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Reduced kappa-opioid activity in a rat model of cholestasis

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Abstract

Increased endogenous opioid activity has been implicated in cholestatic pruritus. In the present study, we have further defined the involvement of opioids in cholestasis. Rats underwent either bile duct ligation or a sham operation. Five days after surgery, brains were removed and agonist-stimulated [^{35}S]GTPγS binding was measured in ten brain regions. Serum endomorphin-2, leu-enkephalin and dynorphin A levels were measured using ELISA on day five. Microdialysis to the dorsal hypothalamic area was conducted in the same animal before and after cholestasis. Dialysate endomorphin-1, leu-enkephalin and dynorphin A levels also were measured. Delta- and kappa-stimulated binding was significantly decreased in cholestasic animals compared to controls in the dorsal hypothalamic area. The serum dynorphin A level was lower in the cholestasic group than in controls (2.56 ± 0.09 and 3.29 ± 0.22 ng/ml, respectively, $P < 0.01$). We propose that pruritus in cholestasis may result from an impaired balance between mu- and kappa-opioid systems.

Keywords: Cholestasis; Pruritus; [^{35}S]GTPγS binding; Opioid system; (Rat)

1. Introduction

Pruritus is often but not always a symptom of cholestatic liver disease. Itch can start on the soles of feet and palms of hands and then becomes more generalized. It can be intractable, very disturbing and hard to manage. Standardized and successful therapies have been elusive. Non-absorbable anion exchange resins (cholestramine, colestipol); hepatic enzyme inducers (phenobarbital, rifampicin); opioid antagonists (nalmefene, naloxone); a serotonin antagonist (ondansetron); an antidepressant (sertraline) and a sesame oil preparation of Δ-9-tetrahydrocannabinol (dronabinol), are medications that have been tried (Bergasa, 2004).

There are both animal and human studies that show involvement of the central nervous system in the pathogenesis of cholestasis-induced itch. Increased opioidergic activity has been implicated in itch. Bergasa et al. (1992) showed down regulation of central mu opioid receptors in a rat model of cholestasis. Additionally, an increase in plasma enkephalin levels (Swain et al., 1992) and an increase in expression of endogenous opioid peptides in liver (Bergasa et al., 1995) have been demonstrated in an animal model of cholestasis. Rats with cholestasis display naloxone-reversible antinociception (Bergasa et al., 1994). When plasma from humans with cholestatic pruritus is injected into the dorsal horn of monkeys, the animals scratch their faces within 10 min. This behavior was alleviated by naloxone. However, plasma from a non-pruritic patient did not induce facial scratching (Bergasa et al., 1993). Also, systemically given naltrexone and nalmefene relieved pruritus and caused an opioid-like withdrawal syndrome in some patients with cholestasis (Jones et al., 2002). Morphine administered intrathecally or subcutaneously induces facial scratching in monkeys (Ko and Naughton, 2000; Ko et al., 2003). Of potential clinical interest are reports that nalfurafine, a selective kappa-opioid receptor agonist, inhibits scratching in mice and monkeys (Togashi et al., 2002; Ko et al., 2003; Inan and Cowan, 2004). This compound has been designated as an orphan drug for uremic pruritus by the European Organization for Rare Diseases (Scrip, 2003).

In view of these findings, we investigated whether both central and peripheral kappa and delta opioid systems, like

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the mu system, are altered in cholestasis. Changes in opioid systems, secondary to cholestasis, may be responsible for the pruritus of cholestasis. For our aims, first we employed in situ agonist-stimulated [^{35}S]GTPγS binding to measure opioid receptor-stimulated activation of G-proteins in response to cholestasis, secondary to common bile duct ligation in rats. Second, we measured serum endogenous opioid levels. Third, central endogenous opioid levels were measured in dialysate samples after microdialysis applied to the dorsal hypothalamus.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (Ace Laboratories, Boyertown, PA) weighing 200–250 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.

2.2. Rat model of cholestasis

There were two experimental groups: sham-operated control rats that underwent a laparatomy with the bile duct left in situ after its manipulation with forceps and bile duct ligated animals that underwent ligation of the common bile duct under general anesthesia (mixture of ketamine HCl 80 mg/kg and acepromazine maleate 0.2 mg/kg, administered i.p.). Cholestasis was overt by 5 days. Bile duct ligated animals had noticeable jaundice (ear skin and more yellowish urine). They looked sick and did not gain weight like control animals. Since we used a standard animal model of cholestasis, we did not check serum and urine bilirubin levels or conduct liver function tests. All experiments took place on the fifth day after surgery. The microdialysis procedure was applied before bile duct ligation, then again 5 days after bile duct ligation, on the same animals.

2.3. In situ [^{35}S]GTPγS binding

All rats (n=8–10) were decapitated after brief exposure to carbon dioxide gas. Brains were quickly removed and flash-frozen in −30 °C isopentane. Prefrontal cortex, caudate putamen, nucleus accumbens core and shell, substantia nigra reticularis and compacta, hippocampus, hypothalamus, thalamus and ventral tegmental area were investigated. Twenty micron cryostat sections were thaw-mounted onto Plus slides (Fisher Scientific, NJ) and stored at −80 °C. The protocol was adapted from Sim et al. (1995) and Schroeder et al. (2003). Sim and Childers (1997) used U50,488H to stimulate kappa-opioid receptors but Schroeder et al. (2003) used dynorphin A instead of U50,488H. Dynorphin A was chosen because of its greater affinity for kappa$_2$ receptors that are more abundant in rat brain. Slides were incubated in assay buffer (50 mM Tris HCl, 3 mM $MgCl_2$, 0.2 mM EGTA and 100 mM NaCl, pH 7.4) for 10 min at 25 °C. Slides were incubated in assay buffer+2 mM GDP (Sigma, MO) for 15 min, then incubated for 2 h in coupling jars in one of the following conditions: (a) basal binding was assessed by incubating slides in assay buffer+0.04 nM [^{35}S]GTPγS (Amersham, IL)+2 mM GDP; (b) total binding was determined by incubating in assay buffer+0.04 nM [^{35}S]GTPγS and (c) non-specific binding slides were incubated in assay buffer+0.04 nM [^{35}S]GTPγS+2 mM GDP+10 μM GTPγS (Sigma, MO). Slides, processed for agonist-stimulated binding, were incubated in assay buffer+0.04 nM [^{35}S]GTPγS+2 mM GDP+the following concentrations of opioid agonist: mu opioid agonist: 5 μM DAMGO [D-Ala^2, *N*-Me-Phe^4, Gly-ol^5]enkephalin (Sigma, MO); delta opioid agonist: 10 μM DPDPE [D-Penicillamine^2, D-Penicillamine^5]enkephalin (Sigma, MO); kappa opioid agonist: 1.0 μM dynorphin A (Sigma, MO). Following 2 h incubation, sections were washed in ice-cold assay buffer twice for 2 min followed by a wash in ice-cold double-distilled water. Slides were dried under cold air and exposed to [^{35}S] sensitive film (βmax Hyperfilm; Amersham, Arlington Heights, IL) together with [^{14}C] standards (Amersham, IL) for 3 days. Films were analyzed for optical density using an AISTM/C Analytic Imaging System (Imaging Research, St. Catherines, Ontario, Canada) and quantified using [^{14}C] standards. For each brain area examined, agonist-stimulated activity was calculated by first subtracting the optical density of nonspecific sections from the optical density of both agonist-stimulated and basal binding sections. Agonist-stimulated activity was expressed as a percentage of basal [^{35}S]GTPγS binding.

2.4. Measurement of serum endogenous opioid levels

Blood (7–8 ml) was drawn by intracardiac injection under light anesthesia (4% isoflurane). Samples (n=8–10) were collected on to chilled glass tubes and left in an ice-filled bucket for 3–4 h. Serum from every animal was divided into three 100 μl samples, added to ice-chilled eppendorf tubes, and kept at −80 °C until the experimental day. Serum levels of endomorphin-2 for mu, leu-enkephalin for delta and dynorphin A for kappa were detected using specific ELISA kits (Phoenix Peptide, CA). Measurements were conducted on duplicate samples.

2.5. Microdialysis

The dorsal hypothalamus was chosen for the microdialysis experiment to measure endogeneous opioid levels in view of our results from [^{35}S]GTPγS binding. Guide cannulas (CMA/12, CMA/Microdialysis, Chelmford, MA) were implanted stereotaxically in each rat skull (n=6) (right side from bregma AP-2.3 mm, L 0.25 mm, and V 7.75 mm) and fixed with dental cement (CMA/Microdialysis, MA) under general anesthesia. Two days after surgery, probes were lowered into the cannulas and animals were placed in observation cages and continuously perfused with artificial cerebrospinal fluid (NaCl 147 mM, KCl 2.7 mM, $CaCl_2$ 1.2 mM, $MgCl_2$ 0.85 mM) (Harvard pump, 2 μl/min). One h later, collection of dialysate commenced. Samples were collected into chilled vials every 30 min and placed in an ice-filled bucket for 5 h. Samples from every animal were divided into three 100 μl aliquots and frozen at −80 °C until measurement of endogenous opioid levels. At the end of the experiment, rats were anesthetized with a mixture of ketamine and acepromazine (80 and 0.2 mg/kg, respectively, i.p.) and cannulas removed. The left side of the skull was stereotaxically implanted as described above and the bile duct ligation procedure was conducted. Five days after surgery, another microdialysis experiment was completed as described above. Dialysate dynorphin A, leu-enkephalin and endomorphin-1 levels

were detected using specific ELISA kits. Duplicate samples were obtained. After euthanasia with carbon dioxide, brains were removed and kept at −80 °C. Sections were sliced with a cryostat and stained with 0.1% cresyl violet. Probe location was confirmed microscopically.

2.6. Statistical analysis

Data are represented as mean±S.E.M. The paired student *t*-test was used for statistical analysis and $P<0.05$ accepted as significant.

3. Results

3.1. [^{35}S]GTPγS binding

[^{35}S]GTPγS autoradiography was used to evaluate mu, delta and kappa opioid receptor activation of G-proteins in the prefrontal cortex, caudate putamen, nucleus accumbens core and shell, substantia nigra reticulata and compacta, hippocampus, hypothalamus, thalamus, and ventral tegmental area of rats. Basal binding values for both sham and bile duct ligated groups were similar (418±29 and 408±22 nCi/g, respectively).There was no difference in DAMGO (mu)-stimulated [^{35}S]GTPγS binding between the two groups of rats for all regions investigated. DPDPE (delta)-stimulated [^{35}S]GTPγS binding showed decreased activity in the dorsal hypothalamus of rats with cholestasis compared to sham-operated controls (129±4% and 175±5% increase from basal binding, respectively, $P<0.05$). Also in the hypothalamus, rats with cholestasis demonstrated less dynorphin A (kappa) stimulated [^{35}S]GTPγS binding activity compared to controls (151±9% and 215±8% increase from basal binding, respectively, $P<0.05$). There was no difference between the two groups of rats for both DPDPE- and dynorphin A-stimulated binding in the other brain areas studied (Fig. 1).

3.2. Serum endogenous opioid levels

Endomorphin-2 (mu) and leu-enkephalin (delta) levels were similar in rats with cholestasis and in control rats. Endomorphin-2 levels were 2.63±0.46 ng/ml in the bile duct ligated group and

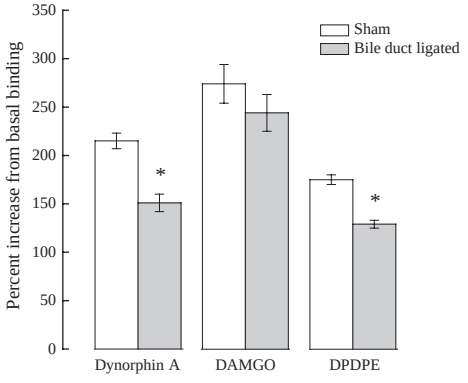

Fig. 1. Dynorphin A-, DAMGO- and DPDPE-stimulated [^{35}S]GTPγS binding in the dorsal hypothalamic area of bile duct ligated and sham-operated rats. Data are expressed as percent increase from basal [^{35}S]GTPγS binding±S.E.M.; *$P<0.05$.

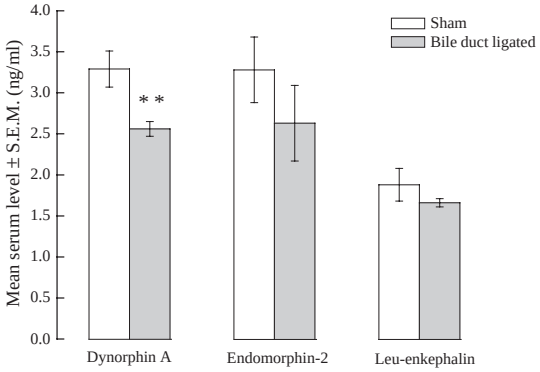

Fig. 2. Serum endomorphin-2 and leu-enkephalin levels are similar in bile duct ligated and sham-operated rats. Serum dynorphin A levels are reduced in bile duct ligated rats compared to sham-operated controls. Data are expressed as mean±S.E.M.; **$P<0.01$.

3.28±0.40 ng/ml in the control group. Leu-enkephalin levels were 1.88±0.20 and 1.66±0.05 ng/ml for bile duct ligated animals and controls, respectively. Dynorphin A levels were significantly less in rats with cholestasis compared to sham-operated controls (2.56±0.09 and 3.29±0.22 ng/ml, respectively, $P=0.008$) (Fig. 2).

3.3. Dialysate endogenous opioid levels

Endogenous opioid levels were detected in the same animal before and after bile duct ligation. Endomorphin-1 (mu), leu-enkephalin and dynorphin A levels were not significantly different due to bile duct ligation. Endomorphin-1 levels were 0.22±0.04 and 0.32±0.07 ng/ml, respectively, before and after the disease. Leu-enkephalin and dynorphin A levels were detected as 0.40±0.03 and 0.39±0.04 ng/ml and 0.15±0.01 and 0.18±0.03 ng/ml, respectively (data not shown).

4. Discussion

We used agonist-stimulated [^{35}S]GTPγS binding autoradiography to investigate changes in mu, delta and kappa opioid systems due to cholestasis, secondary to bile duct ligation in rats. This method visualizes agonist-activated receptors by labeling receptor-coupled G-proteins with a radiolabeled analogue of GTP. Information is provided for both the first step in intracellular signal transduction as well as anatomical distribution of receptors. The procedure is particularly useful for examining coupling efficiency in animal models of behavior or disease and following drug administration to experimental animals. Desensitization can be detected as loss in agonist activity determined as reduced [^{35}S]GTPγS binding (Sovago et al., 2001; Harrison and Traynor, 2003). This method is also employed in humans for postmortem studies to investigate changes in receptor level due to diseases such as Alzheimer's (Ladner et al., 1995), Huntington's (De Keyser et al., 1989) and alcohol dependence (Jope et al., 1998).

We found that both kappa and delta agonist-stimulated [^{35}S]GTPγS binding is decreased in the dorsal hypothalamus area in rats with cholestasis compared to sham-

operated controls. There was no difference between the two groups of rats for mu agonist-stimulated [^{35}S]GTPγS binding in brain areas that we investigated (Fig. 1). This decreased binding activity in kappa and delta opioid receptors might be due to desensitization of receptors secondary to cholestasis. To find out the underlying mechanism of decreased binding activity we checked serum endogenous opioid levels and also conducted microdialysis in the dorsal hypothalamus to determine regional endogenous opioid levels in the brain. Serum endomorphin-2 (mu) and leu-enkephalin (delta) levels were similar in rats with cholestasis and in sham-operated controls. Critically, however, there was a significant difference for serum dynorphin A (kappa) levels between the two groups of rats. Rats with cholestasis had lower dynorphin A levels compared to controls (Fig. 2). We employed microdialysis in the same animal, before and after bile duct ligation, to investigate secondary changes due to cholestasis. Our microdialysis results revealed that dialysate endogenous opioid levels for the three opioid receptors were similar before and after cholestasis. If serum levels of dynorphin A and leu-enkephalin had been higher in cholestatic rats compared to controls, and an increase of regional dynorphin A and leu-enkephalin levels was detected due to cholestasis, we could contend that decreased [^{35}S]GTPγS binding activity is due to receptor desensitization. Decreased binding activity with no change in the regional endogenous opioid levels can result from decreased receptor number, altered expression and functional state of receptors or decreased level of G-proteins. Agonist-stimulated [^{35}S]GTPγS binding autoradiography does not detect receptor number. Expression of G-proteins and the functional state of receptors can be altered due to physiological changes such as development and aging during the life span of organisms (Sovago et al., 2001). Changes in G-protein levels in some diseases also have been detected in several human post-mortem studies. Decreased levels of $G\alpha_i$ in temporal cortex and angular gyrus; decreased G_s stimulated adenyly cyclase activity in hippocampus, temporal cortex and angular gyrus; decreased muscarinic-M_2 receptor agonist stimulated [^{35}S]GTPγS binding and GTP hydrolysis in several cortical regions have all been reported in Alzheimer's disease. Also, Huntington's disease, alcohol dependence and bipolar depression can be given as examples for altered G-protein levels in the pathogenesis of disease (Sovago et al., 2001). In our case, to establish the reason for decreased [^{35}S]GTPγS binding, additional studies such as measurement of receptor number and G-protein levels needed to have been done.

Despite decreased delta-stimulated [^{35}S]GTPγS binding, the serum endogenous delta opioid level was similar between the two groups. The kappa system was associated with both decreased binding and serum levels in our study. It has been postulated that an imbalance between mu and kappa opioid receptors might contribute towards itch in chronic renal failure (Twycross et al., 2003). Our results showed no increase in mu activity but, rather, decreased kappa activity in cholestatic rats. We assume that this decreased kappa activity results in a relative increase in mu activity and subsequently an imbalance between these systems. Pruritus in cholestasis may be a consequence of this imbalance. Nalfurafine hydrochloride (previously known as TRK-820), a kappa opioid receptor agonist, has been evaluated for relieving itch due to chronic renal failure in Europe. Antipruritic activity for nalfurafine has been reported against substance P-induced scratching in mice (Togashi et al., 2002), intrathecal and intravenous administered morphine-induced scratching in monkeys (Ko et al., 2003; Wakasa et al., 2004), compound 48/80-induced scratching in mice (Wang et al., 2005) and chloroquine-induced scratching in mice (Inan and Cowan, 2004). It has been suggested that nalbuphine (which combines mu receptor antagonist and kappa receptor agonist properties) might be superior to opioid antagonists for treating itch (Greaves and Khalifa, 2004). Nalfurafine may also be effective against cholestatic pruritus.

We are the first to investigate the involvement of not just mu, but also kappa and delta opioid receptors, in an animal model of cholestasis. In conclusion, we have shown decreased kappa (but not mu) opioid receptor activity due to cholestasis. This decreased kappa activity may result in a relative increase in mu opioid receptor related endogenous opioid levels by causing an imbalance between mu and kappa opioid systems. We hypothesize that a relative increase in mu (pruritic) activity, along with decreased kappa (antipruritic) activity, contributes towards the pruritus of cholestasis. Decreased kappa activity can be reversed by giving a kappa agonist and the balance between mu and kappa systems may be restored. Despite the absence of definitive data at present, we nevertheless speculate that nalfurafine may be a suitable candidate drug for treating patients with cholestatic pruritus.

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