Short Communication

Application of esterase inhibitors: A possible new approach to protect unsaturated fatty acids from ruminal biohydrogenation

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General nutritional guidelines recommend reducing the consumption of fats originated from ruminant products. This is due to the ruminal biohydrogenation of unsaturated fatty acids (UFAs) which leads to the presence of unhealthy saturated or *trans* fats in ruminant products. Here, for the first time to our knowledge, we have focused on the main biochemical process which results in the saturation of UFAs. Rumen lipolytic activity (RLA) generates non-esterified fatty acids (NEFAs) in the rumen, which are a prerequisite for the biohydrogenation process to occur. We have examined different concentrations of pyridostigmine bromide (PB), of reversible cholinesterase inhibitor, in batch cultures containing 80 mg soybean oil, as a source of triglyceride. PB is the main active compound of an FDA approved drug for the treatment of myasthenia gravis and also pretreatment against nerve gas in humans. Our hypothesis was to evaluate PB as an inhibitor for RLA. In normal conditions, soon after triglycerides enter the rumen, they are hydrolyzed as a result and free fatty acids undergo the biohydrogenation process. Our results indicated that no significant ($P > 0.01$) reduction in linoleic acid (C18:2, ω6) after 6 h incubation was observed for cultures containing PB with concentrations above 0.052 g/dL. We concluded that PB has the potential to inhibit RLA in cultures after 6 h of incubation. Such findings suggest the potential of PB to be utilized in vivo as a feed additive to inhibit biohydrogenation of unsaturated fatty acids in the rumen.

Practical applications: The biohydrogenation of health benefitial unsaturated fatty acids in the rumen cause ruminant products, such as milk and meat, to contain highly saturated fats. Inhibition of rumen microbial lipolytic activity in vivo could increase the flow of unsaturated fatty acids for absorption and therefore, would have the potential to improve fatty acid composition of ruminant milk and meat. This would also have benefits for the animal.

Keywords: Biohydrogenation / Esterase inhibitor / Ruminants / Unsaturated fatty acid

Received: October 26, 2014 / Revised: June 16, 2015 / Accepted: July 3, 2015

DOI: 10.1002/ejlt.201400567

Supporting information available online http://dx.doi.org/10.1002/ejlt.201400567

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Abbreviations: FA, fatty acid; **FAME,** fatty acids methyl ester; **NDF,** neutral detergent fiber; **NEFA,** non-esterified fatty acid; **PB,** pyridostigmine bromide; **PUFA,** polyunsaturated fatty acid; **RLA,** rumen lipolytic activity; **SFA,** saturated fatty acid; **UFA,** unsaturated fatty acid

1 Introduction

Fatty acids (FAs) play an important role in both human and animal health. It is believed that unsaturated fatty acids (UFAs) exert health improving effects likely due to their role in the prevention and treatment of coronary artery disease, hypertension, diabetes, arthritis, other inflammatory and autoimmune disorders, and cancer whereas saturated forms have an adverse effect [1]. Therefore, most of the worldwide dietary guidelines recommend substituting animal fats, rich in saturated fatty acids (SFAs), with vegetable oils, rich in polyunsaturated fatty acids (PUFAs) [2]. Animal fats are mostly of ruminant origin, since both milk and meat are high in SFAs. Although ruminants are consuming feeds with high levels of UFAs, ruminal biohydrogenation converts them into their saturated form. As a result, SFAs are passed to the small intestine, absorbed, and finally with few changes stored in tissues or released in milk.

Although ruminal biohydrogenation has a negative effect on the quality of the ruminant products, it is necessary for normal function of rumen to digest feed. This is because non-esterified fatty acids (NEFAs), especially the unsaturated forms, are toxic for the rumen microbial flora. Therefore, biohydrogenation serves as a detoxifying procedure [3]. Thus to reduce fatty acids problems, it is recommended to feed calcium salts of fatty acids, hydrogenated fats, or encapsulated fats [4].

The key issue for this concern is that toxic form of fats for rumen microflora is NEFAs having a free carboxyl group. FAs can undergo the biohydrogenation process only in this type. However, the main types of dietary lipids entering the rumen are triglycerides, phospholipids, and galactolipids [3, 5]. This means that almost whole FAs entering the rumen are not NEFAs, which would have toxic effects on ruminal microflora. Moreover, these structures are not supposed to be hydrogenated because of lacking the free carboxyl group. Nevertheless, microbial lipases are responsible for producing NEFAs in the rumen by hydrolyzing the ester linkages in complex lipids, causing the release of NEFAs and the establishment of the biohydrogenation process [6, 7]. Therefore, controlling ruminal lipolytic activity (RLA) would potentially be the key approach in protecting UFAs and rumen microbial population from their effects on each other.

Few studies have been conducted with the aim of reducing RLA. Van Nevel and Demeyer [8] tried to decrease RLA, biohydrogenating activity, or both, using antimicrobial compounds. They examined different antibiotics and some other additives in cultures containing 80 mg soybean oil. They found that lipolysis was inhibited by about 10–20%, and the most potent inhibitors were ionophores and amoxicillin. The presence of antimicrobial compounds was unable to reduce biohydrogenation of linoleic and linolenic acids. In another study, the effects of bacteria-specific IgY antibodies and glycerol on the rates of NEFA accumulation during in vitro incubation of mixed rumen microbes were investigated, which lead to several relatively successful results [9].

While the rumen microflora differs greatly in relation to changes in feed type change, feed composition, environmental conditions, etc., controlling the RLA, by methods that affect specific microorganisms, could not provide a practical solution applicable in farms to protect feed UFAs from ruminal biohydrogenation. Fortunately, it has been found that the rumen lipolytic bacteria are expressing two types of enzymes, a cell-bound esterase and an extracellular lipase [3, 10–12], both of them exposed to the outside of the bacteria. These findings led us to examine whether esterase inhibitors are capable in controlling RLA or not.

Esterase is an enzyme that hydrolyzes esters into an acid and an alcohol. In addition to toxicity properties, esterase inhibitors also have several therapeutic effects for humans. Drugs for the treatment of Alzheimer's disease and myasthenia gravis, such as galantamine, donepezil, rivastigmine, tacrine, huperzine, pyridostigmine, and neostigmine, are among therapeutic agents which have esterase inhibitory effects [13]. Our objective in this study was to evaluate the effects of pyridostigmine bromide (PB) on RLA inhibition in vitro.

2 Materials and methods

2.1 Cultures

Here, we examined two acetylcholine esterase inhibitors (pyridostigmine and donepezil) and one pancreatic lipase inhibitor (orlistat) in vitro. Among these esterase inhibitors, pyridostigmine represented inhibitory effects on RLA (Supplemental Fig. S3). To evaluate effects of PB in different concentrations on the RLA, ruminal fermentation was simulated by using batch culture method. The type of cultures we used in this study was adapted from Van Nevel and Demeyer [8]. Several researchers [14, 15–17] recently applied this method to evaluate the effects of ruminal fermentation on feed metabolism in vitro.

Cultures were prepared in glass flasks. Each culture consisted 0.5 g beef cattle commercial concentrate (91.6% DM, 13.8% CP, 34.2% NDF, 13.9% ADF, 3.9% EE, and 9.2% Ash; DM basis), 80 mg soybean oil (Supplemental Table S1), 20 mL rumen fluid, 10 mL buffer solution, and different concentrations of PB. The soybean oil dissolved in diethyl ether was added to the concentrate in the flask. In the next stage, all the flasks were transferred to an incubator at 25°C in the presence of N2 atmosphere for 48 h to omit the diethyl ether. In the experiment day, the rumen fluid was collected from two Korean Hanwoo beef cattle with permanent rumen cannula before morning feeding. The rumen fluid was filtrated by cheesecloth with four layers and transferred to the laboratory in an anerobic condition with the temperature kept at 39°C.

2.2 Concentration preparation

Different concentrations of PB were prepared by serial dilution method (Supplemental Fig. S1). For the first culture in each series, 20 mL of buffer solution [18] containing 2 g PB was mixed with 40 mL rumen fluid. Then 30 mL of the mixture was added to the first flask. As a result, the medium which contains 3.333 g/dL PB was prepared. By mixing the residual from the first solution with 30 mL mixture of the rumen fluid and buffer solution (2:1), the second concentration (1.667 g/dL) was prepared. Half of the former solution was added to the second flask. The dilution process was continued until the 8th flask (0.026 g/dL). Finally the 0 concentration was prepared by adding 30 mL mixture of rumen fluid and buffer solution (2:1) to the last flask. To make the cultures anerobic, the CO_2 was injected immediately after adding the solution to each flask. Then the flasks were caped and transferred to the shaking incubator (39°C, orbital agitation at 70 rpm, LABLINE®, model no. 3529). This procedure was repeated 20 times to have 20 replications for each PB concentrations (Supplemental Fig. S2). At 0 and 6 h incubation, 3 mL 6 *N* HCl was added to each culture to inactivate micro-organisms. For the 0 h, 6 *N* HCl was added immediately before rumen fluid addition to the flasks. Then acidified cultures were kept in −15°C. Half of the replicates (ten replicates) were used for fatty acid analysis and the rest for neutral detergent fiber (NDF) measurement. Among these, three replicates were considered as 0 h (before incubation) and seven were considered as 6 h (after incubation). Then for each concentrations of PB, the amount of fatty acids and NDF was compared between the 0 h replicates and 6 h replicates.

2.3 Fatty acid analysis

Derivatization of fatty acid methyl esters (FAMEs) were performed according to Palmquist and Jenkins [21] with Jenkins [19] modifications. This analytical protocol is specified for ruminant samples. However, no data of any ruminant samples containing PB have ever been published, according to our knowledge. While in our experimental cultures, the PB molecule was also present and we did not know if it has any effect on FA analysis, the derivatization protocol [7, 19] was performed without any modifications and differences among treatments. The GLC-90 FAME mix (Supelco Inc., Bellefonte PA, USA) which contains C17:0 and C19:0 FA was used as the internal standard. FAMEs were separated by gas chromatograph (Shimadzu, GC-17A) equipped with 100 m × 0.25 mm × 0.2 μm column (Supelco SP-2560; Supelco Inc.). Due to the nature of our cultures, for the FAMEs derivatization process, we used the whole dried cultures as the sample for the Palmquist and Jenkins [21] method. According to the method they have developed, 500 mg of sample (dry) should be used for GC analysis. In our cultures, there were 500 mg concentrate, 80 mg soybean oil, 20 mL rumen fluid, 10 mL buffer solution, and different amounts (concentrations) of PB. While in the dried samples, the PB weight which varies between 7.825 to 1000 mg among different concentrations was also included, therefore, for a fair comparison between different treatments, the whole dried cultures were used in FAMEs derivatization. (Fatty acid composition of PB is reported in Supplemental Table S2.)

The mixed FAME standard (Supelco 37 Component, Supelco Inc.) was used as the reference sample which contains common FA from C4 to C24 excluding C18:1n11t (vaccenic acid). Therefore, we were not able to quantify the C18:1n11t in our samples.

2.4 NDF measurement

Neutral detergent fiber (NDF) as an indicator for microbial fermentation was analyzed according to the AOCS Standard methods described by Ankom Technology using the ANKOM A2000 Fiber Analyzer system (ANKOM Technology Corporation, NY).

2.5 Statistical analysis

Data were analyzed statistically using SAS version 9.1 [20]. For each amounts of PB, concentrations of C16:0, C18:0, C18:1n9t, C18:1n9c, C18:2n6c, and NDF were compared between control (0 h) and incubation (6 h) cultures. Analyses were performed by Student's *t*-test ($P < 0.01$) using both quantifications of fatty acids made by comparison to C17:0 and C19:0 internal standards.

3 Results and discussion

The results of cultures stating effect of different concentration of PB on RLA have been reported in Figs. 1–3. Figure 1 illustrates how concentration of linoleic acid (C18:2n6c) were affected in the presence of different concentrations of PB. Figure 1 demonstrates that in concentrations above 0.052 g/dL, PB was capable to protect linoleic acid from disappearing during 6 h incubation with ruminal contents ($P > 0.01$). Van Nevel and Demeyer [8] reported that 6 h incubation is sufficient for RLA and its aftermath fermentation. In the same manner, insignificant difference in linoleic acid concentration between the control (0 h; before microbial activity) and incubation groups (6 h; completed microbial activity) for concentrations above 0.052 g/dL indicates that PB would have the potential to inhibit RLA during the 6 h incubation. But it is assumed that effect of PB on RLA inhibition was not 100% efficient. Because although not significant, some of them remained active since a reduction in linoleic acid concentration was observed in almost all of the PB concentrations above 0.052 g/dL. Moreover, increases were observed in C18:1n9c

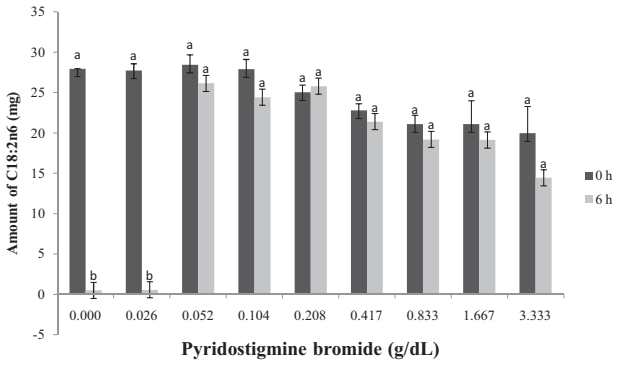

Figure 1. Effect of different concentrations of pyridostigmine bromide on linoleic acid (C18:2n6c) before (0 h) and after 6 h (6 h) incubation. Applications of pyridostigmine bromide in concentrations above 0.052 g/dL were capable to protect linoleic acid from ruminal biohydrogenation. The pooled standard errors are also shown. Significances were tested by Student's *t*-test for $\alpha = 0.01$. $P < 0.01$ was considered statistically significant.

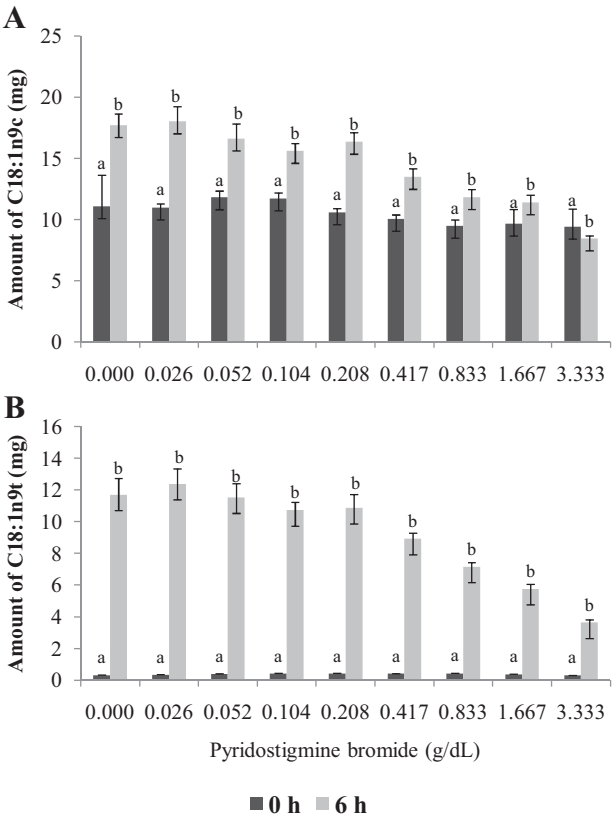

Figure 2. Effect of different concentrations of pyridostigmine bromide on C18:1n9c (A) and C18:1n9t (B) before (0 h) and after 6 h (6 h) incubation. Zero hour bars are the mean of three replicates and the 6 h bars are mean of seven replicates. The pooled standard errors are also shown. Significances were tested by Student's *t*-test for $\alpha = 0.01$. $P < 0.01$ was considered statistically significant.

and C18:1n9t after 6 h of incubation (Fig. 2). These are linoleic acid biohydrogenation intermediates [15], which represent the release of some linoleic acids. Most probably, this increase is secondary to some kind of microbial lipases, which are not inhibited by PB.

Ruminant ability to digest fiber including NDF is due to the rumen microflora. Hence, changes in NDF contents of the cultures were also evaluated to clarify whether the presence of PB was harmful for rumen microflora or not. In other words, this would help us to find out whether the reason for the non-significant differences in linoleic acid concentrations between control (0 h) and incubation (6 h) groups were not secondary to the toxic effects of PB on ruminal micro-organisms. It was hypothesized that 6 h incubation is enough to have some microbial NDF digestibility. In this case, if PB has toxicity for rumen microflora, there would be a similarity in NDF content of cultures between the 0 and 6 h cultures. Similar to previous reports on short-term (8 h) incubations [17], we observed among all treatments including the 0 concentration, a reduction in NDF content (Supplemental Table S3) between the control and incubation groups, which in some of the PB concentrations were significant ($P < 0.05$). Although, it is required to evaluate changes in microbial populations to find out more about the PB effects on rumen microflora, the disappearance of NDF during 6 h incubation could indicate that PB had insignificant effect on rumen microflora.

Unexpected increases in the levels of C18:1 isomers, especially for trans-18:1 was observed (Fig. 2). The increase in trans-9 18:1 was significant for all of PB concentrations ($P < 0.0001$). The increase in biohydrogenation intermediates of linoleic and linolenic acids can be another sign of normal rumen microflora fermentation in the presence of PB. At the same time, it causes concerns related to their accumulation, since some of trans-18:1 isomers have negative effects on both the animal and the humans consuming the ruminant products [15]. Van Nevel and Demeyer [8] also reported a high accumulation of C18:1 after 6 h incubation of 80 mg soybean oil. However, they did not identify different isomers of C18:1 in their report. While we observed a similar increase in 0 concentration for C18:1, it can be postulated that the fatty acid profile of soybean oil resulted in increased levels of trans-18:1 fatty acids, in accordance with the finding of Klein and Jenkins [15], who stated that in cultures containing docosahexaenoic acid, the levels of trans-18:1 fatty acids are elevated.

Results of the experiment also represent an unexpected effect of PB on derivatization of fatty acid methyl esters (FAMEs) from cultures. As indicated in Figs. 1–3, by increase in PB dosage in the cultures, the amount of fatty acids being detected are decreased. This could be due to the effect of PB on the FAMEs derivatization protocol being developed by Palmquist and Jenkins [21] with Jenkins [3] modifications. It is required to conduct some other

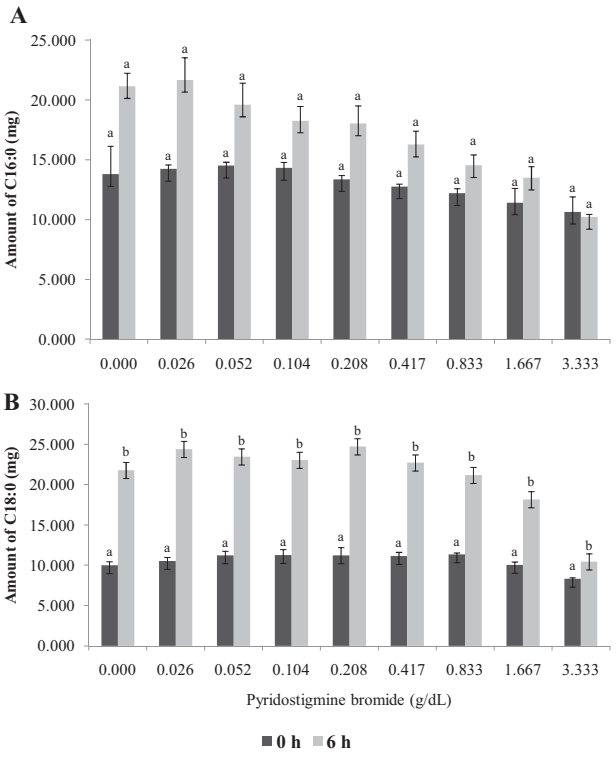

Figure 3. Effect of different concentrations of pyridostigmine bromide on C16:0 (A) and C18:0 (B) before (0 h) and after 6 h (6 h) incubation. Zero hour bars are the mean of three replicates and the 6 h bars are mean of seven replicates. The pooled standard errors are also shown. Significances were tested by Student's *t*-test for $\alpha = 0.01$. $P < 0.01$ was considered statistically significant.

experiments to find out more about the exact effect of PB on the FAMEs derivatization protocol.

4 Conclusions

Rumen-inert fats are used in ruminants' ration to make some compensation for their energy requirements and also increase UFAs in their products. However, commercial rumen-inert fats are limited in the ruminant diet because of feed intake depression, poor fatty acid digestibility, or high cost [4]. Although PB caused an increase in trans-9 18:1 in cultures containing soybean oil which expresses some potential side effects, the protection of almost all of linoleic acids from biohydrogenation in low concentrations (0.052 g/dL) suggests PB as an esterase inhibitor, which is capable to protect UFAs and the rumen microbial population from their negative effects on each other. More experiments are required to be conducted to find the methodology for PB inclusion in the ruminant rations. However, it is concluded that PB would have the potential to be used as an additive in the ruminant rations for RLA inhibition. By utilizing esterase inhibitors including PB in ruminant nutrition, it is expected to protect feed PUFAs from ruminal biohydrogenation. Moreover, it is also expected from inclusion of such materials, to make it possible to add raw seed oils to the ration without negative effects on rumen micro-organisms and feed digestibility. Therefore, UFAs would bypass the rumen intact and reach to the small intestine. The UFAs absorption would lead to accumulation in tissues and secretion into milk. This has the potential to positively affect both human and animal health.

We acknowledge Kangwon National University, College of Animal Life Sciences, Division of Animal Resource Science that allowed this study to be performed. This work was carried out with the support of the "Cooperative Research Program for Agriculture Science and Technology Development (Project No. PJ009766)," Rural Development Administration, Republic of Korea. We give special thanks to Adham Fani Maleki, Mahdi Elahi Torshizi, and Rasoul Soleimani for their help in running the experiment, analyzing samples, statistical analysis, and paper preparation.

The authors have declared no conflicts of interest.

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