Examination of the Catabolic Rates of $^{13}$C-Labeled Fatty Acids Bound to the $\alpha$ and $\beta$ Positions of Triacylglycerol Using $^{13}$CO$_2$ Expired from Mice

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Abstract: Fatty acids in triacylglycerol (TAG) are catabolized after digestion. However, the catabolic rates of several fatty acids bound to the $\alpha$ ($sn$-1, 3) or $\beta$ ($sn$-2) position of TAG have not been thoroughly compared. In this study, the catabolic rates of $^{13}$C-labeled palmitic acid, oleic acid, linoleic acid, $\alpha$-linolenic acid, eicosapentaenoic acid (EPA), or docosahexaenoic acid (DHA) bound to the $\alpha$ and $\beta$ position of TAG were compared using isotope ratio mass spectrometry. The catabolic rates of the studied fatty acids were evaluated using the ratio of $^{13}$C and $^{12}$C in carbon dioxide expired from mice. The results indicated that palmitic acid, oleic acid, or $\alpha$-linolenic acid bound to the $\beta$ position was slowly catabolized for a long duration compared to that when bound to the $\alpha$ position. In contrast, EPA bound to the $\beta$ position was quickly catabolized, and EPA bound to the $\alpha$ position was slowly catabolized for a long time. For linoleic acid or DHA, no difference in the catabolic rates was detected between the binding positions in TAG. Furthermore, EPA and DHA were less catabolized than the other fatty acids. These results indicate that the catabolic rates of fatty acids are influenced by their binding positions in TAG and that this influence on the catabolic rate differed depending on the fatty acid species.

Key words: binding position, catabolism, $^{13}$C-labeled, fatty acid species, triacylglycerol

Abbreviations: $\beta$-OO*DO, 1,2(2,3)-dioleoyl-3(1)-[1-$^{13}$C]-docosahexaenoyl glycerol; $\beta$-OO*EO, 1,3-dioleoyl-2-[1-$^{13}$C]-docosahexaenoyl glycerol; $\beta$-OO*E, 1,2(2,3)-dioleoyl-3(1)-[1-$^{13}$C]-eicosapentaenoyl glycerol; $\beta$-O*EO, 1,3-dioleoyl-2-[1-$^{13}$C]-eicosapentaenoyl glycerol; $\beta$-OO*L, 1,2(2,3)-dioleoyl-3(1)-[1-$^{13}$C]-linoleoyl glycerol; $\beta$-O*LO, 1,3-dioleoyl-2-[1-$^{13}$C]-linoleoyl glycerol; $\beta$-OO*Ln, 1,2(2,3)-dioleoyl-3(1)-[1-$^{13}$C]-$\alpha$-linolenoyl glycerol; $\beta$-O*LnO, 1,3-dioleoyl-2-[1-$^{13}$C]-$\alpha$-linolenoyl glycerol; $\beta$-OO*O, 1,2(2,3)-dioleoyl-3(1)-[1-$^{13}$C]-$\alpha$-oleoyl glycerol; $\beta$-O*OO, 1,3-dioleoyl-2-[1-$^{13}$C]-$\alpha$-oleoyl glycerol; $\beta$-OO*P, 1,2(2,3)-dioleoyl-3(1)-[1-$^{13}$C]-palmitoyl glycerol; $\beta$-O*PO, 1,3-dioleoyl-2-[1-$^{13}$C]-palmitoyl glycerol; DAG, diacylglycerol; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; FFA, free fatty acid; IRMS, isotope-ratio mass spectrometry; LpL, Lipoprotein lipase; 2-MAG, 2-monoacylglycerol; PL, phospholipid; PUFA, polyunsaturated fatty acid; TAG, triacylglycerol; THA, tetracosahexaenoic acid.

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1 INTRODUCTION

Triacylglycerol (TAG) consists of one glycerol molecule and three fatty acid molecules\(^1\). The binding position of the fatty acid to the glycerol backbone is mainly classified as the \(\alpha\) (primary alcohol group of glycerol) and \(\beta\) (secondary alcohol group of glycerol) positions. Natural processes can distinguish between the positions; for example, pancreatic lipase hydrolyzes fatty acids bind to the \(\alpha\) position of TAG in the small intestine to form two fatty acids and 2-monoacylglycerol (2-MAG)\(^2\). This specificity of pancreatic lipase results in the differential absorption and function of fatty acids bound to TAG. TAGs that contain the same species and numbers of fatty acids but in different arrangements are considered positional isomers\(^3\). For example, when TAG contains two different fatty acid species (A and B) and two A molecules and one B molecule are bound to the glycerol backbone, two possible TAG positional isomers exist: TAG with A bound to the \(\beta\) position (\(\beta\)-AAB) and TAG with B bound to the \(\beta\) position (\(\beta\)-ABA). The distribution of fatty acid species bound to the \(\alpha\) and \(\beta\) positions of TAG in natural fats and oils has been investigated\(^4\)--\(^6\). These studies have clarified the importance of the binding position of fatty acids in TAG. In fact, the binding position of fatty acids to TAG is a key consideration in infant nutrition. Moreover, for TAG in human milk, palmitic acid mainly binds at the \(\beta\) position\(^7\)--\(^11\), which improves its absorption in the small intestine\(^12\). The absorption of TAG from human milk is also affected by the formation of fatty acid soap. Milk contains a large amount of calcium ions; therefore, the calcium ions and free fatty acids, formed from the hydrolysis of the fatty acid bound at the \(\alpha\)-position by lipase, react in the small intestine to form the calcium soap of the fatty acid\(^13\). The calcium soap of a fatty acid is not readily absorbed into the small intestinal epithelial cells. Consequently, the binding position of palmitic acid in TAG is very important for human infant nutrition and is mainly bound at the \(\beta\)-position in TAG. Several studies have also suggested that the catabolism rate of a fatty acid is affected by the structure of the fatty acid\(^13\)--\(^15\). Leyton et al. compared the catabolism rates of \(^13\)C-labeled lauric acid, myristic acid, palmitic acid, stearic acid, oleic acid, linoleic acid, \(\alpha\)-linolenic acid, \(\gamma\)-linolenic acid, dihomoy-\(\gamma\)-linolenic acid, and arachidonic acid using \(^13\)C-labeled CO\(_2\) in exhaled gas from Sprague-Dawley rats\(^13\). They found that lauric acid had the highest rate of catabolism among saturated fatty acids and that the rates of catabolism of oleic acid and \(\alpha\)-linolenic acid were equivalent and the highest among unsaturated fatty acids. Kaur et al. compared the catabolic rates of \(^13\)C-labeled oleic acid, eicosapentaenoic acid (EPA), n-3 docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA) using Wister rats\(^15\). They found that the catabolic rate of EPA was the highest among three types of n-3 polyunsaturated fatty acids (PUFAs). These studies indicated that the fatty acid catabolic rate depends on its structure, but they did not take into account of the binding position in TAG because \(^13\)C-labeled free fatty acid was used in the animal studies. These could be ascribed to the difficulty in obtaining the TAG standards such as \(\beta\)-AAB and \(\beta\)-ABA because of their cost and limited number of suppliers. Therefore, few studies have investigated the effect of the fatty acid binding position in TAG on the catabolic rate\(^16\)--\(^18\). We previously compared the catabolic rate of \(^13\)C-labeled palmitic acid bound at the \(\alpha\) and \(\beta\) positions using a CO\(_2\) breath test in mice\(^19\). The results showed that the catabolic rate was different between the \(\alpha\) and \(\beta\) positions and that in the first stage, the fatty acid at the \(\alpha\) position was catabolized faster than that at the \(\beta\) position. However, the rates gradually changed, and 4–6 hours after administration of labeled TAG, the catabolic rate of the fatty acid at the \(\beta\) position was higher than that of the fatty acid at the \(\alpha\) position. Furthermore, we compared the catabolic rates of \(^13\)C-labeled palmitic acid or oleic acid at the sn-1, sn-2, and sn-3 positions of TAG\(^19\). We found differences between the \(\alpha\) (sn-1 and sn-3) position and \(\beta\) (sn-2) position in all experiments, and the fatty acids at the \(\alpha\) position were catabolized faster than those at the \(\beta\) position. This difference could be attributed to the characteristics of pancreatic lipase. This specificity of pancreatic lipase results in differential absorption and different functions of the fatty acids in TAGs. Although these studies revealed the catabolic rates of palmitic acid and oleic acid depending on its binding position in TAG, there is no similar information on other fatty acids such as PUFAs, which are the major fatty acids in foods. In the present study, we compared the catabolic rates of \(^13\)C-labeled palmitic acid, oleic acid, linoleic acid, \(\alpha\)-linolenic acid, EPA, or DHA bound to the \(\alpha\) and \(\beta\) positions of TAG by measuring the \(^13\)C-labeled CO\(_2\) expired by ddY mice that were administrated emulsions of the labeled TAG.

2 MATERIALS AND METHODS

2.1 Chemicals and materials

All of TAG sample including 1,2(2,3)-dioleoyl-3(1)-[1-\(^{13}\)C]-palmitoyl glycerol (\(\beta\)-OO*P), 1,3-dioleoyl-2-[1-\(^{15}\)C]-palmitoyl glycerol (\(\beta\)-O*PO), 1,2(2,3)-dioleoyl-3(1)-[1-\(^{13}\)C]-oleoyl glycerol (\(\beta\)-OO*O), 1,3-dioleoyl-2-[1-\(^{13}\)C]-oleoyl glycerol (\(\beta\)-O*OO), 1,2(2,3)-dioleoyl-3(1)-[1-\(^{15}\)C]-linoleoyl glycerol (\(\beta\)-OO*LO), 1,3-dioleoyl-2-[1-\(^{15}\)C]-linoleoyl glycerol (\(\beta\)-O*LO), 1,2(2,3)-dioleoyl-3(1)-[1-\(^{13}\)C]-\(\alpha\)-linolenoyl glycerol (\(\beta\)-OO*Ln), 1,3-dioleoyl-2-[1-\(^{15}\)C]-\(\alpha\)-linolenoyl glycerol (\(\beta\)-O*Ln*), 1,2(2,3)-dioleoyl-3(1)-[1-\(^{15}\)C]-eicosapentaenoyl glycerol (\(\beta\)-O*E), 1,3-dioleoyl-2-[1-\(^{15}\)C]-eicosapentaenoyl glycerol (\(\beta\)-O*EO), 1,2(2,3)-dioleoyl-3(1)-[1-\(^{13}\)C]-docosahexaenoic glycerol (\(\beta\)-OO*DO), 1,3-dioleoyl-2-[1-\(^{13}\)C]-docosahexaenoic glycerol (\(\beta\)-O*DO)

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were obtained from Tsukishima Foods Industry Co., Ltd. (Tokyo, Japan) (Fig. 1). Other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2 Preparation of emulsion samples

Sample emulsions were prepared as described in our previous study\textsuperscript{17}. In brief, each \textsuperscript{13}C-labeled TAG was mixed with an emulsifying agent (Triton X-100, 5\% of TAG) and

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{structures.png}
\caption{Structures of \textsuperscript{13}C-labeled TAG used in this study.}
\end{figure}
distilled water in a screw-cap vessel to obtain a $^{13}$C-labeled fatty acid concentration of 80 mmol/L. The mixture was vortexed to prepare a rough emulsion and further emulsified using an ultrasonic homogenizer (Ultrasonic Disruptor UD-200, Tomy Seiko Co. Ltd., Tokyo, Japan).

2.3 Animals, administration of sample emulsion, and sampling of expired gas

The animal study was performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee of the Tokyo University of Marine Science and Technology. Male ddY mice (4 wk old; Japan SLC, Shizuoka, Japan) were housed in plastic cages with free access to tap water and a standard MF rodent chow (Oriental Yeast, Tokyo, Japan), under controlled conditions of a 12:12-h light-dark cycle at 22 ± 1°C and 50% humidity. After acclimation for 7 days, 60 mice were divided equally into 12 groups: (i) β-OO*P group, (ii) β-O*PO group, (iii) β-OO*O group, (iv) β-O*OO group, (v) β-OO*L group, (vi) β-O*LO group, (vii) β-OO*Ln group, (viii) β-O*LnO, (ix) β-OO*E group, (x) β-O*EO group, (xi) β-OO*D group, and (xii) β-O*DO group (n = 5 mice/group). Three hours after the start of the light period, an emulsion was orally administered to the mice at the dose volume of 10 mL/kg body weight ($^{13}$C-labeled fatty acid = 0.8 nmol/kg). Mice were individually placed in a 450 mL plastic vessel for 5 min to collect the expired gas at each time point. The gas was collected with a 50 mL syringe and packed into 12 mL evacuated vials (Extainer, Labco, High Wycombe, UK) at 0, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, and 360 min after administration. The mice were fasted during sample collection.

2.4 Analysis of the ratio of $^{13}$CO$_2$ to $^{12}$CO$_2$ in sampled gas

The natural abundances of $^{13}$C and $^{12}$C in G1-grade CO$_2$ manufactured by Taiyo Nippon Sanso Corporation (Tokyo, Japan) were analyzed by SI Science CO., Ltd. (Sugito, Japan, δ$^{13}$C-Vienna Pee Dee Belemnite (VPDB) = −29.6‰). The CO$_2$ was diluted 10 times with nitrogen gas, and the mixed gas was packed into 12-mL evacuated vials. The mixed gas was used as a standard to adjust the measured δ$^{13}$C value using isotope ratio mass spectrometry (IRMS) (ANCA-GSL, SerCon Ltd., Cheshie, UK). The ratio of $^{13}$C to $^{12}$C in the expired CO$_2$ from mice was measured by IRMS. The following parameters were used: injection time, 12 s; gas chromatography column temperature, 100°C; carrier gas, helium; carrier gas flow rate, 60 mL/min; and ionization method, electron impact ionization. The δ$^{13}$C values in each sample of expired gas were calculated using the following equation[13]:

$$\delta^{13}C (_{‰}) = \left[ \left( \frac{^{13}C}{^{12}C} \right)_{\text{sample}} / \left( \frac{^{13}C}{^{12}C} \right)_{\text{standard}} \right] - 1 \times 1000$$

where $\left( \frac{^{13}C}{^{12}C} \right)_{\text{sample}}$ denotes the ratio of $^{13}$C to $^{12}$C in the expired CO$_2$ and $\left( \frac{^{13}C}{^{12}C} \right)_{\text{standard}}$ represents the ratio of $^{13}$C to $^{12}$C in VPDB (0.0112372).

The δ$^{13}$C values at each sampling time were adjusted by subtracting the δ$^{13}$C at 0 h using the following equation, and the resulting values were reported as Δ$^{13}$C.

$$\Delta^{13}C (_{‰}) = \delta^{13}C_t - \delta^{13}C_0$$

where δ$^{13}$C$_t$ represents the δ$^{13}$C value at the time t after sample administration, and δ$^{13}$C$_0$ represents the δ$^{13}$C value before sample administration.

2.5 Statistical analysis

Data are presented as the mean ± S.E. Statistical analyses were performed using two-way repeated-measures ANOVA, followed by the Tukey-Kramer test to identify significant differences among groups or Student’s t-test to identify significant differences between two groups. Differences were considered significant when the p-value was less than 0.05.

3 RESULTS

Figure 2 presents the time course of $^{13}$C-labeled CO$_2$ expiration from the mice after administration of twelve types of TAG binding one $^{13}$C-labeled fatty acid at the α or β position and two oleic acids at remaining positions, namely β-OO*P, β-O*PO, β-OO*O, β-O*OO, β-OO*L, β-O*LO, β-OO*Ln, β-O*LnO, β-OO*E, β-O*EO, β-OO*D, and β-O*DO. In Fig. 2, Δ$^{13}$C (_{‰}) is plotted on the y-axis and represents the ratio of $^{13}$C to $^{12}$C in expired CO$_2$ collected for 5 min at the respective collection times. For all TAG pairs, the Δ$^{13}$C values rapidly increased in the first 90–120 min after administration of the sample emulsion. Thereafter, Δ$^{13}$C values decreased gradually over time. The comparison between β-OO*P and β-O*PO revealed that the rates of $^{13}$CO$_2$ formation were higher in the β-O*PO group than in the β-OO*P group after 120 min. A significant difference between β-OO*P and β-O*PO was detected at 180 min. The trends of the catabolic rates of the β-OO*O and β-O*OO pairs were consistent with those of the β-OO*P and β-O*PO pairs. Namely, the Δ$^{13}$C values of the β-OO*O pair were higher than those of the β-OO*P group after 210 min. A significant difference between the β-OO*O and β-O*OO groups was detected at 270, 330, and 360 min. On the other hand, the differences between the Δ$^{13}$C values of the β-OO*L and β-O*LO groups were not significant. The comparison between the Δ$^{13}$C values of β-OO*Ln and β-O*LnO revealed that the Δ$^{13}$C value of β-OO*Ln at 30 min was significantly higher than that of β-O*LnO. However, after 180 min, the rates of $^{13}$CO$_2$ formation from β-O*LnO increased and surpassed those from β-OO*Ln, and a significant difference was observed at 270 min. In contrast, with β-OO*Ln and β-O*LnO, the Δ$^{13}$C values of β-O*EO were higher in the first 60–90 min than those of

β-OO*E, and the Δ^{13}C values of β-OO*E were higher after the first 120 min than those of β-O*EO. No significant difference in the rates of ^{13}CO_2 formation was observed between β-OO*O and β-O*DO.

As shown in Fig. 2, the area under the curves was calculated in order to compare the catabolism of fatty acid species among TAG positional isomers. The data were analyzed using two-way repeated-measures ANOVA between groups (fatty acid species and their binding position in TAG). Two-way ANOVA revealed the effect of fatty acid species (p < 0.001) but no effect of their binding position (p > 0.05) and no interaction (p > 0.05). Comparisons of the area under the curve among the palmitic acid, oleic acid, linoleic acid, α-linolenic acid, EPA, and DHA groups are shown in Fig. 3. The area under the curve of each fatty acid in Fig. 3 reflects the mean value of the β-AAB and β-ABA type positional isomers. As shown in Fig. 3, the catabolic rates of EPA and DHA were lower than those of the other fatty acids. In particular, the rate of DHA was significantly lower than those of palmitic acid, oleic acid, linoleic acid, and α-linolenic acid.
Fatty acids bound to the α-position of TAGs are incorporated into the chylomicrons in the epithelial cells of the small intestine and circulated through the bloodstream. The TAGs in the chylomicrons are hydrolyzed by lipoprotein lipase (LpL) located on the capillary walls. LpL is expressed in adipose tissue, the heart, the spleen, and the lungs, but it is not active in the liver. Instead of LpL, the liver expresses hepatic lipase, which has different characteristics than those of LpL. Specifically, LpL hydrolyzes the α position of TAG to 2-MAG and FFA, whereas hepatic lipase hydrolyzes fatty acids bound to the β position of TAG. Therefore, FFAs bound to the β position of resynthesized TAGs are ultimately beta-oxidized in the liver. This pathway requires a long time and may be responsible for the long duration of $^{13}$CO$_2$ formation from fatty acids bound to the β position observed after 180 min (Fig. 2).

Dietary PUFAs bound to TAG are incorporated into the sn-2 position of phospholipids (PLs), components of the cell membrane. PUFAs bound to the sn-2 position in PLs are hydrolyzed by phospholipase A$_2$ to synthesize eicosanoids as necessary. PLs are synthesized in the glycerol-3-phosphate pathway, also known as the "Kennedy pathway." In this pathway, glycerol-3-phosphate and two fatty acids are esterified by several enzymes, such as glycerol-3-phosphate acyltransferase. The formed phosphatidic acid is used as a precursor of PL. Diacylglycerol (DAG) and 2-MAG are also used for the synthesis of PLs such as phosphatidycholine and phosphatidylethanolamine. Therefore, it is possible that DAG or 2-MAG with a PUFAs at the β position could be preferentially used for PL synthesis. In fact, we found in this study that the catabolic behaviors of linoleic acid, EPA, and DHA differed from those of palmitic acid and oleic acid (Fig. 2). For example, the catabolic rate of EPA bound to the α position of TAG increased slower and remained higher for a longer time than that of EPA bound to the β position of TAG. In addition, the difference in the catabolic rates of linoleic acid or DHA between the binding positions of TAG was not significant. These results indicate that it is difficult to use PUFAs, except for α-linolenic acid, bound to the β position in the beta-oxidation pathway because they can be preferentially used for PL synthesis. Interestingly, the trends of the catabolic rates of α-linolenic acid were similar to those of palmitic acid and oleic acid. These fatty acids bound to the β position were slowly catabolized for long time compared to fatty acids bound to the α position. Compared with the rates of the n-3 PUFAs EPA and DHA, the catabolic rate of linoleic acid was significantly higher, regardless of the

**Fig. 3** Comparison of the area under the curve from Fig. 2 among palmitic acid, oleic acid, linoleic acid, α-linolenic acid, EPA, and DHA treated groups. Values are means ± SE; n = 5. Different letters indicate significant differences ($p < 0.05$).
binding position in TAG (Fig. 3). These results indicate that α-linolenic acid may be used as an energy source rather than a functional fatty acid.

DHA is an n-3 PUFAs synthesized from EPA by an elongation and desaturation reaction in the body. EPA is first elongated to form DPA. DPA is elongated again and desaturated by Δ6 desaturase to form tetracosahexaenoic acid (THA). THA is β-oxidized to form DHA in peroxisomes. Synthesized DHA is preferentially β-oxidized in peroxisomes rather than used as a substrate for membrane PL synthesis. Madsen et al. compared the metabolism of EPA and DHA in the rat liver and reported that EPA can be oxidized in both peroxisomes and mitochondria, whereas DHA can be oxidized in peroxisomes. They concluded that EPA and DHA are metabolized differently in the rat liver.

In the present study, EPA and DHA were catabolized less than the other fatty acids, and DHA was the least catabolized among them (Fig. 3). These differences can be ascribed to the different metabolic properties of EPA and DHA.

As mentioned above, in the present study, we found that the binding position of each fatty acid in TAG can affect its catabolic behavior (Fig. 2). However, there was no significant difference in the area under the curve for the different fatty acids between the α and β positions of TAG (Fig. 3). These results suggest that the same amounts of the fatty acids used in this study are eventually catabolized, regardless of their binding position in TAG, although their binding position affects the order of 13CO2 formation from 13C-labeled TAG. Consistent with the results of the present study, Tomarelli et al. reported that unsaturated fatty acids were readily absorbed, regardless of their position. However, they also observed that the absorption of palmitic acid and stearic acid bound to the β position of TAG was relatively low compared to that when bound to the α position. The main cause for this low absorption is their high tendency of creating complexes with calcium, which are secreted into the feces. Therefore, further studies in which calcium is added to TAG positional isomers are needed to comprehensively understand this phenomenon.

5 CONCLUSIONS

We compared 13CO2 expired from ddY mice after administration of emulsified 13C-labeled palmitic acid, oleic acid, linoleic acid, α-linolenic acid, EPA, and DHA bound to TAG. Differences between fatty acids were observed, and EPA and DHA were catabolized less than the other fatty acids. This result indicates that EPA and DHA are poorly β-oxidized because of their importance as essential fatty acids. In contrast, α-linolenic acid, a member of the n-3 PUFAs family, was preferentially β-oxidized compared to EPA and DHA. These results suggest that the fatty acid structure, i.e., the total carbon number and degree of desaturation of the fatty acid, can influence the catabolism of fatty acids. We also compared the catabolic rate of TAG between those with fatty acids bound to α and β positions. The results indicated that palmitic acid, oleic acid, or α-linolenic acid bound to the β position was slowly catabolized for a long duration compared with each was bound to the α position. In contrast, EPA bound to the α position was catabolized slower than that bound to the β position. For linoleic acid or DHA, no significant difference was observed between the α and β positions of TAG. These results indicate that the effect of the binding position in the TAG on the catabolic rates of fatty acids differs depending on the fatty acid species. However, the detailed mechanism remains unclear. In our future study, we aim to assess the effect of calcium addition to 13C-labeled TAG positional isomers on their catabolism.

Conflict of interest

The authors declare no conflicts of interest.

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