Novel Functions of Bovine Milk-Derived α-Lactalbumin: Anti-nociceptive and Anti-inflammatory Activity Caused by Inhibiting Cyclooxygenase-2 and Phospholipase A2

Makoto YAMAGUCHI,* Kaori YOSHIDA, and Masayuki UCHIDA

Food Science Institute, Division of Research and Development, Meiji Dairies Corporation; 540 Naruda, Odawara, Kanagawa 250–0862, Japan. Received September 18, 2008; accepted December 6, 2008; published online December 8, 2008

Milk whey proteins contain major components of α-lactalbumin (αLA) and β-lactoglobulin (βLG), and a minor component of lactoferrin (LF). It has been reported that LF reduces noiception and inflammation in various animal models. However, the efficacy of αLA and βLG has not been clarified. This study aimed to assess the efficacy of αLA and βLG in various animal models such as acetic acid-induced writhing, carrageenan-induced paw inflammation, and adjuvant-induced arthritis. Orally administered αLA showed (i) inhibition of writhing induced by acetic acid in mice; (ii) suppression of noiception and inflammation in rat footpads caused by carrageenan in rat; and (iii) therapeutic effects on the development of adjuvant-induced pain and inflammation in rat. In contrast, βLG had no effects in these animal models. To clarify the anti-nociceptive and anti-inflammatory mechanisms of αLA, we examined the levels of interleukin (IL)-6 and prostaglandin (PG)E2 in carrageenan-injected paw exudates. The administration of αLA 1 h before carrageenan injection inhibited the increased formation of IL-6 and PGE2 in paw exudates. Next, we demonstrated in vitro enzyme-inhibition assay; cyclooxygenase (COX), phospholipase A2, and 5-lipoxygenase. αLA inhibited COX and phospholipase A2 activities. Moreover, αLA showed selectivity on COX-2 as compared with COX-1. However, 5-lipoxygenase activity was not affected by αLA. These results suggest that αLA is a safe and useful natural drug for patients that require anti-inflammatory drugs, as αLA is contained in dairy food and is frequently ingested as daily food.

Key words α-lactalbumin; nociception; inflammation; anti-inflammatory drug; cyclooxygenase-2

In recent years, milk constituents have become recognized as functional foods, suggesting that their use has a direct and measurable effect on health outcomes. Milk contains two primary sources of protein, the caseins and whey. After processing, the caseins are the proteins responsible for making curds, while whey remains in an aqueous environment. Whey has been touted as a functional food with a number of health benefits.1) The protein fraction in whey comprises four major protein fractions and six minor protein fractions. The major protein fractions in whey are α-lactalbumin (αLA), β-lactoglobulin (βLG), bovine serum albumin, and immunoglobulins. The biological components of whey demonstrate a range of immune-enhancing properties.2) In addition, whey has the ability to act as an antioxidant,3) antihypertensive,4) antitumor,5) hypolipidemic,6) antiviral,7) antibacterial,8) and chelating agent.9) It is well-known that lactoferrin, the minor component of whey proteins, inhibits production of the inflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 in monocytes. We have confirmed that lactoferrin protects TNF-α production caused by sensitization of hepatic monocytes (kupffer cells) by lipopolisaccaride.9) It has recently been reported that lactoferrin produces analgesia in the thermal, visceral and formalin-evoked noiceptions in rats.10) However, the efficacy of other proteins has not been clarified with regard to analgesic action.

Prostaglandins (PGs) formed by the phospholipase A2 (PLA2) and cyclooxygenase (COX) enzymes are important mediators of nociception and inflammation.11) On the other hand, emerging information has pointed to the role of another arachidonic acid metabolic pathway (the 5-lipoxygenase (5-LO) pathway) in producing and maintaining inflammation.12) There is evidence that COX-2 and 5-LO are co-expressed and up-regulated in a number of inflammatory diseases and that COX-2 as well as 5-LO inhibitors have beneficial effects in inflammatory diseases.13)

The aim of the present study was to assess three points: 1) Can orally administered αLA potentiate an anti-nociceptive response? 2) Can orally administered αLA potentiate an anti-inflammatory response? 3) If so, are COX, PLA2, and 5-LO involved in this potentiation by αLA? In this study, we used acetic acid-induced mouse writhing and for pain, carrageenan-induced rat paw edema for acute inflammation, and adjuvant-induced rat arthritis for chronic inflammation.

MATERIALS AND METHODS

Animals Male ICR mouse weighing from 20 to 25 g and male Wistar rats weighing from 150 to 200 g were obtained from SLC Japan, Inc. (Shizuoka, Japan) and housed in plastic cages for 1 week prior to the commencement of experiments under controlled temperature (21±2°C), humidity (55±15%), and 12-h light/dark cycle, and the light period was from 7:00 to 19:00. Commercially available rat chow and water were provided ad libitum. This study was performed in accordance with “The guidelines of the Meiji Dairies Corporation for the care and use of laboratory animals.”

Acetic Acid-Induced Writhing Model Mouse received an intraperitoneal (i.p.) injection of acetic acid (0.6%) dissolved in saline (0.3 ml/body) that produced typical contractions of the abdominal musculature followed by extension of the hind limbs. Mice were divided into four groups (n=6): 1) vehicle (Control) group; 2) αLA (300 mg/kg)-treated group; 3) βLG (300 mg/kg)-treated group; 4) diclofenac (50 mg/kg)-

* To whom correspondence should be addressed. e-mail: MAKOTO_YAMAGUCHI@MEIJI-MILK.COM © 2009 Pharmaceutical Society of Japan
treated group. A test sample or vehicle (0.9% NaCl) was given orally in a volume of 10 ml/kg through a stainless tube attached to a 1-ml syringe 1 h before acetic acid treatment. The number of writhing motions was determined for 20 min following the acetic acid injection.

Dose Response of αLA on Acetic Acid-Induced Writhing We tested three doses of αLA (30, 100, 300 mg/kg) to determine the most effective dose at exerting physiological activity. αLA solution was given orally 1 h before acetic acid treatment. The number of writhing motions was determined for 20 min following acetic acid injection.

Carrageenan-Induced Inflammation Model Acute inflammation experiments were carried out according to the method described by Winter et al.14) αLA (30, 100, 300 mg/kg), diclofenac (50 mg/kg) or vehicle (0.9% NaCl) was given orally in a volume of 4 ml/kg through a stainless tube attached to a 1-ml syringe. One hour later, 1% carrageenan saline solution (0.1 ml) was injected into the left hind paw under ether anesthesia. The magnitude of the reaction was measured after 3 h (optimal response) and expressed as an increase in paw volume determined by water displacement plethysmometry.

Analgesic Activity by Randall and Selitto Model αLA (30, 100, 300 mg/kg), diclofenac (50 mg/kg) or vehicle (0.9% NaCl) was given orally in a volume of 4 ml/kg through a stainless tube attached to a 1-ml syringe. One hour later, 1% carrageenan saline solution (0.1 ml) was injected into the left hind paw under ether anesthesia. Assessment of pain consisted of measurement of the threshold stimulus for reaction (escape or paw withdrawal) using a weight (maximum limit of 200 g) applied to the pads of hind paws of animals. The threshold for pain sensation was measured before (basal) and 3 h after the intraplantar injection of 1% carrageenan saline solution.

Changes in IL-6 and PGE₂ Concentration in Paw Exudates After the Randall and Selitto test was finished (3 h after carrageenan injection), rats were killed by exsanguination. Then the hind paws were cut at the level of the calcaneus bone and centrifuged at 400 g for 15 min at 4 °C to collect the exudates (edema fluid) for measuring the levels of IL-6 and PGE₂ in paw exudates by using rat enzyme immunoassay kits (GE healthcare Bio-science KK, Tokyo, Japan).

Adjuvant-Induced Arthritis Model The induction of adjuvant-induced arthritis was performed according to the methods described in a previous report.15) On the first day of the experiments (day 0), the rats received a subplantar injection of 100 μl of Freund’s complete adjuvant in the left hind paw under ether anesthesia.

Therapeutic Effects of Orally Administered αLA in Adjuvant Arthritis Rats were divided into four groups (n = 6): 1) vehicle (Control) group; 2) αLA (300 mg/kg)-treated group; 3) βLG (300 mg/kg)-treated group; 4) diclofenac (50 mg/kg)-treated group. Each drug was administered once daily for 3 d, starting at day 14. The hind paw volume was measured by water displacement plethysmometry.

Measurements of Pain Score in Adjuvant Arthritis Rats were divided into four groups (n = 6): 1) vehicle (Control) group; 2) αLA (300 mg/kg)-treated group; 3) βLG (300 mg/kg)-treated group; 4) diclofenac (50 mg/kg)-treated group. The method for measuring pain score (joint hyperalgesia) followed that used in the previous report, with modifications. Each drug was administered at day 16. Measurements of pain-related responses (vocalizations) were performed each day 1 h after drug administration. The number of pain-related responses was determined during ten flexions of the tarsotibial joints of the adjuvant-injected paw.

In Vitro Enzyme Inhibition Assay To assess the mechanisms of αLA as an anti-nociception and anti-inflammation agent, we conducted an in vitro inhibition assay for cyclooxygenase (COX), phospholipase A₂ (PLA₂) and 5-lipoxygenase (5-LO). The analyses of COX, PLA₂, and 5-LO activities were externalized to MDS Pharma Services–Taiwan Ltd. in brief, COX activity was measured by EIA quantitation of PGE₂ produced by SnCl₂ reduction of COX-derived PGH₂. PLA₂ activity was measured by EIA quantitation of [14C]oleate produced by enzymatic conversion of 1-palmitoyl-2-[1-14C]oleoyl-L-3-phosphatidylcholine. 5-LO activity was measured by EIA quantitation of LTB₄ produced by enzymatic conversion of arachidonic acid. Dose-response curves were plotted using nonlinear curve fitting methods and potency (IC₅₀) values calculated for each curve.

Drugs α-Lactalbumin (BioPURE Alphalactalbumin™) and β-lactoglobulin (BioPURE Betalactoglobulin™) were obtained from Davisco Foods International Inc. (Eden Prairie, MN, U.S.A.). Acetic acid, diclofenac, carrageenan, and Freund’s complete adjuvant were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Stock solutions were prepared by dissolving drugs in 0.9% NaCl saline. In the control rats, saline was administered instead of sample solution.

Statistical Analysis All data are expressed as means ± S.E.M. Dunnett’s multiple comparison test (parametric test) or Mann–Whitney’s U test (non-parametric test) were performed. Differences with p < 0.05 were considered statistically significant.

RESULTS

Inhibitive Effects of αLA on Acetic Acid-Induced Writhing After acetic acid (0.6%, 0.3 ml) was injected into the peritoneal cavity, the mouse showed spontaneous writhing (Fig. 1a). Orally administered αLA produced a significant degree of analgesia, from 28.1 ± 1.9 times to 17.2 ± 2.7 times (p < 0.05 vs. controls). βLG, however, had no such effect (29.2 ± 3.5 times). Administration of diclofenac, a Non-Steroidal Anti-Inflammatory Drug (NSAID), significantly decreased spontaneous writhing (5.2 ± 1.3 times, p < 0.01).

αLA dose-dependently and significantly inhibited writhing induced by acetic acid (Fig. 1b). The minimum effective dosage was 300 mg/kg.

Preventive Effects of αLA on Carrageenan-Induced Inflammation to the Hind Paw After carrageenan (1%, 0.1 ml) was injected into the paw, the rats showed the development of edema (increase a paw volume) during the experiment, from 1.14 ± 0.03 ml (0 h) to 1.63 ± 0.10 ml (3 h) in the carrageenan-injected paw (Fig. 2). αLA moderate decreased the edema in the doses tested 30, 100 and 300 mg/kg (1.50 ± 0.10, 1.41 ± 0.09* and 1.34 ± 0.07* ml; * p < 0.05). Orally administered diclofenac significantly decreased the edema (1.31 ± 0.07 ml; p < 0.01).

Analgesic Effects of αLA during the Randall and
Selitto Model  In control rats receiving a sub planter injection of carrageenan, the mean threshold of pain in the inflamed foot 3 h after irritant decreased (47.6 ± 7.5 g compared to 143.9 ± 19.8 g) with the normal non-injected foot. αLA showed moderate analgesic effect in the doses tested 30, 100 and 300 mg/kg (53.9 ± 9.1, 66.6 ± 14.8* and 82.5 ± 10.2** g; *p < 0.05, **p < 0.01) (Fig 3). Orally administered diclofenac significantly increased the mean threshold of pain (96.4 ± 17.4 g; p < 0.01).

In contrast, the mean threshold of pain in normal non-injected foot was not affected by αLA (data not shown).

Changes in IL-6 Concentration in Paw Exudates In control rats receiving a sub planter injection of carrageenan, IL-6 in the inflamed foot 3 h after irritant increased (110.8 ± 45.0 ng/paw as compared to 6.4 ± 0.3 ng/paw) with the normal non-injected foot. αLA showed moderate analgesic effect in the doses tested 30, 100 and 300 mg/kg (55.0 ± 11.7, 35.7 ± 12.4 and 24.5 ± 6.6* ng/paw; *p < 0.05) (Fig 4a). Orally administered diclofenac significantly decreased IL-6 (19.7 ± 2.0 ng/paw; p < 0.01).
Changes in PGE$_2$ Concentration in Paw Exudates In control rats receiving a subplantar injection of carrageenan, PGE$_2$ in the inflamed foot 3 h after irritant increased (393.0±65.2 pg/paw as compared to 188.0±181.1 pg/paw) with the normal non-injected foot. $\alpha$LA showed moderate analgesic effect in the doses tested 30, 100 and 300 mg/kg (201.5±46.3, 135.3±32.5 and 41.7±9.6 pg/paw; * $p<0.05$) (Fig 4b). Orally administered diclofenac significantly decreased PGE$_2$ (29.7±3.1 pg/paw; $p<0.01$).

Therapeutic Effects of $\alpha$LA on Inflammation in Adjuvant Arthritis The control rats (vehicle-treated rats) showed gradual development of arthritis (increase of paw volume) during the experiment, from 2.86±0.15 ml (day 14) to 3.02±0.22 ml (day 17) in adjuvant-injected paw (Fig. 5a). $\alpha$LA-treated rats did not show a decrease in paw volume, with values remaining almost the same throughout the experiment, from 2.81±0.11 ml (day 14) to 2.82±0.36 ml (day 17). A significant difference in paw volume between the control and $\alpha$LA-treated rats was observed on day 17. In contrast, $\beta$LG-administered rats did not show an effect on the development of arthritis in the adjuvant-injected paw. Diclofenac significantly inhibited the development of arthritis, from 2.80±0.12 ml (day 14, $p<0.01$) to 2.53±0.44 ml (day 17, $p<0.01$).

Protective Effects of $\alpha$LA on Joint Hyperalgesia in Adjuvant Arthritis Adjuvant injected into the left hind paw produced extensive joint hyperalgesia (8.2±0.2 times vocalization) in the left hind paw in the control (vehicle-treated) rats (Fig. 5b). $\alpha$LA showed analgesia in the adjuvant-injected paw (4.9±0.4 times), and a significant difference compared to the control was observed ($p<0.05$). In contrast, $\beta$LG administration did not affect the hyperalgesia in the adjuvant-injected paw. Diclofenac showed a significant difference compared with the control (2.8±0.8 times, $p<0.01$).

Effects of $\alpha$LA on COX-1, COX-2, PLA$_2$, and 5-LO in Vitro As shown in Fig. 6a, $\alpha$LA inhibited COX-2 activity. Concentrations of COX-2 after 50% inhibition by $\alpha$LA were 39 $\mu$mol/l. Incidentally, the 50% inhibition concentrations for COX-2 in response to rofecoxib was 0.155 $\mu$mol/l. Moreover, $\alpha$LA showed selectivity on COX-2 as compared with COX-1. Concentrations of COX-1 after 39% inhibition by $\alpha$LA were 704 $\mu$mol/l (maximum dosage that can be added to experiment system).

As shown in Fig. 6b, $\alpha$LA inhibited PLA$_2$ activity. Concentrations of PLA$_2$ after 50% inhibition by $\alpha$LA were 76 $\mu$mol/l. Incidentally, the 50% inhibition concentrations for PLA$_2$ in response to quinacrine was 122 $\mu$mol/l.

In contrast, $\alpha$LA did not inhibit 5-LO activity (data not shown).

DISCUSSION

Milk is an opaque white liquid produced by the mammary glands of female mammals. Cow milk contains, on average, 3.4% protein, 3.6% fat, and 4.6% lactose, 0.7% minerals and supplies 66 kcal of energy per 100 ml. $\alpha$LA exist approximately 120 mg in cow’s milk (100 ml), and is also present in the milk of many other mammalian species.

In an animal model of acute inflammation (injection of carrageenan into the footpad), edema was produced that was associated with marked accumulation of COX mRNA and thromboxane. The injection of adjuvant induced a marked edema of the hind footpads with coincident local production of PGE$_2$ associated with upregulation of COX mRNA and...
protein in the affected paws.\textsuperscript{17)} Non-steroidal anti-inflammatory drugs (NSAIDs) alleviate pain by counteracting the COX enzyme.\textsuperscript{18)} On its own, COX enzyme synthesizes prostaglandins, creating inflammation. On the whole, the NSAIDs prevent the prostaglandins from ever being synthesized, reducing or eliminating the pain. COX-2 selective inhibitor is a form of NSAID that directly targets COX-2, an enzyme responsible for inflammation and pain. Selectivity for COX-2 reduces the risk of peptic ulceration. It has been reported that COX-2-selectivity does not affect other adverse effects of NSAIDs (most notably an increased risk of renal failure).\textsuperscript{19)} In this study, αLA inhibited COX. Moreover, αLA showed selectivity on COX-2 as compared with COX-1. These results suggest that αLA reduces the gastrointestinal side-effects.

It has been reported that αLA fortifies the mucus gel layer by stimulating mucin production and secretion in gastric mucus-producing cells, and that this enhancing effect is independent of endogenous PGE\textsubscript{2}.\textsuperscript{20)} αLA stimulates mucin synthesis and secretion in mucus-producing cells and induces increased thickness of the mucus gel layer in the gastric mucosa, suggesting that stimulation of mucus metabolism by αLA contributes to its gastroprotective actions.

In carrageenan-evoked inflammatory pain, the pro-inflammatory cytokines—including TNF-α, IL-1β, and IL-6—play an early and crucial role in the subsequent inflammatory responses.\textsuperscript{21)} In this study, we demonstrated that αLA has a preventive and therapeutic analgesic effect in inflammatory pain. In addition, αLA inhibits the formation of IL-6, which may contribute to its analgesic and anti-inflammatory effects.

Carrageenan induced paw edema is believed to be biphasic, of which the first phase is mediated by the release of histamine and 5-hydroxytryptamine in the early stage followed by kinin release and then PG in the later phase.\textsuperscript{22)} It has been reported that the second phase (3 h) of edema is sensitive to most clinically effective anti-inflammatory agents. Anti-inflammatory effects of αLA in 3 h of edema suggest involvement of inhibition of PG in the action of αLA.

Writhing test is based on the principle that tissue injury increases the sensitivity to pain and this sensitivity is susceptible to modification by analgesics. In this model of chemical induced tissue injury, αLA showed non-steroidal anti-inflammatory drug like peripheral analgesic activity which was also confirmed by Randell–Selitto test.

The different properties and functions of both isoenzymes, COX-1 and COX-2, can be explained on a molecular level by small structural differences in the active sites of these proteins. In COX-2 in comparison with COX-1 the larger isoleucine-434 and -523 are both replaced by a smaller valine residue. Therefore, the binding pocket in the active site of COX-2 is more spacious than in the case of COX-1. Selectivity of αLA on COX-2 maybe depends on these differences in the structural assembly of the binding site. Further study of these points is needed.

We also examined the protective effects of αLA on joint inflammation (edema) in adjuvant arthritis. Proinflammatory cytokines play a pivotal role in the pathology of rheumatoid arthritis. It has also been reported that the serum levels of TNF-α, IL-6 and interferon (IFN)-γ increase in adjuvant arthritis rats.\textsuperscript{23)} Aspirin is a widely used oral analgesic that acts as an inhibitor of COX. Various proinflammatory cytokines injected into the central nervous system produce pain behavior. It has been reported that aspirin significantly and dose-dependently attenuates the pain behavior induced by TNF-α, IL-6, or IFN-γ administered intrathecally.\textsuperscript{24)} We have recently found that αLA has a marked suppressive effect on pro-inflammatory cytokine release in various animal models, for instance intestinal ischemia/reperfusion model.\textsuperscript{25)} In this study, αLA inhibited IL-6 production in carrageenan-injected paw. These results suggest that αLA may attenuate pain behavior induced by pro-inflammatory cytokines.

In addition to the anti-nociceptive and anti-inflammatory effects presented here, it is known that αLA has many peripheral functions, including immuno-modulation and gut maturation.\textsuperscript{26)} The most common source of exogenous αLA is milk, which is safely optimized for the infants of each mammalian species. Casein clots in the stomach, whereas whey proteins are a soluble protein, which accelerates its gastric emptying.\textsuperscript{27)} These unique characteristic of αLA are useful in maintaining physiological activities in the intestinal tract.

These biological activities are mainly due to the proteins and peptides in milk. However, some of the biological activity of milk protein components is latent, and is released only upon proteolytic action. Bioactive peptides are produced during digestion of milk in the gastrointestinal tract, and also during fermentation and food processing. The physiological effects of bioactive peptides depend on their ability to reach their target sites intact, which may involve absorption through the intestinal epithelium prior to travel to the peripheral organs. The cleavage of latent bioactive peptides from milk proteins normally occurs during digestion by pepsin and pancreatic enzymes (trypsin, chymotrypsin, carboxy and aminopeptidases), thus suggesting that αLA-derived peptides per se may possess remarkable anti-nociceptive and anti-inflammatory activities.

It was reported that the LD\textsubscript{50} of αLA was no less than 2000 mg/kg body weight,\textsuperscript{28)} indicating that the toxicity of αLA was extremely low. Thus, αLA was found to be safe in the anti-nociceptive and anti-inflammatory dose range.

In conclusion, we have reported a novel function of αLA: anti-nociceptive and anti-inflammatory activity is provided by inhibiting COX-2 and PLA\textsubscript{2}. These results suggest that αLA can be a safe and useful natural drug for patients with severe pain that requires anti-inflammatory drugs.

REFERENCES