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Nalfurafine, a kappa opioid receptor agonist, inhibits scratching behavior secondary to cholestasis induced by chronic ethynylestradiol injections in rats

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Abstract

Scratching is the behavioral manifestation of pruritus. The pruritus of cholestasis can be severe, intractable and affect the quality of life. We investigated if ethynylestradiol (EE)-induced cholestasis is associated with scratching in rats and if nalfurafine, a kappa opioid receptor agonist with antipruritic effects in human uremic pruritus, would antagonize such scratching. Chronic injection of EE (2 mg/kg, s.c., for 14 days) induced cholestasis as documented by increased serum concentrations of bile acids and caused a higher incidence of body scratching compared to vehicle, thus providing an animal model to study scratching behavior secondary to cholestasis. Pretreating the rats with nalfurafine (0.005–0.04 mg/kg, s.c.) inhibited EE-induced scratching dose-dependently with an A_{50} value of 0.013 (0.009–0.021) mg/kg. Serum levels of dynorphin A and nitric oxide were decreased in rats with cholestasis compared to control animals. Our data suggest that (a) nalfurafine has the potential to relieve cholestatic pruritus and (b) both kappa opioid and nitric oxide systems are involved, at least in part, in mediating the pruritus of cholestasis.

Keywords: Cholestasis; Itch; Ethynylestradiol; Nalfurafine; Kappa opioid receptor; Nitric oxide; Rat

1. Introduction

Pruritus is an unpleasant sensation that causes the desire to scratch. Pruritus is a symptom of many skin diseases such as atopic dermatitis, contact dermatitis and urticaria. Also, pruritus is associated with cholestatic liver diseases, chronic renal failure and some hematological diseases (Hodgkin's lymphoma, polycythemia vera) (Twycross et al., 2003). Cholestasis is described as impaired hepatocellular secretion of bile. Substances normally secreted in bile, such as bile acids, cholesterol and bilirubin accumulate in plasma in cholestasis. This situation can result from either a functional defect in bile secretion at the level of hepatocytes and/or bile canaliculi as in primary biliary cirrhosis, primary sclerosing cholangitis and hepatitis C or from extrahepatic obstruction such as that secondary to gallstones and pancreatic cancer. Also, during the third trimester of pregnancy, cholestasis may develop and resolves after delivery. Oral contraceptives can induce cholestasis (Elferink, 2003).

Pruritus can be generalized, severe and intractable. It may result in limitation of normal activities, sleep deprivation, depression and even suicidal thoughts (Bergasa and Jones, 1993). Intractable pruritus due to liver disease is one of the indications for liver transplantation (Neuberger, 2003). Increased central opioidergic tone mediated by endogenous opioid peptides may be responsible, in part, for pruritus in cholestasis (Bergasa et al., 1995). The opioid antagonists naloxone, naltrexone and nalmefene have been used to treat pruritus in cholestasis but there is no universally accepted medication available to treat this symptom. Furthermore, the lack of an animal model for the pruritus of cholestasis limits investigation of potential antipruritic agents.

Rodent models of cholestasis can be grouped as follows: (1) application of endotoxin (inflammatory cholestasis), (2) injection of ethynylestradiol, a synthetic estrogen analog (oral contraceptive-induced cholestasis and cholestasis of pregnancy), (3) administration of alpha-naphthylisocyanate (vanishing bile duct syndrome) and (4) common bile duct ligation (extrahepatic biliary obstruction) (Trauner et al., 2005). Scratching behavior has not been reported in any of these

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models. The injection of ethynylestradiol (EE) (5 mg/kg, s.c.) for 5 days in rats induces cholestasis but without associated scratching (Bossard et al., 1993; Pozzi et al., 2003; Fiorucci et al., 2005). Fiorucci et al. (2005) reported that EE elicits changes in basolateral and canalicular transporters by altering the expression of bsep, mrp2 and mdr2, responsible for excretion of toxic metabolites from hepatocytes.

In our present study, we administered EE for 14 consecutive days instead of 5 days and in a smaller dose (2 mg/kg, s.c., instead of 5 mg/kg) to mimic chronic cholestasis. The aim of our work was to investigate first, whether EE-induced cholestasis causes scratching in rats, thus providing an animal model for the study of scratching secondary to cholestasis. Second, we studied if nalfurafine (previously known as TRK-820), a kappa opioid receptor agonist, inhibited this scratching. Nalfurafine decreases scratching behavior in mice induced by compound 48/80 (Wang et al., 2005), chloroquine (Inan and Cowan, 2004) and agmatine (an endogenous amine) (Inan and Cowan, 2006).

We also measured serum dynorphin A levels in rats with cholestasis and in control rats given vehicle. In our previous investigation (Inan and Cowan, 2005), we found that serum dynorphin A is decreased in rats with cholestasis induced by bile duct ligation compared with sham-operated controls. Additionally, we measured serum nitric oxide (NO) levels to investigate the possible role of NO in mediating the pruritus of cholestasis. Andoh and Kuraishi (2003) reported that NO enhances substance P-induced scratching and L-NAME, a nitric oxide synthase (NOS) inhibitor, inhibits this behavior in a dose-dependent manner. In pathologic conditions, inducible NOS can be expressed by hepatocytes, cholangiocytes, stellate cells and Kupffer cells (Trauner, 2003). Dimoulios et al. (2005) reported an increased level of NO in patients with primary biliary cirrhosis. NO, which is involved, at least in part, in the pathogenesis of cholestasis may also be associated with the pruritus of cholestasis.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (Ace Laboratories, Boyertown, PA) weighing 175–200 g were used. The animals were housed 2 per cage with free access to food and water. A standard light–dark cycle was maintained with a timer-regulated light period from 7 a.m. to 7 p.m. The experimental procedure was approved by Temple University Institutional Animal Care and Use Committee. All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health.

Rats were injected s.c. (right or left flank areas) once a day with either vehicle (50% propylene glycol in distilled water) or 17α-ethynylestradiol (EE) (2 mg/kg) for 13 consecutive days. On day 13, before the daily injections, the animals were acclimated in individual rectangular observation boxes for at least 1 h. Immediately after the regular injection of either vehicle or EE, the number of body scratching movements with hind legs was counted for 30 min. The next day, only EE-injected rats were observed. After acclimation, these rats were divided into two groups and pretreated s.c. with either saline ($n=8$) or nalfurafine (0.005–0.04 mg/kg) ($n=8$) 20 min before EE and scratching was again counted for 30 min. Using four doses of nalfurafine, percent inhibition of scratching in a particular rat was calculated from the following formula:

$$100-(\text{Total number of scratches}) \times 100/(\text{Mean number of scratches in saline group}).$$

At the end of 30 min observation, heart blood (4–5 ml) was drawn from both vehicle and EE-injected rats, the animals being under isoflurane anesthesia. Blood was centrifuged at $5\times1000\times g$ for 5 min to obtain serum. Serum samples were kept at −20 °C until used. Serum bile acid levels were measured using a kit (Trinity Biotech, St. Louis, MO) to confirm cholestasis. Serum dynorphin A levels were measured in individual animals ($n=6$–8) using an ELISA kit (Phoenix Peptide, Belmont, CA). For measurement of NO, serum samples were ultra filtered using a 30 kDa molecular weight cut-off filter (Millipore Corporation, Bedford, MA). Serum was centrifuged at 4 °C for 25 min. NO was measured in the filtrate using a colorimetric assay kit (Neogen Corporation, Lexington, KY).

Rats were euthanized by inhalation after exposure to carbon dioxide gas.

2.2. Chemicals

17α-Ethynylestradiol (Sigma, St. Louis, MO) was dissolved in 50% propylene glycol in distilled water. Nalfurafine was a generous gift from Adolor Corporation (Exton, PA) and dissolved in saline.

2.3. Data analysis

Data are expressed as mean±S.E.M. Student's *t*-test was used to compare two groups and linear regression analysis to calculate the A_{50} dose (and 95% confidence limits) of nalfurafine that inhibits scratching by 50% (PharmToolsPro software, The McCary Group, Emmaus, PA). $P<0.05$ was accepted as statistically significant.

3. Results

A total of 104 rats was used; 37 rats received vehicle and 67 rats were given EE. The number of scratches on day 13 for all vehicle- and EE-injected rats is compared in Fig. 1. EE-injected rats scratched significantly more often than vehicle-injected rats during the 30 min observation time on day 13 (24±3 and 12±2 scratches, respectively, $P<0.01$, Fig. 1). The animals scratched not only the area behind the neck, but also face and flanks, with their hind legs. The pH of the solutions was not an obvious causative factor (EE=pH 6.8, vehicle=pH 6.4). The bile acid

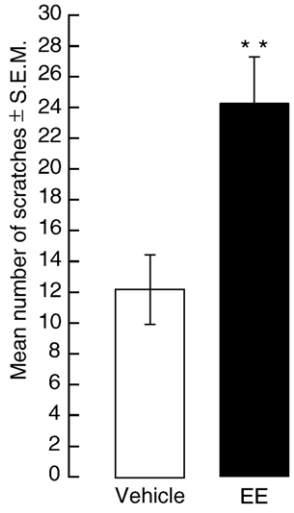

Fig. 1. EE-induced cholestasis causes scratching in rats. EE or vehicle was injected s.c. once daily to rats. Immediately after the 13th injection, the number of scratches was counted for 30 min. Data are expressed as mean±S.E.M. $^{**}P<0.01$.

level for the vehicle group was 24±7.7 μmol/l, whereas that for the EE group was 97±9.7 μmol/l ($P<0.001$), thus verifying cholestasis.

During the 14 day period, EE-injected rats did not gain weight. The mean weights of EE and vehicle groups were similar (218±7 g and 215±7 g, respectively) on day 1. However, on day 14, EE-injected rats had not gained weight (216±5.5 g) but vehicle-injected rats increased weight, as expected (306±6.4 g) ($P<0.001$).

Nalfurafine (0.005–0.04 mg/kg, s.c.) inhibited scratching induced by chronic injection of EE in a dose-dependent manner (Fig. 2). The A_{50} dose of nalfurafine was calculated as 0.013 (0.009–0.021) mg/kg.

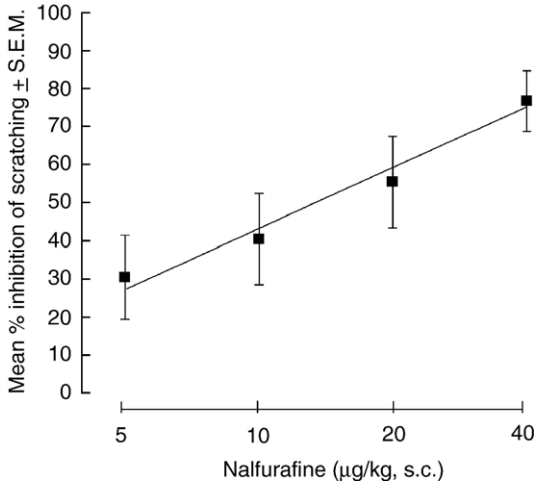

Fig. 2. Nalfurafine inhibits scratching behavior in a dose-dependent manner. On day 14, EE-injected rats were divided into two groups. The animals were injected s.c. with either saline or nalfurafine at −20 min. One minute after the regular injection of EE, the number of scratches was counted for 30 min. Data are expressed as mean±S.E.M.

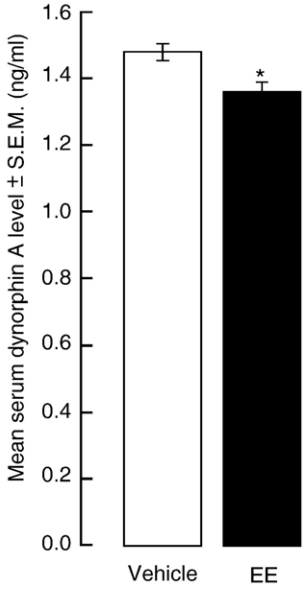

Fig. 3. The serum dynorphin A level was significantly decreased in rats administered EE compared to vehicle-injected rats. Data are expressed as mean± S.E.M. $^{*}P<0.05$.

The mean serum dynorphin A level was lower in EE-injected rats than in vehicle-injected rats (1.36±0.02 ng/ml and 1.48± 0.03 ng/ml, respectively, $n=8$, $P<0.05$, Fig. 3). Also, a lower level of serum NO was detected in rats administered EE compared to vehicle-injected rats (103.5±21 μM and 294.3± 37 μM, respectively, $n=8$, $P<0.001$, Fig. 4).

4. Discussion

The principal findings from the present study are (a) cholestasis induced by chronic injection of EE causes scratching behavior in rats and (b) nalfurafine inhibits such scratching in a dose-dependent manner. Pruritus is a complication of chronic cholestatic liver diseases. Pruritus can vary from mild, with no change in the quality of life, to moderate to severe with sleep deprivation, depression and suicidal thoughts. It can be

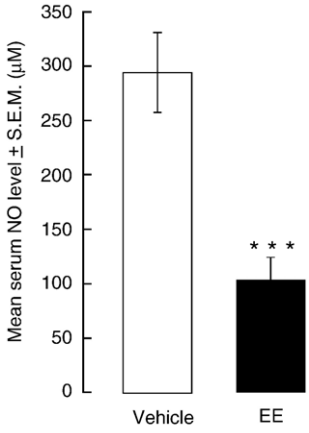

Fig. 4. The serum NO level was lower in cholestatic rats compared to control rats. Data are expressed as mean±S.E.M. $^{***}P<0.001$.

generalized or localized to some parts of the body (Bergasa and Jones, 1993). Presence or absence of pruritus does not always depend on the severity of the disease. Patients with obvious cholestasis may not have pruritus at all; there again, patients with minimal cholestasis may report intense pruritus (Bergasa, 2004, 2005). Increased activity of alkaline phosphatase and increased levels of serum bile acids are laboratory markers of cholestasis. Those rats administered EE had higher bile acid levels than the corresponding vehicle-injected animals. We also conducted a pooled correlation between each rat's number of scratches and the bile acid level for that animal. The correlation coefficient (r) was 0.044 for the EE group and 0.130 for the vehicle group ($n=30$ and 21, respectively), thus suggesting no correlation between the number of scratches and the serum bile acid level.

Our scratching (secondary to cholestasis) model shows similarities to the human situation. First of all, cholestasis is induced chronically with an agent which is also an inducer of cholestasis in humans. Second, scratching behavior is variable (10–100 bouts) among the rats on days 13 and 14 during the 30 min observation sessions. Furthermore, the rats scratched not only the flank areas and behind the neck but also the face and abdomen, suggesting a generalized pruritus. The difference in the mean number of scratches between EE- and vehicle-injected groups is small but nevertheless statistically significant and, we believe, biologically meaningful. Mice are preferred to rats in pruritus studies. Rats, in contrast to mice, are much less sensitive to pruritogens such as compound 48/80 and agmatine (personal observations). For example, a subcutaneous dose of 20 mg/kg of agmatine elicits a mean of 171±25 (S.E.M.) scratches in 30 min in mice ($n=8$) yet only 44±10 scratches in rats ($n=6$) (Inan and Cowan, 2006). Having said that, we had to use rats in our present study because chronic injection of EE did not induce scratching in mice.

Nalfurafine inhibited scratching in a dose-dependent manner in our present study. Nalfurafine was synthesized as an analgesic (Nagase et al., 1998) and acted not only as an antinociceptive agent but also as an antipruritic by inhibiting substance P- and histamine-induced scratching in mice (Togashi et al., 2002). Systemically given nalfurafine was also active against scratching in mice caused by compound 48/80 (an agent releasing histamine from mast cells) (Wang et al., 2005), chloroquine (an antimalarial) (Inan and Cowan, 2004) and agmatine (an endogenous amine) (Inan and Cowan, 2006). Furthermore, Wakasa et al. (2004) reported that nalfurafine suppresses i.v. morphine-induced scratching in monkeys. The ability of nalfurafine to suppress scratching behavior in different species and against different scratch-inducing agents may help define this kappa opioid receptor agonist as a "universal antipruritic". It is notable that Wikström et al. (2005) reported nalfurafine to be a safe and effective antipruritic in uremic pruritus in a multicenter, randomized, double-blind, placebo-controlled human study.

We found that cholestatic rats have lower serum dynorphin A levels than vehicle-injected rats (Fig. 3). In our previous study (Inan and Cowan, 2005), we reported that the kappa opioid system is involved, at least in part, in the pathogenesis of cholestasis. We found that dynorphin A-stimulated [^{35}S] GTPγS binding was lower in the dorsal hypothalamic area of rats with cholestasis (induced by bile duct ligation) compared to sham-operated controls. Also, serum dynorphin A levels were lower in cholestatic rats than in control rats. We propose that reduced kappa opioid activity in cholestasis plays an important role in the development of cholestatic pruritus. In the present study, the lower dynorphin A level in our animal model, together with the inhibition of scratching by a kappa opioid receptor agonist, supports our hypothesis.

Our cholestatic rats had lower serum NO levels than control animals (Fig. 4). NO is involved in the pathogenesis of both cholestasis and pruritus (Andoh and Kuraishi, 2003; Trauner, 2003; Dimoulios et al., 2005). Ma and Wang (1998) reported a low level of serum NO in patients with pruritic intrahepatic cholestasis secondary to pregnancy (ICP) compared to ICP pregnancy without pruritus. This is of interest because EE-induced cholestasis in rodents is thought of as an animal model for ICP (Trauner et al., 2005). Ma and Wang (1998) concluded that the low NO level was associated with pruritus. NO is necessary for kappa opioid receptor-induced effects such as antinociception (Amarante and Duarte, 2002) and hypothermia (Benamar et al., 2002). Our study indicates that NO may be involved in mediating the antipruritic effect of kappa opioid receptor agonists.

In conclusion, we describe in detail, for the first time, an animal model of scratching behavior secondary to cholestasis. This model may be helpful not only to investigate the pathogenesis of this type of pruritus but also to study novel antipruritic agents for therapeutic application. On the basis of our results, we predict that nalfurafine will alleviate the pruritus of cholestasis.

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