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Human Apo A-I and Rat Transferrin Are the Principal Plasma Proteins That Bind Wine Catechins

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The processes of absorption, blood transport, tissular distribution, metabolism, and excretion are at present understood very little. The aim of this study was to investigate blood transport and identify which principal plasma proteins in humans and rats bind to monomeric catechin and procyanidins in red wine ex vivo. Human and rat plasma and serum were incubated with (+)-catechin and procyanidins from grape seed, the origin of red wine catechins. Proteins were separated by SDS-PAGE and native-PAGE to determine which proteins bound to these compounds. The principal protein that bound to (+)-catechin in each species was sequenced. SDS-PAGE showed that (+)-catechin and procyanidins mainly bound to a protein of about 80 kDa in rats and 35 kDa in humans. Their sequencing indicated that these proteins were apo A-I in humans and transferrin in rats. The fact that red wine procyanidins bind to both proteins suggests that they may have a role in reverse cholesterol transport and in the oxidizing action of iron.

KEYWORDS: Human; rat; apo A-I; transferrin; red wine procyanidins; (+)-catechin

INTRODUCTION

Recent advances in science have shown that diet and health are inextricably linked, and this has led to a considerable amount of work on how nutrition can improve health and prevent disease (*1*). Flavonoids are polyphenolic compounds that are contained in a wide variety of plants and which are an integral part of the human diet. They have a wide range of physiological effects in vitro as well in vivo (*1*, *2*). The daily intake of flavonoids depends on dietary habits or countries; it has been estimated to range from 25 mg in northern Europeans (*3*) to 1 g in Japanese tea drinkers (*4*). Grapes and wine are rich dietary sources of phenolic compounds, flavonoids, and non-flavonoids; the most common flavonoids in wine are flavonols, anthocyanins, and monomeric, oligomeric (procyanidins), and polymeric (tannins) catechins (*5*). Moderate wine drinking seems to have healthier effects than the consumption of other alcoholic beverages, and some authors suggest that this additional effect is due to its phenolic content (*6*). It has been suggested that the hydroalcoholic matrix of wine may facilitate the bioavailability of catechins (*5*).

If flavonoids are to have an effect in vivo, they must first be absorbed from the diet and directed to target sites. The processes of absorption, blood transport, tissular distribution, metabolism, and excretion are at present understood very little. The metabolism of flavonoids has been extensively studied in animals, but few data are available for humans. Moreover, nearly all of the studies have been made with quercetin (*7*, *8*) and tea catechins (*9*), whereas few studies have been made with wine catechins (*10*, *11*). Lack of precision and sensitivity in the analytical methods, as well as the lack of commercial markers, has made it more difficult to study the bioavailability of these compounds. Polyphenols have a high affinity for proteins and bind to them by hydrophobic interactions, hydrogen bonds, and covalent bonds (*12*). This binding, however, cannot be generalized because protein−phenol complexes depend heavily on individual structures. The aim of this study was to understand blood transport and to identify the principal plasma proteins in humans and rats liable to bind to red wine procyanidins. For this purpose, we used a pure monomeric (+)-catechin and a mixture of monomeric and oligomeric catechins (MOC) from grape seeds, which are the source of red wine catechins.

MATERIALS AND METHODS

(+)-Catechin Blood Compartmentalization. We used five male Wistar rats from Iffa-Credo (Barcelona, Spain), weighing ~125 g. Blood was drawn from the aorta artery of each anesthetized animal under ether anesthesia with a heparinized syringe, and then each sample was incubated with 2.5 mg of pure monomeric (+)-catechin/mL of blood for 30 min at room temperature. The blood was fractionated by centrifugation. The plasma was removed, and the erythrocytes were washed and resuspended with 0.9% NaCl. (+)-Catechin from the plasma and the erythrocytes was extracted by centrifugation in 9 volumes of methanol/50 mM HCl, dried under N_2 flux, and redissolved with H_2O. Catechin recovery with the extraction method was 75%. Plasma and erythrocyte (+)-catechin was determined by HPLC in a Spherisorb ODS-2 (25 × 0.46 cm) column. The elution system consisted of two solvents: (A) 4.5% HOOCH in H_2O; (B) 10:90 v/v CH_3CN/solvent A, at a flow rate of 1.5 mL min^{-1} (*13*). (+)-Catechin was detected using a diode array detector.

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Monomeric and Oligomeric Catechin Binding to Plasma Proteins. Rat blood was obtained as described above. Human blood was obtained from healthy women between 20 and 30 years old. The blood was used to obtain serum and plasma (by centrifugation at 1500*g* for 10 min) because it has been reported that fibrinogen binds catechin gallates (*14*). Rat and human plasma and serum were incubated with pure monomeric (+)-catechin (Sigma) or a mixture of monomeric and oligomeric catechins (MOC) from grape seed purchased from DRT (Les Dérivés Résiniques et Terpéniques, Dax, France). Because the solubility of the protein−catechin complex depends on a protein/catechin ratio of 59.5 (*15*), the concentration was 4.43 mg/mL of monomeric catechin and 4.86 mg/mL of MOC. MOC has a mean molecular weight of 1246 and, according to the manufacturer, contains essentially monomers (31%), 2-unit oligomers (19%), and 3−10-unit oligomers (50%). Proteins from plasma and serum incubated with (+)-catechin and MOC were fractionated in SDS- and native-PAGE.

The SDS-PAGE was performed in duplicate using 7.5% acrylamide gels (*16*). For bands to be identified, molecular mass markers (MW; Bio-Rad) were loaded (M_r in kDa: myosin, 200; β-galactosidase, 116.25; phosphorylase B, 97.4; serum albumin, 66.2; ovalbumin, 45). After electrophoresis, one of the gels was stained with Coomassie blue and the other with 1% vanillin in 70% H_2SO_4, as specific catechin dye, so that the proteins which bound to the catechins could be identified (*17*).

The native-PAGE was performed in duplicate using 10% acrylamide gels (*18*). After electrophoresis, one of the gels was stained with Coomassie blue and the other blotted onto Immobilon P membranes (Millipore). The blot was developed with Coomassie blue. The mobility of plasma proteins that bind to catechins changes (*19*). Therefore, these proteins can be detected because the band disappears when plasma has been incubated with catechins, whereas the band of plasma with no catechins does not. The major proteins binding to catechins were identified on the membranes by N-terminal sequencing (the Edman degradation procedure) using a Beckman LF3000 sequencer. The analysis was performed by the Protein Sequentation Service (Institut de Biologia Fonamental, Universitat Autonoma, Barcelona, Spain). Sequences for rat transferrin (P12346), human transferrin (P02787), rat apo A-I (P04639), and human apo A-I (P02647) were obtained from the Swiss-Prot database (http://www.expasy.ch/sprot/). Sequences were compared using the Lipman−Pearson algorithm (*20*) in the commercial program MEGALIGN v. 3.16 (1997, Lasergene software package, DNASTAR, Inc., London, U.K.) using a Power Macintosh. Three-dimensional structures for both human and rat proteins were searched for in the Protein Data Bank (http://www2.ebi.ac.uk/pdb). Their codes are 1BP5 for transferrin and 1AV1 for apo A-I. Their structures were visually compared with the Rasmol program (*21*) using a Silicon Graphics Indigo^2 XZ workstation.

RESULTS

(+)-Catechin was measured in plasma and erythrocytes, and the final concentration of catechin in each fraction was calculated by taking into account the recovery of the extraction method and the percentage of each fraction in blood. Compartmentalization, performed with five different experiments, showed that 90 ± 2% of (+)-catechin added to blood was in plasma and that 10 ± 2% was in the cellular fraction.

SDS-PAGE was carried out on plasma and serum to discern whether fibrinogen was the protein that binds catechins. There were no differences between plasma and serum. Both (+)-catechin and MOC bound to the same proteins, indicating that the degree of polymerization did not affect their affinity for these proteins. (+)-Catechin and procyanidins bound to several plasma proteins but mainly to a protein of ~80 kDa in rats (**Figure 1**) and ~35 kDa in humans (**Figure 2**).

To check that catechin−protein complexes were not broken by SDS, electrophoresis was repeated in native conditions (**Figure 3**). The results obtained by native-PAGE again showed that the major plasma protein binding to the compounds was not the same in humans and rats. The N-terminal sequencing of the proteins blotted on an Immobilon membrane identified them as apo A-I in human plasma and as transferrin in rat plasma. The molecular weight of these proteins coincides with the weights obtained from SDS-PAGE analysis.

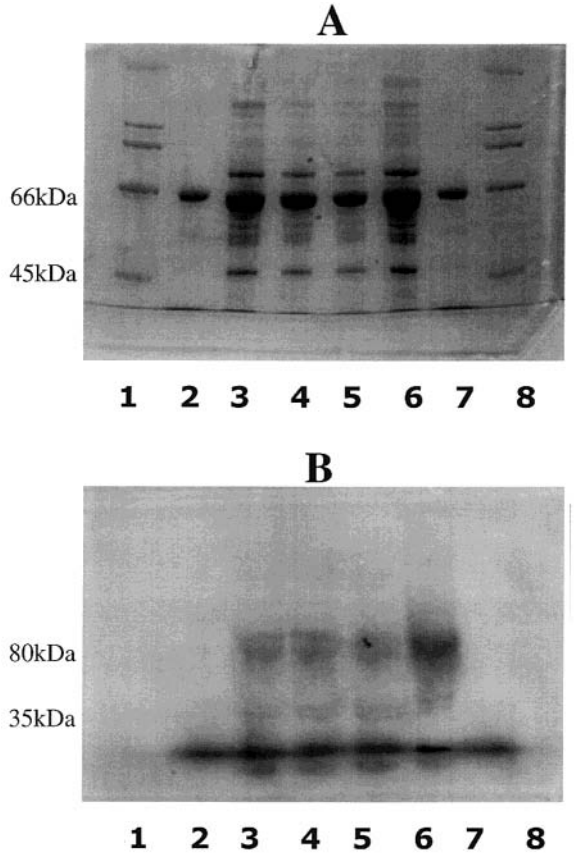

Figure 1. SDS-PAGE analysis of rat plasma proteins that bind to pure monomeric catechin and a mixture of monomeric and oligomeric catechins (MOC) from grape seed. Samples (10 μL) were diluted 1:20 and loaded onto 7.5% polyacrylamide–SDS gels. After electrophoresis, the gels were stained with Coomassie blue or 1% vanillin/70% H_2SO_4 (a specific catechin dye, to identify proteins binding to catechins). Gels with plasma and serum without catechins are not shown because there were no stained bands with vanillin. (A) Staining with Coomassie blue: lane 1, molecular mass markers (MW); lane 2, albumin + monomeric catechin; lane 3, plasma + monomeric catechin; lane 4, plasma; lane 5, serum; lane 6, plasma + MOC; lane 7, albumin + monomeric catechin; lane 8, MW + monomeric catechin. (B) Staining with vanillin: lane 1, MW; lane 2, albumin + monomeric catechin; lane 3, plasma + monomeric catechin; lane 4, plasma + MOC; lane 5, serum + monomeric catechin; lane 6, serum + MOC; lane 7, albumin + monomeric catechin; lane 8, MW + monomeric catechin.

DISCUSSION

It seems clear that the moderate consumption of alcohol reduces mortality from coronary heart diseases (*22*), but wine appears to be more beneficial than alcohol (*6*). Although epidemiological studies (*23*) and some studies carried out on rats (*24*, *25*) indicate that wine flavonoids have effects in vivo, much is still unknown: for example, the mechanism through which they act, how they are transported to target sites, and their bioavailability. It was for this reason that we decided to study the affinity of grape seed catechins—which are abundant in red wine—for plasma proteins and how they are transported in the blood.

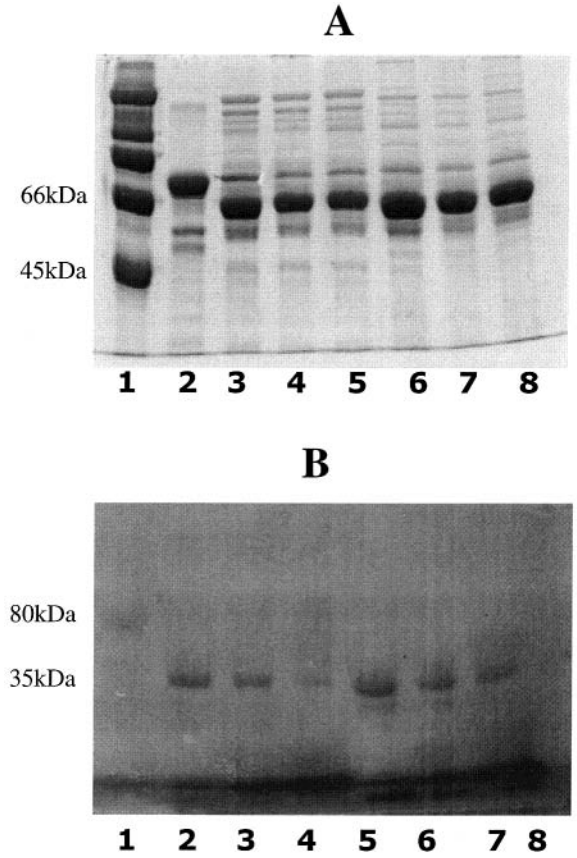

Figure 2. SDS-PAGE analysis of human plasma proteins that bind to pure monomeric catechin and a mixture of MOC from grape seed. Samples (10 μL) were diluted 1:20 and loaded onto 7.5% polyacrylamide–SDS gels. After electrophoresis, the gels were stained with Coomassie blue or 1% vanillin/70% H_2SO_4 (a specific catechin dye, to identify proteins binding to catechins). Gels with plasma and serum without catechins are not shown because there were no stained bands with vanillin. (A) Staining with Coomassie blue: lane 1, molecular mass markers (MW); lane 2, transferrin; lane 3, plasma; lane 4, plasma + monomeric catechin; lane 5, plasma + MOC; lane 6, serum; lane 7, serum + monomeric catechin; lane 8, serum + MOC. (B) Staining with vanillin: lane 1, transferrin; lane 2, plasma + monomeric catechin; lane 3, serum + monomeric catechin; lane 4, plasma + monomeric catechin; lane 5, plasma + MOC; lane 6, serum + MOC; lane 7, serum + MOC.

In accordance with other results (*26*) we found that (+)-catechin is mainly transported by plasma. The small amount that is transported by erythrocytes (10%) is believed to be bound to membrane phospholipids (*27*).

Phenolic compounds generally bind to several proteins (*28*, *29*), and it is believed that they may be transported bound to plasma proteins. To identify the plasma proteins that bind to wine catechins, we carried out SDS electrophoresis on plasma and serum incubated with MOC from grape seed or pure monomeric (+)-catechin. As some researchers have reported that phenol−protein complexes break in the presence of SDS (*19*), we repeated the electrophoresis in native conditions. In both conditions, the proteins that bind to grape procyanidins were the same, so it can be assumed that the SDS does not totally break the phenol−protein complex. Our results show that the principal protein that binds to grape seed procyanidins was a protein with an approximate molecular weight of 35 kDa in humans and 80 kDa in rats. These proteins were identified as apo A-I and transferrin, respectively. The protein/catechin ratio was maintained constant, and vanillin dying (SDS condition)

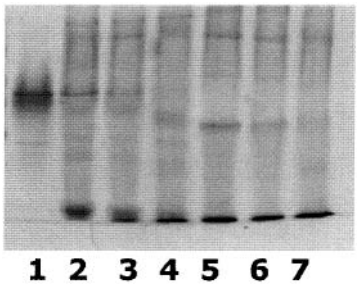

Figure 3. PAGE analysis of human and rat plasma proteins that bind to pure monomeric catechin and a mixture of MOC from grape seed: lane 1, transferrin; lane 2, rat plasma; lane 3, rat plasma + monomeric catechin; lane 4, rat plasma + MOC; lane 5, human plasma; lane 6, human plasma + monomeric catechin; lane 7, human plasma + MOC. Samples (10 μL) were diluted 1:20 and loaded onto 10% polyacrylamide gels in non-denaturing conditions. Proteins were blotted onto Immobilon P membranes after electrophoresis and stained with Coomassie blue. Protein–catechin complexes were identified by band disappearance.

and band disappearance (native condition) were higher with MOC from grape seed than with pure monomeric (+)-catechin. It therefore follows that the affinity of catechins for protein increases as the degree of polymerization of catechins increases, as suggested by other authors (*30*). However, phenolic compounds are metabolized in vivo (sulfates, glucuronides, etc.), and this may modify their binding to plasma proteins.

Other authors have studied flavonoid−protein interactions. Quercetin and its derivatives bind to albumin in the plasma of rats fed a diet supplemented with quercetin (*31*, *32*). Epigallocatechin gallate—a tea catechin—binds to human plasma proteins such as fibronectin, fibrinogen, and a histidine-rich protein when the serum is incubated with tea catechins (*14*). We observed no differences between plasma and serum, which indicates that fibrinogen does not act in the transport of procyanidins from grape seed.

The concentrations of pure monomeric (+)-catechin and MOC from grape seeds that were used in this study are higher than those that have been described in humans and animals after the ingestion of quercetin or tea catechins (*1*, *33*). Such high concentrations were required because the method used needs the system to be saturated if it is to observe the vanillin coloring or the disappearance of the bands. Therefore, the fact that at such high concentrations only certain bands disappear reinforces even further the idea that the catechin−protein binding is highly specific and that even if there is an excess, they do not bind nonspecifically to majority proteins in plasma such as albumin. This shows that in vivo they will probably bind in this specific fashion and that these proteins will interact with wine catechins. There are no studies on whether the oligomeric forms of wine catechins absorb or not, but epigallocatechin gallate of tea catechins has been found in animals that have been given tea catechins (*34*). Thus, it seems that the specific structure of each phenol determines how it interacts with a particular protein, so that each phenol can be specifically transported by plasma and have a particular physiological effect.

As mentioned above, the major protein to bind to grape procyanidins was different in the two species. In humans grape procyanidins bound preferentially to apo A-I, whereas in rats they bound to transferrin. It should be pointed out that other plasma proteins were also bound to a lesser extent in both species. A comparison of human apo A-I and rat transferrin sequences and three-dimensional structures did not clarify why these were the preferred plasma proteins binding to catechins: each protein was very similar in the two species, but the two

proteins were very different. The percentage of similarity between human and rat apo A-I was 60.6%, and that between human and rat transferrin was 71.9%. However, when comparisons were made between transferrin and apo A-I, the similarity dropped to between 12 and 14%. The three-dimensional structure of apo A-I proved to belong to the all-α protein class, and transferrin was shown to be made up of two homologous domains, both belonging to the α-β protein class. The amino acid percentage of each protein was also similar in the two species. The contents of proline, the amino acid that is most related to phenol binding (*35*), were similar in the same protein of each species and higher in transferrin than in apo A-I (5.2 and 4.6% in rat and human transferrin and 2.7 and 3.8% in rat and human apo A-I, respectively). Therefore, this preference to bind to a certain protein in a species must be due to very subtle changes—perhaps in an amino acid or in the conformation—which means that there is a common element between rat transferrin and human apo A-I.

Increases in human serum apo A-I and high-density lipoprotein (HDL) cholesterol are frequently related to moderate alcohol consumption in vivo and, in general, no differences have been detected among different alcoholic beverages (*36*−*38*). This elevated concentration of HDL cholesterol associated with moderate alcohol intake is one of the potential causes for the relative decrease in cardiovascular risk reported in moderate drinkers (*22*). Additional effects have been attributed to wine phenol compounds and are usually related to inhibition of low-density lipoprotein (LDL) oxidation, free radical scavenging, and modulation of the eicosanoid metabolism (*6*), whereas there are no reports on the effects of phenolic compounds on apo A-I. As far as we know, this is the first study that directly relates wine procyanidins and apo A-I. Apo A-I is the major protein in human plasma HDL, and it may actually be a more accurate predictor of cardiovascular risk than HDL cholesterol (*39*). Apo A-I activates lecithin:cholesterol acyltransferase (LCAT) (*40*) and is therefore essential for the reverse cholesterol pathway. LCAT activation is affected by the apo A-I conformation (*40*) so, if apo A-I binds to (+)-catechin and procyanidins, the LCAT may be more active. The fact that the (+)-catechin and procyanidins in red wine bind to apo A-I suggests that they may have a role, until now reserved to ethanol, in the positive effects of wine on the reverse transport of cholesterol from tissues to the liver for excretion.

Iron plays a central role in oxidative damage, and one of the mechanisms for the antioxidant effect of flavonoids is thought to be chelating iron and other metals (*41*, *42*). Transferrin, the other major protein that bound to (+)-catechin and procyanidins, is the plasma iron transporter. It is its iron-binding capacity that makes it able to function as an antioxidant because it means that iron(III) is not available to participate in oxidative iron-catalyzed reactions. Our results suggest that the binding of grape procyanidins to transferrin may contribute to the antioxidant effects observed after wine drinking: rats that drank red wine for 6 months had lower MDA concentrations in the liver and kidney and higher vitamin E in plasma (*25*); humans had a significant increase in serum antioxidant capacity after consuming red wine (*43*).

Further research is required if the meaning of these results is to be fully understood. It is of considerable interest that monomeric catechin and procyanidins in red wine—derived from grape seeds—bind to proteins related to cholesterol and the oxidative metabolism, precisely the areas in which phenols and red wine have been reported to have their greatest effect.

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