Developmental changes in carnitine palmitoyltransferases I and II gene expression in intestine and liver of suckling rats

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Carnitine palmitoyltransferase (CPT) I is expressed in the intestine of suckling rats; its mRNA increases very rapidly after birth, remains on a plateau until day 18 and decreases until weaning, when basal (adult) values are reached, which remain unchanged thereafter. CPT II mRNA values do not show any appreciable change in this period. CPT I and CPT II are expressed mainly in mucosa and, to a lesser extent, in the muscular part of the intestine. Intestinal expression of CPT I is maximal in duodenum and jejunum, whereas CPT II is expressed in a similar pattern throughout the whole intestine. Dam's milk may influence

the intestinal expression of CPT I, since mRNA levels at birth are low but increase after the first lactation. Moreover, rats weaned at either day 18 or 21 decrease their mRNA levels. Apparently, CPT II gene expression is not influenced by the mother's milk. CPT I and CPT II are also expressed in the liver of suckling rats. Hepatic CPT I is maximal at day 3, and levels of CPT II mRNA do not change, in a similar fashion to that in intestine. The profile of expression of CPT I in liver and intestine strongly resembles that previously reported for mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase.

INTRODUCTION

Hepatic ketogenesis in adult in rats is mainly controlled by two systems: (i) entry of fatty acyl-CoA into mitochondria, catalysed by the carnitine palmitoyltransferase (CPT) system [1]; and (ii) enzymic activity of mitochondrial 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase [2]. CPT I, the outer-membrane form of mitochondrial CPT, seems to have a crucial role in the control of entry and β -oxidation of fatty acid in the mitochondria. CPT I activity is regulated through inhibition by malonyl-CoA. Various pathophysiological states such as fasting or diabetes change the absolute concentration of malonyl-CoA and the sensitivity of CPT I to the inhibitor, suggesting a modulatory enzymic system for β -oxidation and ketone-body synthesis in liver [3–5]. In contrast, CPT II, the inner-membrane form of mitochondrial CPT, is not sensitive to inhibition by malonyl-CoA [1].

β -Oxidation and ketogenesis are absent or very low in the foetal liver, but these metabolic pathways emerge after birth [6,7], and so plasma ketone-body concentration is very low in the foetus, but increases markedly after birth [8]. In the suckling rat, physiological hyperketonaemia appears, owing to a stimulation of fatty acid oxidation and ketone-body synthesis in the liver [9,10]. In the switch-on of ketogenesis at birth, several metabolic steps other than the increased activity of hepatic CPT seem to be involved. Activity of the β -oxidation enzymes and mitochondrial HMG-CoA synthase increases; there is a large increase in ketogenesis from both oleate and octanoate during the first 16 h after birth [11]. However, changes in malonyl-CoA concentration are less important for regulation of liver fatty acid oxidation and ketogenesis in the newborn than in the adult [12,13].

Other tissues such as intestine can contribute to the ketone-body level. In this respect Hahn and Taller [14] and Békési and Williamson [15] showed that the intestinal mucosa of neonatal rats has an increased capacity for ketogenesis. This process is accompanied by the increase in CPT I [16,17] and HMG-CoA synthase activities [15]. Mucosa of the rat small intestine has

CPT activity that rises after birth, reaches a peak in the middle of the suckling period and falls at weaning [16].

We have previously presented evidence that, during the suckling period, intestine and liver ketogenesis can be controlled by the expression of the HMG-CoA synthase gene, and that this process depends on an unknown component in the mother's milk [18]. Increased expression of the mitochondrial HMG-CoA synthase gene in liver, intestine and kidney was also reported by Thumelin et al. [19].

To determine whether the developmental pattern in ketone-body synthesis and in HMG-CoA synthase gene expression observed in liver and intestine of suckling rats is accompanied by a similar pattern in CPT gene expression, we measured the mRNA levels for CPT I and CPT II in neonatal rats from birth to weaning. Results presented here show the following.

(i) CPT I and CPT II are markedly expressed in intestinal mucosa and liver of newborn rats.

(ii) The expression of CPT II is similar in stomach, duodenum, jejunum and colon, whereas CPT I is expressed more in duodenum and jejunum.

(iii) In intestine at birth there are low levels of CPT I mRNA, which increase immediately after the first lactation, remain high for the next 18 days and decrease thereafter to minima at weaning.

(iv) CPT I mRNA levels in liver show the highest values at postnatal day 3.

(v) CPT II remains unchanged throughout the developmental period in both liver and intestine.

The relationship between these changes and those in levels of mitochondrial HMG-CoA synthase in this period is discussed.

MATERIALS AND METHODS

Animals and preparation of tissues

Sprague–Dawley rats bred in our laboratory were used. Lactating

rats had litters of 8–10 pups. A group of newborn pups were separated from their mothers before the first lactation and starved for up to 10 h. Most of the pups were weaned on to a chow diet at 21 days of age. In some experiments rats were weaned at day 18. Pups at ages ranging from 0 to 25 days or rats weighing 200 g (60 days of age) were decapitated, and the proximal segment of their small intestine was removed (one-third of total). After rinsing with ice-cold 0.9 % (w/v) NaCl, intestines were cut into pieces and deep frozen. Intestinal mucosa was also obtained, as described elsewhere [20]. In brief, mucosa from the glandular portion of the duodenum and jejunum was separated from the underlying muscle by scraping with a microscope slide. The underlying smooth-muscle samples derived from duodenum and jejunum by this procedure were saved for study. Livers were also removed and frozen in liquid nitrogen.

mRNA analysis

Total RNA from pools from either intestine or liver of four rats was isolated as described elsewhere [21] with minor modifications. Samples (20 µg; determined by the A_{260}) were fractionated on 1 % agarose/formaldehyde electrophoretic gel, transferred and u.v.-cross-linked to Nytran-N membranes (Schleicher and Schuell). Nylon filters were prehybridized for 6 h at 42 °C in 50 % formamide/7.5 × Denhart's solution [21]/1 M NaCl/1 % SDS/50 mM NaH_2PO_4 , pH 6.3, containing 10 % dextran sulphate and heat-denatured salmon sperm DNA (100 µg/ml). cDNA probes were labelled with [α - ^{32}P]dATP by the random-primed method [22]. After denaturing by boiling for 5 min, the labelled probe was added to the pre-hybridization mixture. Hybridization was carried out overnight with the same solution, the radioactivity being 2×10^6 c.p.m./ml. Filters were washed for 3 × 20 min each with $0.2 \times \text{SSC}$ (SSC is 3 M NaCl/0.3 M sodium citrate, pH 7.0)/0.1 % SDS at 65 °C and placed in contact with Kodak X-ray film. The intensity of the blots was determined by densitometry scanning of the autoradiograms in a Bioprofil apparatus (Vilber-Lourmat). Densitometry values were corrected by using rat γ -actin and rat albumin as constitutive probes, which served as controls. Filters were dehybridized at 100 °C for 10 min with the same washing system and then rehybridized.

cDNA probes

The following cDNA fragments were used as probes. A partial fragment of 0.7 kb *Bam*HI–*Sal*I of rat CPT I cDNA, covering the region corresponding to positions 1272–2020 of the rat CPT I mRNA [23] was amplified by PCR as described [24]. A 1.4 kb *Xho*I–*Bam*HI fragment from the cDNA clone (pBKS–CPTII.4) for rat CPT II [25], a specific 1.5 kb probe corresponding to the *Kpn*I–*Kpn*I fragment of the cDNA for rat mitochondrial HMG-CoA synthase, which had been subcloned in Bluescript [26], a fragment from the cDNA (pRSa13) clone for rat serum albumin [27] and the rat γ -actin cDNA [18] were used. All DNA probes were generated by labelling with [α - ^{32}P]dATP to a specific radioactivity of 1.5×10^9 c.p.m./µg of random priming with Klenow polymerase.

RESULTS AND DISCUSSION

To ensure that the mRNA that we intended to measure for CPT I did not cross-hybridize with the CPT II probe, and vice versa, and to determine whether the mRNAs for intestinal CPT I and II were the same size as the corresponding mRNA values for the liver enzymes, Northern blots of intestine from 15-day-old suckling rats were carried out, using the labelled cDNA fragments

Figure 1 Northern-blot analysis of mRNAs for CPT I, CPT II and mitochondrial HMG-CoA synthase from the suckling rat

(a) Total RNA (20 µg/lane) from 15-day-old rats taken from liver (L), small intestine (I), scrapings of intestinal mucosa (S) and intestinal muscle (M) was electrophoresed and transferred to Nytran NY13 N membranes and hybridized with the CPT I, CPT II and mitochondrial HMG-CoA synthase probes as described in the Materials and methods section. Transcript sizes of 4.7, 2.5 and 2.0 kb were obtained, respectively. (b) Distribution of CPT I and CPT II mRNA levels in different parts of the intestinal tract in 15-day-old suckling rats. Total RNA was isolated from various regions of the intestinal tract and analysed by Northern blot (20 µg/lane). The gastrointestinal-tract fragments were stomach, duodenum, jejunum, ileum and colon. The duodenum was taken from the pylorus to the ligament of Trietz. The jejunum was taken from the upper half of the remaining small intestine. The ileum was taken from the lower half.

(described in the Materials and methods section) for CPT I and CPT II as probes. Results presented in Figure 1 show that the intestinal transcripts are clearly different in size: the mRNA for CPT I measured 4.7 kb, whereas that of the gene of CPT II enzyme was 2.5 kb in length; these values are identical with those for the same mRNAs reported for liver CPT I and II respectively [23,25]. Moreover, transcripts of CPT I and CPT II were found not only in the intestinal scrapings, but also in lesser amounts in the muscular part of the intestine, at variance from what was observed for mitochondrial HMG-CoA synthase, which was present only in scrapings. On day 15, when the intestinal expression of CPT I was maximal (see below), the differential expression of this gene and of CPT II was studied throughout the whole intestine. Figure 1(b) shows that there was increased expression in the duodenum and jejunum of CPT I mRNA, a result also found for mitochondrial HMG-CoA synthase (results not shown). CPT II was expressed with similar intensity throughout the whole intestine. The changes in CPT II mRNA levels throughout the intestine were lower than those for CPT I.

mRNA levels for intestinal CPT I and CPT II from foetal, newborn, suckling, weaned and adult rats are shown in Figure 2. It appears that mRNA values from foetal and newborn rats were low, but, from the moment at which suckling began, there was net increase in gene expression up to day 3. Values then reached a plateau until day 18, when they decreased progressively [to approx. 15 % of the highest value (day 3)] after weaning (day 21, on which pups were separated from their mothers) and remained constant in adult-rat intestine. We also determined the mRNA for CPT II from the intestine. The values remained essentially constant throughout the suckling period. To normalize the expression of both sets of values, we used γ -actin mRNA as described [18]. The pattern of expression of CPT I is in agreement with the values of CPT I enzymic activity determined in mitochondria of intestinal mucosa presented by Hahn et al. [16]. However, CPT II activity did not follow the same pattern as mRNA levels. The discrepancy may be due to the difficulty of finding any CPT activity on the inner side of the intestinal mitochondrial membranes after day 18. Hahn et al. [16] reported

Figure 2 Developmental changes in intestine CPT I and CPT II mRNA levels

(a) Representative Northern blot (pooled from four rats) of intestine mRNA of CPT I and CPT II from foetus, newborn, suckling, weaning and adult rats. (b) mRNA levels for CPT I (●) and CPT II (○) were quantitatively determined by densitometric scanning, normalized against γ -actin and represented in arbitrary units (the value corresponding to the 60-day-old rat is taken as 1 unit).

difficulty in obtaining intact mitochondria from enterocytes; so the CPT activity reported was the sum of the enzymic activity in the outer and the inner membrane. The determination of mRNA levels for CPT I and CPT II allows us to establish the relative contribution of these enzymes in intestine from neonatal rats.

We also determined the mRNA values of both CPT genes in separate samples of either scrapings or muscular parts of rat intestine (Figure 3). Indeed, the bell-shaped curve seen for total intestine samples was reproduced in the scrapings, when mRNA samples were probed with CPT I cDNA. In contrast, the muscular region of rat intestine showed a lower, constant, expression. These results are different from those observed for CPT II, in which neither the mucosa nor muscular parts of the small intestine showed changes in the mRNA levels. These data, together with those of Figures 1(a) and 2, suggest that the enterocytes located in the outer part of the brush border express both CPT I and CPT II, but only the CPT I expressed in enterocytes has a pattern that is similar to the expression of mitochondrial HMG-CoA synthase [18], to the activity of HMG-CoA synthase and to ketogenesis production [15]. No results have been reported on CPT I activities either in mucosa or in muscular parts from intestine in suckling rats. In adults rats, the ratios of CPT in intestinal mucosa to muscle are respectively 0.36 and 0.21 $\mu\text{mol}/\text{min}$ per g wet wt. [20], in agreement with our ratios of mRNA levels in these tissues. Kimura and Warshaw [17] determined total CPT activity in isolated mitochondria of rat

Figure 3 Developmental changes in CPT I and CPT II mRNA levels from mucosa and muscle of rat intestine

(a) Representative Northern blot of muscle and mucosa mRNAs from intestine. Mucosa from duodenum and jejunum was separated from the underlying muscle by scraping with a microscope slide. A muscle extract was prepared with the pooled smooth muscle derived from duodenum and jejunum. Total RNA (20 $\mu\text{g}/\text{lane}$) (pooled from four rats) from rats of different ages was electrophoresed, transferred to Nytran NY13 N membranes and hybridized with the CPT I and CPT II probes. (b) mRNA levels from (a) were quantitatively determined by densitometric scanning, normalized, and represented in arbitrary units: ●, CPT I; ○, CPT II. The value corresponding to the 60-day-old rat is taken as 1 unit.

small intestine, and the values obtained change 5-fold between day 10 and day 60.

Fatty acids transported by CPT activity have a dual role. On the one hand, acetyl-CoA produced after β -oxidation forms the substrate of citrate synthase in the tricarboxylic cycle; on the other, acetyl-CoA is the substrate of acetoacetyl-CoA thiolase and mitochondrial HMG-CoA synthase for the synthesis of ketone bodies. In other studies we reported a correspondence between the mRNA and protein profiles for mitochondrial HMG-CoA synthase [18]; moreover, others found a similar pattern for intestinal ketogenesis [15] during suckling. The profiles of enzymic activities of CPT [16] and mitochondrial HMG-CoA synthase [15] are also coincident. This correspondence suggests that the main function of the CPT I expression in suckling rats is to supply acetyl-CoA for ketogenesis. When rats are weaned and intestinal ketogenesis does not take place [15], mitochondrial HMG-CoA synthase expression is abolished, and there is a fall in the mRNA levels for CPT I, but not for CPT II. It seems that the main role of the residual expression of CPT I after weaning is the transport of fatty acids as fuel in the tricarboxylic cycle. A similar conclusion was reached by Kimura and Warshaw [17] with respect to ketogenesis in intestine of suckling rats, when they observed that the rate of removal of [1- ^{14}C]palmitoyl-CoA by mitochondria from the intestine of suckling and adult rats was similar, but that the production of $^{14}\text{CO}_2$ was 3-fold higher in the adult. They deduced that, although intestinal β -oxidation is similar in suckling and adult rats, the oxidation of acetyl-CoA was impaired in the suckling rat, and most of it was converted into ketone bodies.

The high correlation between intestinal ketogenesis, expression

Figure 4 Intestinal CPT I expression in newborn starved rats

(a) Representative Northern blots (pooled from four rats) for CPT I, from newborn starved rats at the indicated ages are shown. Other suckling rats of the same ages were used as a control. (b) mRNA levels of blots in (a) for intestinal CPT I of suckling (▼) or starved pups (■) were quantitatively determined by photodensitometry. The 100% value is taken for both cases as the highest value corresponding to suckling rats 10 h old.

of CPT I and expression of HMG-CoA synthase as the last limiting step in the ketogenic process suggests that the tandem CPT I and mitochondrial HMG-CoA synthase in infant rats are important control points in intestinal ketogenesis, as is found in adult rat liver. In addition, there appears to be a co-ordinate mechanism in the intestine of suckling rats that promotes the expression of both genes in parallel.

Results presented in Figure 2 showing the rapid increase in mRNA levels for CPT I in the first hours after birth posed the question as to whether this increase in gene expression was related to the beginning of ingestion of milk, or to processes which take place after birth itself. It is known that there are multiple adaptations of hormone secretion and metabolic processes in the first few hours of life. To test whether the increase in intestinal CPT I gene expression was produced either by milk ingestion or those processes, or both, some pups were removed before suckling and starved for up to 10 h. Results in Figure 4 show rats fed normally with dam's milk develop a progressive increase in mRNA levels. In addition, rats starved for the same time also increase their mRNA values for CPT I, with subtle differences: (i) the expression is not as high as in suckled rats (at 10 h the mRNA levels in starved rats are 50% of those in milk-fed rats); (ii) there is a delay of approx. 2 h with respect to fed rats.

It is known that newborn mammals, including the rat, mobilize their triacylglycerol stores immediately after birth. Although in rat the body fat content at birth is very low (1%) and devoid of adipose tissue [13], the lipolytic effect produced by the sympathetic-nervous-system activity and by the fall in plasma insulin concentration [28] produce enough non-esterified fatty acids to promote the expression of intestinal CPT I. Alternatively, the fall in plasma insulin concentration [28] and other hormonal effects may also be responsible for the increase in CPT I expression. It is noteworthy, however, that the components of milk apparently induce the CPT I expression sooner and with greater intensity, so that the mRNA levels are clearly higher. It is clear that the levels seen in newborn rats (2–10 h) are the sum of two effects: adaptative phenomena after birth (lipolysis and hormonal effects) and an over-stimulation produced by milk fat and perhaps other components present in milk.

In addition, intestinal mRNA levels were determined in rats before and after (6 h) weaning on day 21, when they were feeding

Figure 5 Intestinal CPT I and CPT II expression in weaned rats

(a) mRNA levels (pooled from four rats) for intestinal CPT I (left panel) and CPT II (right panel), from weaned rats either at the usual weaning day (day 21), or 3 days previously (day 18), were determined by Northern-blot analysis. Asterisk (*) denotes intestinal mRNA levels of rats 6 h after weaning. (b) Photodensitometry values of the blots in (a) for intestine CPT I (left panel) and CPT II (right panel). Weaning day: ■, □, day 21; ▼, ▽, day 18. The 100% value is taken in both cases as the value on day 15.

only on the chow diet. From Figure 5 it is clear that CPT I gene expression decreased sharply at 6 h after weaning to reach only an adult-like residual level in the next and successive days and in adult rats. To confirm these results, a group of rats were weaned on day 18, 3 days before the usual day (day 21), and their mRNA levels for CPT I were determined before weaning, 6 h later and for the following days. Results given in Figure 5 show that, at 6 h after weaning, there was a similar decrease in the CPT I gene expression to that found in rats weaned on day 21, and also only adult-like residual levels of mRNA on days 19, 20 and 21, at which ages the physiological expression is moderately high. The results found for CPT II after premature weaning show no change in mRNA levels. Once more, the decrease in CPT I after premature weaning strongly correlates with the decrease observed in mRNA levels for mitochondrial HMG-CoA synthase. The absence of changes in expression of CPT II after weaning shows that ketogenesis is not regulated at the transcriptional level by CPT II.

In this respect, Casals et al. [29] found that a fat diet increases the synthesis of mitochondrial HMG-CoA synthase mRNA by a factor of 3.3 in adult rat liver. Moreover, Serra et al. [30] found that the same fat diet promoted an identical increase in mitochondrial HMG-CoA synthase protein. We have also found that a fat diet produces an increase in mRNA levels for CPT I that is similar to those of mitochondrial HMG-CoA synthase [31]. Taking into consideration that fat is a major component of milk [13], it may also be the additional stimulus to promote the expression of CPT I, and thus to increase ketogenesis in the intestine of suckling rats, supplying substrate for mitochondrial HMG-CoA synthase, although other factors present in the milk may also promote this expression.

The mRNA levels for CPT I were also determined in liver of newborn, suckling, weaning and adult rats. A developmental pattern of expression through the suckling period was also

Figure 6 Development changes in liver CPT I and CPT II mRNA levels

(a) Representative Northern blot (pooled from four rats) of mRNA levels for CPT I and CPT II are shown from newborn, suckling, weaned and adult rats. (b) mRNA levels for CPT I (●) and CPT II (○) were determined by densitometric scanning, normalized against rat serum albumin and represented in arbitrary units. The basal value for intestine CPT I and CPT II corresponds to the level in the 60-day-old rat.

Figure 7 Liver CPT I expression in newborn starved rats

(a) Representative Northern blot (pooled from four rats) for liver CPT I from newborn starved rats at the indicated ages are shown. Other suckling rats of the same ages were used as a control. (b) Photodensitometry values of the blot in (a). mRNA levels for CPT I in suckling (▼) or starved (■) rats are shown. The 100% value is taken for both cases as the highest value corresponding to suckling rats 10 h old.

observed (Figure 6). mRNA levels for rat CPT I were highest on day 3 after birth, decreasing progressively until weaning, when the rat shows similar mRNA levels to those of the normal fed adult. Liver CPT II mRNA levels did not change throughout the suckling period. These results confirm and extend those of

Thumelin et al. [32], in which mRNA for CPT I and CPT II was determined by Northern blotting in foetus at 21 days, and in neonatal rats 1 and 15 days old.

To test whether the increase in gene expression in liver could be related either to the birth process and the associated lipolytic changes itself, or to the ingestion of milk, the liver expression of CPT I of the pups removed before suckling and starved for up to 10 h was determined and compared with the expression of normally suckled pups of the same ages. Results in Figure 7 show that in milk-fed pups a large increase in liver mRNA levels for CPT I is produced, clearly seen at 4 h. Moreover, fasted pups also vigorously express liver CPT I at lower levels and with a delay of 4 h. At 10 h mRNA levels of both groups are similar. Comparison of these results with those of intestinal CPT I indicates that, for starved pups, liver expression in the first few hours of life is stronger than that of the intestine.

A fat diet increases the liver mRNA levels for CPT I in adult rats [31]. It is possible that an increase in non-esterified fatty acids after internal hepatic lipolysis after birth may produce the same effects in suckling rats. It is known that several mammalian species (rabbits, guinea pigs, humans) accumulate triacylglycerol stores in foetal liver at term and that these are rapidly mobilized after birth for local utilization rather than for export of non-esterified fatty acids to peripheral tissue [33–37]. Data from this study suggest that these non-esterified fatty acids could be involved in liver CPT I expression in the first few hours of life. Alternatively, other hypotheses (for instance hormonal effects) cannot be ignored.

Saggerson and Carpenter [38] showed that CPT I activity increased 6-fold between the late-foetal stage (20 days gestation) and the first day after birth, a similar value to the increase seen in mRNA levels. Similar values of CPT I activity (an increase of 4.5-fold) were observed by Thumelin et al. [32]. CPT II activity did not differ significantly between foetal and neonatal rats [32,38]. Comparison between the development pattern of mRNA for CPT I and CPT I enzymic activity [9,39] shows similar profiles, i.e. a rapid increase after birth that remains high during the suckling period and falls to the adult values at weaning.

In the suckling period, the time courses of the increase in liver CPT I activity and of the decrease in liver malonyl-CoA concentration suggest that this effector is not directly responsible for the initiation of fatty acid oxidation or ketogenesis in the neonatal period and that additional mechanisms must be operating [38]. The comparison between liver profiles of CPT I mRNA (Figure 6), and mitochondrial HMG-CoA synthase of suckling rats [18] suggests that a similar mechanism controls the co-ordinated expression of both genes during development. It is possible that a common *trans*-acting factor may interact with *cis*-elements that regulate the intestinal expression of these genes, and that the improvement to the mechanism for transcription could depend on an unknown factor present in the milk. To date only the promoter for mitochondrial HMG-CoA synthase has been reported, but not that for CPT I. When this promoter is known, such structural and mechanical relationships between the two genes may be revealed. Other mechanisms such as modifications in the sensitivity of CPT I to malonyl-CoA inhibition [40] and succinylation of mitochondrial HMG-CoA synthase [41] may contribute to the regulation of ketogenesis in this period.

All these results, those on the expression of CPT I and mitochondrial HMG-CoA synthase, suggest that the intestine may contribute to the overall rate of ketogenesis in higher proportions than liver in suckling rats. These results also agree with those reported by Békési and Williamson [15], who determined the rate of ketogenesis in intestinal mucosa of infant

rats. It appears that on weaning, when the brain is almost fully developed, ketone-body synthesis remains under the exclusive control of the liver, as it does during the whole life of the adult animal.

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