LYPRINOL® : ANTI-INFLAMMATORY AND UTERINE-RELAXANT ACTIVITIES IN RATS, WITH SPECIAL REFERENCE TO A MODEL FOR DYSMENORRHOEA

Summary

Lyprinol exhibits anti-inflammatory activity distinct from that of most NSAIDs, controlling chronic but not acute inflammation. Unlike COX-1 inhibitors (aspirin, meclofenamic acid) it is not gastro-toxic. Preadolescent rats with Lyprinol can modify both (i) the spontaneous and (ii) the oxytocin-induced contractions of the uterus. In humans there is anecdotal evidence that Lyprinol can relieve dysmenorrhea.

This report explores the concept that the uterotrophic actions of Lyprinol are conditioned by:
• the intrinsic profile of estrogenic hormones and progestagens and,
• certain extrinsic stimuli.

Evidence from in vitro studies indicates that Lyprinol is not a smooth muscle relaxant and that its uterotrophic mechanism is not that of a cyclo-oxygenase inhibitor, but may mimic that of a leukotriene receptor antagonist.

Key-words: Lyprinol - Dysmenorrhea - Anti-inflammatory - Anti-leukotriene.

INTRODUCTION

The evolution and development of Lyprinol for the treatment of arthritis has been discussed elsewhere (1, 2). Clinical and experimental studies have clearly indicated both its beneficial effects upon arthritic inflammation in rats, dogs and humans and general safety.

Mechanistic studies conducted in vitro (1, 3) indicated that Lyprinol might regulate enzymatic oxygenation of unsaturated fatty acids in vivo by inhibiting 5-, 12- and perhaps other lipooxygenases (LO). Since arachidonate-derived leukotrienes and lipoxins can also promote inflammation in extra-articular tissues, it was suggested that Lyprinol might also help control some of the symptoms of asthma, inflammatory bowel disease, etc. There is now a considerable body of anecdotal evidence to support this conjecture.
Dysmenorrhea is another painful condition associated with increased levels of prostaglandins and/or lipoygenase products. Fifty percent of menstruating women suffer primary dysmenorrhea (painful menstruation) at significant economic and social cost. In the United States it has been estimated that 600 million work hours are lost due to dysmenorrhea each year (5). Primary dysmenorrhea is not associated with any pathology. Prostaglandins and the leukotrienes are physiological mediators of normal uterine contraction (6), but in some women their production exceeds physiological requirements and clinical signs of dysmenorrhea then develop (7).

As part of its normal physiological function, the uterus contracts rhythmically and the force and frequency of these contractions are regulated, in the first instance, by the sex hormones. Oxytocin, a hormone synthesized in the pituitary that stimulates strong uterine contraction, acts to increase the local release of eicosanoids (6, 8, 9). There is a relationship between the stage of the sexual cycle and the sensitivity of the uterus to oxytocin (10).

Elevated concentrations of all classes of eicosanoids (prostacyclins, leukotrienes, platelet activating factor and HETEs) have been identified in women suffering dysmenorrhea (7, 11, 12). These mediators increase the force of uterine contraction (cramp), constrict blood vessels with resultant anoxia of tissues (pain) and then sensitizes pain receptors in pelvic nerve terminals to other pain-inducing chemicals and physical stimuli (13). The eicosanoids enter the circulation and cause general malaise with diarrhea, headache, dizziness and nausea. In approximately 80% of women with dysmenorrhea the symptoms are relieved effectively by treatment with one of the NSAIDs that inhibit the cyclooxygenase enzymes that synthesize prostaglandins. In approximately 20% of primary dysmenorrhea patients, NSAIDs do not give satisfactory relief (5, 14).

Dysmenorrhea as a clinical entity only occurs in humans because only primates menstruate. Animal models of these conditions can only allude to likely changes in the human uterus. To evaluate potential therapeutic agents for the treatment of dysmenorrhea an in vitro model was developed using the uteri removed from ovariectomized rats that were pre-treated with sex hormones and also with and without Lyprinol.

**METHODS**

- **Anti-inflammatory assays** (1): Lyprinol and other marine lipids were evaluated in a model of chronic systemic inflammation, namely the adjuvant-induced polyarthritis in rats (4). At first signs of disease onset (day 10 post-adjuvant) animals were dosed orally once daily for 4 days. Signs of disease were scored on days 10, 14, and 17; the latter assessment giving a measure of the rebound in symptoms following cessation of dosing on day 13. Products that showed little/no activity in this therapeutic assay were also tested for possible prophylactic activity by dosing rats orally for at least 15 days, beginning one day before inoculating the adjuvant (day 0).

Acute anti-inflammatory activity was assessed using the standard carrageenan paw edema assay, routinely used to evaluate Cox-1 inhibitors for aspirin-like activity,

Lipids were administered orally both as freshly prepared aqueous dispersions prepared with 0.04% Tween-20 (10 ml/kg) and also as solutions in olive oil (1 ml/kg).

- **Gastro toxicity assay**: this was conducted in rats with an imposed disease stress that sensitizes the gastric mucosa to the noxious effects of aspirin and other NSAIDs. Untreated animals with adjuvant arthritis (day 15 post-adjuvant), or animals pre-inflamed by a tail base injection of 0.1 ml 1% acetic acid 5 days previously, were fasted overnight and then dosed with the test lipids. After 3 hours their stomachs were excised, rinsed with saline, and examined for macroscopic hemorrhagic lesions.

- **Ex vivo Dysmenorrhea model**: female Wistar rats approximately 6 weeks old (n = 18) were ovariectomized and allowed to recover for at least 7 days before hormone treatment was given. To mimic the effects of changing hormone levels during the sexual cycle, estrogen 10μg/kg (Oestradiol benzoate, Illum, Australia), methyl progesterone acetate 25μg/kg (MPA, Illum, Australia), estrogen 10mg/kg plus MPA 25μg/kg or olive oil vehicle was injected subcutaneously into the ovariectomized rats. No attempt was made to reproduce physiological stages of the rat estrus cycle. Rats were killed 5 days later and the uterus removed, divided into 4 segments and placed in organ baths. The tissue was maintained in Krebs's solution at 37°C and aerated with 5% CO₂ in air. The force and frequency of longitudinal contractions was measured with a strain gauge and the data captured on a computerized recording system (Moc Lab). The area under the curve was measured to determine uterine work (mN·sec).

The oily Lyprinol was not water-soluble so it could not be added directly to the organ bath preparations. Lyprinol (150 μg/kg) was administered to the conscious rats by stomach tube on 3 occasions at 24-
hour intervals, the last being 2 hours before euthanasia. A group of hormone-treated rats without Lyprinol was also included in each experiment.

When uteri were placed in the organ bath they demonstrated spontaneous contractions, the magnitude depending on the hormone pre-treatment. Oxytocin (1 μU - 1 μU) was then added to the baths to induce contractions of greater force and frequency, measured as increased uterine work.

After approximately 1 hour in the organ bath the contractile effects of the Lyprinol pre-treatment disappeared and control measurements of spontaneous activity and induced activity were recorded. Indomethacin 1 μM was then added to the bath and the spontaneous activity was measured. The oxytocin challenge was repeated.

**RESULTS**

- **Anti-inflammatory activity** (table 1): Lyprinol was unique among the marine oils tested in suppressing the development of arthritic inflammation. Other OTC marine products sold as nutraceuticals to help relieve pain and symptoms of arthritis were generally ineffective.

Two leukotriene receptor antagonists (LRA) were not as effective as Lyprinol in treating this model polyarthritis. The doses (mg/kg) of these LRAs were the human daily dose (for a 75 kg adult) and should have proved more than ample to block LT receptors in the rat.

- **Gastro toxicity** (table 2): even at massive doses, Lyprinol was not gastro-irritant in contrast to many NSAIDs used to treat dysmenorrhea.

- **Uterotrophic properties of Lyprinol** (tables 3 and 4): in a preliminary study it was showed that one Lyprinol treatment 24 hours before excising the uterus had no effect and that treating rats for 7 consecutive days did not increase the effect of Lyprinol beyond that of a 3 day pre-treatment. Lyprinol depressed ex vivo contractions in uteri from rats pre-treated with the oil vehicle and estraogen. It had no detectable effects on the spontaneous contractile activity of uteri from rats pre-treated with progesterone or the combination of estrogen plus prostagen. Lyprinol pre-treatment reduced the uterine work induced by oxytocin (1 μU - 1 μU) in rats also pre-treated with estrogen alone or progestagen alone, but had no detectable effect on uterine contractility from rats pre-treated with the oil vehicle. Lyprinol caused a rise in the uterine work in animals pre-treated with the hormone combination (table 3).

- **The injection of vehicle had no effect**: There was no paradox inasmuch as Lyprinol treatment tended to increase spontaneous uterine contraction activity in animals given the estrogen plus progestagen yet reduced the magnitude of the oxytocin-induced contractions (table 3).

- A similar paradox was evident when a leukotriene receptor antagonist (Montelukast 10 nM) was included in the organ bath with uteri from animals also pre-treated with the estrogen plus progestagen combination (which may mimic oral contraceptives).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose/kg &amp; regimen</th>
<th>Mean percentage inhibition</th>
<th>Incidence arthritis</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Olive Oil)</td>
<td>2 ml P/1/</td>
<td>0</td>
<td>37/40</td>
</tr>
<tr>
<td>GI Musel lipid (Lyprinol)</td>
<td>20 mg P</td>
<td>01</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>16 mg T</td>
<td>96</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6/22</td>
</tr>
<tr>
<td>Cod liver Oil (2 Seas, UK)</td>
<td>2 ml P</td>
<td>07</td>
<td>12</td>
</tr>
<tr>
<td>Cod liver Oil (Twin Labs US)</td>
<td>2 ml P</td>
<td>04</td>
<td>04</td>
</tr>
<tr>
<td>Norw.Salmon (Carlton US)</td>
<td>2 ml P</td>
<td>38</td>
<td>25</td>
</tr>
<tr>
<td>Pikosol (ibko AS, Nk)</td>
<td>2 ml P (-114)</td>
<td>(-11)</td>
<td>5/5</td>
</tr>
<tr>
<td>Mutton bird (Yolla, Aust)</td>
<td>2 ml P (-27)</td>
<td>(-27)</td>
<td>5/5</td>
</tr>
<tr>
<td>LRA Zafirukast (Zeneca)</td>
<td>20 mg T</td>
<td>33</td>
<td>19</td>
</tr>
<tr>
<td>LRA Montelukast (Merck)</td>
<td>10 mg T</td>
<td>71</td>
<td>38</td>
</tr>
</tbody>
</table>

Table 1: Anti-inflammatory activity of Lyprinol, some OTC marine oils, and 2 leukotriene receptor antagonists (LRA) in rats developing polyarthritis.

Footnotes to table 1:
1. Relative to controls dosed orally with Spanish olive oil (2 ml/kg).
2. Dose schedule: P = prophylactic for 13 days; T = therapeutic for 4 days (days 10 through 13).
3. As measured with a strain gauge micrometer.
4. Assessed by 2 observers on a scale of 0-5, incorporating clinical signs, weight changes etc. as well as paw/tail swelling.
5. On day 14.
6. Lyprinol = 1 part mussel lipid and 2 parts olive oil (w/w).
Table 2: Gastro toxicity of Lyrpinol and some NSAIDs used to treat dysmenorrhea.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body Wei.</th>
<th>Gastric Lesion Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyrpinol</td>
<td>03</td>
<td>0</td>
</tr>
<tr>
<td>Aspirin</td>
<td>74</td>
<td>52</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>50</td>
<td>49</td>
</tr>
<tr>
<td>Naproxen</td>
<td>23</td>
<td>38</td>
</tr>
<tr>
<td>Na mefenamate</td>
<td>40</td>
<td>70</td>
</tr>
</tbody>
</table>

* The lesion index determined in groups of 3 rats with either (i) pre-established arthritis or (ii) alkyl alcohol-induced inflammation, fasted overnight.

Table 3: Effects of Lyrpinol administration in vivo on uterine work, as measured in vitro.

<table>
<thead>
<tr>
<th>Hormonal pre-treatment</th>
<th>Spontaneous activity (mN/sec)</th>
<th>Spontaneous activity after Lyrpinol (mN/sec)</th>
<th>Oxytocin-induced activity (mN/sec)</th>
<th>Oxytocin-induced activity after Lyrpinol (mN/m/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrogen (E)</td>
<td>6.6 ± 2.5</td>
<td>2.6 ± 1.3</td>
<td>14.1 ± 1.8</td>
<td>9.2 ± 3.0*</td>
</tr>
<tr>
<td>Progestagen (P)</td>
<td>10.0 ± 0.9</td>
<td>6.2 ± 1.4</td>
<td>6.2 ± 2.1</td>
<td>6.1 ± 2.5</td>
</tr>
<tr>
<td>E + P</td>
<td>9.0 ± 1.5</td>
<td>12.0 ± 1.8</td>
<td>45.1 ± 9.9</td>
<td>30.8 ± 8.7**</td>
</tr>
</tbody>
</table>

Table 4: Effects of indomethacin in vitro on uterine work. Data from groups of 3-5 animals; all data significant (P > 0.05).

<table>
<thead>
<tr>
<th>Hormone treatment</th>
<th>Uterine work - Indomethacin 10^-5 M % Control (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrogen (E)</td>
<td>7.2 ± 1.9</td>
</tr>
<tr>
<td>Progestagen (P)</td>
<td>31.9 ± 6.7</td>
</tr>
<tr>
<td>E + P</td>
<td>25.0 ± 15.5</td>
</tr>
</tbody>
</table>

Indomethacin (10 µM) reduced uterine work induced by oxytocin (1mM/ml) in animals pre-treated with the vehicle, progestagen and estrogen & progestagen combination. It had no effect on rats pre-treated with estrogen only (table 4).

**Discussion**

Currently it is a common practice to treat women with dysmenorrhea, who are refractory to conventional NSAID therapy (aspirin, mefenamate, ibuprofen, ketoprofen, nimesulide etc) with the contraceptive pill.

The experiments reported here further support the important role of sex hormones in regulating the profile of endogenous contractile mediators within the uterus. The NSAID indomethacin only reduced uterine contraction after some hormone treatments, suggesting prostaglandins are not necessarily the dominant mediators of contraction. The data presented here indicates that Lyrpinol is not acting as a relaxant of the myometrium. Rather exceptionally it still allowed increased spontaneous activity in uteri taken from rats pre-treated with estrogen and progesterone. It is hypothesised that Lyrpinol modulates activity in the arachidonic acid cascade in the uterus leading to an alteration in the profile of contractile mediators. These data indicate that Lyrpinol is certainly not acting by the same mechanism as indomethacin (which specifically inhibits cyclo-oxygenase).

Uterine contraction is only one of several physiological events triggered by these mediators. They also induce local vasoconstriction, the primary cause of pain in dysmenorrhea. This in vivo experimentation did not measure vascular spasm.

Anecdotal evidence from patients taking Lyrpinol for arthritis suggests that the severity of their dysmenorrhea is concordantly reduced. The fact that Lyrpinol relaxed the uteri pre-treated with estrogen, when indomethacin did not, suggests that Lyrpinol either alone, or perhaps in combination with an NSAID may provide a useful treatment for dysmenorrhea that is refractory to NSAIDs alone. Further insights will be gained if the Lyrpinol-derived uterus relaxant can be isolated in a more water-soluble form, so that it can be added directly to organ baths for concentration-response studies in vivo.

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