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Novel anti-inflammatory mechanism of action of Lyprinol® in the AIA rat model

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TITOLO

Un nuovo meccanismo anti-inflammatorio dell'azione del Lyprinol® nel modello di ratto dell'AIA

KEY WORDS

Lyprinol®, *Perna canaliculus*, adjuvant-induced arthritis, pain, cytokines, proteomics

PAROLE CHIAVE

Lyprinol®, *Perna canaliculus*, artrite indotta da adiuvante, dolore, citochine, proteomica

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Summary

Lyprinol® is the oil of *Perna canaliculus*, the green-lipped mussel of New Zealand, extracted by liquid CO₂ (super-critical extraction); it is free of protein and carbohydrate. A large number of studies have been published on its composition, complex mode of action, activity in animal models, and efficacy in controlling osteoarthritis and moderate asthma in patients; one seminal and critical study was published in this very journal (5). This review article summarizes and presents to the clinicians the results of a comprehensive evaluation of the mechanism of action of Lyprinol in the accepted rat model of AIA. The study was conducted in ABCT (HKPU) under the direction of Samuel CL Lo, PhD. Lyprinol® was shown to: 1. Control pain in the animals; 2. Modulate cytokines with a decrease in cytokines associated with inflammation, and an increase in IL-10; 3. Decrease the synthesis of some proteins associated with inflammation, while increasing the synthesis of MDH. This use of proteomics casts a new light on the anti-inflammatory mechanism of action of Lyprinol®, and differentiates it positively from other omega-3 PUFAs-rich products.

Riassunto

Lyprinol® è l'olio di *Perna canaliculus*, una varietà di mitile della Nuova Zelanda, estratto con CO₂ liquido (estrazione supercritica). Sono assenti proteine e carboidrati. Sono stati pubblicati un gran numero di studi sulla sua composizione, il complesso meccanismo d'azione, l'attività in modelli animali e l'efficacia sui pazienti nel controllo dell'osteoartrite e dell'asma moderato; è stato pubblicato su questa stessa rivista (5) uno studio critico. Questa review riassume e presenta ai medici i risultati di una valutazione globale del meccanismo d'azione del Lyprinol® nel modello di ratto dell'AIA. Lo studio è stato condotto in ABCT (HKPU) sotto la direzione del Prof. Samuel CL Lo. È stato dimostrato il ruolo del Lyprinol®: 1) nel controllo del dolore negli animali; 2) nella modulazione delle citochine con una diminuzione di quelle associate all'infiammazione, e un aumento della IL-10; 3) nella diminuzione della sintesi di alcune proteine associate all'infiammazione, mentre aumenta la sintesi di MDH. L'uso della proteomica getta una nuova luce sul meccanismo d'azione anti-infiammatorio del Lyprinol® e lo differenzia in modo positivo dagli altri prodotti omega-3 ricchi in PUFAs.

Abbreviations:

Adjuvant-induced arthritis: AIA; Department of Applied Biology & Chemical Technology: ABCT; Hong Kong Polytechnic University: HKPU; Interleukin-10: IL-10; Malate dehydrogenase: MDH; Poly-unsaturated fatty acids: PUFAs; Fatty acids: FAs; High pressure liquid chromatography: HPLC.

Introduction

A lipid-rich extract, prepared by supercritical fluid [CO₂] extraction of freeze-dried stabilized New Zealand green-lipped mussel *Perna canaliculus* powder (NZGLM, Lyprinol®), has been shown to have significant anti-inflammatory (AI) activity when given to animals and humans (1-12). According to Murphy et al. (13) and Wolyniak et al. (14), Lyprinol® is a mixture of five main lipid classes including sterol esters, triglycerides, free fatty acids, sterols and polar lipids. The total fatty acid content of the lipid extract was 0.664 g/ml. Fifty-three unsaturated fatty acids (UFAs) were found and 37 were poly-unsaturated fatty acids (PUFAs). Omega-3 PUFAs are the most abundant PUFAs present. Eicosapentaenoic acid (20 carbons and 5 double bonds, shorthand =20:5 omega-3) (EPA) and docosahexaenoic acid (22:6 omega-3) (DHA) are the major omega-3 PUFAs present in Lyprinol. According to Wolyniak et al. (14), another 21 UFAs were detected but their concentrations were

too low for assignment of double-bond positions. Moreover, there were 17 saturated FAs, with 14:0, 16:0, and 18:0 present in the greatest concentration. The 10 n-3 PUFAs detected included 20:5n-3 and 22:6n-3, the two main n-3 FAs; n-3 PUFAs at low concentrations were 18:3, 18:4, 20:3, 20:4, 21:5, 22:5, 24:6, and 28:8. There were 43 UFAs from the n-4, n-5, n-6, n-7, n-8, n-9, n-10, n-1 families, with 16:2n-4, 16:1n-5, 18:1n-5, 18:2n-6, 20:4n-6, 16:1n-7, 20:1n-7, 16:1n-9, 18:1 n-9, and 20:1 n-9 being the most abundant. In total, 91 FAs were detected in the extract of the NZGLM (14).

Although the exact active ingredients that brought about this AI effect are unknown, Wistar and Dark Agouti rats treated p.o. with this lipid extract did not develop adjuvant-induced polyarthritis or collagen (II)-induced auto-allergic arthritis (4). This was achieved with doses inferior to the ones of NSAIDs, and 200 times lower than other seed or fish oils (4). Further, omega-3 PUFA subfractions of this lipid extract inhibited LTB₄ biosynthesis by polynuclear white blood cells *in vitro*, and PGE₂ production by activated macrophages (15). Subfractions of this extract containing natural antioxidants [e.g. carotenoids] also exhibited AI activity (6). In contrast to NSAIDs, Lyprinol® is non-gastrotoxic in disease-stressed rats at 300

mg/kg p.o. (2), and does not affect platelet aggregation in both humans and rats (16).

Clinical studies, either controlled or randomized, have demonstrated that Lyprinol® has very significant AI activity in patients with osteoarthritis (OA) (5, 8, 9, 17), asthma (18), and other inflammatory conditions (11). Therefore, it seems that Lyprinol® is a reproducible, stable source of bioactive lipids with much greater potency than plant/marine oils currently used as nutritional supplements to ameliorate signs of inflammation (4, 11, 19). More importantly, in humans and animal subjects taking Lyprinol®, there are no reported side-effects, even at doses up to 2,500 mg/day in patients.

Materials and methods*Chemicals*

Unless stated otherwise, all chemicals were purchased from Sigma (St. Louis, MO, USA). All chemicals were at least of AR grade. Organic solvents used were at least of HPLC grade.

Induction of inflammation in Sprague-Dawley (SD) rats

Four groups of six six-week-old male SD rats were purchased from the Central Animal Facility (CAF)

of Hong Kong Polytechnic University (HKPU). All the rats were kept and cared under conditions that fully met the requirements of the Procedures for the Care of Laboratory Animals or Animals (Control of Experiments) Regulations Chapter 340 of the Hong Kong SAR government. Ethics approval (ASESC No.04/9) had been obtained from The Animal Subjects Ethics subcommittee of the HKPU. Arthritis was induced in anesthetized animals by administration of adjuvant according to a method previously described (20, 21). Briefly, on day 0, each rat was injected in the paw of the right hind limb with 100 µl of Freund's complete adjuvant containing 10 mg/ml of *Mycobacterium butyricum* (Difco, Livonia, MI, USA). Another six rats without arthritis induction were observed as normal group.

Products/drug tested fed to the treatment and control groups of rats

300 µl (25 mg/kg of rat) of lipid extract of *Perna canaliculus* (Lyprinol®, Pharmalink International Ltd.) was mixed with chow and fed daily to the treatment group. 300 µl of olive oil (Virgin® Bertolli, Italy), and 20 mg/kg per rat of Naproxen were fed as vehicle/negative and positive control, respectively. Normal chow was provided to the normal control group.

Based on this model, we asked three questions:

1. Is Lyprinol an effective pain-controller, and how does it compare to Naproxen, a reference NSAID and pain-killer?
2. Does Lyprinol influence the production and release of cytokines associated with inflammation (both pro-inflammatory and anti-inflammatory)?
3. Are there specific changes in proteomics attributable to Lyprinol?

First Question: Is Lyprinol an effective pain-controller, and how does it compare to Naproxen, a reference NSAID and pain-killer?

Pain Score Measurement

The measurement of pain score was performed according to Hayashida et al. (22). The number of pain-related responses, represented by vocalizations, was recorded during ten flexions of the tarsotibial joints of the adjuvant-injected paw. Results were expressed as the mean number of vocalizations.

Results

Photographic and radiographic analysis of AIA rat paws

Figure 1 shows: the photograph of the right hind paw of rat 14 days after AIA induction (A), and a radiograph (B) 21 days after AIA induction [note the swelling of soft

tissues and the bone deformation around the ankle region]. However, 1 year after the induction of AIA, deformation of joints and lesions are still observable in the olive oil control group (C). Conversely, deformation of joints and lesions are not visible in the Lyprinol® treatment group (D).

Pain score measurement

Pain score measurement is a widely used and reliable method to reflect the effectiveness of different treatments on AIA (22). As shown in figure 2, the NSAID Naproxen effectively maintained the pain score of AIA rats at a relatively low level during the whole course of the experiment. Compared to the ones of the control and olive oil-treated groups, Lyprinol® effectively lowered the pain score of AIA rats from day 4 to day 26 after induction of arthritis. The effect of Lyprinol® was most pronounced between day 4 to day 14, with an effect comparable to that of Naproxen. The effect of Lyprinol® began to wane after day 14, but still maintained the pain score at a significant lowered level when compared to the control and olive oil-treated groups.

These results, published in 2007 in eCAM (23), clearly demonstrated that Lyprinol® is very useful in controlling pain of severe arthritis, especially during the early and intermediate phase.

Figure 1 - See text for legend (Adapted from Ref. 23, with permission from the authors)



(A)



(B)

(C) With olive oil only



(D) With Lyprinol



Second Question: Does Lyprinol influence the production and release of cytokines associated with inflammation (both pro-inflammatory and anti-inflammatory)?

The splenocyte preparation, cell count and viability staining, and ELISA assays for cytokines were described in detail previously (23, 24).

Results

Levels of pro-inflammatory cytokine interleukin-6 (IL-6)

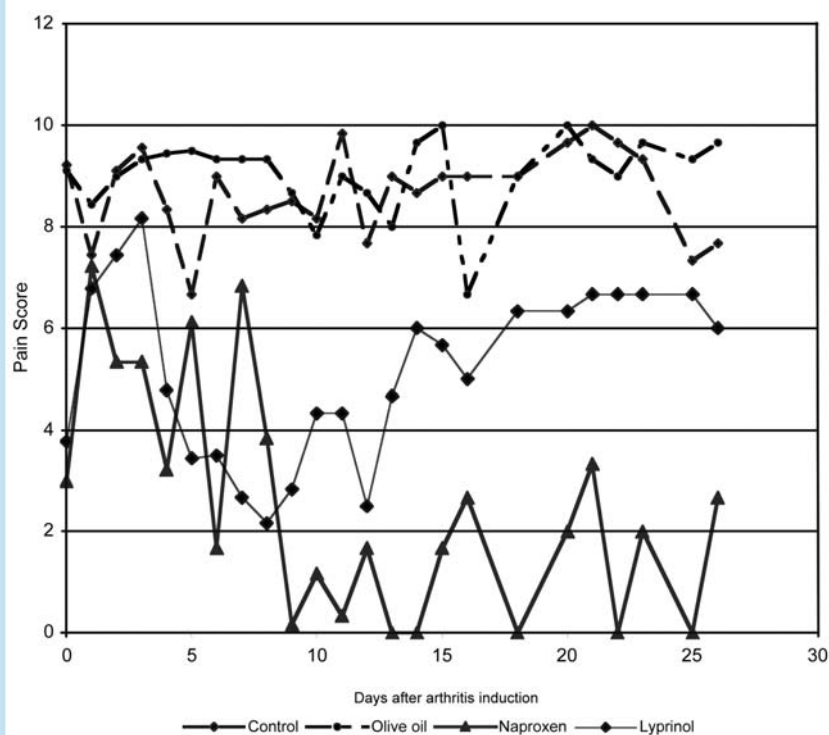
The level of the pro-inflammatory cytokine Interleukin-6 (IL-6) was decreased at day 7. The level of IL-6 in the Lyprinol® group was significantly lower than the one of the control and olive oil groups. The level of IL-6 in the Lyprinol® group is close to the one observed in the NSAID Naproxen group.

These results clearly demonstrated that Lyprinol® can decrease the production of the pro-inflammatory cytokine IL-6 in the early phase of AIA.

Levels of pro-inflammatory cytokine interleukin-1 α (IL-1 α)

The level of IL-1 α at day 7 and especially at day 14 was reduced significantly in the Lyprinol® group when compared to the control and olive oil groups. This effect on IL-1 α production was not seen in rats fed with the NSAID Naproxen.

Figure 2 - Mean pain score measured by ten flexions of the tarsotibial joints of adjuvant-injected paw (n=6), as described in Materials and Methods. Note that Lyprinol effectively controlled pain between day 4 and day 12 when compared to control and olive oil groups (Adapted from Ref. 23, with permission of the authors)



The results demonstrated that Lyprinol can reduce the production of the pro-inflammatory cytokine IL-1 α in AIA rats.

Levels of cytokines tumor necrosis factor- α (TNF- α) and interferon-gamma (IFN- γ)

The levels of TNF- α in the Lyprinol[®] group on day 14 were greatly decreased when compared to those

of the control group. Indeed, the level of TNF- α detected was even lower than the one found in AIA rats treated with the NSAID Naproxen. Besides, the production of another pro-inflammation cytokine, IFN- γ , was also found to be significantly decreased when compared to the control and olive oil-treated groups. Again, the level of IFN- γ in the Lyprinol[®] group was

even lower than the one observed in the AIA rats treated with the NSAID Naproxen.

These results show that Lyprinol[®] can decrease the production of IL-6, TNF- α , IL-1 α and IFN- γ in AIA rats.

Level of anti-inflammatory cytokine interleukin-10 (IL-10)

The anti-inflammatory cytokine Interleukin-10 (IL-10) was found to be increased at day 28 after AIA. Although there is no statistically significant difference between the levels observed in the control group and the Lyprinol[®] group, the increase of interleukin-10 in the Lyprinol[®] group almost reached levels observed in the positive control group (Naproxen).

Third Question: Are there specific changes in proteomics attributable to Lyprinol?

To address this question, our group examined the possible effects of oral administration of Lyprinol on protein expression of splenocytes in rats with AIA. We studied splenocytes (a mixture of large and small lymphocytes including macrophages, B-cells and T-cells, etc. that were usually used to assess cytokines secretion post-challenged by lipopolysaccharides) to see if Lyprinol would induce differential protein expressions in these cells. Protein expression profiles from

splenocytes isolated from AIA rats taking Lyprinol were compared to those isolated from control AIA rats. Any differential protein expression identified would be a lead candidate for follow-up studies on biochemical mechanisms of Lyprinol beyond the level of secretion of cytokines.

The different specific techniques: 2-Dimensional electrophoresis (2DE) of the splenocytes, protein visualization and image analysis, and in-gel tryptic digestion and subsequent protein identification by matrix-assisted-laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) were described and discussed in detail previously (25).

Results

We found that, in splenocytes of AIA rats taking Lyprinol, six proteins were down-regulated while malate dehydrogenase (MDH) was increased. Most of the down-regulated proteins -POMT2, Tdrd 7, TCP, dynactin 2 and PDI- are related to metabolism, while MDH is specifically related to glucose metabolism. If this increase of MDH protein expression, coupled with decreased metabolism, is equated to a decreased level of glucose available for MHC-I activation (which is known in other models), it will provide a logical explanation of the

anti-inflammatory activities of Lyprinol. These results point to a totally new and original mechanism of action of Lyprinol, explaining, at least in part, its amazing anti-inflammatory efficacy at doses much lower than other PUFAs preparations.

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