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Does hepatic vagus nerve modulate the progression of biliary fibrosis in rats?

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ABSTRACT

Recent studies have shown that vagus nerve activation inhibits cytokine production in a variety of non-neural cells though activation of α7 nicotinic acetylcholine receptor (α7nAChR). Since chronic inflammation plays a pivotal role in liver fibrosis, this study was designed to investigate the role of hepatic vagus nerve in the progression of hepatic fibrosis in rats. Cirrhosis was induced by chronic ligation of the bile duct. Hepatic hydroxyproline level, portal pressure, serum transaminase level, hepatic TIMP-1 (tissue inhibitor of metalloproteinase-1) and MCP-1 (monocyte chemoattractant peptide-1) expression were measured in order to assess the progression of liver cirrhosis. α7nAChR expression was assessed using RT-PCR as well as immunostaining. RT-PCR analysis of the liver showed that α7nAChR mRNA is expressed in rat liver. Immunostaining study demonstrated that hepatic α7nAChR is mainly expressed in the hepatocytes of cirrhotic liver with minimum α7nAChR expression in biliary epithelium or myofibroblasts. Bile duct ligation was associated with portal hypertension, increased hepatic hydroxyproline level as well as TIMP-1 and MCP-1 expression in the liver. However neither selective hepatic vagotomy nor methyllycaconitine (an α7nAChR antagonist) could significantly affect development of portal hypertension or hepatic fibrosis in rats. Selective hepatic vagotomy could only attenuate serum aspartate aminotransferase level in bile duct ligated rats but did not have a significant effect on hepatic inflammation as assessed by MCP-1 mRNA expression. Our study provides evidence against a crucial role for the hepatic vagus nerve as an intrinsic protective mechanism in modulation of hepatic fibrosis in a rat model of biliary cirrhosis.
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1. Introduction

Liver is the largest solid organ in the body and plays a central role in the regulation of homeostasis. Chronic liver injury leads to expression and secretion of chemokines such as monocyte chemoattractant peptide-1 (MCP-1) that are responsible for infiltration of inflammatory cells in the liver (Marra et al., 1998). Inflammatory mediators can trigger trans-differentiation of hepatic stellate cells as well as bone marrow-derived cells to myofibroblasts (Ebrahimkhani et al., 2008). Myofibroblast formation is associated with remodeling of extracellular matrix and fibrosis via expression of mediators such as tissue inhibitor of metalloproteinase-1 (TIMP-1) (Ebrahimkhani et al., 2008). Therefore, extensive liver fibrosis (cirrhosis) is the most common feature of chronic hepatic inflammation. Although, liver fibrosis is a major cause of liver failure worldwide, the available treatments remain ineffective. A logical way to reduce hepatic inflammation is to potentiate the physiological anti-inflammatory mechanism(s) that modulate inflammation in the liver. The "cholinergic anti-inflammatory pathway" has recently been described by Tracey and colleagues and refers to the role that autonomic nervous system plays in regulation of host defense and in physiologic responses to pathogens (Tracey, 2007). Their studies have shown that the vagus nerve activation can reduce inflammation via activation of alpha7-nicotinic acetylcholine receptors (α7nAChR) that are located on inflammatory cells (Wang et al., 2003). There are reports to show that there is a cross-talk between liver and central nervous system during neural inflammation (Campbell et al., 2008). However the role of such cross-talk during the course of hepatic inflammation is not well understood.

Liver is innervated by both sympathetic and parasympathetic nerves that contain afferent and efferent aminergic, cholinergic, peptidergic, and nitrergic components (McCuskey, 2004). There is evidence to show that hepatic innervation plays a role in the regulation of hepatic metabolism and hemodynamics (Lautt, 1983; Bioulac-Sage et al.,

Abbreviations: α7nAChR, α7 nicotinic acetylcholine receptor; α-SMA, α smooth muscle actin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BDL, bile duct ligated; DAB, diaminobenzidine; ISPP, intrasplenic pulp pressure; MLA, methyllycaconitine; MCP-1, monocyte chemoattractant peptide-1; SHV, selective hepatic vagotomy; TIMP-1, tissue inhibitor of metalloproteinase-1.

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1990; Bockx et al., 2012). In the process of hepatic fibrosis, cholinergic nervous fibers are known to increase in the fibrous areas (Ungváry and Donáth, 1975; Akiyoshi, 1989; Akiyoshi and Terada, 1998). It is also known that both muscarinic (Vatamaniuk et al., 2003) and nicotinic (Soeda et al., 2012) acetylcholine receptors are expressed in the liver. Recent reports demonstrated that in humans, the highest accumulation of α7nAChR in the body was observed in the liver (Sakata et al., 2011). α7nAChR has been reported as the major target for the "cholinergic anti-inflammatory pathway" in a variety of tissues including gastrointestinal tract (Matteoli et al., 2013) and liver during acute hepatic injury (Hiramoto et al., 2008; Park et al., 2013). Hiramoto et al. demonstrated that the hepatic vagus nerve attenuates Fas-induced apoptosis in the mouse liver via α7nAChR (Hiramoto et al., 2008). Park et al. has also shown that activation of α7nAChR ameliorates hepatic ischemia/reperfusion injury in mice (Park et al., 2013). Although these reports indicate that α7nAChR might modulate hepatic inflammation during acute hepatic injury, the role of this receptor in modulation of hepatic fibrosis remains elusive.

α7nAChR is an ion channel and is expressed in a variety of cells such as macrophages (Matteoli et al., 2013), endothelial cells (Mazloom et al., 2013), hepatic stellate cells (Soeda et al., 2012), hepatic cholangiocytes (Jensen et al., 2013) as well as hepatic mitochondria (Gergalova et al., 2012). All above mentioned cells are present in hepatic tissue and play roles during the process of liver fibrosis (Ebrahimkhani et al., 2008). However, our understanding of the role of α7nAChR in hepatic fibrosis is very limited. Likewise, the role of the hepatic branch of the vagus nerve in modulation of chronic hepatic inflammation/fibrosis is not well understood. The present study was aimed to investigate the effect of hepatic branch of the vagus nerve on the process of hepatic fibrosis as well as expression of MCP-1 and TIMP-1 in a rat model of biliary fibrosis.

2. Material and methods

2.1. Ethics statement

All animal maintenance and procedures were in accordance with recommendations established by the Animal Ethics Committee of Tarbiat Modares University as well as NIH guidelines (publication no. 85-23). The protocol was approved by the Ethics Committee of Tarbiat Modares University. All surgeries were performed under deep anesthesia, and all efforts were made to minimize suffering of the animals.

2.2. Chemicals

Anti-α7nAChR antibody was purchased from Abcam (Cambridge, MA, USA). The rest of the materials were purchased from Sigma-Aldrich (Poole, UK), unless otherwise specified in the text.

2.3. Animals

Male Sprague–Dawley rats weighting 250–300 g were obtained from Razi Institute (Hesarak, Iran) and used in the experiments. Rats were kept four per cage under standard room temperature condition (22–25 °C) on a 12 h light/dark cycle, with wood chip bedding and ad libitum access to normal rodent chow and water. Six to eight animals were used in each experimental group.

2.4. Bile duct ligation

Bile duct ligation (BDL) was used as an animal model of chronic liver fibrosis. A midline abdominal incision was made under general anesthesia induced by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). The peritoneal cavity was opened and the common bile duct was isolated and triply ligated as described (Mani et al., 2006). Sham procedure involved a similar operation, but without ligation of the bile duct.

2.5. RNA extraction and RT-PCR

Pieces of liver tissue from rats were minced to obtain fractions smaller than 30 mg, which were then homogenized using sterile syringes. Total RNA extraction was performed using the RNeasy Fibrous Tissue mini kit (QIAGEN, Germany) according to the manufacturer's protocol. DNA elimination was achieved by adding DNase (Qiagen, Germany) during RNA extraction. Reverse transcription was performed using cDNA reverse transcription kit (Parstous, Tehran, Iran). Quantitative RT-PCR was used to study the expression of TIMP-1, MCP-1 and α7nAChR in the experimental groups. For quantitative RT-PCR, 18 s ribosomal RNA was used as internal standard (housekeeping gene). Oligonucleotide primers used for PCR were as follows:

a. *Rattus norvegicus* TIMP-1, NM_053819.1, PCR product size: 250 bp
Forward: 5′-AGCCTGTAGCTGTGCCCCAA-3′, Reverse: 5′-AACTCCTC GCTGCGGTTCTG-3′
b. *Rattus norvegicus* MCP-1, NM_031530.1, PCR product size: 157 bp
Forward: 5′-GGGCCTGTTGTTCACAGTTGC-3′, Reverse: 5′-GGGACA CCTGCTGCTGGTGAT-3′
c. *Rattus norvegicus* α7nAChR, NM_012832.3, PCR product size: 414 bp
Forward: 5′-CCTGGCCAGTGTGGAG-3′, Reverse: 5′-TAAGCAAAGTCT TTGGACAC-3′
d. *Rattus norvegicus* 18S, NR-046239.1, PCR product size: 109 bp
Forward: 5′-ATCACCTTTCGATGGTAGTCG-3′, Reverse: 5′-TCCTTGAT GTGGTAGCC-3′.

PCR reactions comprised of 2 μl of cDNA template, 10 pmol each of forward and reverse oligonucleotide primers, 10 μl of optimized PCR Master Mix (Ampliqon, Denmark) in a total reaction volume of 20 μl. After 15 min incubation at 95 °C, PCRs were done using a 60 second annealing step, followed by a 45 second elongation step at 72 °C and a 45 second denaturation step at 95 °C.

The expression of TIMP-1, MCP-1 and α7nAChR were assessed by real-time PCR using a Rotor-Gene Q machine (Qiagen, Hilden, Germany). The level of transcriptional difference between the experimental group and control group was evaluated relative to the level of 18S RNA expression.

2.6. Immunohistochemical localization of α7nAChR

We used immunohistochemistry technique for localization of α7nACHR in rat liver. Immunohistochemistry was performed on paraffin sections (5 μm) as described (Iredale et al., 1998). In brief, slides were deparaffinized with xylene and dehydrated in alcohol and subjected to antigen retrieval by pretreating for 25 min in a microwave (800 W) at medium power in 0.01 M citrate buffer (pH 6). Specimens were then incubated at room temperature in 10% normal goat serum (in PBS) for 1 h. The primary antibody was rabbit anti rat α7nAChR antibody (1:700 dilutions) and incubated for 2 h at room temperature. Incubation with biotinylated goat anti-rabbit antibody (Bioidea, Tehran, Iran) was carried out for 1 h at room temperature. HRP-linked streptavidin was then used for staining of the immune complex. Sections were photographed by Olympus BX51 microscope with a DP27 digital camera.

2.7. Immunofluorescence study of α-smooth muscle actin (a-SMA) and α7nAChR

Immunofluorescence study for α-SMA, a marker for myofibroblasts and α7nAChR were performed as previously described (Iredale et al., 1998). The primary antibodies were monoclonal anti-α-SMA (clone 1A4) (diluted 1:500 in PBS) and rabbit anti-rat α7nAChR antibody (1:700 in PBS dilution). Fluorescent-dye labeled secondary antibodies were goat anti-mouse IgG-TR and goat anti-rabbit IgG-FITC (diluted 1:200 in PBS; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and used for detection of primary antibodies respectively. Then the sections

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