

Distribution of epicatechin metabolites in lymphoid tissues and testes of young rats with a cocoa-enriched diet

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An increasing number of scientific studies support that flavanol-rich foods and beverages such as cocoa can promote human health, and are beneficial agents for the prevention of some diseases. Our previous studies showed that long-term cocoa intake enhances the antioxidant status in lymphoid organs and also modulates lymphocyte functionality in healthy young rats. Cocoa polyphenolic antioxidants seem to be the best candidates for those effects. However, data regarding polyphenol metabolites in tissues after a long-term cocoa intake are scarce. In the present study we mainly focus on the uptake and accumulation of epicatechin metabolites in lymphoid organs, including the thymus, spleen and mesenteric lymphoid nodes, as well as in the liver and testes after a diet rich in cocoa. Ten young weaned Wistar rats were fed randomly with a 10% (w/w) cocoa diet or a control diet for 3 weeks, corresponding to their infancy and youth. Tissues were treated with a solid-phase extraction and analysed by liquid chromatography–tandem MS. The major compounds recovered in these tissues were glucuronide derivatives of epicatechin and methyl-epicatechin. The highest concentration of these metabolites was found in the thymus, testicles and liver, followed by lymphatic nodes and spleen. The high amount of epicatechin metabolites found in the thymus supports our previous findings showing its high antioxidant capacity compared with other tissues such as the spleen. Moreover, this is the first time that epicatechin metabolites have been found in high concentrations in the testes, confirming other studies that have suggested the testes as an important site of oxidation.

Epicatechin metabolites: Cocoa: Lymphoid tissues: Testes

Cocoa (*Theobroma cacao*) has been consumed since 600 BC, when the Mayans and Aztecs considered it to be a divine food⁽¹⁾. Nowadays, cocoa is consumed as chocolate and other confectionery products throughout the world. Cocoa has become of increasing interest due to its high content of polyphenolic compounds, particularly flavonoids – mainly flavanols, including (–)-epicatechin, (+)-catechin and polymeric procyanidins⁽²⁾.

Cocoa flavonoids have been linked to beneficial health effects, mainly in chronic diseases such as CVD⁽³⁾, but also preventing certain kinds of cancer⁽⁴⁾, improving brain function⁽⁵⁾ and modulating immune response^(6,7). The effects of cocoa on the immune system are less well known and most of the studies are performed *in vitro* showing the modulatory effects of cocoa extracts and its isolated polyphenols on stimulated macrophages⁽⁸⁾ and lymphocytes^(9,10). However, these *in vitro* effects from cocoa cannot be extrapolated to *in vivo* function since the bioavailability and metabolism

of its polyphenols must be taken into account. Monomeric flavanols are stable during gastric transit⁽¹¹⁾, although controversial data in the degradation or not of procyanidin oligomers in the stomach have been reported^(11,12). After gastric transit, flavanols are absorbed from the jejunal lumen into the epithelial cell layer and metabolised in the intestine in methyl and glucuronide conjugates, with an additional glucuronidation, methylation and sulfation in the liver, both in rats and humans^(13,14). Consequently, flavanol conjugates are found in the plasma and urine of experimental animals after their intake⁽¹⁵⁾. Therefore, studies on the bioavailability of flavonoids and their metabolites are now playing a crucial role in the understanding of the health-promoting properties of flavonoids.

We previously found that long-term cocoa intake by healthy young rats enhances antioxidant status in lymphoid organs. Certainly, cocoa intake promotes the progression of immature thymocytes towards more mature T cell stages in the thymus,

Abbreviation: MRM, multiple reaction monitoring.

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and modulates lymphocyte functionality in both intestinal and systemic compartments^(6,16). Taking into account the effects of a cocoa diet on the immune function, the aim of the present study was to investigate which are the specific epicatechin metabolites from regular 10% cocoa diet consumption, and their distribution and concentration in young rat lymphoid organs. Furthermore, we have considered the accumulation of metabolites in the testes as a specific target organ since previous studies have shown that flavonoids such as quercetin affect sperm quality⁽¹⁷⁾.

Methods

Materials

Reagents and standards were obtained from the following sources: methanol and acetonitrile (HPLC grade) from Scharlau (Barcelona, Spain); formic acid, (–)-epicatechin and ethyl gallate from Sigma-Aldrich-Fluka (St Louis, MO, USA); taxifolin from Extrasynthèse (Genay, France). Standards of epicatechin-5-*O*-glucuronide, 3'-*O*-methylepicatechin and 4'-*O*-methylepicatechin were chemically synthesised and characterised as previously published^(18,19). Ultrapure water (Milli-Q) was obtained from Millipore (Bedford, MA, USA). The extraction cartridge plates were Waters Oasis[®] Hydrophilic-Lipophilic Balance (HLB) (3 ml, 60 mg; Waters Corp., Milford, MA, USA).

Animals and experimental design

Wistar rats of 2 weeks of age (50% male, 50% female), with their dam, were obtained from Harlan (Barcelona, Spain). They were housed in cages of one dam with ten pups on a 12 h light–dark schedule. At the age of 3 weeks, pups were weaned and randomly distributed in two different groups (*n* 10) receiving, over a period of 3 weeks, (a) chow containing 10% (w/w) natural cocoa or (b) control chow, and both groups had free access to water. The chow used in the present study was an American Institute of Nutrition (AIN)-93G formulation. The 10% (w/w) cocoa diet was produced from modified AIN-93G containing 100 g cocoa per kg as described previously⁽¹⁶⁾. Studies were performed in accordance with the institutional guidelines for the care and use of laboratory animals, and experimental procedures were approved by the Ethical Committee for Animal Experimentation of the University of Barcelona (reference 3131).

Phenolic content of chow containing 10% (w/w) natural cocoa

The determination and quantification of individual phenolic compounds (mg/g) in chow containing 10% (w/w) natural cocoa were analysed by HPLC as previously described⁽²⁰⁾ and were found to be 0.34 (SD 0.01) mg (–)-epicatechin/g, 0.10 (SD 0.004) mg (+)-catechin/g and 0.23 (SD 0.01) mg procyanidin B2/g⁽¹⁶⁾.

Sample preparation

After 3 weeks of the cocoa or control diet, 6-week-old rats were anaesthetised and the thymus, spleen, liver, lymphoid

nodes and testes were excised. Organs were immediately frozen in liquid N₂, and then stored at –80°C until analysis. Flavan-3-ol metabolites were extracted three times (0.75 ml × 3) with an ice-cold solution of 1.5 M-formic acid with 5% of methanol, using a Mixer Mill (Retsch MM 300, Qiagen, Hilden, Germany) at 20 Hz during 0.5 min and centrifuged each time at 14 000 rpm. Solid-phase extraction was carried out using HLB cartridges (Waters Corp.). Pooled supernatant fractions were loaded into preconditioned cartridges with the internal standard (ethyl gallate) and washed with 2 ml of 1.5 M-formic acid in water and with 2 ml of 5% methanol in water. Elution was performed with 2 ml of 0.1% formic acid in methanol. Eluate was evaporated under an N₂ stream and reconstituted with 100 μl of taxifolin, as an additional external standard, dissolved in mobile phase A (0.1% formic acid in water). Recoveries of the metabolites were 85 (SD 13)% for epicatechin-5-*O*-glucuronide, 84 (SD 15)% for 3'-*O*-methylepicatechin, 84 (SD 15)% for 4'-*O*-methylepicatechin and 88 (SD 10)% for epicatechin aglycone.

Liquid chromatography–tandem MS

Liquid chromatography analyses were performed using an Agilent 1200 (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump and a refrigerated autosampler. A triple quadrupole mass spectrometer (API 3000) from Applied Biosystems (PE Sciex, Concord, ON, Canada), equipped with a Turbo IonSpray source operated in the negative-ion mode, was used to obtain the MS/MS data. Liquid chromatography–MS/MS methodology was performed as previously published⁽¹³⁾. Optimum MS/MS parameters were optimised for each compound by infusion experiments. Data were collected under the multiple reaction monitoring (MRM) mode, tracking the transition of parent and product ions specific for each compound. The MRM monitored the following transitions in each analysis: epicatechin (289/245), epicatechin glucuronide (465/289), epicatechin sulfate (369/289), epicatechin sulfoglucuronide (545/289), methylepicatechin (303/137), methylepicatechin glucuronide (479/303), methylepicatechin sulfate (383/303), methylepicatechin sulfoglucuronide (559/303), ethyl gallate as an internal standard (197/169) and taxifolin (303/285) as an additional external standard. For quantification purposes, calibration curves were prepared in control tissues, in the range of expected concentrations, by supplementation with known concentrations of epicatechin-5-*O*-glucuronide: 0, 0.2, 1, 2 and 4 μmol/l. As no other glucuronide conjugates were available, both epicatechin and methylepicatechin glucuronides were quantified and expressed as epicatechin-5-*O*-glucuronide equivalents. The concentration of metabolites was expressed as mean values with their standard errors of the mean (nmol/g tissue).

Results

After regular 3-week consumption of a 10% (w/w) cocoa diet, epicatechin metabolites were identified in rat tissue samples. As shown in Fig. 1, one epicatechin glucuronide (MRM 465/289) and two methylepicatechin glucuronides (MRM 479/303) were identified in all tissues after the administration of cocoa. Because the concentrations of these metabolites

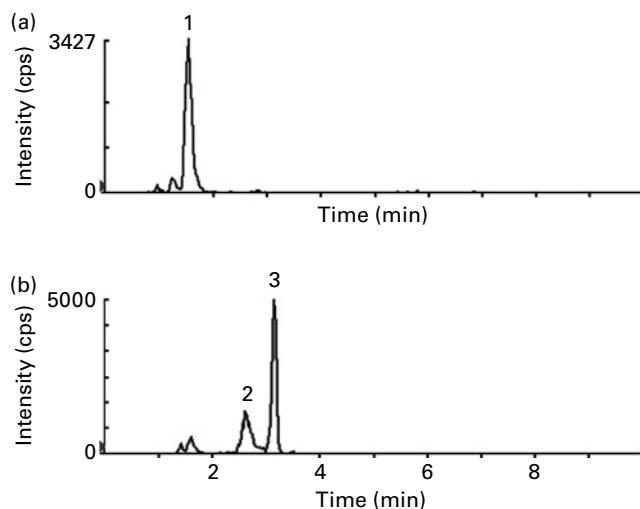


Fig. 1. Representative multiple reaction monitoring (MRM) chromatograms of epicatechin metabolites in the testicles of rats after regular cocoa consumption. (a) Epicatechin-7-*O*-glucuronide (tentatively) at peak 1 (MRM 465/289). (b) Methylepicatechin glucuronides 1 and 2 at peaks 2 and 3, respectively (MRM 479/303). cps, Counts per s.

were in the order of nmol/g tissue, identification was achieved by the product ion scan of the deprotonated molecules in the urine samples of the rats, as previously published⁽¹⁶⁾. Free methyl derivatives of epicatechin and sulfate and sulfoglucuronide conjugates were not detected in these tissues. No epicatechin metabolites were found in animals fed the control diet.

The identified metabolites were quantified in the testicles, liver, and in lymphoid tissues including the thymus, spleen and lymphatic nodes (Table 1) by comparison with calibration curves of epicatechin-5-*O*-glucuronide obtained in control tissues. As far as we know, this is the first time that epicatechin glucuronides have been quantified using an actual standard of epicatechin glucuronide. Previously, metabolites were quantified with regard to the corresponding aglycone^(13,21) or after sample hydrolysis⁽¹⁵⁾. The highest concentrations of total metabolites, including glucuronide conjugates of epicatechin and methylepicatechin, were found in the thymus, testicles and liver in about 2-fold higher concentrations than in lymphatic nodes and spleen.

Taking into account the predominant metabolites in these tissues, epicatechin glucuronide was found in the testicles and thymus in higher concentrations than in the liver and

lymphoid nodes (about 1.5-fold). Moreover, the liver and thymus were the organs with major amounts of total methylepicatechin glucuronides. Methylepicatechin glucuronide 2 was the major methylepicatechin glucuronide in all the tissues. A significantly lower concentration of this compound was observed in lymphoid nodes with respect to the liver and thymus (about 2.7-fold lower). Methylepicatechin glucuronide 1 was found in the liver and thymus at significantly higher concentrations than in the lymphoid nodes and spleen (about 7- and 6-fold, respectively), while significantly higher concentrations (of only about 2-fold) were observed in the testicles when compared with the liver.

Discussion

The present study shows for the first time that epicatechin metabolites are distributed in young rat lymphoid tissues, testes and liver after exposure to a 10% (w/w) cocoa diet over 3 weeks. The cocoa used in the present study contained mainly (–)-epicatechin as the major flavanol. This compound, which is stable during gastric transit⁽¹¹⁾ or comes from the degradation of procyanidin oligomers⁽¹²⁾, is absorbed in the jejunal part of the intestine, where it is methylated and glucuronidated, and then, in the liver, undergoes further conjugation^(13,14). In the present study, glucuronide conjugates of epicatechin and methylepicatechin are the main metabolites found in rat tissues. Positive identification of the methyl and glucuronide metabolites of epicatechin detected in the sample tissues was not possible as no standards of all of them are available. In the present study, the glucuronide standard used (epicatechin-5-*O*-glucuronide) showed shorter retention time than the glucuronide conjugate found in the rat tissues. Natsume *et al.*⁽²²⁾ purified and elucidated the structure of epicatechin metabolites in human and rat urine after oral administration of (–)-epicatechin as (–)-epicatechin-3′-*O*-glucuronide, 4′-*O*-methyl(–)-epicatechin-3′-*O*-glucuronide, and 4′-*O*-methyl(–)-epicatechin 5 or 7-*O*-glucuronide in human urine, and 3′-*O*-methyl(–)-epicatechin, (–)-epicatechin-7-*O*-glucuronide, and 3′-*O*-methyl(–)-epicatechin-7-*O*-glucuronide in rat urine. In accordance with Natsume’s findings, we tentatively identified the glucuronide conjugate found in tissues of the present study as (–)-epicatechin-7-*O*-glucuronide, which is also coherent with its late elution as compared with the epicatechin-5-*O*-glucuronide standard⁽¹⁸⁾. The HPLC elution behaviour of epicatechin-glucuronides

Table 1. Distribution of epicatechin metabolites in lymphoid organs and testes after a 10% (w/w) cocoa diet (Mean values with their standard errors)

Tissues	Samples (n)	Epicatechin glucuronide (nmol/g tissue)*		Methylepicatechin glucuronide 1 (nmol/g tissue)*		Methylepicatechin glucuronide 2 (nmol/g tissue)*	
		Mean	SEM	Mean	SEM	Mean	SEM
Liver	10	1.76	0.24	1.40	0.13	1.47	0.16
Lymphoid nodes	10	1.71	1.09	0.18	0.02	0.53	0.18
Spleen	10	0.80	0.61	0.23	0.03	1.22	0.39
Testicles	5	2.92	0.52	0.58	0.19	1.22	0.50
Thymus	10	2.43	0.29	1.27	0.13	1.39	0.18

* Expressed as epicatechin-5-*O*-glucuronide equivalents.

found in human subjects and rats reported by Natsume *et al.*⁽²²⁾ would also be in accordance with our previous results in which, after cocoa consumption, a major glucuronide with higher retention time in human subjects than in rats was found⁽¹³⁾. Furthermore, following Natsume's observations, we tentatively identified the major methylglucuronide derivative (peak 3, Fig. 1) as 3'-*O*-methyl(-)-epicatechin-7-*O*-glucuronide.

The present results are also consistent with previous studies. Thus, one epicatechin glucuronide and one 3'-*O*-methyl-epicatechin glucuronide were found in rat brain tissue after the administration of 100 mg(-)-epicatechin/kg per d over 10 d⁽²³⁾. Furthermore, the metabolites detected in rat tissues in the present study matched the findings of other authors who detected epicatechin-glucuronide and methyl-epicatechin glucuronide in the plasma, gastrointestinal tract, liver and kidneys of rats after feeding with grapeseed extract⁽²⁴⁾. Predominant metabolites in rat plasma and bile after oral administration of (-)-epicatechin were found to be glucuronide conjugates of 3'-*O*-methyl(-)-epicatechin at concentrations about 7-fold higher than other (-)-epicatechin conjugates that were secondary metabolites⁽²⁵⁾.

There are no studies about the accumulation of epicatechin metabolites in tissues after cocoa intake but the presence of catechins after tea consumption has been reported in several tissues. After maternal exposure to green tea extracts, tea catechins were detected in most of the fetal rat organs (brain, eyes, heart, lungs, kidneys and liver), catechin gallates being more readily taken up than catechins⁽²⁶⁾. Tea catechins and theaflavins were found in the small and large intestine, liver and prostate in conjugated and free forms after administration of black tea to mice⁽²⁷⁾. After administration of 10 μ mol [³H]epicatechin to rats, major amounts of radioactivity were observed in the large intestine and caecum at 24 h, and little radioactivity was observed in the stomach, plasma, small intestine, blood and testes⁽²⁸⁾.

As shown here, epicatechin metabolites were accumulated in concentrations 2-fold higher in the thymus, testes and liver than in lymphoid nodes and spleen. The high amounts of epicatechin metabolites accumulated in the thymus correlate well with previous findings demonstrating an increase in thymus superoxide dismutase and catalase activities after cocoa consumption in rats⁽¹⁶⁾. Furthermore, the fact that the activity of these antioxidant enzymes is not affected in the liver or spleen⁽¹⁶⁾ might be explained by the minor concentration found in the spleen and lower epicatechin glucuronide levels in the liver.

The accumulation of epicatechin metabolites in the thymus could affect lymphocyte composition in this tissue, because a cocoa diet in rats seems to favour T cell development⁽¹⁶⁾. Moreover, although the tissue accumulation was less, rats fed cocoa also exhibited modifications in spleen cell composition and, interestingly, changes in lymphocyte functionality, increasing proliferation rate and decreasing IL-4 secretion⁽⁶⁾.

In addition, epicatechin metabolites have also been observed in mesenteric lymph nodes, which belong to gut-associated lymphoid tissue (GALT) compartments. Ramiro-Puig *et al.*⁽⁷⁾ demonstrated changes in GALT lymphocyte composition in young rats, such as decreases in the Th percentage and increases in $\gamma\delta$ T cell percentage. These changes observed in

mesenteric lymph nodes and in lymphocyte composition could result from epicatechin metabolites accumulated in these tissues.

The relatively high concentration of epicatechin metabolites found in the testes, mainly epicatechin glucuronide, shows that an accumulation could occur. To our knowledge, there are not many studies about the accumulation of epicatechin metabolites and their effect on the testes. Previously, a diet containing 0.5 to 2% cocoa-rich flavanols given to male rats over a period of 2 weeks showed a dose-dependent reduction in oxidative DNA damage in rat testes, as concluded from the decrease of levels in a marker of DNA oxidation, 8-hydroxy-2'-deoxyguanosine⁽²⁹⁾. This reduction in oxidative damage in the testes after cocoa intake could be due to the accumulation of epicatechin metabolites in the testes. To our knowledge, only low activities for epicatechin metabolites have been described until now. With regards to structure-activity, it has been shown that substitution of the hydroxyls in the catechol B-ring of catechins results in a decrease of the antioxidant activity⁽¹⁹⁾. Flavonoids containing an unsubstituted catechol B-ring do not inhibit NADPH oxidase, but scavenge the superoxide radical^(30,31), suggesting that the *o*-dihydroxy catechol structure in the B-ring is the most important factor for protecting against oxidation⁽³¹⁾. The *O*-methylation of catechol arrangement in the B-ring converts the flavonoid to an NADPH oxidase inhibitor⁽³⁰⁾, affording a plausible explanation for the improvement of endothelial function and bioavailability of NO in human subjects after the intake of epicatechin products⁽³²⁾.

In conclusion, in the present study we show that epicatechin is absorbed from the complex matrix that is cocoa, it is metabolised in glucuronide and methylated forms and distributed in the testicles, liver and lymphoid organs (thymus, spleen and lymphoid nodes). Further studies should be done to evaluate the bioactivity of these metabolites, which could contribute to explain whether that accumulation is related to the biological activity associated with flavanol consumption.

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