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Influence of fatty acid patterns on the intestinal absorption pathway of quercetin in thoracic lymph duct-cannulated rats

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Abstract

Since it is known that dietary fats improve the bioavailability of the flavonol quercetin, we purposed to investigate whether this effect is due to increased lymphatic transport of quercetin. In rats with implanted catheters in the thoracic lymph duct, we administered quercetin into the duodenum with TAG emulsions containing either long-chain fatty acids (LCT) or medium-chain fatty acids (MCT). Controls received quercetin together with a glucose solution. LCT administration increased the lymphatic output of quercetin (19·1 (SEM 1·2) nmol/8 h) as well as the lymph-independent bioavailability of the flavonol, determined as area under the plasma concentration curve (1091 (SEM 142) μM × min). Compared with glucose administration, MCT neither increased the lymphatic output (12·3 (SEM 1·5) nmol/8 h) nor the bioavailability of quercetin (772 (SEM 99) μM × min) significantly (glucose group: 9·8 (SEM 1·5) nmol/8 h and 513 (SEM 55) μM × min, respectively). Because LCT are released within chylomicrons into the intestinal lymph while MCT are mainly released into the portal blood, we conclude from the present results that dietary fats that are mainly composed of LCT improve quercetin bioavailability by increasing its transport via the lymph, thereby circumventing hepatic first-pass metabolism of the flavonol. In addition, LCT could enhance quercetin absorption by improving its solubility in the intestinal tract.

Key words: **Quercetin: Long-chain TAG: Medium-chain TAG: Lymph**

Quercetin is one of the major dietary flavonoids distributed in a variety of plant foods. As it occurs mainly conjugated to glycosides in plants, hydrolysis by enzymes in the small intestine or by bacterial enzymes in the hindgut is a prerequisite for epithelial uptake[1,2]. Besides its chemical structure, the food with which quercetin is ingested influences the magnitude of its bioavailability. For example, dietary fat has been shown to have a positive effect in this regard[3]. The enhancing effect of dietary fat on the bioavailability of quercetin could be explained either by an improved solubility of the flavonol within the intestinal lumen or by increasing quercetin transport via the lymph. By using the lymph route, hepatic first-pass metabolism and biliary excretion of quercetin metabolites could be circumvented[4]. Indeed, we previously reported that intragastrically administered quercetin was transferred into the lymph in rats[5]. However, the contribution of lymphatic transport for the systemic availability of quercetin has not been fully elucidated. In the present study, we investigated the effect of dietary fat on intestinal quercetin absorption using thoracic lymph duct-cannulated rats. The administered quercetin dose (10 mg/kg body weight) can be easily achieved with commercially available quercetin supplements. We administered the flavonol within a TAG emulsion consisting of either medium-chain fatty acids (medium-chain TAG, MCT) or long-chain fatty acids (long-chain TAG, LCT). The latter ones are predominantly incorporated into chylomicrons and released into the intestinal lymph, while the former ones are absorbed via the portal vein[6]. If quercetin is transported in a large part within chylomicrons via the lymph, the concentration of this flavonol should be significantly higher in the lymph fluid after the administration of LCT compared with MCT.

Abbreviations: C_{max}, maximum plasma concentration; LCT, long-chain TAG; MCT, medium-chain TAG; T_{max}, time to reach maximum plasma concentration.

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Experimental methods

Chemicals

The TAG emulsion consisting of long-chain fatty acids was prepared from commercial soyabean oil. The other oil containing medium-chain fatty acids was a kind gift from Unilever. It consisted solely of octanoic and decanoic acids with a mean chain length of C8·8[7]. β-Glucuronidase/sulfatase (β-glucuronidase type H-1 from *Helix pomatia*) was obtained from Sigma-Aldrich Company. Standard quercetin and kaempferol (the internal standard) were from Extrasynthese. HPLC solvents were all of analytical grade.

Animal experiments

Male Wistar/ST rats, 8 to 10 weeks old (from Japan SLC Company), with a body weight of 250–300 g were used for the experiment. The present study was performed according to the guidelines for the care and use of laboratory animals of the University of Tokushima Graduate School, Institute of Health Biosciences and the approved experimental procedure by the Committee for the Care and Use of Laboratory Animals at Kinki University. Rats were anaesthetised with sodium pentobarbital for the surgical procedure. The thoracic lymph duct was cannulated with vinyl tubing (0·8 mm outer diameter). For infusion of compounds, a polyethylene tube (1·2 mm outer diameter) was inserted via the abdominal wall into the stomach and the tip of the tube was placed in the duodenum about 1·5 cm after the pylorus. After an overnight recovery in restraining cages with free access to a glucose–NaCl solution (25 g glucose and 4·97 g NaCl/l), quercetin was administered at a dose of 10 mg/kg body weight as a single injection within a volume of 1 ml of the test liquids via the duodenal tubes. The test liquids consisted of either a saturated glucose solution (0·52 g/ml) or emulsions prepared with either LCT (10 mg lecithin and 0·21 ml soyabean oil/ml) or MCT (10 mg lecithin and 0·22 ml MCT oil/ml). We chose an amount of lipids that was similar to our previous experiment with pigs[7]. After injection of these test liquids, the duodenum was continuously perfused with the glucose–NaCl solution at a rate of 1·5 ml/h until the end of the experiment. Lymph collection started 2 h before quercetin administration and was continued until 8 h thereafter. The complete lymph was collected in glass tubes containing 30–100 μl of an EDTA solution (10 mM) to avoid coagulation and was fractionated at 0·25, 0·5, 0·75, 1, 1·5, 2, 3, 4, 6 and 8 h. At each of these time points, about 100 μl of blood were collected from the tail vein into heparinised glass capillaries and centrifuged (2000 ***g*** for 5 min) in order to separate plasma and blood cells.

Quercetin analysis

An aliquot of the lymph fractions (50–100 μl) was treated with β-glucuronidase/sulfatase (100 U (54 pKat) β-glucuronidase) together with ascorbic acid (final concentration 5 mM) at pH 5·0 for 30 min at 37°C in order to hydrolyse conjugated quercetin metabolites. These conditions and the enzyme mixture yielded the highest amount of free flavonols from the plasma of quercetin-fed rats and also released nearly 100 % of free quercetin from quercetin-3-*O*-glucuronide in preliminary analyses. However, because we did not specifically check the hydrolysis of other conjugates, it is possible that the total concentration of flavonol metabolites might be slightly higher than that reported here.

After enzyme incubation, the solution with the obtained flavonol aglycone was mixed with the same volume of methanol–acetic acid (100:5, v/v), centrifuged at 2000 ***g*** for 10 min at 4°C, and the supernatant was applied to HPLC analysis. In a subset of samples, chylomicrons were separated by centrifugation (20 000 ***g*** for 20 min at 4°C) from the lymph, and quercetin was analysed in the nominally chylomicron-free lymph fluid as described previously. From each blood sample, 40 μl of plasma were diluted ten times with 0·2 M-sodium acetate buffer (pH 5·0), then mixed with the same volume of the enzyme solution (1 U (0.54 pKat) β-glucuronidase/μl) together with ascorbic acid, and incubated for 30 min. Then,

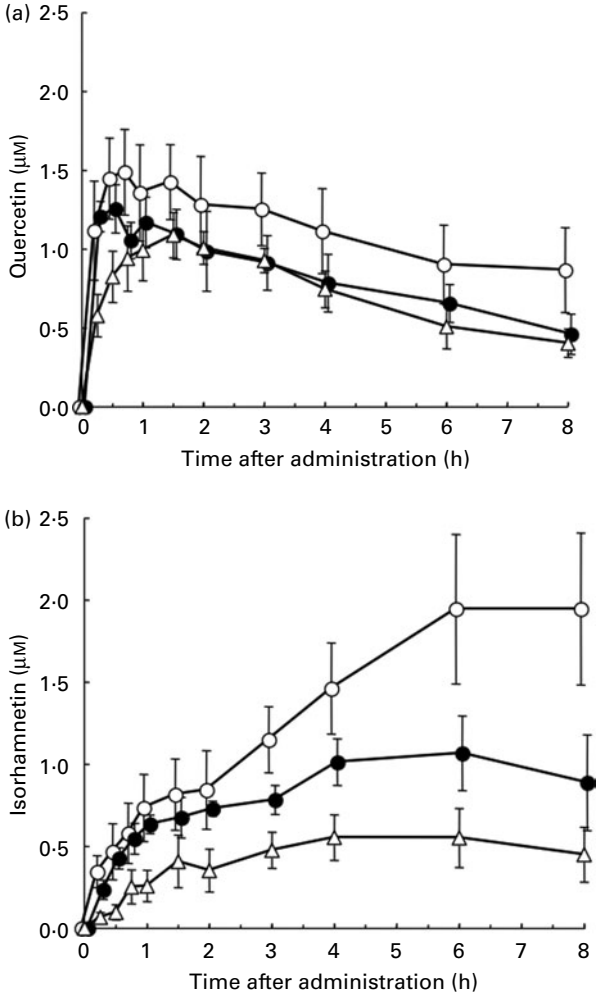

Fig. 1. Blood plasma concentrations of (a) quercetin and (b) isorhamnetin of rats after intraduodenal administration of quercetin (10 mg/kg body weight). At time 0, quercetin was administered either within a saturated glucose solution (Δ) or together with an emulsion containing either long-chain TAG (○) or medium-chain TAG (●). Values are means (*n* 5 animals per group), with their standard errors represented by vertical bars. Plasma was treated with β-glucuronidase/sulfatase before analysis.

Table 1. Pharmacokinetic parameters of quercetin metabolites in the peripheral plasma‡
(Mean values with their standard errors, *n* 5)

	C_{max} (μM)		T_{max} (h)		$AUC_{0-8\,h}$ (μM × min)	
	Mean	SEM	Mean	SEM	Mean	SEM
Quercetin						
Glucose	1·275	0·126	2·65	0·91	328·8	27·8
LCT	1·935*	0·237	2·25	1·04	512·8	82·9
MCT	1·431	0·073	0·65	0·15	367·0	41·7
Isorhamnetin						
Glucose	0·740	0·134	5·00	1·10	184·0	33·7
LCT	2·075*	0·412	5·40	1·17	577·9**	73·3
MCT	1·142	0·205	5·00	0·89	404·9	64·8
Total flavonols (quercetin + isorhamnetin)						
Glucose	1·767	0·205	2·85	0·90	512·7	54·5
LCT	3·727***†	0·351	4·35	1·47	1090·7**	142·2
MCT	2·215	0·147	2·10	1·02	771·9	98·6

C_{max}, maximum plasma concentration; T_{max}, time to reach maximum plasma concentration; LCT, long-chain TAG; MCT, medium-chain TAG.
Mean values were significantly different compared with that of glucose: * $P<0·05$, ** $P<0·01$, *** $P<0·001$.
Mean values were significantly different compared with that of MCT: † $P<0·01$.
‡ Plasma was treated with β-glucuronidase/sulfatase before analysis.

quercetin and isorhamnetin were extracted with ethyl acetate. The extract was dried under N_2, and solubilised with HPLC mobile-phase solution for analysis. Unhydrolysed lymph samples were also treated similarly to detect free quercetin and isorhamnetin. Kaempferol was added to HPLC samples as an internal standard. The mobile phase was composed of methanol–water–acetic acid (46:52:2, by vol.). Quercetin and isorhamnetin were determined by HPLC at a flow rate of 1·0 ml/min using a C18 column (TSK gel ODS-80Ts; Tosoh Company). The eluate was monitored with an UV detector at 365 nm (SPD-10A, AUFS 0.002; Shimadzu Company). The limit of detection of quercetin and isorhamnetin in the lymph fluid was 0·03 and 0·06 pmol/μl, respectively. In plasma, the limit of detection of quercetin and isorhamnetin was 0·075 and 0·15 pmol/μl, respectively. In plasma and lymph samples drawn before the administration of quercetin, baseline flavonol levels were determined. Baseline lymph quercetin concentrations after hydrolysis were below the limit of detection, and in plasma, quercetin concentrations were around the limit of detection. A substantial peak close to the retention time of isorhamnetin was detected in baseline samples of both lymph and plasma probably originating from the enzyme solution. All isorhamnetin data from lymph and plasma samples were thus corrected by subtraction of this unspecific peak.

Determination of TAG

Lymphatic TAG levels were determined using the Triglyceride *E*-test Wako Kit (Wako Pure Chemical Industries Limited) that is based on a published method[8].

Statistics

Results are expressed as means with their standard errors (n 4–5). Maximum plasma concentrations (C_{max}) and time to reach maximum plasma concentrations (T_{max}) were directly taken from the analysed data. AUC was calculated by using the trapezoidal rule. Data among the three treatment groups were compared with ANOVA, followed by a Bonferroni correction *post hoc* test. Statistical calculations were carried out using InStat (GraphPad Software, Inc.). Data were considered as statistically significant at $P<0·05$.

Results

Quercetin metabolites in plasma

We could not quantify the flavonol aglycone in plasma samples without prior β-glucuronidase/sulfatase treatment because of the limited plasma volume obtained from the tail vein at each time point. In previous experiments, the levels of free quercetin and isorhamnetin in rat plasma were below our detection limit after administration of an even higher quercetin dose (50 mg/kg), though[9]. Based on these data, we considered all of the analysed flavonols to be conjugates (glucuronides and/or sulphates) of quercetin or isorhamnetin, respectively.

After duodenal administration of quercetin, the first flavonol detected in plasma was quercetin (Fig. 1(a)). C_{max} of quercetin were significantly higher after administration of LCT compared with glucose (Table 1). The area under the plasma concentration curve (AUC) was also numerically higher in the LCT group compared with the other two groups, but this difference did not reach statistical significance. The absorption of quercetin from the glucose solution seemed to be somewhat delayed in comparison with the two TAG emulsions (Fig. 1(a)), although T_{max} values were not significantly different between the treatments (Table 1). Quercetin plasma levels were decreasing in all groups towards the end of the sampling period of 8 h.

Besides quercetin, we detected isorhamnetin as a major metabolite in the plasma of all groups. Due to the similar retention time of tamarixetin in our HPLC analysis, we were

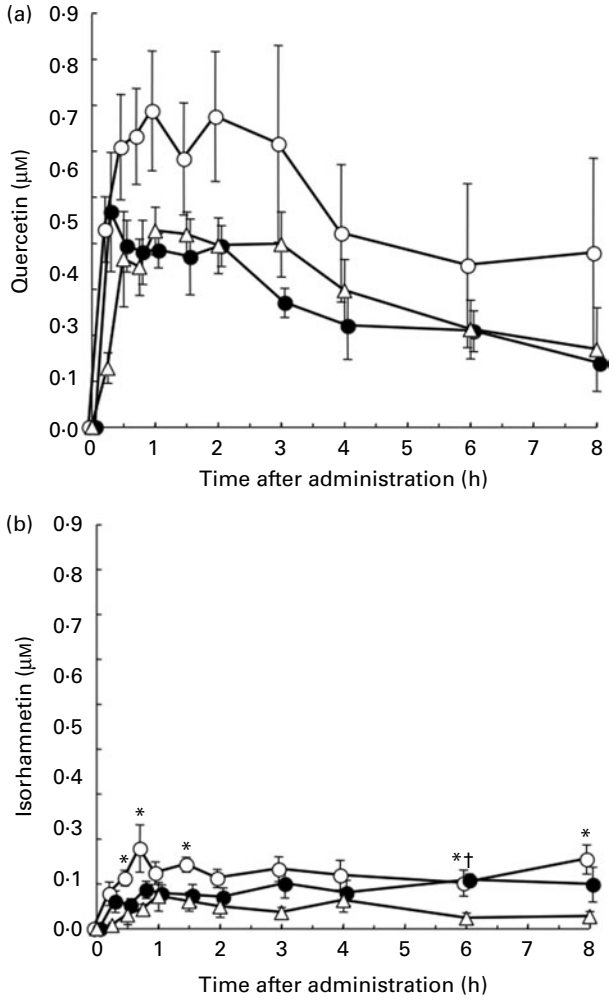

Fig. 2. Concentrations of (a) quercetin and (b) isorhamnetin in the lymph obtained from the thoracic lymph duct of rats after intraduodenal administration of quercetin (10 mg/kg body weight). At time 0, quercetin was administered either within a saturated glucose solution (Δ) or together with an emulsion containing either long-chain TAG (LCT, ○) or medium-chain TAG (MCT, ●). Values are means (*n* 5 animals per group), showing concentrations in the total fraction of the lymph fluid sampled between two time points, with their standard errors represented by vertical bars. The lymph fluid was treated with β-glucuronidase/sulfatase before analysis. * Mean value for LCT was significantly different from that of glucose ($P<0.05$). † Mean value for MCT was significantly different from that of glucose ($P<0.05$).

not able to differentiate between this 4′-methyl-metabolite and isorhamnetin. However, from previous studies, it is known that the relative amount of tamarixetin in the blood plasma of rats is rather negligible compared with isorhamnetin[10,11]. Thus, we can assume that the respective peak in our chromatograms represented mainly isorhamnetin.

In contrast to quercetin, plasma levels of isorhamnetin increased slowly and reached maximal plasma levels in all three groups not before 5 h after quercetin administration (Table 1). Although C_{max} of isorhamnetin was not significantly different between MCT and glucose, concentrations of this metabolite at each sampling point were higher in the MCT group than in the glucose group (Fig. 1(b)). Rats that had received LCT showed the highest isorhamnetin concentrations; their C_{max} value was significantly different from rats that had received glucose. In the LCT group, the AUC for isorhamnetin was also significantly higher than in the glucose group (Table 1).

Accordingly, C_{max} of the sum of both quercetin and isorhamnetin differed between LCT, on the one side, and MCT and glucose, on the other side, while the difference between the latter two groups was not statistically significant. The AUC of quercetin plus isorhamnetin was also higher in LCT rats than in glucose rats.

Quercetin metabolites in the lymph fluid

The mean lymph flow rates during 8 h after quercetin administration were similar in all groups (glucose group 4·7 (SEM 1·0) ml/h, LCT group 4·9 (SEM 0·8) ml/h and MCT group 4·7 (SEM 0·5) ml/h; *n* 5 per group). In lymph samples collected during a 2 h period before quercetin administration, flavonols could not be detected. In lymph samples that had not been treated with β-glucuronidase/sulfatase before HPLC analysis, quercetin and isorhamnetin were below the limit of detection at all sampling points. Thus, flavonols in the lymph fluid consisted mainly of conjugated metabolites.

Lymph concentrations of quercetin were similar in animals from the glucose and MCT groups (Fig. 2(a)). Thus, the cumulative lymphatic output of this flavonol was not different between those two groups (Table 2). In contrast, mean concentrations of quercetin in the LCT group were higher at most sampling points, though this was not significant. However, the resulting cumulative lymphatic quercetin output was significantly higher in animals that had received the flavonol together with LCT (Table 2).

Table 2. Pharmacokinetic parameters of quercetin metabolites in the lymph‡

(Mean values with their standard errors, *n* 5)

	C_{max} (μM)		Cumulative lymphatic output (nmol/8 h)	
	Mean	SEM	Mean	SEM
Quercetin				
Glucose	0·525	0·069	8·3	1·0
LCT	0·832	0·167	14·1*†	2·0
MCT	0·593	0·093	8·5	1·0
Isorhamnetin				
Glucose	0·104	0·029	1·5	0·5
LCT	0·235*	0·038	5·0	1·1
MCT	0·130	0·023	3·8	1·1
Total flavonols (quercetin + isorhamnetin)				
Glucose	0·607	0·091	9·8	1·5
LCT	0·985	0·159	19·1**†	1·2
MCT	0·684	0·094	12·3	1·5

C_{max}, maximum plasma concentration; LCT, long-chain TAG; MCT, medium-chain TAG.
Mean values were significantly different compared from that of glucose: * $P<0.05$, ** $P<0.01$.
Mean values were significantly different compared from that of MCT: † $P<0.05$.
‡ The lymph fluid was treated with β-glucuronidase/sulfatase before analysis.

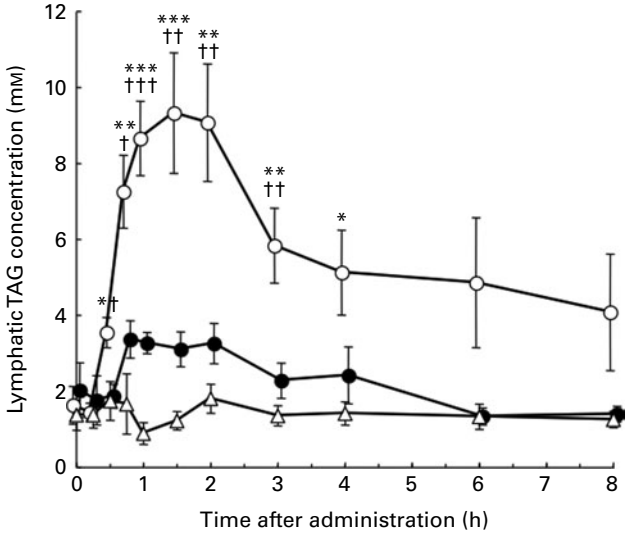

Fig. 3. Concentration of TAG in the lymph obtained from the thoracic lymph duct of rats after intraduodenal administration of quercetin (10 mg/kg body weight). At time 0, quercetin was administered either within a saturated glucose solution (Δ) or together with an emulsion containing either long-chain TAG (LCT, ○) or medium-chain TAG (MCT, ●). Values are means (*n* 4 animals per group), showing concentrations in the total fraction of the lymph fluid sampled between two time points, with their standard errors represented by vertical bars. Mean values for LCT were significantly different from those of glucose: * $P<0{\cdot}05$, ** $P<0{\cdot}01$, *** $P<0{\cdot}001$. Mean values for LCT were significantly different from those of the MCT group: † $P<0{\cdot}05$, †† $P<0{\cdot}01$, ††† $P<0{\cdot}001$.

Lymph concentrations of isorhamnetin conjugates were notably lower than those of quercetin in all three groups (Fig. 2(b)). Isorhamnetin concentrations were higher in the LCT group compared with the controls at several sampling periods (Fig. 2(b)). The C_{max} of isorhamnetin was also significantly higher in this group (Table 2).

The cumulative lymphatic output of quercetin and isorhamnetin together was highest in the LCT group.

TAG in the lymph fluid

TAG concentration in the lymph was markedly increased by the administration of LCT and significantly higher than in the glucose and MCT groups up to 4 h after administration of quercetin (Fig. 3). Fatty acids in the lymph from LCT rats consisted nearly exclusively of linoleate, oleate, palmitate and stearate. The administration of MCT induced a small increase in the concentration of TAG (mainly caprate and caprylate) that was not significantly different from the glucose administration. TAG levels in the lymph of glucose rats remained at basal levels during the whole 8 h sampling period.

Quercetin concentration in chylomicrons

To investigate whether quercetin conjugates were incorporated into chylomicrons, the lipoproteins were removed from an aliquot of the lymph samples collected from the LCT group between 1 and 2 h after administration of quercetin. Concomitant to analysis in the original lymph, quercetin was also analysed in this nominally chylomicron-free lymph fluid. While TAG concentration in the latter fluid was only 20 % of that in the total lymph, the concentration of quercetin did not differ between the two fluids (Table 3). Thus, quercetin conjugates did not seem to be specifically associated with the chylomicrons in the lymph.

Discussion

It is known that the bioavailability of quercetin is enhanced when it is administered together with lipids and emulsifiers[9,12]. In dietary sources, quercetin is mainly found in glycosylated form. Before absorption, the aglycone has to be released by enzymes in the small intestine or by bacterial enzymes in the hindgut[1,2]. Fat-containing diets increase the bioavailability of this flavonol[3,7]. A simple explanation for this observation could be the lipophilic nature of quercetin. A better solubility in the intestinal tract in the presence of lipids should enhance absorption. However, the bioavailability of more hydrophilic quercetin glycosides such as quercetin-3-*O*-glucoside is positively influenced by the dietary fat content as well[3,13]. As already mentioned, hydrolysis of quercetin-3-*O*-glucoside is prerequisite for the absorption of the flavonol aglycone. This is realised by the brush-border enzyme lactase-phloridzin hydrolase in the small intestine[14,15]. Thus, the hydrophilic quercetin-3-*O*-glucoside should not be so dependent on the presence of lipids to increase its absorption, because quercetin aglycone is released directly at the enterocyte membrane through which it is absorbed. However, as mentioned above, fats enhance the bioavailability of this glucoside as well[3,13].

The absorption of lipids could also enforce the release of quercetin into the intestinal lymph. This could circumvent hepatic first-pass metabolism and biliary excretion of the flavonol and thereby increase its bioavailability. Indeed, we could show in rats that quercetin is transported via the lymph route[5]. In order to test whether dietary fats mainly improved quercetin absorption via this latter mechanism, we used thoracic lymph duct-cannulated rats from which we collected the lymph after duodenal administration of quercetin together with glucose, MCT or LCT. Since medium-chain fatty acids are mainly released into the portal blood and are not incorporated into chylomicrons[6], only the LCT emulsion

Table 3. Distribution of quercetin conjugates in the lymph†
(Mean values with their standard errors, *n* 4)

	Original lymph		Chylomicron-deprived lymph	
	Mean	SEM	Mean	SEM
Quercetin (μM)‡	0·524	0·106	0·516	0·150
TAG (mM)§	8·07	0·51	1·55*	0·08

Mean value was significantly different compared from that of original lymph: * $P<0{\cdot}01$.
† The lymph fluid was treated with β-glucuronidase/sulfatase before analysis.
‡ Quercetin was administered with long-chain TAG and the lymph was collected between 1 and 2 h after administration.
§ TAG concentration in the lymph before administration was 1·01 (SEM 0·00) mM.

should enhance quercetin bioavailability if flavonol transport together with chylomicrons in the lymph were a relevant factor.

In line with our hypothesis, LCT administration caused the highest lymphatic output of quercetin that was matched with the highest lymphatic transport of TAG in all groups. In contrast to LCT, the slight increase in lymphatic TAG concentration after administration of MCT was not significantly different from animals receiving the glucose solution. Similarly, the cumulative lymphatic output of quercetin and isorhamnetin and the bioavailability of the flavonol were only barely increased after MCT administration and, again, not significantly different from the respective data of the glucose group. Considering the differences between the absorption mechanisms of LCT and MCT, these findings support our hypothesis that dietary fat increases the bioavailability of quercetin mainly by enhancing its transport via the intestinal lymph.

While a previous study in which pigs were fed quercetin-containing diets with either LCT or MCT led to ambiguous results[7], the present evidence of a significant quercetin transfer into the intestinal lymph after administration of LCT highlights the importance of this pathway, at least in rats.

Regarding the absorption of long-chain fatty acids, enterocytes resynthesise TAG which are incorporated into chylomicrons that are released into the intestinal lymph. Therefore, we checked the association of quercetin with these lipoproteins in the lymph samples from rats of the LCT group. Interestingly, quercetin concentration did not differ between the original lymph and the lymph fluid that had been depleted of chylomicrons by about 80 %. This argues against an incorporation of quercetin into chylomicrons. Because we could hardly detect any lipophilic quercetin or isorhamnetin aglycone in the lymph fluid, flavonols must have been almost entirely conjugated in the enterocytes before they were released into the lymph. The resulting conjugates are more hydrophilic than free quercetin and isorhamnetin. This could explain why they were obviously not enriched in chylomicrons.

Interestingly, LCT also increased the absorption of quercetin independent of the lymph route, as indicated by the higher plasma AUC values of quercetin and isorhamnetin. After administration of LCT, plasma concentrations of the metabolite isorhamnetin were increasing towards the end of the 8 h sampling period, concomitant with a decrease in plasma quercetin levels. This argues for post-absorptive methylation of quercetin to isorhamnetin most probably occurring in the liver. Accordingly, the observation that the concurrent lymphatic isorhamnetin concentrations were comparatively small indicates that methylation of quercetin by catechol-*O*-methyltransferases in the intestinal epithelium seems to be only of minor relevance in comparison with the liver. This *in vivo* observation is in accordance with another study that showed a rather low contribution of methylation to phase II metabolism of quercetin in the rat small intestine[16].

In contrast to LCT, MCT was not able to increase the lymph-independent bioavailability of quercetin significantly. The administration of the lipophilic quercetin together with TAG composed of medium-chain fatty acids may provide less suitable solubilisation conditions, because the capacity of such fatty acids and their monoacylglycerols to swell biliary mixed micelles is considerably lower than that of long-chain fatty acids and their respective monoacylglycerols[17,18]. More favourable solubilisation conditions for quercetin with LCT co-administration could improve the absorption of the flavonol and, thus, quercetin uptake into the blood irrespective of lymphatic transport.

In summary, the present results clearly support a role of dietary fats, especially of LCT, in enhancing quercetin transport via the lymph route. Intraduodenal administration of quercetin together with an emulsion containing LCT increased the bioavailability of the flavonol by increasing its transport via the intestinal lymph. LCT might also improve intestinal solubility and the absorption of quercetin independent of lymph transport. Administration of quercetin together with a fat consisting of MCT had only a minor impact on the bioavailability of the flavonol. This is probably due to the fact that medium-chain fatty acids, in contrast to long-chain fatty acids, hardly enhance lymphatic TAG transport.

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R. C. and S. W. designed the study; K. M. carried out the experiments and analysed the data; K. M. and R. C. performed the statistical analysis and wrote the manuscript; S. W. and J. T. contributed to the interpretation of the results and discussion; K. M. and R. C. had primary responsibility for the final content. All authors read and approved the final manuscript. None of the authors had any conflict of interest.

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