Title:
Melatonin inhibits alcohol-induced increases in duodenal mucosal permeability in rats in vivo

Authors:
Anna Sommansson, Wan Salman Wan Saudi, Olof Nylander and Markus Sjöblom

Affiliation:
Division of Physiology, Department of Neuroscience, Uppsala University,
PO Box 593, SE-751 24 Uppsala, Sweden

Running head:
Melatonin provide intestinal barrier functions

Address for correspondence:
Markus Sjöblom, Ph.D.
Department of Neuroscience
Division of Physiology
Uppsala University
BMC, PO Box 593, SE - 751 24 Uppsala, Sweden
Phone: +46-18 4714186
Fax: +46-18 4715021
E-mail: Markus.Sjoblom@neuro.uu.se
ABSTRACT

Increased intestinal permeability is often associated with epithelial inflammation, leaky gut or other pathological conditions in the gastro-intestinal tract. We recently found that melatonin decreases basal duodenal mucosal permeability suggesting a mucosal protective mode of action of this agent.

The aim of the present study was to elucidate the effects of melatonin on ethanol-, wine- and HCl-induced changes of duodenal mucosal paracellular permeability and motility.

Rats were anaesthetized with thiobarbiturate and a ~30-mm segment of the proximal duodenum was perfused in situ. Effects on duodenal mucosal paracellular permeability, assessed by measuring the blood-to-lumen clearance of $^{51}$Cr-EDTA, motility and morphology were investigated.

Perfusing the duodenal segment with ethanol (10% or 15% alcohol by volume), red wine or HCl (25-100 mM) induced concentration-dependent increases in paracellular permeability. Luminal ethanol and wine increased, while HCl transiently decreased duodenal motility. Administration of melatonin significantly reduced ethanol- and wine-induced increases in permeability by a mechanism abolished by the nicotinic receptor antagonists hexamethonium (i.v.) or mecamylamine (luminally). Signs of mucosal injury (edema and beginning of desquamation of the epithelium) in response to ethanol exposure were seen only in a few villi, an effect that was histologically not changed by melatonin. Melatonin did not affect HCl-induced increases in mucosal permeability or decreases in motility.

Our results show that melatonin reduces ethanol- and wine-induced increases in duodenal paracellular permeability partly via an enteric inhibitory nicotinic-receptor dependent neural pathway. In addition, melatonin inhibits ethanol-induced increases in duodenal motor activity. These results suggest that melatonin may serve important gastrointestinal barrier functions.
Key words: duodenal barrier, intestinal barrier dysfunction, alcoholic liver disease, leaky gut syndrome, duodenal bicarbonate secretion
INTRODUCTION

The most important physiological function of the small intestinal epithelium is to absorb nutrients, ions and water. At the same time the epithelium must prevent or restrict the uptake of antigens, pathogens and water-soluble noxious agents from the lumen. Disruption or dysfunction of the epithelial barrier may increase infiltration of harmful substances but also change epithelial secretory and absorptive functions contributing to tissue inflammation and injury. Indeed, increased intestinal permeability is a common clinical observation in patients with alcoholic liver disease, celiac disease, diabetes mellitus and inflammatory bowel disease (13, 24, 34, 39, 52).

The passive entry of solutes across the duodenal epithelium is restricted mainly by intercellular tight junctions. Tight junctions are heterogeneous protein complexes that forms plastic, ion-selective pores and pores for small non-charged solutes in the apical portion of the epithelium and are crucial for the maintenance of barrier integrity (6). The transmembrane components of the tight junction complex include occludin, tricellulin and proteins from the junction adhesion molecule- (JAM) and claudin-family (16, 25). The transmembrane components are linked to the actin cytoskeleton with intracellular scaffold proteins, zona occludens (ZO-1 and ZO-2) and cingulin, which provides structural integrity to the cells (25, 40). Tight junctions are not static barriers but highly dynamic areas that are continuously modified in response to various luminal stimuli (5, 6, 27).

Chronic alcohol consumption is associated with intestinal barrier dysfunction, and elevated intestinal permeability is one of the main causes of endotoxemia in alcoholic liver disease (37). High concentrations of ethanol (>40%) induce epithelial damage and causes increased intestinal permeability. However, moderate concentrations of ethanol increases paracellular permeability by the displacement and disruption of tight junction proteins, and removal of ethanol re-closes the paracellular gaps in vitro (20). Also acetaldehyde, the first
metabolite of ethanol induces increased paracellular permeability associated with redistribution of tight junction proteins (7). Ethanol easily crosses biological membranes and is absorbed by passive diffusion. In man ~10% of the ingested alcohol is absorbed by the gastric epithelium in a fasting state, whilst the largest fraction is absorbed in the proximal small intestine (18). It is therefore likely that the ethanol concentration in the proximal duodenum will be almost the same as that ingested (9, 10). Previous experiments in our laboratory have shown that luminal perfusion with hydrochloric acid or hypo- as well as hypertonic solutions (30, 31, 41) increases duodenal epithelial paracellular permeability. Interestingly, the increase in permeability in response to hypotonic saline (50 mM NaCl) appears to be mediated by 5-HT acting on 5-HT₃ and 5-HT₄ receptors, as well as by nicotinic acetylcholine receptors (31) strongly suggesting regulation of duodenal epithelial paracellular permeability.

Melatonin is a close derivative of 5-HT and the gastrointestinal (GI) tract is the largest extrapineal source of melatonin, with amounts that may be up to ~400 times larger than in the pineal gland (11, 14). In the GI tract melatonin is synthesized by enterochromaffin cells and released melatonin exerts its actions either by binding to the melatonin membrane receptors MT₁ and MT₂, the cytosolic MT₃ receptor, or by acting as a potent scavenger of free radicals (47). The MT₁ and MT₂ receptor subtypes are expressed throughout the GI tract in rats (38, 49) and in the human small intestine (36).

Previously we have found that central nervous administration of phenylephrine increases the release of melatonin by the duodenal segment. In the duodenal segment melatonin was found to be a potent stimulant of mucosal bicarbonate secretion. The effect of phenylephrine was abolished by intravenous administration of the melatonin receptor antagonist luzindole or by sectioning the vagal trunk and the sympathetic chains (43). Recent data from our laboratory show that melatonin decreases basal duodenal mucosal permeability.
However, the effect of melatonin on experimentally-induced increases in duodenal paracellular permeability has previously not been studied. The aim of the present study was to elucidate the ability of melatonin to influence ethanol- and acid-induced increases in duodenal mucosal paracellular permeability and duodenal motor activity in anesthetized rats. Duodenal epithelial paracellular permeability was assessed by measuring the blood-to-lumen clearance of $^{51}$Cr-EDTA and duodenal contractions were determined by measuring changes in intraluminal pressure. For comparison, we also determined the effect of melatonin on duodenal permeability and motility in animals exposed to red wine. Our results clearly show that melatonin markedly attenuates the increases in permeability in response to both ethanol and wine, but not to acid, via a nicotinic receptor-dependent neural pathway.
MATERIALS AND METHODS

Chemicals and drugs

Melatonin (M5250), hexamethonium chloride (H2138), bovine albumin (A2153), mecamylamine hydrochloride (M9020) and the anesthetic 5-ethyl-5-(1’-methyl-propyl)-2-thiobarbiturate (Inactin®) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol 95.5 vol-% (Etax A®) was purchased from Solveco Chemicals AB, Täby, Sweden. Parecoxib (Dynastat®) was obtained from Apoteket AB, Uppsala, Sweden. N-Acetyl-2-benzyltryptamine (Luzindole, N-0774) were obtained from Tocris Bioscience, Ellisville, MO, USA. $^{51}$chromium-labelled ethylenediaminetetraacetate ($^{51}$Cr-EDTA) was purchased from PerkinElmer Life Sciences Inc. (Boston, MA, USA). Red wine (Shiraz Mourvèdre Viognier, Robertson Winery, South Africa, 2009, 14.5 alcohol vol-%) was purchased at Systembolaget, Sweden.

Ethics Statement

All experiments in the present study were approved by the Uppsala Ethics Committee for Experiments with Animals (Permit Number: C309/10).

Animals

Male Sprague Dawley rats weighing 210–260 g were obtained from Scanbur AB (Sollentuna, Sweden) or from Taconic (Ejby, Denmark). The animals were maintained under standardized temperature and light conditions (12:12-h light-dark cycle; temperature, 21-22°C). The rats were kept in cages in groups of two or more and had access to tap water and pelleted food (Ewos, Södertälje, Sweden) ad libitum. The animals were deprived of food (fasted) for 16 hours (overnight) before experiments, but had free access to drinking water. Experiments were started by anesthetizing the animal at about 8.00 am with Inactin®, 120 mg kg$^{-1}$ body
weight given intraperitoneally. To minimize preoperative stress, anesthesia was performed by experienced personal at the Animal Department, Biomedical Center, Uppsala, Sweden.

**Surgical procedure**

In the laboratory, animals were tracheotomised and fitted with a tracheal tube to facilitate respiration. Body temperature was maintained at 37-38°C throughout experiments by a heating pad controlled by a rectal thermistor probe. The left femoral artery and vein were catheterized with PE-50 polyethylene catheters (Becton, Dickinson & Co., Franklin Lakes, NJ, USA). For continuous recordings of the systemic arterial blood pressure, the arterial catheter containing 20 IU·ml$^{-1}$ heparin isotonic saline was connected to a transducer operating a PowerLab system (AD Instruments, Hastings, UK). The vein was used for drug injections and for infusion of saline and $^{51}$Cr-EDTA at a rate of 1.0 ml·hr$^{-1}$.

A laparotomy was performed and the common bile duct was catheterized with a PE-10 polyethylene tubing close to its entrance into duodenum (2-3 mm) to prevent pancreatico-biliary juice from entering the duodenum. A soft silicone tubing (Silastic®, 1.0 mm ID, Dow Corning, Midland, MI, USA) was introduced into the mouth and pushed gently along the esophagus, guided through the stomach and pylorus, and secured by ligatures 2-5 mm distal to the pylorus. A PE-320 tubing was inserted into duodenum about 2.5-3.5 cm distal to the pylorus and secured by ligatures. The proximal duodenal tubing was connected to a peristaltic pump (Gilson minipuls 3, Villiers, Le Bel, France) and the segment continuously perfused with a 154 mM sodium chloride solution (saline) at a rate of ~0.4 ml·min$^{-1}$. To complete the surgery, the abdominal cavity was closed with sutures and the wound covered with plastic foil. 30 min after surgery, parecoxib 10 mg·kg$^{-1}$ was given intravenously (i.v.) to reverse the surgery-induced paralysis of the intestine. After surgery, ~60 min was allowed for
cardiovascular, respiratory, and intestinal functions to stabilize before experiments were commenced.

Measurement of duodenal mucosal permeability

After completion of surgery $^{51}$Cr-EDTA was administered i.v. as a bolus of ~75 µCi followed by a continuous infusion at a rate of ~50 µCi per hour. The radioactive isotope was diluted in saline and infused at a rate of 1.0 ml·hr$^{-1}$. One hour was permitted for tissue equilibration of the $^{51}$Cr-EDTA. Two blood samples (~0.3 ml each) were collected during the experiment; the first was collected ten minutes before starting the experiment and the second after ending the experiment. The blood volume loss was compensated for by injection of a 0.3 ml 7% bovine albumin solution. After centrifugation 50 µl of the plasma was removed for measurements of radioactivity. The duodenal segment was perfused with saline at a rate of 0.4 ml·min$^{-1}$, and the perfusate was collected in 10-min samples. The luminal perfusate and the blood plasma were analyzed for $^{51}$Cr-activity in a gamma counter (1282 Compugamma CS, Pharmacia, Uppsala, Sweden). A linear regression analysis of the plasma samples was made to obtain a corresponding plasma value for each perfusate sample. The clearance of $^{51}$Cr-EDTA from blood-to-lumen was calculated as described previously (30) and are expressed as ml·min$^{-1}$·100 g$^{-1}$.

Measurement of duodenal wall contractions

Measuring the intraluminal pressure assessed duodenal wall contractions. The inlet perfusion tubing was connected, via a T-tube, to a pressure transducer and intraluminal pressure was recorded on an IBM PC compatible computer. The outlet tubing was positioned at the same level as the inlet tubing. An upward deflection of at least 2 mmHg above baseline was defined as a motor response. The changes in intraluminal pressure were recorded, via a digitizer, on a
computer using PowerLab® and the software Labchart7® (ADInstruments Ldt. Hastings, East Sussex, UK). The duodenal motility was assessed in intervals of 10 min by planimetry to measure the total area under the pressure curve (area under the curve; AUC) during the sample period. The values given are means ± SEM of three 10-min intervals (unless stated otherwise).

Experimental protocol

In all experiments duodenal paracellular permeability, motility (motor activity), systemic arterial blood pressure (mmHg) and body temperature (°C) were monitored continuously and recorded at 10-min intervals.

Control experiments were performed by measuring the parameters above for 110-min perfusing the duodenal segment with isotonic saline at a rate of ~0.4 ml·min⁻¹.

In animals exposed to ethanol luminally, the experiments started with the perfusion of the duodenum with saline for 30-min to collect basal data. Thereafter, the duodenum was perfused for 30-min with either a 10%- or a 15%-ethanol-solution with a final sodium chloride concentration of 154 mM (a solution isotonic with blood plasma). The experiment was terminated after another 50-min perfusion with saline.

For animals pretreated with melatonin and exposed to ethanol luminally, the experimental protocol was exactly the same as above except that melatonin was administered i.v. as a bolus dose of 10 mg·kg⁻¹ or 20 mg·kg⁻¹ 10 min before start of the ethanol perfusion.

In the case of rats pretreated with luzindole and exposed to ethanol luminally, the experimental protocol was the same as above except that the melatonin antagonist luzindole was administered i.v. as a bolus dose of 0.17 mg·kg⁻¹ 10 min before start of the ethanol perfusion.
In animals pretreated with hexamethonium and exposed to ethanol luminally, the experiments was the same as above except that the nicotinic acetylcholine receptor antagonist hexamethonium was administered i.v. as a bolus dose of 10 mg·kg⁻¹ 10 min before start of the ethanol perfusion followed by a continuous hexamethonium infusion of 10 mg·kg⁻¹·h⁻¹ throughout the experiment. In the first set of experiments the protocol was the same as above for animals pretreated with melatonin and hexamethonium and exposed to ethanol luminally, except that melatonin was administered i.v. as a bolus dose of 20 mg·kg⁻¹ just before the administration of hexamethonium. In the next set of experiments a different nicotinic acetylcholine receptor antagonist, mecamylamine, was tested. The duodenum was perfused for 30-min with saline to collect basal data. Thereafter, mecamylamine was added to the luminal perfusate to a final concentration of 0.1 mM, and perfused for another 30-min. Subsequently, the duodenum was perfused for 30-min with isotonic 15%-ethanol-solution containing 0.1 mM mecamylamine. 10-min before the ethanol exposure melatonin was administered i.v. as a bolus dose of 20 mg·kg⁻¹. After ethanol perfusion the experiment was terminated after another 50-min perfusion with saline.

In animals pretreated with capsazepine and exposed to ethanol luminally, the duodenum was perfused with saline for 40-min to collect basal data. Thereafter, capsazepine was added to the luminal perfusate to a concentration of 0.25 mM and perfused for another 10-min. The duodenum was then perfused for 30-min with isotonic 15%-ethanol-solution containing 0.25 mM capsazepine. After ethanol perfusion the experiment was terminated after another 60-min perfusion with saline.

In the case of rats exposed to wine luminally, the duodenum was perfused with saline for 30-min to collect basal data followed by the perfusion of red wine (14.5 alcohol vol-%) for 30-min. The experiment was terminated after another 50-min perfusion with saline.
In animals pretreated with melatonin and exposed to wine luminally, the experimental protocol was exactly the same as above except that melatonin was administered i.v. as a bolus dose of 20 mg·kg$^{-1}$ 10 min before starting perfusing the duodenal segment with wine.

In the groups of rats exposed to luminal HCl, the duodenal segment was perfused with saline for 30 min to collect basal data followed by the perfusion of with either 25 mM, 50 mM or 100 mM HCl for 5 min. The experiment was terminated after another 65 min perfusion with saline.

In animals pretreated with melatonin and exposed to luminal HCl, the experimental protocol was the same as “Animals exposed to luminal HCl” except that melatonin was administered i.v. as a bolus dose of 20 mg·kg$^{-1}$ 10 min before start of the HCl perfusion.

**Histology**

In three separate series of experiments, specimens from the duodenal segment were examined histologically. One hour after completion of the duodenal operation (described in detail above), the experiment was started.

Group I: The duodenal segment was perfused with saline for 60 min (n = 4).

Group II: The duodenal segment was first perfused with saline for 30 min and subsequently with 15% ethanol made isotonic with NaCl for 30 min (n = 4).

Group III: The same protocol as for group II except that melatonin was injected i.v. at a dose of 20 mg·kg$^{-1}$ 20 min after start of the experiment (n = 4).

After the experiment the duodenal segment was immediately fixed in a 10% neutral buffered formalin solution. After fixation, the segment was cut along its length and embedded in paraffin. Sections from the middle part of the segment (~1.5 cm from the pylorus), 4 µm thin,
were stained with haematoxylin–eosin. All villi in each section were evaluated. Duodenal
morphology was assessed by light microscopy by an experienced pathologist who was
uninformed of the treatment regimes.

Statistical analysis
Descriptive statistics are expressed as means ± SEM, with the number of experiments given in
parentheses. The statistical significance of data was tested by repeated measures analysis of
variance. To test differences within a group significance was tested either by Student's *t*-test
(two-tailed test) or 1-factor repeated measures ANOVA followed by Tukey post-hoc test,
when appropriate. Between groups a 2-way repeated measures ANOVA was used, followed
by a Bonferroni post-hoc test. All statistical analyses were performed on an IBM-compatible
computer using GraphPad Prism 5.03 software (San Diego, CA, USA). A *p*-value less than
0.05 was considered significant.
RESULTS

In the control group, i.e. animals perfused luminally with isotonic saline during the whole experiment, duodenal epithelial paracellular permeability and duodenal motor activity were stable throughout the experiment (Figs. 1 and 3) and neither the permeability, motor activity nor the mean arterial blood pressure (MABP) were influenced by intravenous or luminal administration of vehicle (isotonic saline) alone (not shown). The overall mean $^{51}$Cr-EDTA clearance was $0.13 \pm 0.03 \text{ml}\cdot\text{min}^{-1}\cdot\text{100g}^{-1}$ (n=6) and the motility index, i.e., AUC, averaged $438 \pm 65 \text{AUC 10 min}^{-1}$ (n=6). MABP and body temperature remained stable in all groups except experiments with hexamethonium where the MABP decreased immediately from $119 \pm 3.4 \text{mmHg}$ to $77 \pm 5.1 \text{mmHg}$ (n=15, p<0.05) and remained at that level throughout the experiment.

Perfusing the duodenal lumen with 10% ethanol during 30 min increased $^{51}$Cr-EDTA clearance from $0.15 \pm 0.04$ to $0.35 \pm 0.05 \text{ml}\cdot\text{min}^{-1}\cdot\text{100g}^{-1}$ (p<0.05, n=6, Fig. 1 A). 10% ethanol induced a transient (during the first 10 min of exposure) increase in duodenal motor activity from $355 \pm 102$ to $891 \pm 256 \text{AUC 10 min}^{-1}$ (p<0.05, n=6, Fig. 1 B). Increasing the luminal concentration of ethanol to 15% induced greater changes in duodenal paracellular permeability and motor activity than during 10% ethanol. With 15% ethanol for 30 min $^{51}$Cr-EDTA clearance increased from $0.18 \pm 0.06$ to $1.67 \pm 0.29 \text{ml}\cdot\text{min}^{-1}\cdot\text{100g}^{-1}$ (p<0.05, n=9, Fig. 1 A), and the duodenal motility increased from $537 \pm 46$ to $1258 \pm 174 \text{AUC 10 min}^{-1}$ (p<0.05, n=9 Figs. 1B and 3). However, the motility-increase remained high throughout the 30-min ethanol exposure at a level of $1089 \pm 178 \text{AUC 10 min}^{-1}$ (p<0.05, n=9, Fig. 1 B).

Previously, we have shown that intravenous administration of melatonin, up to a dose of 20 mg$\cdot$kg$^{-1}$, did not affect basal duodenal permeability or basal duodenal motility (48). In the first set of experiments, melatonin 10 mg$\cdot$kg$^{-1}$ was administered i.v. 10 min before perfusing the duodenal lumen with 15% ethanol. In these experiments ethanol increased $^{51}$Cr-EDTA
clearance from 0.24 ± 0.08 to 1.26 ± 0.21 ml\cdot min^{-1}\cdot100g^{-1} (Fig. 2 A), an increase that was
smaller (p<0.05, n=5) than during 15% ethanol alone (1.67 ± 0.29 ml\cdot min^{-1}\cdot100g^{-1}, Fig. 2 A).
In addition, melatonin significantly (p<0.05) reduced the ethanol-induced increase in
duodenal motility (Fig. 2 B).

Increasing the melatonin dose to 20 mg\cdot kg^{-1} (i.v.), reduced the 15% ethanol-induced
increase in $^{51}$Cr-EDTA clearance even further (0.65 ± 0.23 ml\cdot min^{-1}\cdot100g^{-1}, p<0.05, n=6,
Fig. 2 A). Furthermore, this increase was significantly (p<0.05) lower than that observed with
10 mg\cdot kg^{-1} melatonin. In animals pretreated with 20 mg\cdot kg^{-1} melatonin i.v. 15% ethanol failed
to augment duodenal motility (Figs. 2 B and 3).

Similarly, pretreatment with melatonin, at a dose of 20 mg\cdot kg^{-1} (i.v.), reduced the 10%ethanol-induced rise in $^{51}$Cr-EDTA clearance (which increased from 0.12 ± 0.02 to 0.54 ±
0.13 ml\cdot min^{-1}\cdot100g^{-1}) and abolished the ethanol-induced increase in duodenal motility (which
changed from 666 ± 103 to 568 ± 73 AUC 10 min^{-1}, n=3, not shown).

To assess whether endogenous melatonin take part in the mediation of the ethanol-
induced increase in duodenal epithelial permeability and motility, the melatonin MT_{1}/MT_{2}
receptor antagonist luzindole was used. It has previously been shown that a single i.v. bolus
dose of 0.17 mg\cdot kg^{-1} luzindole abolish melatonin-induced stimulation of duodenal mucosal
bicarbonate secretion (45, 46). In addition, we have also shown that luzindole (0.17 mg\cdot kg^{-1})
does not affect basal duodenal paracellular permeability or duodenal motility (48). In the
present study the antagonist was injected as a bolus dose (0.17 mg\cdot kg^{-1}, i.v.) 10 min prior to
the start of perfusing the duodenal lumen with either 10% or 15% ethanol. Luzindole did not
affect the increases in $^{51}$Cr-EDTA clearance or motility, and the increases were similar to
values seen in 10% ethanol alone ($^{51}$Cr-EDTA clearance from 0.10 ± 0.02 to 0.29 ± 0.09
ml\cdot min^{-1}\cdot100g^{-1} and motility from 242 ± 28 to 595 ± 201 AUC 10 min^{-1}, n=4, not shown).
Similarly, luzindole did not affect the increases in $^{51}$Cr-EDTA clearance (from 0.13 ± 0.03 to
1.34 ± 0.15 ml·min⁻¹·100g⁻¹ (p<0.05, n=5) or motility (from 419 ± 191 to 707 ± 234 AUC 10 min⁻¹ (p<0.05, n=5) in response to 15% ethanol (not shown).

The important roles of enteric nerves and vagal stimulation in control of duodenal function are well established. Hexamethonium is a nonselective nicotinic receptor antagonist blocking transmission in autonomic ganglia. In the present study, hexamethonium caused an instant and significant (p<0.05) drop in the MABP (from 119 ± 3.4 mmHg to 77 ± 5.1 mmHg, not shown) but did not change the 15% ethanol-induced increase in permeability. Similar to ethanol alone ⁵¹Cr-EDTA clearance increased from 0.23 ± 0.05 to 1.63 ± 1.23 ml·min⁻¹·100g⁻¹ (p<0.05, n=9, Fig. 4 A). Furthermore, hexamethonium abolished the ethanol-induced increase in duodenal motility (p<0.05, n=9, Fig. 4 B).

Interestingly, hexamethonium-treatment abolished the inhibitory effect of melatonin (20 mg·kg⁻¹) on the 15% ethanol-induced increases in permeability. The ⁵¹Cr-EDTA clearance increased from 0.10 ± 0.03 ml·min⁻¹·100g⁻¹ to 1.57 ± 0.24 ml·min⁻¹·100g⁻¹, an increase not different from values with 15% ethanol alone (Fig. 4 A).

In addition to hexamethonium, the effects of mecamylamine, a nonselective and noncompetitive nicotinic acetylcholine receptor antagonist, were evaluated. In contrast to hexamethonium, which is positively charged and passes cell membranes very poorly, mecamylamine can be administered luminally to induce local effects in the intestine and thus avoid a decrease in MABP. Mecamylamine at a luminal concentration of 0.1 mM had no effect on MABP or the basal permeability, but inhibited the effect of 20 mg·kg⁻¹ melatonin i.v. on the ethanol-induced (15%) increase in permeability. During mecamylamine administration the ⁵¹Cr-EDTA clearance increased from 0.10 ± 0.02 to 0.92 ± 0.26 ml·min⁻¹·100g⁻¹ (p<0.05, n=4, not shown), an increase that was significantly higher compared to values obtained with melatonin plus 15% ethanol-treatment (c.f. 0.65 ± 0.23 ml·min⁻¹·100g⁻¹ (p<0.05, n=6).
Mecamylamine also reduced duodenal motility from 332 ± 130 AUC 10 min\(^{-1}\) to 79 ± 36 AUC 10 min\(^{-1}\) (not shown).

The ethanol-induced changes in duodenal epithelial permeability and motility may be mediated via activation of capsaicin receptors as ethanol previously has been shown to potentiate vanilloid receptor 1 function (51). In addition, luminal administration of the capsaicin antagonist capsazepine (1 µM-1 mM) has previously been shown to inhibit acid-induced increase in duodenal blood flow (3) and capsaicin-induced increase of duodenal mucosal bicarbonate secretion (17). It was therefore of interest to investigate whether the ethanol-induced changes in permeability and motility could be affected by capsaicin-receptor blockade. In the present study luminal capsazepine (0.25 mM) had no significant (p>0.05) effect on 15% ethanol-induced increase in permeability (Fig. 5A). However, the capsazepine treatment abolished the ethanol-induced increase in motility (Fig. 5B).

It was of interest to examine whether wine, with an ethanol concentration of 14.5 vol-%, induced the same effects on permeability and motility as 15% ethanol. Similar to the effect of 15% ethanol, red wine induced a marked increase in \(^{51}\)Cr-EDTA clearance (Fig. 6A) but red wine did not induce changes in duodenal motility (Fig. 6B).

Pretreatment with melatonin (20 mg·kg\(^{-1}\) i.v.) attenuated the increase in paracellular permeability in response to perfusion with red wine (p<0.05, Fig. 6A).

It has previously been shown that HCl increases duodenal mucosal permeability in a concentration-dependent manner (28) and it was therefore of interest to examine whether melatonin could affect this response. Perfusing the duodenal lumen with 25 mM HCl during 5 min increased \(^{51}\)Cr-EDTA clearance from 0.36 ± 0.04 to 0.61 ± 0.09 ml·min\(^{-1}\)·100g\(^{-1}\) (p<0.05, n=6, Fig. 7). Furthermore, 25 mM HCl exposure transiently depressed duodenal motility (Fig. 7A). Increasing the luminal HCl concentration to 50 mM increased the \(^{51}\)Cr-EDTA clearance
to a greater extent than did 25 mM. Similarly to 25 mM HCl, perfusion with 50 mM HCl transiently reduced duodenal motility (p<0.05, n=9, Figs. 7C and D).

Perfusion with 100 mM HCl increased $^{51}$Cr-EDTA clearance from 0.33 ± 0.07 to 2.00 ± 0.32 ml/min•100g$^{-1}$ (p<0.05, n=5, Fig. 7E) and induced a more pronounced reduction of motility than did 25 or 50 mM HCl.

In contrast to the effects of melatonin on the ethanol-induced responses, melatonin did not affect the acid-induced increases in duodenal epithelial paracellular permeability (Figs. 7A, C, and E). Melatonin did, however, abolish the 25 mM acid-induced decrease in motility (Fig. 7B), and significantly attenuated the transient decrease in motility in response to 100 mM HCl (Fig. 7F).

**Histology**

The duodenum perfused with isotonic NaCl for 60 min (group I) had a normal morphological appearance (n=4, Fig. 8A) and in high-magnification of the villus tip (Fig. 8D). Perfusion of the duodenal segment with 15%-ethanol for 30 min (group II) caused mild villous tip damage observed as edema and the beginning of desquamation of the epithelium at the tip of a few villi tips (n=4, Fig. 8B) and in high-magnification of the villus tip (Fig. 8E). Administration of melatonin, injected i.v. at a dose of 20 mg·kg$^{-1}$ 20 min after start of the experiment in animals perfused with isotonic NaCl for 30 min and subsequently with 15%-ethanol for 30 min (group III) caused minor degenerative changes at the tips of the villi in less number of cells (n=4, Fig. 8C) and in high-magnification of the villus tip (Fig. 8F). However, no obvious histologically differences were seen between group II and group III.
DISCUSSION

Previous studies from our laboratory demonstrate that melatonin is a potent stimulant of duodenal mucosal bicarbonate secretion (44-46) and very recent data show that luminal melatonin decreases basal duodenal epithelial paracellular permeability in rats in vivo (48). In the present study we show, for the first time that experimentally induced increases of duodenal mucosal permeability by luminal perfusion of ethanol or wine, is markedly reduced by administration of melatonin. Interestingly, the permeability-reducing effect of melatonin was abolished by pretreating the animals with hexamethonium or mecamylamine, two nonselective nicotinic receptor antagonists, suggesting that the permeability-reducing effect of melatonin is mediated via stimulation of an inhibitory nicotinic receptor-dependent neural pathway. The doses of melatonin needed for these, potentially protective, effects are similar to those previously found in the duodenal luminal perfusate in response to central nervous stimulation (43). In contrast, melatonin did not affect the HCl-induced increase in duodenal paracellular permeability suggesting that ethanol and HCl increase duodenal mucosal permeability via different mechanisms. Taken together, these observations strongly suggest that melatonin possesses the ability to affect not only basal but also ethanol-induced increases in mucosal permeability.

The major rate-limiting pathway for passive transport of solutes and fluid across the epithelium is the lateral intercellular spaces i.e. the paracellular route. The spaces between epithelial cells are linked together at the apical junctional complex by tight junctions (6). Tight junctions consist of a complex protein structure that forms plastic pores in the epithelium. Interestingly, data from several studies, both in vitro and in vivo, indicate changes in tight junction permeability in response to various physiological and external stimuli (5, 6, 27). However, an increase in paracellular permeability may also reflect disturbance of epithelial integrity. Indeed, increased intestinal paracellular permeability is a common clinical
finding in patients with alcoholic liver disease, coeliac disease, Crohn’s disease, diabetes mellitus and ulcerative colitis (13, 24, 34, 39, 52).

In the present study we showed that luminal perfusion of the duodenum with ethanol induced an increase in $^{51}$Cr-EDTA clearance suggesting that ethanol causes dilatation of the epithelial paracellular pathway. The most rationale explanation for the ethanol-induced increase in permeability is epithelial damage. The finding that 15% ethanol caused a greater increase in epithelial permeability than did 10% ethanol supports this notion. However, the histological examinations of the duodenal segment in the present study after exposure of 15% ethanol for 30 min revealed minor superficial epithelial changes in only a few villi, and these minor changes were limited to the very tip of the villi. Similarly, in a previous study it was shown that perfusing the duodenal segment with a hypotonic solution (50 mM NaCl) also induces mild villous tip damage in some, but not all, rat duodena. However, the lesion score, which reflects the degree of mucosal damage, turned out to be very small and almost identical in control and in parecoxib-treated rats although the hypotonicity-induced increase in duodenal mucosal permeability was markedly greater in the latter group of animals. Hence, this type of superficial injury seems to contribute very little to the increased clearance of $^{51}$Cr-EDTA (35). Furthermore, in this study we found that the paracellular permeability returned to baseline level after cessation of the perfusion with the ethanol solution indicating that the response was reversible. Interestingly, in a previous study, using a Caco-2 cell line it was shown that noncytotoxic concentrations of ethanol increases paracellular permeability by displacement and disruption of tight junction proteins and that the paracellular gaps re-closed when ethanol was removed (20). Also acetaldehyde, the first metabolite of ethanol induces increases in paracellular permeability associated to redistribution of tight junction proteins (7). Although it is not likely that acetaldehyde is responsible for the increased permeability in
the present study since ethanol was administered directly into the duodenal lumen and the activity of alcohol dehydrogenase is very low in this epithelium (19). If the ethanol-induced increase in epithelial paracellular permeability is not primarily caused by mucosal damage, what is then the mechanism? One explanation may be that the increase in epithelial paracellular permeability reflects activation of a physiological defense mechanism. Theoretically, dilatation of the tight junctions in conjunction with increased capillary filtration pressure, due to dilatation of submucosal arterioles, will increase the lateral interstitial fluid pressure, which in turn increase convective fluid transport through the paracellular pathway thereby diluting the aggressive factors in the vicinity of the epithelial surface. However, in the present study we did not observe any significant changes in net fluid secretion in response to ethanol or HCl (unpublished observations, Sommansson A.), which makes this explanation unlikely. Hence, based on the data in the present study combined with those previously published from our laboratory, it is postulated that the ethanol-induced increase in permeability reflects a general alarm reaction elicited by a minor disturbance of mucosal integrity rather than being caused by a damaged epithelium per se.

An interesting finding of the present study was that intravenous administration of melatonin before ethanol exposure reduced the ethanol-induced increase in epithelial paracellular permeability. The intravenous doses of melatonin used in this study have previously been shown not to influence the basal duodenal permeability (48). However, when the duodenal mucosa is exposed to ethanol or wine, systemic administration of melatonin dampens the intensity of the postulated alarm reaction, possibly due to activation of mucosal protective mechanisms. Support for a physiological regulation of this response is the finding that hexamethonium or mecamylamine abolishes the permeability-reducing effect of melatonin. This finding strongly suggests that inhibitory neural pathways, involving nicotinic receptors, participate in the permeability reducing mechanism of melatonin. Support for the
existence of an inhibitory neural pathway regulating duodenal epithelial paracellular permeability is the finding that luminally applied lidocaine, a widely used neural blocker, increases mucosal permeability in a concentration-dependent manner (29). Interestingly, there is data to support an association between melatonin and the activity of nicotinic acetylcholine receptors. Melatonin increases the expression of α-bungarotoxine sensitive nicotinic receptors and also potentiates their effects (21). However, in neurons of plexus submucosus, melatonin reversibly decrease the amplitude of nicotinic excitatory postsynaptic potentials suggesting a blockade of nicotinic receptors (8). Furthermore, we show that the melatonin receptor antagonist luzindole have no effect on the ethanol-induced increases in permeability and motility suggesting that endogenous release of melatonin from duodenal enteroendocrine cells is not sufficient to influence the response.

In the present investigation it was also tested whether the vanilloid receptor 1 participates in the mediation of the ethanol-induced increase in duodenal mucosal permeability. For that purpose we used the vanilloid receptor 1 antagonist capsazepine at a luminal concentration previously shown to inhibit the hyperemic response to luminal CO₂ in the rat duodenum (2). However, the ethanol-induced increase in permeability was more or less insensitive to the luminal administration of capsazepine suggesting that capsaicin-sensitive neurons are not involved.

The data in the present investigation may hint that melatonin reduces the paracellular permeability by a structural change of the intestinal epithelial tight junctions. This is supported by a recent study where melatonin ameliorated diclofenac-induced increases in small intestinal permeability by restoring the tight junction structure (22). In addition, melatonin has been shown to ameliorate radiotherapy-induced intestinal damage (23, 33), protect against postburn gut inflammation (4) and reduce bacterial translocation in colitis and after ischemia-reperfusion injury (1, 26, 42, 50).
In contrast to ethanol-induced increase in duodenal mucosal permeability, which was attenuated by melatonin, the HCl-induced increase was not. Furthermore, it has previously been shown that nicotinic receptor blockade almost abolish the hypotonicity-induced increase in mucosal permeability (32) but not that induced by acid or ethanol as shown in the present study (15). These data suggest that the increase in duodenal epithelial permeability in response to ethanol, acid or hypotonicity is mediated by different mechanisms supporting the notion of physiological regulation of duodenal epithelial paracellular permeability. Hence, it appears that the duodenal mucosa possesses the ability to identify different constituents in the luminal content and to respond accordingly.

The effects of melatonin on gastrointestinal motor activity is not conclusive since published data show that the motility response vary with the melatonin dose (12). Low doses (1 or 10 µg/kg) of melatonin, administered i.p., have been shown to increase intestinal transit, whilst higher doses (100 or 1000 µg/kg) decreases the transit time (12). Recently we found, in anesthetized rats, that duodenal motility was unaffected by luminal administration of melatonin or the melatonin antagonist luzindole (48). Hence during basal conditions, i.e. perfusion with isotonic saline, melatonin does not appear to affect duodenal motility, at least not in anesthetized rats. It is therefore interesting to note that melatonin markedly inhibited the ethanol-augmented duodenal motility. It is thus tempting to suggest that the ethanol-induced changes in duodenal motility and mucosal permeability occur by similar mode of action. However, because capsazepine markedly reduced the ethanol-induced increase in duodenal motility but not the increase in mucosal permeability it is suggested that the mechanisms mediating the change in duodenal motility and mucosal permeability are quite different. It thus appears that ethanol augments duodenal motility but not mucosal permeability, via activation of capsaicin-sensitive vanilloid receptors.
Even though the ethanol concentration in red wine was roughly the same as that in the ethanol solution, the motility response differed between the two solutions since red wine did not affect duodenal motility. The reason for this difference in effect is not clear but it is possible that some constituent in red wine counteracts the ethanol-stimulating effect on motility.

Previous experiments in our laboratory have shown that HCl reversibly inhibits duodenal motility in rats pretreated with the nonselective COX-inhibitor indomethacin. In the present investigation we could extend this finding by showing that HCl inhibits duodenal motility also in COX-2 inhibited animals. Apparently, ethanol and HCl have opposite effects on duodenal motility, at least in COX-2 inhibited rats. The reason for the divergent effect of HCl and ethanol on duodenal motility may relate to the fact that HCl, but not ethanol, is a dominant component of gastric juice. Since the duodenum has a better capacity to neutralize the acid than the jejunum, the physiological significance of the HCl-induced decrease in duodenal motility may be to prevent a rapid discharge of the acidic content into the jejunum thereby allowing time for neutralization of the acid by alkaline juices from the duodenal epithelium, liver and the pancreas. Melatonin had minimal, if any, effect on the HCl-induced motility response suggesting that the mechanisms mediating the effect of ethanol and HCl on duodenal motility differ considerably.

In conclusion, we demonstrate for the first time that melatonin has the capability to reduce ethanol- and wine-, but not acid-induced increases in paracellular permeability in the duodenal mucosa via neural pathways involving nicotinic receptors. Furthermore, melatonin was shown to inhibit ethanol-induced increases in duodenal motor activity. These results indicate that melatonin may serve important gastrointestinal barrier functions.
ACKNOWLEDGMENTS

We thank Gunilla Jedstedt, Marie Heidenvall and Philip Svanfeldt for their excellent technical assistance. We thank Dr. Erik Wilander (Uppsala University, Uppsala, Sweden) for the histological evaluation.

GRANTS

Support was given by the Emil and Ragna Börjesson Foundation, and the Lars Hiertas Foundation.
REFERENCES


FIGURE LEGENDS

Figure 1. Effects of ethanol on duodenal epithelial paracellular permeability and duodenal motility.

A). Ethanol caused a concentration-dependent increase in duodenal epithelial blood-to-lumen clearance of \(^{51}\)Cr-EDTA. In controls (isotonic NaCl) permeability was stable throughout experiment. B). 10% ethanol perfusion caused a transient increase in motility the first 10 min of exposure and then returned to baseline. 15% ethanol perfusion caused a robust and sustained motility increase as long as ethanol was present in the lumen.

Values are mean ± SEM. * indicates a significant (p<0.05) increase compared to baseline in the same group and # indicates a significant larger increase compared to 10% ethanol treated animals.

Figure 2. Effects of melatonin on ethanol-induced increases in duodenal paracellular permeability and motor activity.

Melatonin dose-dependently reduced ethanol-induced increases in duodenal permeability (A) and duodenal motility (B). Values are mean ± SEM. * indicates a significant (p<0.05) increase compared to baseline in the same group and # indicates a significantly reduced response compared to the 15% ethanol treated group.

Figure 3. Effects on intraduodenal pressure (motility) in anesthetized rats.

Representative experiments are shown. All rats are treated with parecoxib 10 mg·kg\(^{-1}\) (i.v.) about 60 min before start of experiment to reverse the surgery-induced paralysis of the intestine. A). A control rat. B). The effects of luminal perfusion of 15% ethanol. C). The effects of luminal perfusion of the duodenal segment with 15% ethanol pretreated with 20 mg·kg\(^{-1}\) (i.v.) melatonin.
Figure 4. Nicotinic receptor inhibition abolish the effects of melatonin.

The effects of luminal perfusion of the duodenum with 15% ethanol pretreated with 20 mg·kg$^{-1}$ i.v. melatonin and/or 10 mg·kg$^{-1}$·h$^{-1}$ i.v. hexamethonium. A). Hexamethonium alone had no effect on ethanol-induced increase in permeability, but completely abolished the effects induced by melatonin. B). As shown previously, hexamethonium significantly reduced motility. In addition, during hexamethonium administration ethanol perfusion did not give rise to an increased motility. Values are mean ± SEM. * indicates a significant (p<0.05) increase compared to baseline in the same group, # indicates a significantly reduced increase compared to the hexamethonium treated groups and § indicates a significantly reduced motility compared to the melatonin and 15% ethanol treated group.

Figure 5. Capsazepine has no effect on ethanol-induced increases in duodenal permeability or motility.

The effects of luminal perfusion with 0.25 mM capsazepine had no effect on ethanol-induced increases in duodenal paracellular permeability (A) but reduced the ethanol-induced increase in motility (B). Values are mean ± SEM. * indicates a significant (p<0.05) increase compared to baseline in the same group and # indicates a significantly reduced response compared to the 15% ethanol treated group.
Figure 6. Effects of red wine on duodenal epithelial paracellular permeability and duodenal motility.

Luminal perfusion with red wine increased the blood-to-lumen clearance of $^{51}$Cr-EDTA and melatonin (20 mg·kg$^{-1}$ i.v.) significantly reduced the wine-induced increase in duodenal paracellular permeability (A). The motility did not alter during the red wine perfusion but was significantly increased after the wine perfusion and melatonin did not alter this (B).

Values are mean ± SEM. * indicates a significant (p<0.05) increase compared to baseline in the same group and # indicates a significant reduced increase in the wine and melatonin treated group compared to the wine treated group.

Figure 7. Effects of HCl on duodenal epithelial paracellular permeability and duodenal motility.

The effects of luminal perfusion of the duodenum with 25 mM, 50 mM and 100 mM with and without melatonin pretreatment (20 mg/kg i.v.). HCl caused a concentration-dependent increase in duodenal paracellular permeability, that were not altered by melatonin pretreatment (A, C and E). The motility significantly decreased shortly after HCl exposure (B, D and F). The decrease in motility was abolished by melatonin in the group perfused with 25 mM HCl (B) and significantly reduced in animals exposed to 100 mM HCl (F). In the response to 50 mM HCl melatonin had no significant influencer (D).

Values are mean ± SEM. * indicates a significant (p<0.05) increase compared to baseline in the same group and # indicates a significant (p<0.05) decrease compared to baseline in the same group.
Figure 8. Histology of the duodenal mucosa.

Three different specimens from the morphological examination of the duodenum are shown. A). Group I, i.e. the duodenum was perfused with isotonic NaCl for 60 min and in D) high-magnification of the villus tip. The morphological appearance was normal. B). Group II, i.e. the duodenal segment was perfused with isotonic NaCl for 30 min and subsequently with 15%-ethanol for 30 min caused mild villous tip damage observed as edema and beginning of desquamation of the epithelium at the tip in some of the villi, and in E) high-magnification of the villus tip. C). Group III, i.e. same as group II but in addition melatonin was injected i.v. at a dose of 20 mg·kg$^{-1}$ 20 min after start of the experiment, and in F) high-magnification of the villus tip. In group III only minor degenerative changes in the cells of the tip of the villous was observed. The scale in figures A to C indicates 200 µm, and in D to F 20 µm.