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sPhospholipase A_2 is inhibited by anthocyanidins

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Abstract Epidemiological studies suggest that nutritional antioxidants may reduce the incidence of neurodegenerative disorders and age-related cognitive decline. Specifically, protection against oxidative stress and inflammation has served as a rationale for promoting diets rich in vegetables and fruits. The present study addresses secretory phospholipase A_2 ($sPLA_2$) as a novel candidate effector of neuroprotection conferred by anthocyanins and anthocyanidins. Using a photometric assay, 15 compounds were screened for their ability to inhibit PLA_2. Of these, cyanidin, malvidin, peonidin, petunidin, and delphinidin achieved K_i values $\leq$18 μM, suggesting a modulatory role for berry polyphenols in phospholipid metabolism.

Keywords Phospholipase A_2 · Flavonoids · Anthocyanin · Anthocyanidin

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Introduction

Phospholipases A_2 form a superfamily of esterases that specifically cleave the acyl ester bond at the *sn*-2 position of membrane phospholipids, generating free fatty acids and lysophospholipids (Dennis 1994). These hydrolases are involved in a complex network of signaling pathways, linking receptor agonists, oxidants, and proinflammatory cytokines to the release of arachidonic acid and to eicosanoid synthesis (Sun et al. 2004). Eisosanoids include prostaglandins, thromboxanes, prostacyclins, and leukotrienes (Granstrom 1984), which act as inflammatory mediators. Moreover, oxidative metabolism of arachidonic acid and disruption of the mitochondrial respiratory chain, mediated by phospholipase A_2 (PLA_2) cardiolipin hydrolysis, may contribute to the generation of reactive oxygen species (ROS) and oxidative stress (Muralikrishna Adibhatla and Hatcher 2006).

PLA_2s may be grouped into at least three major classes, Ca^{2+}-dependent cytosolic PLA_2 ($cPLA_2$), Ca^{2+}-independent cytosolic PLA_2 ($iPLA_2$) and secretory PLA_2 ($sPLA_2$) (Tibes and Friebe 1997), which are expressed in the central nervous system (CNS) (Sun et al. 2004). Of these, $sPLA_2$s are major contributors to the excessive production of arachidonic acid in inflammatory conditions (Yedgar et al. 2000) and comprise the 14 kDa "group V" PLA_2 with high affinity for phosphatidylcholine-rich plasma membranes (Murakami and Kudo 2004). In mammalian brain, group V

PLA_2 is found primarily in cortical neurons (Nardicchi et al. 2007) and in the hippocampus (Molloy et al. 1998). Inhibitors of PLA_2 hold promise in the treatment of brain disorders that involve oxidative stress, changes in phospholipid metabolism, accumulation of lipid peroxides, and inflammation including ischemia, multiple sclerosis, epilepsy, and Alzheimer's disease (Farooqui et al. 2006).

Emerging neuroprotective properties of anthocyanins from berry fruits (Kang et al. 2006; Joseph et al. 2007; Shukitt-Hale et al. 2007; Tarozzi et al. 2007; Duffy et al. 2008), have renewed the interest in dietary compounds' potential for PLA_2 inhibition. Anthocyanins are polyphenolic constituents of many fruits and vegetables that are particularly abundant in bilberries, black raspberries, and chokeberries, where they occur mostly as glycosides (anthocyanins) at concentrations of 600, 700, and 1500 mg per 100 g fresh weight, respectively (Nyman and Kumpulainen 2001; Wu et al. 2006). On average, daily anthocyanin consumption may reach 180–215 mg in western societies (Kuhnau 1976) but recent calculations from U.S. American surveys have alerted to variability due to sociodemographic and lifestyle factors (Chun et al. 2007). In animals, ingestion of anthocyanins has been associated with reversal of age-related cognitive and motor deficits (Joseph et al. 1999), with protection from ischemia-induced damage (Sweeney et al. 2002; Wang et al. 2005), and with decreased vulnerability to oxidative stress (Galli et al. 2002).

As oxidative stress and inflammation are modulated by PLA_2 activity (Farooqui et al. 2006) we hypothesized a role for PLA_2 in conferring neuroprotection by berry constituents. The present study investigates the in vitro impact of anthocyanidins, anthocyanins' aglycons, on PLA_2-V activity using enzyme kinetic parameters.

Materials and methods

Chemicals

1,2-Bis(heptanoylthio)-phosphatidylcholine, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), thioetheramide phosphatidylcholine and recombinant human PLA_2-V were obtained from Cayman Europe (Tallinn, Estonia). $CaCl_2$, KCl, and HCl (25%) were purchased from Merck (Darmstadt, Germany), TritonX-100 from ICN Biomedicals (Aurora, Ohio), Tris from Carl Roth (Karlsruhe, Germany), and protocatechuic acid from Sigma-Aldrich (Steinheim, Germany).

Cyanidin, cyanidin-3,5-*O*-diglucoside, cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, delphinidin, malvidin, malvidin-3,5-*O*-diglucoside, malvidin-3-*O*-galactoside, malvidin-3-*O*-glucoside, peonidin, pelargonidin, pelargonidin-3,5-*O*-diglucoside, petunidin, and catechin were purchased from Extrasynthese (Genay, France).

Flavonoids were dissolved and diluted with DMSO. From the ethanolic solution of thioetheramide phosphatidylcholine, the solvent was evaporated under a stream of nitrogen and a DMSO solution was reconstituted and vortexed vigorously before further dilution.

PLA_2 assay

Enzyme kinetics analysis was performed using a photometric assay based on the Ellman method (Ellman et al. 1961). Briefly, hydrolysis of the *sn*-2 ester bond of the substrate 1,2-bis(heptanoylthio)-glycerophosphocholine by PLA_2-V is followed by the exposure of free thiols. These trigger the conversion of DTNB to 2-nitro-5-thiobenzoic acid which is detected photometrically at 405 nm. Experiments were performed at least twice in duplicate.

Prior to performing inhibition studies, linearity of product formation was investigated with regard to incubation time and various substrate, DTNB, and enzyme concentrations to optimize assay conditions. Thereupon, the assay was carried out in an aqueous buffer solution (pH 7.5) containing KCl, $CaCl_2$, Tris and Triton-X 100 at final assay concentrations of 94, 9, 24 mM, and 280 μM, respectively. Immediately before the assay was performed, substrate and PLA_2-V were resuspended in assay buffer and DTNB was dissolved in an aqueous solution of Tris–HCl (pH 8) with enzyme and DTNB yielding final concentrations of 100 ng/ml and 87 μM, respectively. For enzyme kinetic analysis, at least five substrate concentrations between 0.15 and 1.2 mM were used per concentration step. For non-linear regression analysis of catechin and protocatechuic acid effects, substrate was applied at a concentration of 0.3 mM.

Assays were performed in 96-well microtiter plates at room temperature, containing DTNB, substrate solution plus the respective test substance. Thioetheramide phosphatidylcholine was used as a reference PLA_2 inhibitor and DMSO served as a negative control. This solvent was shown to be inactive at the concentration used in the assay (1.7% v/v). The phospholipase reaction was initiated by adding PLA_2-V, or assay buffer for control measurements. With respect to enzyme kinetic experiments, inhibition was measured at test compound concentrations ranging from 4 to 80 μM for anthocyanidins, and from 32 nM to 5 μM for thioetheramide phosphatidylcholine. Absorption at 405 nm was recorded at intervals of 30 s between 5 and 10 min thereafter with a Tecan Spectra Mini Photometer (Crailsheim, Germany).

Data analysis

Following normalization, the absorption was plotted against the incubation time. The resulting slope served as a

measure of enzyme initial velocity (v) and was plotted against the respective substrate concentration [S] to obtain a substrate–velocity curve. Curves were then linearized by creating a reciprocal plot, or Lineweaver–Burk plot (L–B plot), which gave a family of intersecting lines for results of inhibition and control experiments. From this plot, the Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) were calculated, while the line intersection point served to determine the mode of inhibition. The linear fit of the negative control was extrapolated to the point of x-axis intersection, with the negative abscissa intercept $-1/K_m$ and the ordinate intercept equaling $1/V_{max}$.

Kinetic models considered for PLA_2 inhibition by anthocyanidins are outlined in supplementary Fig. 1. For calculation of further kinetic constants, i.e., the dissociation constant K_i, plus coefficients α and β for discrimination between complete and partial inhibition, secondary diagrams were generated plotting slope L–B plot versus [I], 1/v versus [I] (Dixon plot), [S]/v versus [I] (Cornish–Bowden plot), $1/\Delta$ y-axis intercept L–B plot versus 1/[I] and $1/\Delta$ slope L–B plot versus 1/[I]. For enzyme kinetics analysis, we assumed rapid equilibrium of the enzyme–substrate binding reaction, allowing us to use K_m and K_s as equivalents (Copeland 2000). Prism v. 4.00 (GraphPad Software, CA, USA) and Microsoft Office Excel 2003 (Microsoft Corporation, WA, USA) were used for non-linear regression and kinetic analysis. ISIS/Draw v. 2.1.4 (MDL Information Systems, CA, USA) served to illustrate chemical structures of anthocyanidins.

Results

Of the 15 compounds examined, anthocyanidins exhibited the best inhibitory effects on PLA_2 in a first round of experiments (data not shown). Inhibitory properties of anthocyanins, in contrast, were less pronounced and could not be quantified as absorption interfered with the photometric assay at millimolar concentrations. Catechin, the flavan-3-ol analog of cyanidin, and protocatechuic acid, a potential cyanidin metabolite, reached 50% inhibition at concentrations of 2.5 and 3.3 mM, respectively. Further investigations of enzyme kinetics were therefore restricted to cyanidin, malvidin, peonidin, petunidin, delphinidin, and pelargonidin (Fig. 1). For these agents, K_m (0.3 mM) and V_{max} (14 μmol/min ml) were determined from L–B plots. With regard to the mode of interaction with PLA_2, only the reference compound thioetheramide phosphatidylcholine ($K_i = 0.59$ μM) exhibited complete competitive inhibition. For malvidin ($K_i = 6.4$ μM), a hyperbolic slope L–B plot versus [I] replot was obtained, indicating partial competitive PLA_2 inhibition at $\alpha = 1.8$ and assuming $\beta = 1$. L–B plots for pelargonidin ($K_i = 325$ μM) and delphinidin ($K_i = 18$ μM) met criteria for mixed competitive and non-competitive PLA_2 inhibition. For both compounds, linearity of the L–B plot slope versus [I] replot confirmed complete inhibition at α values of 14.8 and 1.6 for delphinidin and pelargonidin, respectively. Petunidin ($K_i = 14$ μM), peonidin ($K_i = 10$ μM) and cyanidin (2.1 μM) were also identified as mixed competitive and non-competitive inhibitors from L–B plots. However, their L–B plot slope versus [I] replots indicated a partial (hyperbolic) type of inhibition. For these flavonoids, the ternary complex rate coefficients α and β (supplementary Fig. 1) were calculated from the linear plots of $1/\Delta$ slope versus 1/[I] and $1/\Delta$ ordinate intercept versus 1/[I] (Segel 1993), yielding values of 1.6, 1.6, and 2.9 (α) and 0.62, 0.79, and 0.7 (β) for petunidin, peonidin, and cyanidin, respectively.

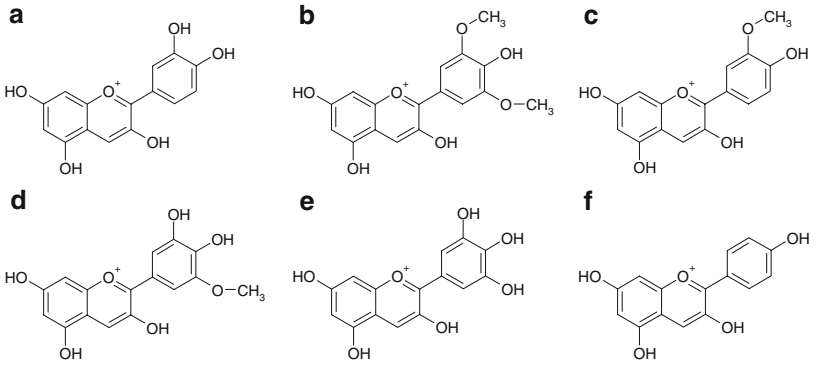
Discussion

The present study is the first to demonstrate $sPLA_2$-V inhibition by anthocyanidins in the low micromolar range ($K_i = 2.1$–18 μM), with the exception of pelargonidin ($K_i = 325$ μM). For cyanidin, inhibition approached that of the reference $sPLA_2$ inhibitor, thioetheramide phosphatidylcholine, with K_i values differing by a factor of 4. Anthocyanidin–glycosides, in contrast, were weak PLA_2-V inhibitors for which K_i values could not be estimated as anthocyanins' absorption at higher concentrations interfered with the photometric assay. Thus, with the exception of pelargonidin, the aglycons of prevalent anthocyanins from food sources are potent PLA_2 inhibitors. Anthocyanidins can be formed from anthocyanins at the intestinal level by epithelial cell and microflora β-glucosidases (Tsuda et al. 1999; Keppler and Humpf 2005; Tarozzi et al. 2007), and have also been identified in brain (Talavera et al. 2005; El Mohsen et al. 2006).

Other than anthocyanidins, a limited number of flavonoids have been tested for PLA_2 inhibitory activity. Among these, the flavonols quercetin, quercetagetin and kaempferol-3-O-galactoside, plus the flavon scutellarein inhibited PLA_2-II with IC_{50} values ranging from 2 to 18 μM (Lindahl and Tagesson 1993; Gil et al. 1994). For PLA_2-V, the flavonol derivate papyriflavonol A and the biflavonoids amentoflavone and ochnaflavone showed 50% inhibition at concentrations between 5 and 42 μM (Kwak et al. 2003; Moon et al. 2007), but K_i values are lacking.

Four of the six tested anthocyanidins exerted only partial inhibition of PLA_2, as has also been observed for PLA_2-I with the flavonol quercetin and its 3-rutinoside rutin (Lindahl and Tagesson 1993; Lindahl and Tagesson 1997).

With regard to structural features, similar K_i values for most anthocyanidins investigated argue against a major role of anthocyanidins' B-ring substitution pattern in



substance		type of inhibition	Ki (μM)	α	β
Thioetheramide-PC		competitive	0.59		
Cyanidin	(a)	partial mixed	2.1	2.9	0.70
Malvidin	(b)	partial competitive	6.4	1.8	
Peonidin	(c)	partial mixed	10	1.6	0.79
Petunidin	(d)	partial mixed	14	1.6	0.62
Delphinidin	(e)	mixed	18	14.8	
Pelargonidin	(f)	mixed	325	1.6	

Fig. 1 Chemical structures of anthocyanidins under study and kinetic parameters of PLA_2 inhibition. Thioetheramide phosphatidylcholine (PC) served as a reference inhibitor

predicting $sPLA_2$-V inhibitory potential. To judge by weak inhibitory activity of catechin, the flavan-3-ol analogon of cyanidin ($IC_{50} = 2.5$ mM), anthocyanidins' unsaturated C-ring or their electric charge may prove more informative. With respect to the type of PLA_2 inhibition, however, B-ring substitution patterns deserve further study.

As natural anthocyanins are reportedly unstable in the intestinal environment, the role of phenolic acid metabolites generated by intestinal microflora and metabolic degradation is of particular interest (Aura et al. 2005; McGhie and Walton 2007; Vitaglione et al. 2007). However, follow-up experiments conducted with protocatechuic acid, a potential cyanidin metabolite, elicited only very weak $sPLA_2$-V inhibition ($IC_{50} = 3.2$ mM). Bioavailability of individual parent compounds therefore deserves further study prior to assuming in vivo inhibitory effects.

For those agents that exhibit in vitro activities in the low micromolar range, a number of possible CNS functionalities may be discussed. Recent studies implicate increased PLA_2 activity and PLA_2-generated mediators in the acute inflammatory response of the brain, e.g., to ischemia (Farooqui et al. 2006), in kainic acid-induced neurotoxicity (Thwin et al. 2003), and in chronic pathologies associated with Alzheimer's disease, Parkinson's disease and multiple sclerosis (Farooqui et al. 2006), schizophrenia (Tavares et al. 2003; Barbosa et al. 2007), and bipolar affective disorder (Ross et al. 2006). It is believed that PLA_2 cellular effects manifest at multiple levels: Phospholipid breakdown increases membrane permeability and, consequently, Ca^{2+} influx, lipolysis, and proteolysis (Farooqui et al. 1997). Lysophospholipids, in turn, may exert detergent-like effects on neuronal membranes (Farooqui et al. 1999) and act as precursors of the platelet-activating factor (PAF), a strong mediator of the inflammatory process (Yedgar et al. 2000). Free fatty acids released from phospholipids can alter mitochondrial polarization state (Pompeia et al. 2000), cause mitochondrial dysfunction and may trigger an uncontrolled arachidonic acid cascade, followed by synthesis of inflammatory mediators, production of ROS (Farooqui et al. 1997) and neurotoxic 4-hydroxynonenal (Farooqui and Horrocks 2006). Released arachidonic acid, finally, may alter membrane fluidity (Villacara et al. 1989), inhibit glutamate uptake (Barbour et al. 1989), and modulate activities of protein kinases (Katsuki and Okuda 1995).

Neuroinflammation, oxidative stress, and altered phospholipid metabolism are involved in the pathophysiology of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and multiple sclerosis, leading to neuronal loss via a complex sequence of events that comprise an upregulation of complement, cytokines, and acute phase reactants among other mediators (Gilgun-Sherki et al. 2001; Minghetti 2005; Farooqui et al. 2006; Farooqui et al. 2007). In this context, there is growing support for strategies that prevent inflammatory reactions during neurodegeneration. Mixed results have been achieved by inhibiting selective pathways of eicosanoid production, i.e. the lipoxygenase (LOX) and cyclooxygenase (COX) pathways (Yedgar et al. 2000). Control of arachidonic acid production currently holds

promise in the treatment of phospholipid pathologies. A challenge in maintaining basal levels of arachidonic acid, lysophospholipids, and PAF, however, is posed by the multiplicity of PLA_2s, the interplay among downstream mediators and the recognition that many PLA_2 functionalities are also essential for normal cell function (Balsinde et al. 1999).

Moreover, with regard to the etiology of most disorders, it remains to be established whether phospholipid breakdown is present early in neurodegenerative disease or whether it is only an epiphenomenon of cell death (Klein 2000). Pending an improved understanding of cause and effect, the utility of candidate PLA_2 inhibitors in counteracting phospholipid degradation is difficult to predict by in vitro data.

Should PLA_2 inhibition occur at the concentrations achieved by dietary intake of anthocyanins, this may help explain certain fruits' role in lowering age-related neurodegenerative disease (Ramassamy 2006; Joseph et al. 2007). In support of this notion, ingestion of blueberry constituents enhanced hippocampal plasticity (Casadesus et al. 2004), memory (Goyarzu et al. 2004), and motor performance (Joseph et al. 1999), plus induced changes in CNS signal transduction and receptor sensitivity (Joseph et al. 1999). Anthocyanins and their corresponding aglycons are found in animal brains within minutes after oral uptake (Andres-Lacueva et al. 2005; El Mohsen et al. 2006). In animals fed with blueberries, anthocyanin concentrations in brain correlated with cognitive performance (Andres-Lacueva et al. 2005). Although oxidative stress (Cantuti-Castelvetri et al. 2003) and inflammatory reactions (Perry et al. 2007) both contribute to age-related pathologies, antioxidant activity alone does not explain the potency of berry constituents in protecting against neurodegeneration (Shukitt-Hale et al. 2008). Anthocyanin effects on phospholipid metabolism may help explain such benefits as does inhibition of lipid peroxidation (Wang et al. 1999) and modulation of inflammatory mediators COX I and II (Seeram et al. 2001).

The present findings on anthocyanidins' $sPLA_2$-V inhibitory functionality encourage further investigations addressing other PLA_2 isoforms. To date, dietary supplementation with anthocyanins is considered safe and unlikely to interfere with drug metabolism (Dreiseitel et al. 2008).

Partial inhibition of PLA_2-V by most compounds under study may prove advantageous in vivo in that basal levels of phospholipid-derived mediators could be maintained for normal brain function.

Taken together, beneficial effects of fruit antioxidants on aging and neurodegeneration warrant investigations at multiple levels. Our findings on $sPLA_2$-V inhibition by anthocyanidins provide further evidence to rationalize antioxidative and antiinflammatory activities. More studies are invited to explore PLA_2 isoform specificity of these properties, and to define their behavioral correlates.

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