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ORIGINAL PAPER

Effect of Naloxone-3-Glucuronide and *N*-Methylnaloxone on the Motility of the Isolated Rat Colon After Morphine

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Abstract The effect of the opioid antagonists naloxone-3-glucuronide and *N*-methylnaloxone on rat colon motility after morphine stimulation was measured. The rat model consisted of the isolated, vascularly perfused colon. The antagonists (10^{-4} M, intraluminally) and morphine (10^{-4} M, intra-arterially) were administered from 20 to 30 and from 10 to 50 min, respectively. Colon motility was determined by the luminal outflow. The antagonist concentrations in the luminal and venous outflow were measured by high-performance liquid chromatography. Naloxone-3-glucuronide and *N*-methylnaloxone reversed the morphine-induced reduction of the luminal outflow to baseline within 10 and 20 min, respectively. These antagonists were then excreted in the luminal outflow and could not be found in the venous samples. Naloxone, produced by hydrolysis or demethylation, was not detectable. In conclusion, highly polar naloxone derivatives peripherally antagonize the motility-lowering effect of morphine in the perfused isolated rat colon, are stable, and are not able to cross the colon-mucosal blood barrier.

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Introduction

Opioid therapy is the mainstay of treatment for cancer-related pain affecting approximately 9 million people worldwide annually [1], and 55 to 95% of patients with advanced cancer have severe pain [2]. Morphine (MO) is the drug of choice for the management of moderate to severe chronic cancer pain because of its effectiveness, tolerability, ease of use, and low cost [3]. Unfortunately, constipation is one of the most troublesome side effects of opioid therapy and often adds to the distress of patients being treated for chronic pain [4]. It is even a problem when opiates are administered on a short-term basis for postoperative pain relief [5]. In addition to constipation, patients receiving opioids have other aspects of opioid bowel dysfunction, including lower abdominal discomfort, fecal impaction with overflow diarrhea and incontinence, nausea, vomiting, and inadequate absorption of oral drugs [6]. Furthermore, it has been demonstrated that functional deterioration in patients with advanced cancer correlates closely with poor bowel function [7].

Whereas the analgesic action of opioids is mediated through the central nervous system, constipation is a consequence of their action on the gut wall, reducing intestinal secretion and motility in both small and large bowel, with a lengthening of gut transit time [8]. These adverse gastrointestinal opioid effects are produced through the activation of central and peripheral μ-opioid receptors [9, 10]. The activation of peripheral receptors within the gastrointestinal tract appears to be particularly important because the magnitude of bowel dysfunction correlates more closely with opioid

concentrations in the enteric nervous system than with concentrations in the central nervous system [11]. Similarly, selective activation of gastrointestinal μ-opioid receptors by poorly absorbed μ-opioid agonists, such as loperamide, results in profound inhibition of gastrointestinal transit [12]. The adverse effects of opioids on gastrointestinal function can be reversed by antagonism of μ-receptors with currently available μ-opioid antagonists such as naloxone (NX) and nalmefene. However, these antagonists are centrally active, therefore they also antagonize analgesia and precipitate symptoms of opioid withdrawal in MO-dependent subjects.

The development of μ-opioid receptor antagonists with an action restricted to the periphery is therefore necessary to prevent the effects of opioids on the gastrointestinal tract without antagonizing the central effects, such as analgesia, and producing opioid withdrawal symptoms.

In humans NX undergoes an extensive first-pass liver effect with formation of the main metabolite naloxone-3-glucuronide (NXG). In contrast to morphine-glucuronides [13–15], the binding affinity and potency of NXG for opioid receptors are not known. Whereas NX is easily absorbed, we hypothesize that the highly polar NXG should not reach the systemic circulation and, therefore, not reduce analgesia. A similar pharmacokinetic behavior is assumed by *N*-methylnaloxone (MNX), like NXG, a peripherally acting opioid receptor antagonist [16–20].

The aim of this study was to investigate in an isolated, arterially perfused rat colon whether intraluminally (i.l.) administered NXG and MNX are able to normalize MO-delayed colon transit without being absorbed into the systemic circulation.

Methods

Preparation of the isolated perfused rat colon

The model of an isolated, vascularly perfused rat colon has been described in detail elsewhere [21, 22]. Each colon preparation was used just for one perfusion trial. Briefly, adult Wistar rats of either sex weighing 250–300 g were used. They were cared for in accordance with laboratory animal care and use guidelines established by the Veterinary Services of the Canton of Bern (animal assay permit no. 83/00). Before operation, rats were fasted for 24 hr, with free access to water. The animals were anesthesized with pentobarbital, 50 mg kg^{-1} body weight intraperitoneally, before midline laparotomy. The hindgut and the upper small intestine were freed up to the duodenum and separated after ligation of the supplying vessels.

A silastic tube was inserted into the cecum. Similarly, the distal descending colon was opened by a transverse incision, and the lumen was flushed with 20 ml of prewarmed isotonic saline from the proximal end before a second silastic tube was inserted into the distal descending colon. Subsequently, the inferior mesenteric artery was divided distally of the left colic artery. Finally, the distal descending colon was transected. At this point, the canulated colon (i.e., cecum, ascending, transversing, and descending colon) receives its arterial blood supply only from the superior mesenteric artery. The superior mesenteric artery and the superior mesenteric vein were next dissected and canulated with polyethylene tubes (OD, 0.067 in.; Intramedic polyethylene tubing, Becton Dickinson, Parsippany, NJ). Vascular perfusion was started immediately after cannulation of the superior mesenteric artery at a perfusion rate of 3 ml min^{-1} (Gibson Miniplus 3; Abimed, UK) to avoid any ischemia of the colon. Finally, the canulated colon was freed from the abdominal cavity by dividing the posterior attachments. Vascular and luminal perfusion (LKB Microperpex Peristaltic Pump Model. 2132; Pharmacia Biotech, Sweden) of the colon was performed in a thermostatically controlled perfusion chamber (GFL Laborbedarf, Burgwedel, Germany) [23]. Luminal perfusion was performed at a starting perfusion rate of 0.75 ml min^{-1}, resulting in an perfusion pressure of 60 $\pm$ 5 mm Hg at the beginning of the experiments and in the control animals. There was no apparent distention of the colon at this perfusion rate. The luminal perfusion pressure was continuously measured and kept constant by altering the luminal perfusion rate if necessary. The luminal outflow represented the sum of motility actions, secretions, and resorptions of the isolated perfused colon. Therefore we assumed that the luminal outflow reflected colon motility. The vascular and luminal perfusion fluid (modified Krebs-Ringer-Henseleit solution) used was isosmolar with rat plasma and had the following composition (mM): NaCl, 125; KCl, 4.3; $MgCl_2$, 1; $CaCl_2$, 2.5; $NaHCO_3$, 25; NaH_2PO_4, 1; and glucose, 5. The perfusion fluid was freshly prepared, filtered through Schleicher & Schuell 597 filter paper (Dassel, Germany) before use, had a pH of 7.4, and was bubbled with O_2 (95%) and CO_2 (5%; Evaporator Type 2362; Liebich, Bielefeld, Germany). The temperature was maintained at 37° $\pm$ 0.3°C. Viability of each colon was tested at the end of the experiment by its ability to contract after a mechanical stimulus.

Colon motility and outflow measurements

The agonist solution consisted of 10^{-4} M MO (morphine hydrochloride trihydrate Ph. Eur.; Merck, provided by Grogg, Bern, Switzerland) dissolved in perfusion fluid. This concentration resulted from a preceding dose-finding study with 10^{-6}, 10^{-5}, and 10^{-4} M MO. The solution was applied intra-arterially (i.a.) from 10 to 50 min after T0 (= time point of steady state). The equimolar antagonist solutions

were prepared by dissolving 10^{-4} M NXG (naloxone-3-β-D-glucuronide hydrate; Lipomed, Arlesheim, Switzerland) or 10^{-4} M MNX (naloxone methiodide; Sigma-Aldrich, Buchs, Switzerland) in perfusion fluid. Perfusion fluid was used as placebo solution. NXG ($n = 9$), MNX ($n = 6$), and placebo ($n = 3$) solutions were applied intraluminally (i.l.) from 20 to 30 min after T0. Luminal and venous outflow samples were collected during 5-min intervals starting 5 min before (baseline) and 5, 15, 25, 35, and 45 min after the administration of MO. The luminal and venous outflows were determined by weighing. There was no significant difference between arterial inflow and venous outflow, indicating the absence of significant resorption (data not shown).

Determination of NX, NXG, and MNX in the luminal and venous outflow by HPLC

An ASPEC XL robotic system (Gilson, Villiers Le Bel, France) was used for extraction of the biological samples. To 1 ml luminal outflow, diluted 1:10 with perfusion fluid and filtrated, or 1 ml venous outflow, undiluted and filtrated, 100 μl internal standard solution (1.5 mg ethylmorphine hydrochloride Ph. Eur. [Merck], dissolved in 100 ml bidistillated water) and 3.0 ml 0.5 M carbonate buffer, pH 9.3, were added. After vortexing, the mixture was applied to solid phase extraction (SPE) columns (Chromabond C-18 ec, 3 ml, 500 mg; Macherey-Nagel, Oensingen, Switzerland), preconditioned with two aliquots of 2.5 ml methanol, 40% acetonitrile in 0.01 M phosphate buffer pH 2.1 (v/v), and bidistillated water, respectively. After washing with six aliquots of 2.5 ml 0.005 M carbonate buffer, pH 9.3, 0.5 ml water, and 0.5 ml 40% acetonitrile in 0.01 M phosphate buffer, pH 2.1 (v/v), elution was performed with three aliquots of 1.0 ml 70% acetonitrile in 0.01 M phosphate buffer, pH 2.1 (v/v). The eluate was then concentrated at 45°C under N_2 to dryness, redissolved in 120 μl HPLC mobile phase A (see below), sonicated, and filtrated. Eight microliters of the SPE extracts was then used for HPLC analysis. An Agilent 1100 Series Capillary HPLC System (Agilent Technologies, Waldbronn, Germany) was used. The extracts were separated at 45°C on a Zorbax XDB C-8 3-μm column (Agilent Technologies; 150 × 0.5 mm i.d.) using a multistep gradient (mobile phase A, bidistilled water + 0.05% trifluoroacetic acid [v/v]; B, acetonitrile + 0.05% trifluoroacetic acid; 3% B [0–6 min], 3%–12% B [6–14 min], 12%–20% B [14–20 min], 20%–100% B [20–21 min], 100% B [21–30 min], 100%–3% B [30–35 min], 3% B [35–45 min]; flow rate of 15 μl min^{-1}). Quantitation of NX, NXG, and MNX was performed at 205 nm (diode array detector) based on peak areas and using the internal standard method. Validation data were as follows: SPE extraction efficiency, 66–91%; calibration graph, linear in the range of 50–9000 ng ml^{-1}, $r^2 > 0.993$; intra- and interassay precision, ±1.2%–7.0% RSD; intra- and interassay accuracy, −1.8% to −1.3%; limits of quantitation and detection, 20 and 10 ng ml^{-1}, respectively (signal-to-noise ratio, 5:1).

Statistics

Statistical analysis was performed with SPSS version 11 (Mac) using the Mann-Whitney test (nonparametric). Significance was assessed at the $P < 0.05$ level (two-tailed).

Results

Effect of NXG on the isolated rat colon motility inhibited by MO

Figure 1 shows that the luminal outflow of the isolated, perfused rat colon was reduced by 10^{-4} M i.a. MO, from 7.74 ± 0.41 to 5.10 ± 0.32 g (mean ± SEM; $n = 9$) within 10 min. Therefore, 20 min after T0 the colon motility was decreased to 66.8% ± 2.2% of the baseline value. With a luminal outflow of 7.62 ± 0.48 g ($P = 0.009$), this colon motility-inhibiting effect was, from 20 to 30 min, almost reversed to baseline by 10^{-4} M i.l. NXG. The MO effect reappeared 10 min after stopping the NXG administration. When using placebo (perfusion fluid; $n = 3$) the colon motility-inhibiting effect by MO was not much influenced between 20 and 40 min. After 50 min the outflow was similar for NXG and placebo.

Effect of MNX on the isolated rat colon motility inhibition by MO

The luminal outflow was reduced by 10^{-4} M i.a. MO from 6.73 ± 0.31 to 4.06 ± 0.24 g (mean ± SEM; $n = 6$) within 10 min (Fig. 2), corresponding to 60.4% ±2.7% of the baseline motility. Fourty minutes after T0, 10^{-4} M i.l. MNX increased the luminal outflow to 7.53 ± 0.28 g ($P = 0.02$), corresponding to 97.7% ± 4.6% of the baseline value.

NXG and MNX levels in the luminal and venous outflow of the isolated rat colon

In the luminal outflow samples collected after i.l. NXG, the NXG concentrations measured by a fully validated HPLC method ranged from 0 (baseline) to 88.38 μg ml^{-1}. In the venous outflow no NXG and NX could be detected. After i.l. MNX, the luminal MNX concentrations varied between 0 (baseline) and 33.69 μg ml^{-1}. Again, no MNX was detectable in the venous outflow.

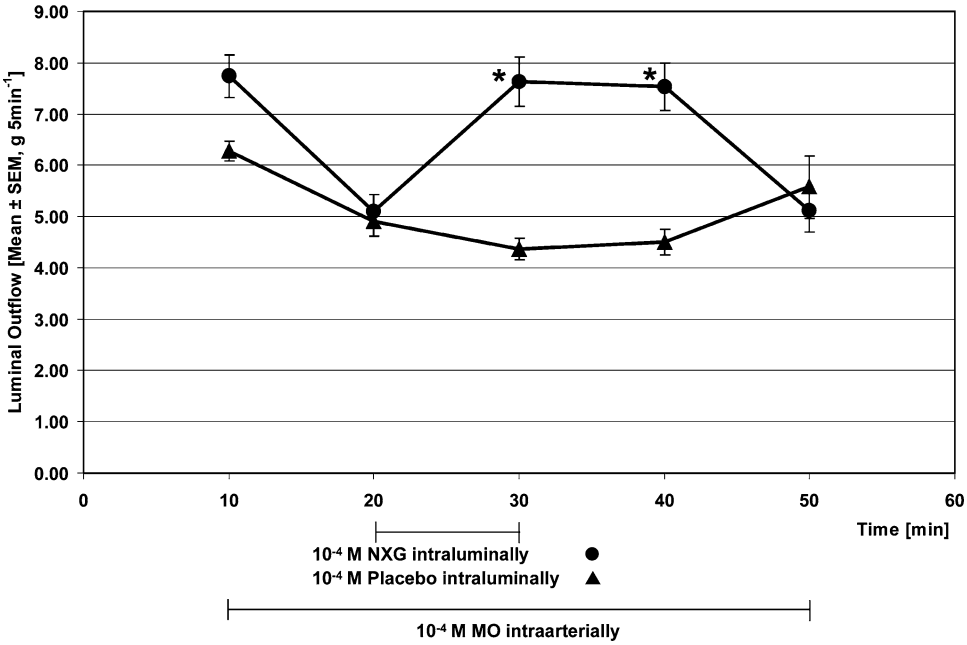

Fig. 1 Luminal outflow of the isolated, perfused rat colon after i.a. administration of 10^{-4} M MO from 10 to 50 min, 10^{-4} M i.l. NXG from 20 to 30 min (●; $n = 9$), and 10^{-4} M i.l. placebo (perfusion fluid) from 20 to 30 min (▲; $n = 3$). The graphs show the reversal effect of the opioid antagonist NXG on the MO-stimulated colon motility inhibition vs. placebo. *Significant at $P < 0.05$ vs. placebo

Discussion

The ex vivo model of the isolated, vascularly perfused rat colon used in this study proved to be suitable and robust for measuring agonistic and antagonistic effects of opioids on the colonic motility. In a dose-finding study, micromolar concentrations of MO resulted in the best organ response. In addition, these rather high concentrations are in accordance with those used in previous opioid experiments on isolated rat colons [24, 25] as well as guinea pig ileal circular muscle [26].

To the best of our knowledge, the present data show, for the first time, that the opioid glucuronide NXG exerts a peripheral effect on colon motility similar to that of the quarternary opioid MNX. In contrast to placebo, NXG and MNX administered i.l. significantly reversed the effects of i.a. MO on colonic motility. Neither NXG or MNX given i.l. nor NX could be detected in the venous outflow of the organ preparation. These findings suggest that the highly polar opioid antagonists are not able to pass the colonic mucosal blood barrier. Furthermore, the absence of NX in the venous outflow indicates that NXG and MNX are luminally not hydrolyzed and demethylated, respectively, by

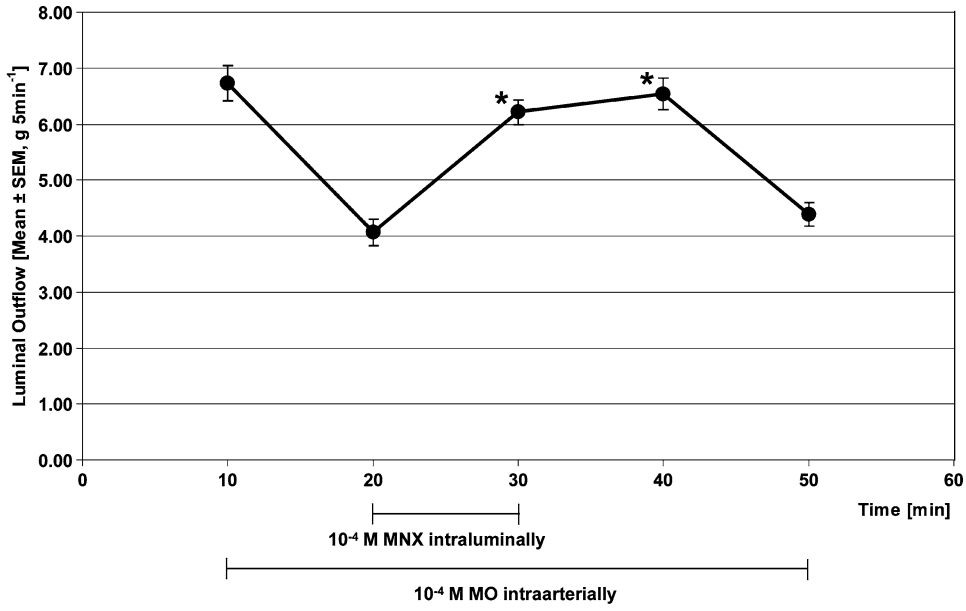

Fig. 2 Luminal outflow of the isolated, perfused rat colon after i.a. administration of 10^{-4} M MO from 10 to 50 min and of 10^{-4} M i.l. MNX from 20 to 30 min ($n = 6$). The graph shows the reversal effect of the opioid antagonist MNX on the MO-stimulated colon motility inhibition. *Significant at $P < 0.05$ vs. placebo

enzymes or bacteria to the easily absorbable NX. The latter phenomenon has been described for chronic pain patients treated with MO, where NX reduced constipation but caused opioid withdrawal symptoms [27]. This indicates a central effect of NX despite the well-known first-pass glucuronidation of NX in the liver. Similar observations were made in advanced cancer patients, where NX at doses >20% of the 24-hr dose of MO had a marked laxative effect but also produced opioid withdrawal signs in some of the patients [28]. Further studies were postulated evaluating the ideal dose of oral NX which exerts the peripheral laxative effect without producing withdrawal symptoms.

In rats where oral NX reversed the constipating effects of oral MO, no influence on nociception was observed [29]. It was concluded that an extensive metabolization of NX is responsible for the lack of central activity.

Our results support the findings that in MO-dependent rats, peroral NXG induced diarrhea without signs of MO-induced withdrawal, as observed with NX [5]. It was demonstrated in rats that subcutaneously administered NX, in contrast to quartenary opioid antagonists, decreased peripheral as well as central effects of MO-dependent rats [30].

Human studies performed in healthy volunteers and in human subjects on a methadone program showed that oral *N*-methylnaltrexone shortened the orocecal transit time without producing withdrawal symptoms [12, 31–33].

Quarternary opioid antagonists are suggested, according to results of subcutaneous, oral, and intravenous administration, to be unable to cross the blood-brain barrier and thus to explain the lack of opioid withdrawal symptoms under the respective treatment. Our study with MNX and NXG in the rat organ preparation indicates that both substances are unable to cross the colon-mucosal blood barrier.

However, further in vitro and in vivo studies are needed, for example, to further characterize the receptor binding affinity of NXG and to clinically elucidate the present study findings in healthy volunteers and patients.

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