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Effects of Modulating M3 Muscarinic Receptor Activity on Azoxymethane-Induced Liver Injury in Mice

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Abstract

Previously, we reported that azoxymethane (AOM)-induced liver injury is robustly exacerbated in M3 muscarinic receptor (M3R)-deficient mice. We used the same mouse model to test the hypothesis that selective pharmacological modulation of M3R activity regulates the liver injury response. Initial experiments confirmed that giving a selective M3R antagonist, darifenacin, to AOM-treated mice mimicked M3R gene ablation. Compared to vehicle controls, mice treated with the M3R antagonist had reduced survival and increased liver nodularity and fibrosis. We next assessed AOM-induced liver injury in mice treated with a selective M3R agonist, pilocarpine. After pilocarpine treatment, stimulation of post-M3R signaling in the liver was evidenced by ERK and AKT activation. In contrast to the damaging effects of the M3R antagonist, administering pilocarpine to AOM-treated mice significantly attenuated hepatic stellate cell activation, collagen deposition, bile ductule proliferation, and liver fibrosis and nodularity. As anticipated from these findings, livers from pilocarpine-treated mice exhibited reduced expression of key players in fibrosis (α 1 collagen, α -smooth muscle actin, TGF- β 1, PGDF, TGF- β 1R, PGDFR) and decreased mRNA levels for molecules that regulate extracellular matrix formation (TIMP-1, TIMP-2, MMP-2, MMP-13). Cleaved caspase-3, nitrotyrosine and BrdU immunostaining provided evidence that pilocarpine treatment reduced hepatocyte apoptosis and oxidative stress, while increasing hepatocyte proliferation. Collectively, these findings identify several downstream mechanisms whereby M3R activation ameliorates toxic liver injury. These novel observations provide a proof-of-principle that selectively stimulating M3R activation to prevent or diminish liver injury is a therapeutic strategy worthy of further investigation.

Keywords

liver injury; muscarinic receptors; pilocarpine; darifenacin; azoxymethane

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1. Introduction

In both humans and animal models, cholinergic input, primarily from the vagus nerve, modulates liver injury responses. In rodents, for example, vagus nerve transection reduces oval cell reaction after galactosamine-induced liver injury, and attenuates hepatocyte proliferation and liver regeneration following partial hepatic resection [1-3]. In contrast, vagus nerve stimulation in rats attenuates hepatic ischemia-reperfusion injury [4]. In humans, denervated liver grafts used for transplantation exhibit reduced ductular reaction compared to innervated native livers [3]. Although acetylcholine-induced activation of hepatic muscarinic and nicotinic receptors has been implicated, the mechanisms underlying cholinergic regulation of liver injury are uncertain [3, 5]. Muscarinic cholinergic G protein-coupled receptors are expressed widely and modulate a variety of biological functions [6, 7]. Of five muscarinic receptors designated M1R-M5R, M3R is the subtype primarily expressed in human and rodent liver [3, 8]. Although M3R expression and activation was shown to promote cell survival in various organ systems, its role in regulating liver injury is incompletely understood.

Administration to rodents of high-dose azoxymethane (AOM), an ingredient of cycad palms and a by-product of oxidation of the industrial compound methylamine, induces acute hepatic failure, whereas repetitive low doses induce chronic liver injury that mimics human disease [9-15]. AOM-induced changes in stellate cell activation, collagen deposition, and expression of cytokines and regulators of the extracellular matrix are similar to those observed in other chemically-induced chronic liver injury models [16]. Using mice with genetic ablation of *Chrm3*, the gene encoding M3R, we showed that M3R-deficiency augments AOM-induced liver nodularity, fibrosis, bile ductular proliferation, oval cell expansion and hepatocyte apoptosis [17]. Compared to wild-type mice, treating M3R-deficient mice with AOM markedly increased liver injury and reduced animal survival [17]. Treatment with scopolamine butylbromide, a non-selective muscarinic receptor antagonist, also worsened AOM-induced liver injury but the effects were much less severe than those seen with *Chrm3* gene ablation [17], perhaps because of confounding effects of this agent on other muscarinic receptor subtypes.

Based on these observations we hypothesized that selective modulation of M3R activity alters the liver injury response, thereby identifying M3R as a novel therapeutic target. The goal of the present study was to test this hypothesis using the AOM-induced liver injury model. First, we examined the actions of an M3R-selective *antagonist*, darifenacin, anticipating that, like *Chrm3* gene ablation, this agent would greatly increase the severity of AOM-induced liver injury. Then, we examined the effects of a selective M3R *agonist*, pilocarpine, on AOM-induced liver injury in mice, anticipating that this would have the opposite action and ameliorate AOM-induced liver injury. In our previous studies inhibition of M3R activation preceded (M3R-deficient mice) or coincided (scopolamine butylbromide treatment) with induction of liver injury (AOM administration). To more closely mimic the clinical setting in which treatment is instituted only after liver injury is recognized, in the present study we investigated the effects of modulating M3R activity *after* AOM treatment was completed. To gain insight into the molecular mechanisms underlying the effects of M3R activation in the liver, we examined stellate cell activation, collagen deposition, bile ductule and hepatocyte proliferation, hepatocyte apoptosis and the expression of key molecules regulating liver fibrosis and extracellular matrix formation. Our results show that treatment with pilocarpine attenuates AOM-induced liver injury, thereby providing proof-of-principle for the therapeutic potential of selective activation of M3R in chronic liver injury.

2. Materials and methods

2.1. Animals

Animal studies were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* prepared by the U.S. National Academy of Sciences (National Institutes of Health publication 86-23, revised 1985) and were approved by both the Institutional Animal Care and Use Committee at the University of Maryland School of Medicine and the Research and Development Committee at the VA Maryland Health Care System. All mice [genetic background: 129S6/SvEv X CF1 (50%:50%), Taconic Labs, NY] were housed under identical conditions in a pathogen-free environment with a 12:12-hour light/dark cycle and free access to standard mouse chow and water. Mice were acclimatized for two weeks prior to any treatment.

2.2. Experimental Designs

The actions of darifenacin, an M3R-selective antagonist, and pilocarpine, an M3R-selective agonist, were studied in the AOM model of liver injury; 6-week-old male mice treated with intraperitoneal AOM (10 mg/kg once each week for 6 weeks; Midwest Research Institute, Kansas City, MO). After a one-week interval, to deliver test agents, mice were implanted subcutaneously with osmotic mini-pumps (Alzet model 2006, DURECT Corporation, Cupertino, CA). In the first treatment group mice were treated with darifenacin (3 mg/kg/day; US Biological, Swampscott, MA; n = 19 mice) or vehicle (50% DMSO, n = 20 mice). In the second treatment group mice were treated with pilocarpine (2.5 mg/kg/day; Sigma-Aldrich; n = 14 mice) or vehicle (PBS; n = 14 mice). To achieve 14 weeks of treatment, after the first 7 weeks pumps were removed and replaced with new pumps freshly-loaded with test agents.

Mouse weight and mortality were recorded weekly. Twenty-one weeks after the first AOM dose surviving mice were euthanized. Additional control mice that did not receive AOM were euthanized at the same time. To assess hepatocyte proliferation, two hours before euthanasia, mice were injected with 50 mg/kg 5-bromo 2'-deoxyuridine (BrdU; Sigma, St. Louis). At euthanasia, two investigators masked to study group graded gross liver appearance (0, normal; 1, mild liver surface nodularity; 2, intermediate liver surface nodularity; 3, marked nodularity and/or ascites) [17]. Livers were removed, weighed and fixed in 4% para-formaldehyde. For later measurement of mRNA levels, a portion of liver was stored in RNAlater.

2.3. Liver Histology and Morphometry

Paraffin-embedded liver sections were stained with hematoxylin and eosin (H&E) and assessed for fibrosis using the Ishak fibrosis scale by a pathologist (WT) masked to study group [18]. Hepatic collagen was quantified using morphometric analysis of Sirius red-stained sections. Briefly, after dewaxing and hydration, liver sections were stained with picroSirius red solution for 1 h followed by washing in acidified water. Resulting sections were dehydrated, mounted and examined using a Nikon i80 photo-microscope (100 × total magnification). Ten different areas were examined from each section. Since the degree of Sirius red staining measured by the saturation of the red channel correlates well with chemically-determined collagen content and morphometrically-determined fibrosis, fibrosis was assessed as the proportion of summed pixels per unit area of liver section (in arbitrary units) determined using Image Pro-plus software (version 5.0; Media Cybernetics, Silver Spring, MD).

2.4. Immunohistochemistry (IHC)

IHC was performed utilizing the avidin-biotin reaction with vectastatin elite ABC kit (Vector Labs, Burlingame, CA) per manufacturer's recommendations. After deparafinization, hydration and endogenous peroxidase blockade (10% H₂O₂), heat-induced antigen retrieval was performed using citrate buffer. Sections were incubated at room temperature with 5% normal goat serum (20 min), avidin and biotin blocking reagent (15 min each), and washed 3 times with 0.1% Tween-20 in PBS between each step. After overnight treatment with primary antibody at 4°C, sections were incubated with biotinylated secondary antibody (30 min) followed by incubation with streptavidin-HRP (30 min). Further, sections were stained and then counterstained with DAB (2 min) and hematoxylin (4 min), respectively. Hepatocyte proliferation and apoptosis were assessed using primary antibodies against BrdU (BD Bioscience, San Jose, California; dilution 1:100) and cleaved caspase-3 (Cell Signaling Technology, Beverly, MA; dilution 1:100), respectively. Ductular proliferation was assessed using anti-EpCAM antibodies (Abcam, Cambridge, MA; dilution 1:100). To assay BrdU-stained nuclei, at least 1000 hepatocyte nuclei were counted at 200X magnification. Cleaved caspase-3 staining was assessed using Image Pro-plus software. Bile ductule cells were defined as EpCAM-stained cuboidal cells forming ductular structure with lumens. At least 5 fields were examined at 200X magnification and results expressed as the number of bile ducts/high-power field (HPF). To determine the impact of treatments on oxidative stress and activation of post-M3R signaling pathways, livers sections were stained for 3-nitrotyrosine (3-NT, 1:200) (EMD Millipore), phosphorylated extracellular signal-regulated kinases (p-ERK, 1:100) (Cell Signaling Technology, Beverly, MA) and p-AKT (1:50, Cell Signaling Technology, Beverly, MA). Liver sections stained for 3-NT and p-ERK were graded semi-quantitatively by masked investigators for: 0-occasional, 1-minimal, 2-moderate and 3-extensive staining. p-AKT-stained hepatocytes were counted at 200X magnification by examining at least 5 fields per section.

2.5. Quantitative RT-PCR (qPCR)

Liver samples were homogenized using a Polytron PT2100 homogenizer (Kinematica, Switzerland) and RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) per manufacturer's instructions. cDNA was synthesized using RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific, USA). Assays were performed in 96-well plates using Sybr green master mix (12.5 µl, Qiagen, USA), template (1 µl), each primer (1 µL, 10 pmol/µl) and nuclease-free water (9.5 µl). Primer sequences are listed in Table 1. The following two-step thermal cycling profile was used for qPCR analysis (StepOnePlus™ Real-Time PCR, Applied Biosystems, USA): Step I (cycling): 95°C for 5 min, 95°C for 10 s and 60°C for 30 s for 40 cycles. Step II (melting curve): 60°C for 15 s, 60°C 1 min and 95°C for 30 s. To determine fold changes in mRNA expression, the $\Delta\Delta C_t$ method was used.

2.6. Statistical Analysis

Data are expressed as mean $\pm$ the standard error (SE). To determine statistical significance, analysis was performed using Student's unpaired *t*-test (normally distributed data) or the Mann-Whitney U test (nonparametric data). Nominal data were analyzed using a χ^2 test with Fisher's test. Correlations were determined using Spearman's test. Survival was analyzed using the Kaplan-Meier method. Significance was defined as $P < 0.05$.

3. Results

3.1. Treatment with darifenacin worsens the severity of AOM-induced liver injury

Initially, to improve our understanding of the specific role of M3R activation, we determined whether a selective M3R antagonist, darifenacin, mimicked the actions of *Chrm3* gene ablation on AOM-induced liver injury [17]. Figure 1A illustrates our study

design (details in Methods). As shown in Fig. 1B-D, compared to vehicle controls (50% DMSO), darifenacin-treated mice gained less weight, were more likely to die before attaining the study end-point and had reduced liver-weight-to-body-weight ratios. Additionally, darifenacin-treated mice had increased liver nodularity (Fig. 1E), a nearly four-fold increase in fibrosis measured using Sirius red staining (Fig. 1F) and increased Ishak fibrosis scores (not shown). Our previous study indicated that inhibiting M3R activation promotes AOM-induced ductular proliferation [17]. Thus it was also not surprising that we observed a greater than two-fold increase in ductular proliferation in darifenacin-treated compared to vehicle-treated mice (Fig. 1F). Collectively, these findings suggest that darifenacin treatment closely mimics the pattern of AOM-induced liver injury seen with *Chrm3* gene ablation than treatment with the non-selective muscarinic receptor antagonist, scopolamine butylbromide [17].

3.2. Treatment with pilocarpine stimulates post-M3R signaling

Next, we determined the effects of a selective M3R agonist, pilocarpine, on AOM-induced liver injury in mice. Based on our results with darifenacin, we anticipated that M3R activation would have the contrary effect and ameliorate AOM-induced liver injury. Figure 2A illustrates our study design (details in Methods). First, to confirm that treatment with pilocarpine stimulated M3R activation, we examined the phosphorylation of two key signaling molecules downstream of the receptor, ERK and AKT [19, 20]. As shown in Fig. 2B and C, at the end-point of the study, immunohistochemical analysis revealed significantly greater ERK and AKT activation in liver sections from pilocarpine-treated compared to control mice, thus providing evidence that pilocarpine activated post-M3R signaling.

3.3. Treatment with pilocarpine attenuates gross liver injury and the likelihood of developing ascites

During the course of the study, there were no significant differences in the weights of PBS- and pilocarpine-treated mice (Fig. 3A) or in their liver-weight-to-body-weight ratios (Fig. 3C). Compared to control mice, pilocarpine-treatment was associated with a trend towards improved survival (Fig 3B; $P = 0.2$) and reduced risk of developing ascites (Fig. 3D; $P = 0.08$). However, liver nodularity was significantly reduced in pilocarpine-treated compared to PBS-treated mice (Fig. 3E).

3.4. Treatment with pilocarpine reduces liver fibrosis

To determine the effect of pilocarpine treatment on AOM-induced liver fibrosis, we analyzed Sirius red- and H&E-stained liver sections. Representative photographs of Sirius red-stained liver sections are shown in Figure 4A. Pilocarpine-treated mouse livers had significantly reduced collagen deposition (morphometric analysis of Sirius red-stained sections) and Ishak fibrosis scores (H&E-stained sections) compared to those from PBS-treated mice (Fig. 4A and B). Strong correlation was observed between liver nodularity and Sirius red-stained area ($r=0.56$, $P<0.01$). In concert with these changes, livers from pilocarpine-treated mice had significantly reduced expression of mRNA for $\alpha 1$ -collagen and α -smooth muscle actin (α -SMA) (Fig. 4C and D). These findings validated our assessment of liver injury by gross examination. qPCR analysis of mRNA isolated from mouse livers revealed that compared to those from PBS-treated mice, liver sections from pilocarpine-treated mice had modest, but significant, reductions in the expression of TGF- $\beta 1$, TGF- $\beta 1R$, PDGF and PDGFR (Fig. 4E), key mediators of hepatic stellate cell activation [21]; there was no effect on TGF- $\beta 2$ expression (not shown).

3.5. Treatment with pilocarpine reduces ductular proliferation

To determine the effect of pilocarpine on ductular proliferation, we examined EpCAM-stained liver sections and counted bile ducts in five or more randomly-selected high-power fields. The numbers of ductules in livers from pilocarpine-treated mice were significantly reduced compared to those from PBS-treated mice (Fig. 4F). These findings indicate that stimulating M3R activation *after* AOM induction of liver injury attenuates ductular proliferation. There was strong correlation between ductular proliferation and liver fibrosis; bile ductule number in EpCAM-stained liver sections correlated with Sirius red staining ($r=0.52$, $P<0.01$). Thus, data from our previous [17] and current studies indicate that the state of M3R activation modulates AOM-induced ductular proliferation.

3.6. Treatment with pilocarpine alters the expression of tissue inhibitors of metalloproteinases (TIMP) and matrix metalloproteinases (MMP)

Liver fibrosis results from an imbalance between matrix production and degradation. TIMPs and MMPs, which derive primarily from activated stellate cells, are major regulators of extracellular matrix deposition and reorganization. Therefore, we determined the effect of pilocarpine treatment on expression of mRNA for TIMPs and MMPs that are recognized as playing major roles in matrix reorganization in cirrhosis. As shown in Fig. 5A, pilocarpine treatment markedly reduced expression of TIMP-1 and TIMP-2, and MMP-2 and MMP-13; mRNA expression of each of these genes was reduced by at least 50%.

3.7. Treatment with pilocarpine attenuates hepatocyte apoptosis, and TNF- α and FasL expression

Hepatocyte apoptosis is the principal initiator of liver fibrosis. In liver injury, TNF- α induces hepatocyte apoptosis and primes quiescent hepatocytes for replication [16, 22, 23]. Hence, we assessed the effect of pilocarpine treatment on hepatocyte apoptosis, using immunohistochemical staining for cleaved caspase-3 as a marker, and also measured levels of TNF- α and FasL mRNA. As shown in Fig. 5B, cleaved caspase-3 staining was significantly reduced in pilocarpine-treated compared to PBS-treated mice. In addition, pilocarpine treatment significantly reduced hepatic mRNA levels of both TNF- α and FasL (Fig. 5C).

3.8. Treatment with pilocarpine promotes hepatocyte restoration

Hepatocyte proliferation is an important restorative response in liver injury. In the absence of injury, hepatocytes remain quiescent. Previously, we showed that immunochemical staining of liver sections from untreated mice revealed rare BrdU-stained nuclei, whereas sections from AOM-treated mice had evidence of increased hepatocyte proliferation [17]. To characterize further the effects of pilocarpine on hepatocyte restoration following injury, we examined BrdU-stained liver sections. Compared to those from PBS-treated mice, livers from pilocarpine-treated mice had a modest, but statistically significant, increase in the numbers of BrdU-stained hepatocytes (Fig. 6A). These findings suggest that pilocarpine-induced M3R activation strengthens the restorative response to liver injury by stimulating hepatocyte proliferation.

3.9. Treatment with pilocarpine reduces hepatic nitrosative stress

Finally, to gain additional mechanistic insight into the therapeutic potential of activating M3R to modulate toxic liver injury, we evaluated the effects of pilocarpine treatment on peroxynitrite generation, an indicator of nitrosative stress that plays a role in hepatocyte apoptosis and fibrogenesis [24-27]. As shown in Fig. 6B, we found that treatment with pilocarpine reduced 3-nitrotyrosine staining in AOM-treated mouse livers (~50% reduction

in 3-nitrotyrosine staining). This finding indicates that pilocarpine treatment reduces AOM-induced nitrosative stress in mouse liver.

4. Discussion

In the present study, by showing that darifenacin, the most M3R-selective antagonists available [28], augmented AOM-induced liver injury, we validated our previous findings with M3R-deficient mice and mice treated a broad-spectrum muscarinic receptor antagonist scopolamine butylbromide [17]. More importantly, we demonstrate the therapeutic potential of stimulating M3R activation to mollify liver injury. Specifically, we found that treating mice with pilocarpine, an agonist with the greatest affinity for M3R among the five muscarinic receptor subtypes [29, 30], robustly attenuated AOM-induced liver injury as evidenced by: 1) reduced liver nodularity, fibrosis and ascites; 2) reduced hepatocyte apoptosis and increased hepatocyte proliferation; 3) reduced ductular proliferation; 4) down-regulated expression of α 1-collagen, α -SMA and other initiators and perpetuators of hepatic fibrosis; 5) reduced expression of liver injury cytokines e.g. TNF- α ; 6) reduced oxidative stress and 7) increased activation of hepatocyte survival signaling, e.g. increased activation of ERK and AKT. Our findings with pilocarpine support further investigation of the therapeutic potential of M3R activation to prevent or treat reduce chronic liver injury.

The role of cholinergic input in regulating liver injury was previously evaluated by other investigators using vagus nerve transection or stimulation, experimental approaches with important disadvantages. Acetylcholine, the major neurotransmitter released by the vagus, activates both muscarinic and nicotinic receptors; both receptor classes are expressed in the liver [5, 8]. Also, in addition to acetylcholine, the vagus releases other neurotransmitters that may modulate injury responses (e.g. vasoactive intestinal peptide) [31]. Hence, approaches utilizing selective M3R ablation and inhibition [17], or selective M3R activation as shown herein, provide a clearer representation of the role that muscarinic receptors play in regulating liver injury. Additionally, our experimental strategy suggests that pharmacological modulation of M3R activation *after* liver injury (that is, after completion of AOM treatment) alters the liver injury response, again speaking to its therapeutic potential.

DMSO, the vehicle for darifenacin, is reported to attenuate concanavalin-, thioacetamide- and acetaminophen-induced acute liver injury, and thioacetamide-induced liver fibrosis [32-35]. By inhibiting NF- κ B activation, DMSO, a highly effective free-radical scavenger, reduces the transcription of inflammatory cytokines, particularly TNF- α , thereby modulating immune cell function [35-38]. Despite these potentially protective effects of DMSO, darifenacin strongly augmented AOM-induced liver injury; a finding that reveals the major regulatory role of M3R in liver injury. To avoid masking therapeutic benefit with pilocarpine, we used a different vehicle, phosphate-buffered saline, for experiments with the M3R agonist.

Pilocarpine treatment reduced fibrosis in conjunction with markedly attenuating expression of markers of stellate cell activation, i.e. α 1-collagen and α -SMA. Pilocarpine-treated mice had reduced hepatic expression of ligands and receptors that promote stellate cell activation and proliferation, i.e. TGF- β 1, PDGF and their receptors. In AOM-treated mice, increased hepatic expression of extracellular matrix regulators such as TIMP-1 and TIMP-2, and MMP-2 and MMP-13, consistent with other models of chronic liver injury, was markedly blunted by pilocarpine treatment [39]. These data indicate that one mechanism whereby treatment with pilocarpine attenuates chronic liver injury is by reducing fibrogenesis.

Activation of M3R was shown to promote survival and reduce apoptosis in other cell types [6]. Since ongoing hepatocyte apoptosis is a strong contributor to hepatic fibrosis and cirrhosis [40], and rodent hepatocytes express M3R, we assessed hepatocyte survival in our

treatment groups. AOM-treated mice that received pilocarpine demonstrated reduced hepatocyte apoptosis and reduced expression of liver injury cytokines, TNF- α and FasL. Moreover, livers from pilocarpine-treated mice showed increased hepatocyte proliferation and activation of ERK and AKT, key pro-survival ligands downstream of M3R activation.

The data shown here provide insight into the mechanisms whereby M3R activation reduced chronic liver injury. However, our study does have limitations. Pilocarpine crosses the blood-brain-barrier [41] and central nervous system (CNS) effects modulating the liver injury response cannot be excluded. In this context, it is reassuring that AOM-treated mice that received darifenacin had more severe AOM-induced liver injury, similar to that observed previously with M3R ablation [17]; darifenacin has minimal brain penetration. This makes it unlikely that M3R expressed in the CNS plays an important role in the liver injury response. Collectively, our findings suggest that M3R activation within the liver itself is most important for regulating injury.

In chronic liver diseases, ductular reaction correlates with fibrosis [42, 43]. Recent studies suggested that ductular reaction can also drive fibrosis [44, 45]. Chobert et al. showed that CK-19-positive cells express TGF- β 1, which may promote stellate cell activation and hepatic fibrosis [45]. Previously, we reported strong correlation between CK-19 and Sirius red staining [17]. In the present study we observed strong correlation between Sirius red and EpCAM staining. Taken together, our previous and current findings are consistent with the notion that ductular proliferation plays an important role in fibrogenesis [45]. Since bile ductular cells express M3R, we speculate that the modest reduction in ductular proliferation seen in pilocarpine-treated mice results from a combination of pilocarpine-induced M3R-mediated proliferation and reduced compensatory reaction due to augmented hepatocyte proliferation and hepatic parenchyma restoration.

In conclusion, the key observation in our study is that pharmacological activation of M3R attenuates chronic liver injury. This effect is associated with reduced hepatocyte loss and fibrogenesis, and increased hepatocyte survival. While our study provides mechanistic insights into M3R-mediated regulation of liver injury, additional studies are required to gain a better understanding of M3R-mediated regulation of inflammatory cytokines. Our findings should encourage the development and evaluation of M3R-based therapeutic approaches to prevent liver injury and promote repair.

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Figure 1. Treatment with an M3R-selective antagonist increases the severity of AOM-induced liver injury. (A) Study Design. Mice were treated with azoxymethane (AOM, 10 mg/kg ip) once each week for 6 weeks. After a one-week interval, mice were allocated to treatment with darifenacin (3 mg/kg/day; n=19 mice) or vehicle (50% DMSO; n=20 mice) for 14 weeks, administered via subcutaneous osmotic mini-pumps. (B) Mouse weights. Mice were weighed weekly; during the last 6 weeks of the study, darifenacin-treated mice weighed significantly less than vehicle-treated controls. (C) Mouse survival. Darifenacin-treated mice had significantly reduced survival compared to vehicle-treated controls. (D) Liver weights (expressed as percent body weight). Livers harvested from darifenacin-treated mice were modestly, but significantly, lighter than those from control mice. (E) Gross liver nodularity. Livers from darifenacin-treated mice had significantly higher liver nodularity scores than those from vehicle-treated control mice. (F) Liver fibrosis and bile ductule hyperplasia. Representative images of Sirius red- and EpCAM-stained liver sections from darifenacin- and vehicle-treated mice are shown. Livers from darifenacin-treated mice had increased

fibrosis as determined by measuring the area of collagen (Sirius red)-stained sections. The numbers of bile ductules per high power field (HPF) were increased in liver sections from darifenacin-compared to those from vehicle-treated mice. Bars represent means $\pm$ SE. * P < 0.05, ** P < 0.01, *** P < 0.001 compared to mice treated with vehicle.

Figure 2.

Treatment with an M3R agonist stimulates post-M3R ERK and AKT activation. (A) Study design. Mice were treated with AOM (10 mg/kg ip) once each week for 6 weeks. After a one-week interval, mice were allocated to treatment with pilocarpine (2.5 mg/kg/day; n=14 mice) or vehicle (PBS; n=14 mice) for 14 weeks, administered via subcutaneous osmotic mini-pumps. (B) ERK activation. Representative images of phospho-ERK-stained liver sections from mice treated with pilocarpine and vehicle are shown. ERK activation (phospho-ERK staining) was increased in livers from pilocarpine-treated mice compared to those from PBS-treated mice. (C) AKT activation. Representative images of phospho-AKT-stained liver sections from mice treated with pilocarpine and vehicle are shown. Liver sections from pilocarpine-treated mice had increased numbers of phospho-AKT-stained hepatocytes (arrows) compared to sections from PBS-treated animals. Bars represent means $\pm$ SE. ** P < 0.01, *** P < 0.001 compared to mice treated with vehicle (PBS) alone.

Figure 3. Treatment with an M3R-selective agonist lessens the severity of AOM-induced liver injury. (A) Mouse weights. Mice were weighed weekly; for the duration of the study, there was no difference in the weights of pilocarpine-treated and PBS-treated control mice. (B) Mouse survival. Pilocarpine-treated mice trended towards increased survival compared to PBS-treated controls. (C) Liver weights (expressed as percent body weight). There were no significant differences in the weights of livers harvested from pilocarpine-treated mice compared to those from control animals. (D) Ascites. Mice treated with pilocarpine were less likely to develop ascites than those treated with PBS alone. (E) Gross liver nodularity. Livers from pilocarpine-treated mice had significantly lower liver nodularity scores than those from PBS-treated mice. Bars represent means $\pm$ SE. * $P < 0.05$ compared to mice treated with PBS alone.

Figure 4. Effects of treatment with an M3R-selective agonist on AOM-induced liver fibrosis and bile ductile hyperplasia. (A) Liver fibrosis. Representative images of Sirius red-stained liver sections from PBS- and pilocarpine-treated mice are shown. Livers from pilocarpine-treated mice had reduced fibrosis as determined by measuring the area of collagen (Sirius red)-stained sections. (B) Ishak fibrosis scores. Liver sections from pilocarpine-treated mice had significantly reduced Ishak scores compared to those from PBS-treated mice. (C) $\alpha 1$ collagen expression was significantly reduced in livers from pilocarpine-treated mice compared to those from PBS-treated mice. (D) Expression of α -SMA, a marker of stellate cell activation, was significantly reduced in livers from pilocarpine-treated mice compared to those from PBS-treated mice. (E) Expression of TGF- β 1, TGF- β 1R, PDGF and PDGFR was significantly reduced in livers from pilocarpine-treated mice compared to those from PBS-treated mice. (F) Representative images of EpCAM-stained liver sections from PBS-

and pilocarpine-treated mice are shown. Liver sections from pilocarpine-treated mice had significantly reduced numbers of bile ductules/HPF. Bars represent means $\pm$ SE. * P <0.05 and ** P <0.01 compared to mice that received PBS alone.

Figure 5. Effects of treatment with an M3R-agonist on hepatic expression of regulators of extracellular matrix formation and hepatocyte apoptosis. **(A)** Treatment with pilocarpine significantly reduced expression of TIMP-1, TIMP-2, MMP-2 and MMP-13. **(B)** Representative sections show cleaved caspase-3 staining in livers from AOM-treated mice treated with PBS or pilocarpine. Arrowheads indicate cleaved caspase-3-stained hepatocytes. Treatment with pilocarpine reduced cleaved caspase-3 staining. **(C)** TNF- α and FasL expression. Expression of these liver injury cytokines was reduced in livers from pilocarpine-treated mice compared to those from vehicle-treated mice. Bars represent means $\pm$ SE. * P <0.05, ** P <0.01, *** P <0.001 compared to mice treated with PBS alone.

Figure 6.

Effects of treatment with an M3R-selective agonist on hepatocyte proliferation and oxidative stress in AOM-treated mice. **(A)** Hepatocyte proliferation. Representative sections show BrdU staining in livers from AOM-treated mice that received PBS or pilocarpine. Arrowheads indicate BrdU-stained hepatocytes. Treatment with pilocarpine significantly increased the numbers of BrdU-stained hepatocytes. **(B)** Oxidative stress. Representative images of nitrotyrosine-stained liver sections from AOM-treated mice that received PBS or pilocarpine are shown. Treatment with pilocarpine significantly reduced AOM-induced peroxynitrite generation. Bars represent means $\pm$ SE. * $P < 0.05$, *** $P < 0.001$ compared to mice that received PBS alone.

Table 1

Primer Sequences

Gene	Primer sequence	
	Forward	Reverse
<i>TGFβ1</i>	GCCTGAGTGGCTGTCTTTTGA	GCTGAATCGAAAGCCCTGTATT
<i>TGFβ1-R</i>	CCACTTGCGACAACCAGAAGTC	GTCGTTCTTCCTCCACACGG
<i>PDGF</i>	GGTCCCATGCCATTAACCAT	CCGTCCTGGTCTTGCAAACCT
<i>PDGF-R</i>	CTTTGTGCCAGATCCCACCA	TCACTCGGCACGGAATTGTC
<i>MMP-2</i>	CCCTCAAGAAGATGCAGAAGTTC	TCTTGGCTTCCGCATGGT
<i>MMP-13</i>	GTTCAAGGAATTCAGTTTCTTTATGGT	GGTAATGGCATCAAGGGATAGG
<i>TIMP1</i>	GCATGGACATTTATTCTCCACTGT	TCTCTAGGAGCCCCGATCTG
<i>TIMP2</i>	TTCCGGGAATGACATCTATGG	GGGCCGTGTAGATAAACTCGAT
<i>α1 collagen</i>	GATGACGTGCAATGCAATGAA	CCCTCGACTCCTACATCTTCTGA
<i>α-SMA</i>	CTCTGCCTCTAGCACACAACT	CCAGGGCTACAAGTTAAGGGT
<i>FasL</i>	AAGAAGGACCACAACACAA	TAATCCCATTCCAACCAGAG
<i>TNF-α</i>	GTGGAAGTGGCAGAAGAG	AATGAGAAGAGGCTGAGAC
<i>GAPDH</i>	ACAACTTTGGCATTGTGGAA	GATGCAGGGATGATGTTCTG