Effects of Melatonin on Gallbladder Neuromuscular Function in Acute Cholecystitis

Pedro J. Gomez-Pinilla, Pedro J. Camello, and María J. Pozo

Department of Physiology, Nursing School, University of Extremadura, Caceres, Spain

Received March 22, 2007; accepted July 3, 2007

ABSTRACT

Gallbladder stasis is associated to experimental acute cholecystitis. Impaired contractility could be, at least in part, the result of inflammation-induced alterations in the neuromuscular function. This study was designed to determine the changes in gallbladder neurotransmission evoked by acute inflammation and to evaluate the protective and therapeutic effects of melatonin. Experimental acute cholecystitis was induced in guinea pigs by common bile duct ligation for 2 days, and then the neuromuscular function was evaluated using electrical field stimulation (EFS; 5–40 Hz). In a group of animals with the bile duct ligated for 2 days, a deligation of the duct was performed, and after 2 days, the neuromuscular function was studied. The EFS-evoked isometric gallbladder contraction was significantly lower in cholecystitic tissue. In addition, inflammation changed the pharmacological profile of these contractions that were insensitive to tetrodotoxin but sensitive to atropine and ω-conotoxin, indicating that acute cholecystitis affects action potential propagation in the intrinsic nerves. Nitric oxide (NO)-mediated neurotransmission was reduced by inflammation, which also increased the reactivity of sensitive fibers. Melatonin treatment prevented qualitative changes in gallbladder neurotransmission, but it did not improve EFS-induced contractility. The hormone recovered gallbladder neuromuscular function once the biliary obstruction was resolved, even when the treatment was started after the onset of gallbladder inflammation. These findings show for the first time the therapeutic potential of melatonin in the recovery of gallbladder neuromuscular function during acute cholecystitis.

Gallbladder tone is mainly regulated by both myogenic mechanisms and neurohormonal inputs. The neural control of gallbladder motility involves reflexes that include both efferent and afferent nerve fibers as well as the intrinsic plexus in the gallbladder wall (Mawe et al., 2006). Acetylcholine (ACh) released from cholinergic neurons induces contraction of the gallbladder smooth muscle through muscarinic receptors (Parkman et al., 1999b), and it has neuromodulatory functions, promoting or inhibiting the release of other neurotransmitters (Parkman et al., 1999b). Cholinergic neurons coexpress other neurotransmitters such as NO and several neuropeptides (Talmage et al., 1992). Afferent nerve fibers containing calcitonin gene-related peptide and tachykinins have also been described in the ganglionated plexus of the gallbladder (Mawe and Gershon, 1989).

Acute acalculous cholecystitis (AC) is characterized by gallbladder inflammation in the absence of gallstones. Although its pathogenesis is unknown, gallbladder stasis is always present, probably as the result of the deleterious neural and muscular actions of inflammatory mediators such as reactive oxygen species and prostaglandins (Pozo et al., 2004). In animal models, it has been described that cholecystitis reduces gallbladder contractile responses to agonists that act directly on smooth muscle cells (Parkman et al., 1999a; Xiao et al., 2001) and that it also causes alterations in calcium signaling and contractile machinery (Gomez-Pinilla et al., 2006b). In addition, EFS-induced contractions are also impaired in inflamed gallbladder, mainly due to the reduction in the function of cholinergic nerves and the up-regulation of the inhibitory nitrergic component (Parkman et al., 2000). The effect of cholecystitis on afferent fibers has not yet been explored.

Melatonin (MEL), the main product of pineal gland, is a potent free radical scavenger, and it activates a broad group of antioxidant cellular mechanisms (Tan et al., 2002). These properties made melatonin efficacious against different diseases where oxidative stress is the main cause (Karasek, 2004). The gastrointestinal tract is an important source of melatonin (Kvetnoy et al., 2002). The liver and the gallblad-
under are especially exposed to high levels of the hormone, because hepatic metabolism is the major pathway for deactivation of melatonin (Lane and Moss, 1985), which is also present in active form in bile and concentrated in the gallbladder (Tan et al., 1999). In the gastrointestinal tract, melatonin has a gastroprotective function (Konturek et al., 1997) and therapeutic effects against malignancy associated with irritable bowel syndrome (Head and Jurekna, 2003).

The aims of this study were to investigate the effects of acute cholecystitis in the neuromuscular transmission and to evaluate the impact of melatonin treatment. Our results indicate that melatonin restores neuromuscular function in inflamed gallbladder, which can be of importance to recover gallbladder contractility in this pathological condition.

**Materials and Methods**

**Design: Animal Preparations.** Male guinea pigs, weighing 400 to 600 g, were used in the study. AC was induced to animals by common bile duct ligation (CBDL) for 2 days, as described previously (Gomez-Pinilla et al., 2006b). This method was approved by the Animal Care and Ethical Committees of the University of Extremadura (Caceres, Spain). In brief, after anesthesia with 20 mg/kg i.p. ketamine hydrochloride and 5 mg/kg i.p. xylacine, a laparotomy was performed, and the distal end of the common bile duct was ligated. Two days after, the animals were sacrificed for tissue harvest (n = 28). In the model used in this study, the gallbladder is stretched as the result of bile duct ligation and the continuous bile output. Taking this into account, it would be difficult to see any improvement in the neuromuscular function by keeping the bile duct ligated, which represents a remarkably extreme pathological condition. To solve this dilemma, in a group of animals 2 days later, CBDL, the common bile duct, was deligated (DL) under anesthesia with microsurgical scissors, and 2 days later, the animals were sacrificed (n = 28). For both experimental models, a group of guinea pigs were sham-operated (n = 4), which included all of the surgical steps, with the exception of common bile duct ligation.

**Melatonin and Tempol Administration.** Guinea pigs were treated orally with melatonin (2.5 or 30 mg/kg/day). Melatonin was dissolved in glucose solution (1.5%), and it was placed in the oropharynx by using a syringe. This treatment was applied daily at the same time, just before the light in the animal house was switched off (7:00 PM). Melatonin was administered 14 days before the sacrifice of the animals in both experimental groups, AC and DL. In a group of animals subjected to DL, melatonin treatment (30 mg/kg) started 12 h after CBDL was performed, and it continued until the sacrifice of the animal. Tempol was administered in the drinking water at 1 mM for 14 days before the animal was sacrificed.

**Functional Studies.** At the appropriated time, the animals were killed with deep halothane anesthesia and cervical dislocation. Gallbladders were removed, and they were immediately placed in ice-cold Krebs-Henseleit solution (KHS-H) for 2 min. After construction of a frequency-response curve (10 mm; emission, 425 nm). To determine the neural and myogenic components of the EFS-evoked contractions, the nerve Na\(^+\) channel inhibitor tetrodotoxin (TTX) was used. In control strips that was significantly decreased in animals subjected to CBDL compared with sham controls (Fig. 1, A and B). The diminished response was reflected by reductions in both the amplitude and the duration of the contractions (P < 0.01 and P < 0.001, two-way ANOVA for both; Fig. 1, C and D).

To determine the neural and myogenic components of the EFS-evoked contractions, the nerve Na\(^+\) channel inhibitor tetrodotoxin (TTX) was used. In control strips, 1 μM TTX abolished EFS-elicited responses (Fig. 2A). In inflamed strips, tetrodotoxin was not effective (3.6% enhancement at 25 Hz) (Fig. 2B), but when the strips were coincubated with 1 μM tetrodotoxin plus 0.1 μM α-conotoxin GVIA, an N-type channel inhibitor.
calcium channel blocker, there was a reduction (63% inhibition at 25 Hz) in the contractile response evoked by EFS (Fig. 2B). These results indicate that in inflamed gallbladder, the transmission of the action potential along neural fibers is impaired and that EFS stimulates neurotransmitter release directly from nervous terminals.

To elucidate the neurotransmitters involved in the EFS-induced contraction, we tested several antagonists/inhibitors on this neural response. In control animals, we found that 1 μM atropine reduced the EFS-elicited contractile response (82% inhibition at 25 Hz; Fig. 3A), but the strips from cholecystitic animals were less sensitive to atropine blockade (30% inhibition at 25 Hz; Fig. 3D). The impact of inflammation on the contribution of NO was tested by using the inhibitor of the nitric-oxide synthase, L-NAME, at 100 μM. This inhibitor enhanced EFS-induced contraction in strips from control guinea pigs, especially at the lowest frequencies assayed (90% enhancement at 5 Hz; Fig. 3B), but it had little effect in inflamed strips (17% enhancement at 5 Hz; Fig. 3E).

Small-diameter sensory neurons that are sensitive to capsaicin play a major role in the generation of neurogenic inflammation (Sann et al., 1996). When we induced sensory nerve desensitization by treatment with a high concentration of capsaicin (10 μM), we found no effect in control strips (Fig. 3C), but this treatment induced an inhibition of EFS-elicited contractile responses in cholecytistic strips (66% inhibition; Fig. 3F).

**Effects of Melatonin on Neuromuscular Function in Acute Cholecystitis.** We have reported previously that melatonin treatment was able to restore gallbladder neuromuscular function in aging (Gomez-Pinilla et al., 2006a). To determine whether this hormone had beneficial effects in the alterations described above, we treated the animals with 2.5 and 30 mg/kg melatonin (MEL 2.5; MEL 30) as described under Materials and Methods. Under these conditions, none of melatonin doses used enhanced the amplitude of the contractile responses evoked by EFS (Fig. 4A), but the contractions partially recovered the sensitivity to TTX (85 and 77% inhibition for MEL 2.5 and MEL 30, respectively, at 25 Hz; Fig. 4B). Although the treatment dose-dependently increased the inhibitory effects of atropine and it decreased the inhibitory effects of capsaicin significantly at some frequencies (Fig. 4, C and E), these changes were small. However, melatonin was able to protect nitrergic nerves, because when L-NAME was added to the organ bath, the EFS-evoked con-
tractile responses were enhanced in similar proportions to those found in control strips (91 and 85% enhancement for MEL 2.5 and MEL 30, respectively, at 5 Hz; Fig. 4D). These results indicate that melatonin had some effects on inflammation of the gallbladder, but there are some contractile disabilities related to the experimental method used in this study, as indicated under Materials and Methods, that melatonin treatment cannot restore.

Thus, we tested melatonin effects in animals that underwent the deligation protocol. When deligation was performed in animals that were not treated with melatonin, the neuromuscular function worsened, as indicated by the reduction in the EFS-induced contraction (Fig. 5, A and B). Taking into account the small amplitude of these contractions, we did not apply antagonists/inhibitors of the neurotransmitters to determine the nature of this response. However, when the animals were treated with melatonin 10 days before performing the surgical procedures and until the animal was sacrificed, there was a very noticeable improvement in gallbladder neuromuscular function. As shown in Fig. 5C, melatonin treatment increased the gallbladder neurogenic responses in a dose-dependent way. In the strips from animals treated with melatonin, the EFS-elicted responses recovered the sensitiveness to TTX (70 and 73% inhibition for MEL 2.5 and MEL 30, respectively, at 25 Hz; Fig. 6A) and atropine (73 and 76% inhibition for MEL 2.5 and MEL 30, respectively, at 25 Hz; Fig. 6B) to a level comparable with that seen in control tissue. Although capsaicin still induced a small inhibition of EFS-induced responses (5 and 10% inhibition for MEL 2.5 and MEL 30, respectively, at 25 Hz; Fig. 6D), the reduction was significantly smaller than that found in inflamed tissue; at the highest frequencies, this effect was not different from that registered in control tissue. In this experimental group, 30 mg/kg melatonin also re-established the sensitivity to L-NAME (Fig. 6C), but this was not the case for 2.5 mg/kg melatonin, suggesting that the effects of CBDL in the nitrergic function are exacerbated by the deligation procedure.

These results suggest that melatonin has prophylactic effects preventing neuromuscular damage during cholecystitis. To check whether melatonin also has a therapeutic role in the management of acute cholecystitis, melatonin treatment (30 mg/kg) was initiated after the onset of gallbladder inflammation. As represented in Figs. 5C and 6, melatonin recovered gallbladder contractility in response to EFS and the pharmacological profile of the neurotransmission, indicating that indoleamine can ameliorate the neuromuscular damage induced by acute cholecystitis.

These effects of melatonin could be related to its antioxidant and scavenger properties as indicated by the reduction in the lipidic peroxidation and the increase in the levels of GSH induced by melatonin treatment (Table 1).
Effects of Tempol on Neuromuscular Function in Acute Cholecystitis. To test whether other antioxidants/scavengers also have beneficial effects on the impaired neuromuscular transmission in acute cholecystitis, we also tested the effects of tempol, a membrane-permeable superoxide dismutase mimetic (Krishna et al., 1996). Administration

**Fig. 4.** Melatonin treatment protects intrinsic neurons, but it does not improve EFS-induced contraction. A, effects of melatonin treatment (2.5 and 30 mg/kg) on EFS-induced gallbladder contractions in acute cholecystitic animals. Histograms represent the effects of 1 μM TTX (B), 1 μM atropine (C), 100 μM L-NAME (D), and 10 μM capsicain (E) on EFS-elicited contractile response in control, AC, and AC melatonin-treated gallbladder strips. Data are mean ± S.E.M. n = 5 to 18 strips. Note that EFS-induced responses recover TTX and L-NAME sensitivity, whereas melatonin has less effect on cholinergic and sensory fibers (∗, P < 0.01 AC versus control; †, P < 0.05 MEL30 versus AC; and ‡, P < 0.01 MEL 30 versus AC; ANOVA).

**Fig. 5.** Melatonin treatment improves the neurogenic damage exacerbated by the deligation procedure. A, original recordings showing guinea pig gallbladder contractile responses elicited by EFS (0.3-ms duration, 5–40 Hz, 350 mA, for 10 s every 3 min) applied to AC and DL strips. Traces are typical of 16 and 17 strips for AC and DL strips, respectively. B, summary data of EFS-elicited responses (peak amplitude) in both experimental groups (+++ P < 0.001; ANOVA). C, effects of melatonin treatment (2.5 and 30 mg/kg; DL + MEL 2.5 and DL + MEL 30, respectively; 30 mg/kg for 4 days after the onset of AC, DL + MEL 4 days) on EFS-elicited contractile response gallbladder strips from animals that underwent the deligation procedure. Data are mean ± S.E.M. (n = 12–28 strips; ∗, P < 0.05; **, P < 0.01; †††, P < 0.001 versus AC; ANOVA).
of tempol in the drinking water for 14 days to guinea pigs that underwent the protocol of deligation prevented the functional impairment of EFS-induced contraction, although to a lesser extent than 30 mg/kg melatonin (63 and 26% recovery for MEL 30 and tempol, respectively; 30 mg/kg for 4 days after the onset of AC, DL + MEL 4 days). After EFS was performed under control conditions, strips were incubated for 20 min with the antagonist/inhibitor, and EFS was repeated. Data are mean ± S.E.M. (n = 7–9 strips; *, P < 0.01 versus control; δ, P < 0.01 versus AC; ANOVA).

**TABLE 1**

Effect of acute cholecystitis, melatonin, and tempol treatment on oxidative stress markers

Data are expressed as mean ± S.E.M. of fold increase respect to levels found in control tissue. n = 5 to 6 animals.

<table>
<thead>
<tr>
<th></th>
<th>AC</th>
<th>DL</th>
<th>DL + MEL 2.5</th>
<th>DL + MEL 30</th>
<th>DL + MEL 4 Days</th>
<th>DL Tempol (1 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA level</td>
<td>4.82 ± 0.43</td>
<td>4.32 ± 0.41</td>
<td>1.90 ± 0.37 *<em>,,</em></td>
<td>1.44 ± 0.26 *<em>,,</em></td>
<td>1.20 ± 0.26 *<em>,,</em></td>
<td>1.20 ± 0.21 *<em>,,</em></td>
</tr>
<tr>
<td>GSH level</td>
<td>0.31 ± 0.05</td>
<td>0.35 ± 0.05</td>
<td>0.76 ± 0.07 *<em>,,</em></td>
<td>0.91 ± 0.06 *<em>,,</em></td>
<td>0.89 ± 0.06 *<em>,,</em></td>
<td>0.96 ± 0.04 *<em>,,</em></td>
</tr>
</tbody>
</table>

* P < 0.05; ** P < 0.01 vs. AC.
* P < 0.05; ** P < 0.01 vs. DL.

The current report shows that the impairment in guinea pig gallbladder neurotransmission evoked by inflammation was associated with a decrease in the contribution of the efferent plexus and the up-regulation of sensory afferent fibers. In addition, melatonin treatment caused an improvement in the neurogenic contractile response and the normalization of the different neural components that were probably related to its antioxidant and scavenger properties.

Our results indicate that EFS evokes a gallbladder re-
Fig. 7. Tempol treatment improves the neuromuscular damage exacerbated by the deligation procedure, and it normalizes the contribution of the different neural components in the inflamed gallbladder. A, original recordings showing guinea pig gallbladder contractile responses elicited by EFS (0.3-ms duration, 5–40 Hz, 350 mA, for 10 s every 3 min) applied to DL strips from animals treated with 1 mM tempol (DL + tempol 1 mM). Traces are typical of 17 and 20 strips for DL and DL + tempol 1 mM, respectively. B, summary data of EFS induced-responses (peak amplitude) in those experimental groups (\(P < 0.001\) and \(P < 0.01\) versus DL; ANOVA). C to F, histograms showing the effects of 100 \(\mu\)M TTX, 1 \(\mu\)M atropine, 100 \(\mu\)M L-NAME, and 10 \(\mu\)M capsaicin on EFS-elicited contractile responses in control, AC, and DL + tempol groups. Data are mean ± S.E.M. (n = 10–21 strips; * \(P < 0.05\) versus control; ** \(P < 0.01\) versus AC; ANOVA).
sponse by the release of different inhibitory and excitatory neurotransmitters. The smaller contractile responses to EFS in cholecystitic strips suggest the existence of an inflammation-induced impairment in the gallbladder intrinsic nerves, in agreement with previous results in human and animal models (McKirdy et al., 1994a; Parkman et al., 2000). However, the reduced gallbladder smooth muscle contractility to ACh found in cholecystitis (Parkman et al., 1999a, 2000; Gomez-Pinilla et al., 2006b) could also contribute to the impaired neuromuscular function in inflamed tissue. The most striking finding in our study was the lack of sensitivity shown by cholecystitic strips to TTX, which could be explained by a direct release of neurotransmitter from nervous terminal. This was confirmed by the sensitivity of the EFS-induced responses to \( \omega \)-conotoxin GVIA, a blocker of N-type calcium channel located in the presynaptic membrane whose activation is necessary for neurotransmitter release. These results suggest that inflammation evokes a functional denervation in the gallbladder that avoids the genesis or propagation of action potential through efferent fibers. Alterations in the properties and/or expression levels of voltage-dependent Na\(^+\) channels have been implicated in a variety of pathological states, including inflammation of the viscera (Yoshimura et al., 2001; Stewart et al., 2003; Beyak et al., 2004). In this regard, the more common effect of inflammation on Na\(^+\) channels is the up-regulation of TTX-resistant slow (Nav1.8) type (Yoshimura et al., 2001; Beyak et al., 2004). Alterations in the pharmacological profile of Na\(^+\) channels could also explain the TTX-resistant contractions reported in this study.

Classically, ACh released in response to EFS is the main excitatory component of the gallbladder contraction (Yau and Youther, 1984; Parkman et al., 1997). Here, we show that in control conditions, atropine abolished EFS-induced contraction, whereas in inflamed tissue, it just reduced EFS-elicited contraction approximately 50\%, indicative of a functional denervation of the cholinergic component, similar to results described previously in inflamed gallbladder (Parkman et al., 2000).

Nonadrenergic noncholinergic neurotransmission in guinea pig gallbladder was described more than a decade ago (Moureelle et al., 1993), and NO is the main nonadrenergic noncholinergic neurotransmitter involved (McKirdy et al., 1994b; Alcón et al., 2001). Inflammation evokes a functional impairment in gallbladder nitrergic innervation as demonstrated by the lack of effects of l-NAME in cholecystitic strips compared with control tissue. This result does not support the study from Parkman et al. (2000), where l-NAME only had an effect in inflamed tissue, indicating that normal gallbladder does not release NO from the intrinsic plexus. This is in conflict with the presence of nitrergic nerves described in guinea pig gallbladder (Mawe et al., 2006) and with the functional data reported above.

Neurotransmitters released from sensory nerves evoked contraction or relaxation of the gallbladder (Maggi et al., 1989). In our study, sensory denervation with capsaicin had no effect in control conditions, whereas it reduced EFS-elicited contractile response in inflammation, suggesting excitatory neurotransmitter release from sensory nerves in inflamed gallbladder. The major participation of the sensory innervation is a common finding in neurogenic inflammation (Sann et al., 1996). In the gallbladder, we have shown that aging, which is also related to increased oxidative stress, is associated with over-reactivity of sensory fibers (Gomez-Pinilla et al., 2006a).

The most important finding of our study is that melatonin has prophylactic and therapeutic effects on inflammation-induced impairment in gallbladder neuromuscular function. Thus, with 14-day melatonin treatment, the EFS-induced contractile response recovered the sensivity to TTX, indicating that melatonin protects the voltage-dependent Na\(^+\) channels involved in the neural transmission of the action potential. Furthermore, the nitrergic innervation recovered its functionality and sensory fibers became less sensitive to EFS. However, melatonin itself did not improve the contractile response to EFS unless the obstruction of the bile duct was relieved. Under these conditions, melatonin reversed the impairment in contractility in a dose-dependent manner, and it fully recovered the different neural components stimulated by EFS. It must be pointed out that 2.5 mg/kg melatonin had no effects on the nitrergic innervation after deligation, although this treatment was efficacious in increasing the participation of these inhibitory nerves with the bile duct-ligated animals. Deligation itself worsened gallbladder contractility, as consequence probably of an increase in oxidative stress insult due to reperfusion of the organ once the mechanical stretch was alleviated. This is supported by the increase in the MDA levels indicative of lipidic peroxidation and oxidative injury found after deligation. On this basis, it seems that nitrergic innervation is especially sensitive to the enhanced oxidative stress after deligation. In agreement with this, we have recently reported a minor participation of nitrergic nerves in neuromuscular transmission in aging and its recovery after melatonin treatment (Gomez-Pinilla et al., 2006a). Furthermore, melatonin has been shown to have neural and sensory innervation (Barajas-López et al., 1996; Reiter et al., 2003).

According to our results, melatonin not only protects against inflammation but also resolves the inflammation-induced impairment of neuromuscular function. Thus, when melatonin treatment started after the onset of gallbladder inflammation, there was an enhancement of the contractile response to EFS that also recovered the neurotransmission pattern. However, the prophylactic administration of melatonin was more effective than the therapeutic administration, which could be related to the increase in the antioxidant defenses induced by the administration of melatonin before the oxidative insult.

It is well accepted that melatonin administration at pharmacological doses decreases free radical formation and leads to a substantial recovery of the major antioxidant enzymes (Reiter, 1998). Recent evidence has shown that melatonin has protective effects on liver and hepatic injury after extrahepatic bile duct ligation in rats (Shiess et al., 2000; Esrefoğlu et al., 2005; Ohta et al., 2005). In addition to liver and hepatic damage, free radical accumulation associated with bile duct ligation has been implicated in the genesis of gallstone (Eder et al., 1996). In this regard, antioxidant treatment with melatonin not only reversed the increased oxidative stress but also prevented gallstone formation (Shiess et al., 2000). In our preparation, either prophylactic or therapeutic melatonin treatments were effective in reducing MDA levels and in increasing the endogenous antioxidant defense.
GSH, indicating that melatonin antioxidant effects can be responsible for the improvement in the neuromuscular function. In fact, the treatment of the animals with the membrane-permeant superoxide dismutase mimetic tempol also induced a significant improvement in the neuromuscular function of inflamed gallbladder, which is in agreement with other reports showing that tempol reduces the dysfunctions associated to oxidative stress insult (Chatterjee et al., 2000; Mehta et al., 2004). Collectively, our data suggest a prophylactic and therapeutic role of melatonin in experimental acute cholecystitis, a remarkable finding due to the lack of an effective pharmacological treatment for acute cholecystitis.

In conclusion, the results obtained in the present study indicate that inflammation impairs gallbladder neuromuscular function as the result of changes in the neural inputs to smooth muscle. These changes can be summarized as a dereviliation of effenter nerves together with a hyperactivity of afferent fibers. Melatonin significantly ameliorated the inflammation-related changes in gallbladder neuromuscular transmission, indicating its potential to combat inflammation-induced gallbladder damage.

Acknowledgments

We thank Rosario Moreno for technical assistance.

References