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Protective activities of *Vaccinium* antioxidants with potential relevance to mitochondrial dysfunction and neurotoxicity

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Abstract

Both the neurotransmitter dopamine (DA) and a neurotoxic metabolite, 6-hydroxy DA, can be oxidized to generate hydrogen peroxide and other reactive species (ROS). ROS promote oxidative stress and have been implicated in dopaminergic neurodegeneration, e.g., Parkinson's disease (PD). There is also evidence for a relation between catecholamine-mediated oxidative damage in dopaminergic neurons and the effects of these neurotransmitters on the redox state of cytochrome *c* (Cytc). In neurons and other cells, oxidative stress may be enhanced by abnormal release of Cytc and other mitochondrial proteins into the cytoplasm. Cytc release can result in apoptosis; but sub-apoptogenic-threshold release can also occur, and may be highly damaging in the presence of DA metabolites. Loss of mitochondrial membrane integrity, a pathological situation of relevance to several aging-related neurodegenerative disorders including PD, contributes to release of Cytc; and the level of such release is known to be indicative of the extent of mitochondrial dysfunction. In this context, we have used a Cytc-enhanced 6-hydroxy DA oxidation reaction to gauge dietary antioxidant activities. Anthocyanin-rich preparations of *Vaccinium* species (*Vaccinium myrtillus*, *Vaccinium corymbosum*, and *Vaccinium oxycoccus*) as well as a purified glycosylated anthocyanidin were compared. The most potent inhibition of oxidation was observed with *V. myrtillus* preparation: 50% inhibition with 7 μM of total anthocyanins. This activity was 1.5–4 times higher than that for the other preparations or for the purified anthocyanin. Ascorbate (Vitamin C), at up to 4-fold higher concentrations, did not result in significant inhibition in this assay. Antioxidant activity in the assay correlated strongly ($r^2 > 0.91$, $P < 0.01$) with reported *Vaccinium* content of anthocyanins and total cyanidins, but not quercetin or myricetin. The results provide evidence for the high potency of anthocyanins towards a potentially neurotoxic reaction, and provide a basis for *in vivo* testing of these flavonoids and their physiological metabolites in the context of neuro- and mitochondrio-protective effects.

Keywords: Neurotoxic; Dopamine; Cytochrome *c*; Mitochondria; Anthocyanins; *Vaccinium*

1. Introduction

Reactive oxygen species (ROS) and other oxidizing agents generated *in vivo* can damage tissues and affect the activities of biological molecules. Excessive production, deficient elimination, or a combination of both reactions involving ROS may, over time, contribute to aging, cardiovascular and neurodegenerative disorders, and cancer (e.g., Ames, 2004; Ames et al., 1993; Benzie, 2003; Galli et al., 2002; Jenner, 1996; Joseph et al., 2005; Medina et al., 2002; Migliaccio et al., 1999). In addition to the body's endogenous antioxidants, dietary antioxidants such as vitamins E and C are critical factors in controlling oxidative damage. There is also much current interest in the potential protective effects of other, non-vitamin, dietary antioxidants; one class of these is the anthocyanins, glycosylated anthocyanidin flavonoids abundant in many fruits and vegetables (e.g., Kahkonen and Heinonen, 2003; Wang et al., 1997). These blue–red pigments are potent antioxidants: they readily donate hydrogen to form relatively stable unpaired-electron structures, and chelate transition metals such as iron (Rice-Evans et al., 1996). In the context of the nervous system, vitamin and non-vitamin dietary antioxidants, including anthocyanins, have been shown to improve motor and cognitive functions in experimental animals (Bickford et al., 2000; Galli et al., 2006; Jenner, 1996; Joseph et al., 1999; Socci et al., 1995) and to prevent ROS-mediated apoptotic death in neurons (Jones

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et al., 2000; Levites et al., 2002; Luo et al., 1998; Medina et al., 2002).

There is evidence that cytochrome *c* (Cytc), a mitochondrial protein that functions in the electron transport chain, can contribute to neurodegenerative disorders along with other apoptogenic mitochondrial proteins (Hashimoto et al., 2003; Oliver et al., 2005; and references therein). When mitochondrial membrane integrity is compromised and Cytc release occurs into the cytoplasm of cells, the result may be increased oxidative stress, respiratory chain dysfunction, and apoptotic cell death (Berman and Hastings, 1999; Cai et al., 1998; Dessolin et al., 2002; Oliver et al., 2005). Non-apoptogenic release of mitochondrial Cytc can also occur (Chen et al., 1998; Clayton et al., 2005; Deshmukh and Johnson, 1998; Martinou et al., 1999; Oliver et al., 2005; Von Ahsen et al., 2000) and is likely to promote cellular oxidative stress. There is also evidence that dopamine (DA) oxidation in neurons can result in the production of reactive quinone species and ROS such as hydrogen peroxide; this lowers brain antioxidant defences and increases oxidative stress (Adams and Odunze, 1991; Berman and Hastings, 1999). Such oxidative stress mechanisms are also the basis of the neurotoxic effect of 6-hydroxy dopamine (6-OHDA) used to model Parkinson's disease (PD) in experimental animals (Adams and Odunze, 1991; Ames, 2004; Cohen and Heikkila, 1974; Levites et al., 2002; Von Ahsen et al., 2000; Woodgate et al., 1999). Moreover, recent evidence suggests that catecholamines, DA and 6-OHDA, have Cytc-reducing properties and, by interfering with normal cytochrome redox cycling, may contribute to dopaminergic neurodegeneration (Mazzio et al., 2004), e.g., generation of highly destructive hydroxyl radicals by Fe(II)–Cytc and hydrogen peroxide. Also, in relation to PD, alpha-synuclein aggregation is known to be induced by co-incubation with Cytc and hydrogen peroxide (Hashimoto et al., 1999).

In this study, the antioxidant potencies of anthocyanin-rich preparations of three *Vaccinium* species and, for comparison, of a purified glycosylated anthocyanidin, cyanidin 3-glucoside, were gauged using an assay based on cytochrome *c*-enhanced oxidation of 6-OHDA. In the context of the neurotoxic aspects of Cytc and dopamine metabolites presented above, this oxidation reaction may be of relevance to the pathology of some neurodegenerative diseases, especially the dopaminergic neurodegeneration characteristic of Parkinson's disease, and more generally to diseases involving mitochondrial dysfunction.

2. Materials and methods

2.1. Materials

Cytochrome *c* (equine heart), 6-hydroxydopamine (6-OHDA), ascorbic acid, and buffers were obtained from Sigma–Aldrich. Cyanidin 3-glucoside was obtained from Polyphenols. A 6-OHDA stock solution was prepared in the reaction buffer, argon-saturated Hepes (50 mM, pH 7.4); the stock was continuously flushed through with argon gas and prepared fresh for each experiment. Cyanidin 3-glucoside was of HPLC purity grade as analyzed using a 10–100% linear gradient of (v/v) HCO_2H–H_2O–MeOH (1:9:10) into HCO_2H–H_2O (1:10) (Polyphenols). Anthocyanin-rich aqueous extract was obtained from the berries of *Vaccinium myrtillus* (bilberry; product of northeastern Finland), and from *Vaccinium corymbosum* (blueberry; product of Canada, southwestern British Columbia) and *Vaccinium oxycoccus* (cranberry; Ocean Spray® product of northeastern USA) by the methods previously described (Yao et al., 2004). The total anthocyanin concentration in the extracts was obtained from a standard absorption curve for known amounts of cyanidin 3-glucoside (Yao et al., 2004). *Vaccinium* extracts were stored frozen, −80 °C.

2.2. 6-OHDA and TMPD oxidation assays

Assays with 6-OHDA as the indicator for oxidative activity were performed using similar procedures and reagent concentrations as those reported for assays with *N*,*N*,*N*′,*N*′-tetramethy-1,4-phenylenediamine (TMPD) as the indicator (Yao et al., 2004). Briefly, the putative antioxidants to be tested (see concentration in figure legends) were added to the reaction buffer in a cuvette containing cytochrome *c* and hydrogen peroxide after a 10 pre-incubation of the latter two reagents. The cuvette contents were flushed with argon for 2 min before addition of 6-OHDA from the argon-saturated stock. Based on total amounts of reagents added to the assay, the final concentrations of 6-OHDA, cytochrome *c*, and hydrogen peroxide in the assay were 200, 10 μM, and 5 mM, respectively. Progression of the oxidation reactions was followed spectrophotometrically at 490 nm. Typically, results at 60 or 120 s were used for the analyses. A time course up to 160 s is shown in Fig. 1. Concentrations were the same for both

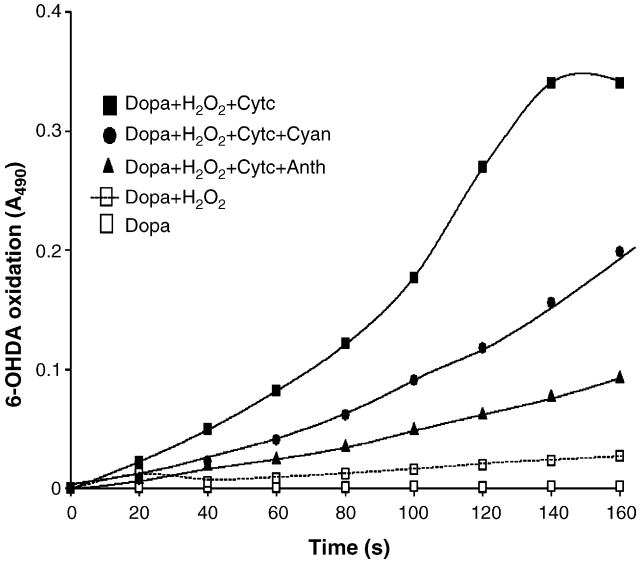

Fig. 1. Kinetics of 6-OH Dopamine oxidation and the effects of anthocyanins. The oxidation of 6-OH dopamine (Dopa) in the presence of the reagents indicated in the figure was followed over a time period of 160 s (see Section 1 for assay reagent concentrations). The oxidation of 6-OH dopamine due to the combined effects of H_2O_2 and cytochrome *c* was attenuated by both purified cyanidin 3-glucoside (6.9 μM) and an anthocyanin-rich extract (8.6 μM total anthocyanins). This figure represents the results of one, typical assay; results of all the assays are presented in the figures that follow.