC for an experiment. All experiments were replicated at least twice. In total, HSC were obtained from about 72 mice and two rats. Cell identity was confirmed by autofluorescence and expression of alpha smooth muscle actin (ASMA) and glial fibrillary acidic protein (GFAP). HSC preparation purity was always >95%. HSC were routinely cultured in 10% serum supplemented RPMI 1640 medium. To assess catecholamine production, HSC and conditioned media were harvested after four days in culture; cells were washed twice with ice cold phosphate buffered saline. Cells or conditioned medium were then rapidly analysed for catecholamine content by high pressure liquid chromatography (HPLC).

**Apoptosis assays**

Equal numbers of HSC were plated on 6 mm petri dishes with or without 10 μM prazosin. Fresh prazosin was added every two days when the medium was changed. At harvest, apoptotic activity was assessed with the Vybrant (annexin V) apoptosis assay kit (Molecular Probes, Eugene, Oregon, USA).

**Cell proliferation assay**

Freshly isolated HSC were seeded into culture flasks, grown to subconfluence (~day 7), and then harvested, resuspended in serum free medium, and re-plated on 96 well plates at 5000 cells/well. Twenty four hours later, when the cells had become quiescent, NE (10 μM) with or without various inhibitors (for example, prazosin (10 μM), pertussis toxin (100 ng/ml), wortmannin (100 nM), SB202190 (10 μM), PD98059 (20 μM), or RO-32-0432 (1 μM)) were added to some wells. After 44 hours, cell numbers were assessed by a further four hour incubation with WST-8 tetrazolium reagent (Dojindo Molecular Technologies, Gaithersburg, Maryland, USA). In viable cells, the tetrazolium salt is metabolised to a colorimetric dye and cell number is proportional to the signal intensity at 450 nm. As detailed in information that the manufacturer provides with the reagents, the use of tetrazolium dyes has been validated against thymidine incorporation for a variety of cell types, including stellate cells. Therefore, we and others have used this assay to evaluate HSC proliferation.

**RT-PCR**

RNA was extracted from HSC using RNeasy kits (Qiagen, Valencia, California, USA). One step reverse transcription-polymerase chain reaction (RT-PCR) was performed with...
Superscript one step RT-PCR with platinum Taq kits (Invitrogen, Carlsbad, California, USA) and Ambion's QuantumRNA Classic II 18S internal standard (Ambion, Austin, Texas, USA). Products were separated by electrophoresis on a 1.5% agarose gel using published primer sequences and conditions.20 31

**HPLC analysis**

Catecholamines were extracted from HSC pellets and HSC conditioned medium with perchloric acid. NE, dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), serotonin (5-hydroxytryptamine, 5-HT), 5-hydroxyindoleacetic acid (5-HIAA), and homovanillic acid (HVA) were analysed by HPLC,32 using dihydroxybenzilamine as the internal standard. Catecholamine concentrations were calculated with a Hewlett Packard integrator.

**Immunoblot**

Net protein content of HSC homogenates was quantified by BSA assay (Pierce, Rockford, Illinois, USA) using bovine serum albumin standards. Proteins (10 μg/lane) were then resolved by polyacrylamide gel electrophoresis and transferred to nylon membranes. After membranes were incubated with primary antibodies to ASMA (1:3000; Sigma), tyrosine hydroxylase (TH 1:100; Novus Biologics, Littleton, Colorado, USA), dopamine beta-hydroxylase (1:250;...
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Norepinephrine (NE) increases hepatic stellate cell (HSC) protein positive cells were counted in five randomly selected high power fields/liver section from each animal. Data are mean (SD). NE (n = 5) or vehicle (n = 10). After four weeks, glial acidic fibrillary activation in NE deficient ob/ob mice. Ob/ob mice were treated with ob/ob control versus lean control; *p<0.05 for ob/ob control versus lean control; †p<0.05 for ob/ob-NE versus ob/ob control.

Research Diagnostics, Flanders, New Jersey, USA). Reduced activation of hepatic stellate cells (HSC) in norepinephrine (NE) deficient Dbh−/− mice. Twelve Dbh−/− and six of their control Dbh+/+ littermates were fed methionine choline deficient (MCD) diets. Half of the Dbh−/− mice were also infused with isoproterenol (ISO) for four weeks. (A) Alpha smooth muscle actin (ASMA) immunopositive sinusoidal cells were counted in five randomly selected fields/liver section from each mouse. Mean (SD) results of one experiment are shown. *p<0.05 for Dbh+/+ versus Dbh−/− control; †p<0.05 for Dbh−/− control versus Dbh+/++ISO. Identical results have since been obtained in a second experiment that studied an additional 12 mice (four mice/group). (B) Photomicrograph from representative Dbh+/+ mice. (C) Photomicrograph from typical Dbh−/− mice. Arrows indicate typical ASMA+ HSC (B) or vessel wall (C).

(6) ng/ml). In contrast, NE was not detected in conditioned medium from Dbh−/− HSC or in unconditioned medium that had not been exposed to HSC. In addition to NE, normal murine HSC lysates contained dopamine, 5-HT, catecholamine metabolites (DOPAC and HVA), and the 5-HT metabolite 5-HIAA (fig 2).

HSC express multiple adrenoceptor subtypes

Although HSC are known to express α1-adrenoceptors, it is not known which α-adrenoceptor subtypes are expressed, or if HSC express β-adrenoceptors. RT-PCR analysis demonstrated that primary HSC expressed α1A, α1D, β1, and β2...
by prazosin (fig 5B). Proliferative activity was also significantly reduced in HSC from Dbh−/− mice (fig 5C). Exogenous NE rescued the growth of Dbh−/− HSC (fig 5D), confirming the importance of endogenous NE for HSC growth.

**NE is an autocrine growth factor for HSC**

Normal HSC become spontaneously activated during culture and proliferate at a greater rate than they die. Therefore, increases in proliferative activity normally drive HSC growth in culture. To determine to what extent, if any, NE related differences in cell number might also reflect differences in apoptotic activity, HSC were harvested, incubated with annexin V, and analysed by flow cytometry. After one day in culture, a slightly greater proportion of apoptotic HSC were detected in cultures from Dbh−/− mice (12.4 (0.26)% compared with Dbh+/− mice (9.96 (0.15)% (p = 0.007). Therefore, endogenous NE promotes the viability of HSC. This antiapoptotic effect may help to explain why endogenous NE is required for optimal HSC growth.

**NE activates adrenoceptor G protein coupled mechanisms that induce mitogenic and survival pathways**

Previously, we showed that adding NE to cultures of murine HSC caused a dose related increase in proliferation that was blocked by coadministration of prazosin. To be certain that this response was not an unusual property of murine HSC, we cultured rat HSC with NE, with and without prazosin. Incubation with NE for 48 hours significantly (p<0.05) increased rat HSC numbers, and prazosin significantly (p<0.05) blocked these trophic effects, demonstrating that NE generally promotes HSC proliferation.

We next addressed potential mechanisms for this effect. Adrenergic agonists are known to be coupled to G proteins in other mesenchymal cells in which they induce proliferation by activating phosphoinositide triphosphate and mitogen activated protein kinases. Therefore, primary HSC from normal mice were cultured with NE in the presence and absence of pertussis toxin, a G protein inhibitor; Wortmannin, a PI3-kinase inhibitor; SB202190, an inhibitor of p38 MAP kinase; PD98059, a MEK inhibitor; and Ro-32-0432, a protein kinase C inhibitor. Pertussis toxin, Wortmannin, and PD98059 significantly reduced NE induced proliferation (fig 6). Thus the proliferative effects of NE on HSC are mediated by G protein coupled receptors and the downstream effects involve PI-3 kinase and the Erk family of mitogen activated protein kinases.

**Adrenergic agonists regulate HSC activation in intact mice**

To determine if NE had any impact on HSC function in intact animals, NE was infused chronically into ob/ob mice, which have low levels of NE17, 20 and are resistant to fibrosis despite having chronic steatohapatitis. Immunohistochemistry was used to demonstrate GFAP(+) cells in livers because this marker provides a reliable estimate of both quiescent and activated HSC. Control ob/ob mice have significantly fewer HSC than their lean littermates. Continuous subcutaneous infusion of NE for four weeks markedly stimulated HSC proliferation in ob/ob mice such that the numbers of GFAP(+) HSC in NE treated ob/ob mice doubled, leading to HSC numbers that approached those of their control littermates (fig 7).

Ob/ob mice are genetically incapable of producing leptin, a factor that is known to activate HSC proliferation by direct interaction with HSC receptors. Therefore, it is conceivable that the effects of NE in this strain might have been confounded by leptin deficiency. To exclude this possibility, we studied Dbh−/− mice, which also have reduced NE but are
not leptin deficient. We fed Dbh−/− mice and Dbh+/− mice antioxidant depleted diets. Normal mice develop steatohepatitis and fibrosis after eating these diets for 4–8 weeks. Consistent with this literature, after four weeks of treatment, control Dbh+/− mice exhibit striking accumulation of HSC that express ASMA, an accepted marker of HSC activation (fig 8A, B). In contrast, ASMA+ve HSC cannot be demonstrated in Dbh−/− mice (fig 8A, C). This finding is unlikely to be a staining artefact because ASMA+ve vessel walls are easily identified (fig 8C). Moreover, compared with Dbh+/− controls, Dbh−/− mice exhibit significantly less hepatic expression of TGF-β1 and collagen, two other indicators of HSC activation (fig 9A, B). Ribonuclease protection analysis also demonstrated a small (~30–40%) but statistically significant (p<0.05) reduction in induction of TIMP-2 transcripts in the Dbh−/− group. The diet barely increased expression of other genes (for example, MMP-2, MMP-9, and TIMP-1) that regulate matrix degradation, and therefore no group related differences in these transcripts were observed.

To verify that it was reduced adrenoceptor activity that prevented HSC activation, we implanted osmotic minipumps that contained vehicle or isoprenaline, a β-adrenoceptor agonist, into Dbh−/− mice and repeated the feeding experiment. Infusion of isoprenaline rescued HSC activation in Dbh−/− mice and returned numbers of ASMA+ve HSC to levels exhibited by Dbh+/− mice that were also fed the hepatotoxic diet (fig 8A). Isoprenaline infusion similarly normalised induction of TGF-β in Dbh−/− mice (fig 9B). In

![Image](http://www.gutjnl.com/group.bmj.com/Downloaded from http://gutjnl.com/group.bmj.com)
parallel experiments, we examined the effect of NE, an α-adrenoceptor agonist, on HSC activation in ob/ob mice. Compared with control ob/ob mice, ob/ob mice that were treated with NE minipumps for four weeks had significantly increased liver expression of TGF-β1 and collagen mRNA (fig 10A, B). Indeed, in two of five ob/ob mice, NE treatment increased hepatic fibrosis sufficiently for scar formation to be detected simply by staining liver sections with Mason trichrome reagent (fig 10C). No such fibrosis was detected in twice as many ob/ob controls. Therefore, both α-predominant (NE, fig 7) and β-predominant (isoprenaline, figs 8A, 9B) adrenoceptor agonists affect HSC activation in vivo. Despite this increase in HSC activation, alanine aminotransferase values in NE treated ob/ob mice were lower than in their littermate controls (fig 10D). Therefore, NE related increases in fibrogenesis are not easily attributed to NE exacerbation of liver injury in leptin deficient mice.

**DISCUSSION**

We have shown that HSC express key enzymes for catecholamine biosynthesis, and produce NE and other catecholamines. Moreover, HSC use catecholamines to autoregulate their growth because increases in HSC number are significantly attenuated by culturing normal HSC with α- or β-adrenoceptor antagonists. Inhibitor studies suggest that the trophic actions of NE require activation of kinases that promote cell viability and proliferation. This finding is intriguing because leptin, another factor that promotes HSC growth, was recently shown to activate similar signalling mechanisms.

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It is also conceivable that leptin promotes HSC activation by inducing expression or function of adrenoreceptors or components of the post-receptor signalling pathways that mediate NE effects. More work is required to delineate the exact nature of leptin-NE interactions. Nevertheless, our findings in Dbh−/− mice, which produce leptin and have normal leptin receptors but are deficient in NE, support the general importance of adrenoceptor agonists in HSC activation. In these mice that are genetically incapable of converting dopamine into NE, hepatotoxic diets only activate HSC when adrenoceptor activity is increased by treatment with adrenergic agents. Thus when NE is deficient, leptin cannot fully activate HSC, and TGF-β1 and collagen are not induced normally in response to liver injury.

That NE may subserve functions other than its classically assigned role of neurotransmission is becoming well established in other organs. For example, cardiac remodelling in heart failure involves mitogenic and fibrogenic actions of NE that are mediated via adrenoceptors. The present studies provide novel evidence that HSC are both a source and a direct cellular target of NE. This extends our understanding of the mechanisms that mediate the profibrogenic actions of catecholamines in the liver and suggests that targeted interruption of catecholamine signalling in hepatic stellate cells may be a useful therapeutic approach to constrain the fibrogenic response to liver injury.
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