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Identification and in Vitro Biological Activities of Hop Proanthocyanidins: Inhibition of nNOS Activity and Scavenging of Reactive Nitrogen Species

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Oligomeric proanthocyanidins constitute a group of water-soluble polyphenolic tannins that are present in the female inflorescences (up to 5% dry wt) of the hop plant (*Humulus lupulus*). Humans are exposed to hop proanthocyanidins through consumption of beer. Proanthocyanidins from hops were characterized for their chemical structure and their in vitro biological activities. Chemically, they consist mainly of oligomeric catechins ranging from dimers to octamers, with minor amounts of catechin oligomers containing one or two gallocatechin units. The chemical structures of four procyanidin dimers (B1, B2, B3, and B4) and one trimer, epicatechin-(4β→8)-catechin-(4α→8)-catechin (TR), were elucidated using mass spectrometry, NMR spectroscopy, and chemical degradation. When tested as a mixture, the hop oligomeric proanthocyanidins (PC) were found to be potent inhibitors of neuronal nitric oxide synthase (nNOS) activity. Among the oligomers tested, procyanidin B2 was most inhibitory against nNOS activity. Procyanidin B3, catechin, and epicatechin were noninhibitory against nNOS activity. PC and the individual oligomers were all strong inhibitors of 3-morpholinosydnonimine (SIN-1)-induced oxidation of LDL, with procyanidin B3 showing the highest antioxidant activity at 0.1 μg/mL. The catechin trimer (TR) exhibited antioxidant activity more than 1 order of magnitude greater than that of α-tocopherol or ascorbic acid on a molar basis.

KEYWORDS: Proanthocyanidins; procyanidins; catechin; nitric oxide synthase; peroxynitrite; SIN-1; TBARS; antioxidants; hops; *Humulus lupulus*

INTRODUCTION

The proanthocyanidins (condensed tannins) are defined comprehensively as oligomers and polymers built up from polyhydroxy flavan-3-ol units such as catechin, gallocatechin, and their epimers (*1*, *2*). They form one of the two major categories of vegetable tannins that are widely distributed in the plant kingdom. Proanthocyanidins are also known to occur in the female inflorescences ("hops" or "hop cones") of the hop plant, *Humulus lupulus* L. (*3*). The estimated amounts of total proanthocyanidins reported for hops range from 0.5 to 5% on dry weight basis, depending on the variety and on the analysis method used (cf. *3* and references cited). Human intake of hop proanthocyanidins is mainly through beer. It is estimated that about 70−80% of the beer proanthocyanidins are derived from barley while only 20−30% originate from the hops. The proanthocyanidins from hops are structurally very similar to those found in barley, the main difference being the higher proportion of gallocatechin units in the barley oligomers.

Proanthocyanidins from various natural sources exhibit a wide range of biological activities. They are effective antioxidants and offer protection against cardiovascular disease, immune disorders, and neurodegenerative diseases (*4*−*8*). The potential beneficial effects of proanthocyanidins on human health have been attributed mostly to their strong free radical scavenging and strong antioxidant activities. Proanthocyanidins are believed to be superior antioxidants as compared to flavones and flavonols, whose quinones are more likely to redox-cycle and act as prooxidants (*9*). Pycnogenol, a commerical extract composed mainly of oligomeric proanthocyanidins from the bark of French maritime pine, *Pinus maritima*, has strong free radical scavenging activity against reactive oxygen and nitrogen species (*6*, *10*). Pycnogenol also protected human endothelial cells from oxidative stress (glutathione depletion) induced by reactive nitrogen species derived from 3-morpholinosydnonimine-*N*-

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ethylcarbamide or SIN-1 (*11*). Oligomeric procyanidins from *Vitis vinifera* seeds (CAS 85594-37-2) showed a dose-dependent antioxidant activity in the iron-promoted model of lipid peroxidation in phosphatidylcholine liposomes, with an IC50 of 2.5 μmol/L (*12*). The oligomeric procyanidin had antioxidant activity more than 1 order of magnitude greater than that of the monomeric unit, catechin (IC50 = 50 μM).

Other studies have focused on the relation between chain length and antioxidant activity of proanthocyanidins (*5*, *8*, *13*). When concentrations of the various compounds are compared by weight, the monomers, epicatechin and epigallocatechin, provided greater protection than the oligomers against peroxynitrite-dependent oxidation of dihydrorhodamine 123 (*13*). However, the higher molecular weight procyanidins (proanthocyanidins consisting exclusively of catechin and epicatechin units) were more effective than the shorter-chain procyanidins against liposome oxidation initiated in the lipid domain by 2,2′-azobis-2,4-dimethylvaleronitrile (*8*). In contrast, oligomeric chain length had no effect on the antioxidant protection against copper-mediated LDL oxidation (*8*). In another study, the antioxidant activity of procyanidins in the lipid phase decreased with polymerization, whereas the antioxidant action in the aqueous phase increased from monomer to trimer and then decreased from trimer to tetramer (*5*). Using the mouse liver homogenate lipid peroxidation assay, Wang et al. (*14*) showed that procyanidin dimers isolated from the seeds of *Vitis amurensis* were found to be more potent than (+)-catechin. The antioxidant activity of the 4→6 linked dimer seemed higher than that of the 4→8 linked dimer. Procyanidin oligomers of different sizes isolated from the seeds of *Theobroma cacao* were reported to be highly effective in inhibiting oxidation and nitration reactions mediated by peroxynitrite (*13*). The cocoa procyanidins were also effective in preventing liposome oxidation initiated by peroxyl radical generators, copper, and iron/ascorbate (*8*). However, the specific structures of the dimers and trimers used in these antioxidant studies were not established.

The increasing interest in the search for therapeutic agents for nitric oxide (NO)-related disorders has led other research groups to evaluate the potential of proanthocyanidins as scavengers of reactive nitrogen species and as inhibitors of NO synthase (see below). Nitric oxide (NO), a gaseous free radical, has been associated with a wide range of physiological processes (*15*). NO is a neurotransmitter in the nervous system and it modulates the β-adrenergic signaling pathway and other signaling pathways. However, excess production of NO may be involved in glutamate- and oxygen-induced toxicity in the brain (*16*, *17*). NO may react with superoxide anion to form the powerful prooxidant, peroxynitrite ($ONOO^-$). Peroxynitrous acid (ONOOH, pK_a 6.8) and/or its primary decomposition products, $ONO^\bullet$ and $^\bullet OH$ radicals, can induce the oxidation of low-density lipoprotein (LDL) and may also react with other biological molecules resulting in the nitration of protein-bound tyrosine and deamination of DNA (*13*, *18*, *19*). Excess amounts of NO and peroxynitrite may be involved in the pathophysiology of neurological disorders such as stroke, Parkinson's disease, Huntington's disease, and amylotrophic lateral sclerosis (*15*, *20*). Endogenously derived NO is synthesized from L-arginine by nitric oxide synthases (NOS) present in different cell types (*15*). Three different NOS isoforms have been identified, namely NOS I (NOS1, ncNOS, nNOS); NOS II (NOS2, iNOS); and NOS III (NOS3, ecNOS, eNOS). NO derived from nNOS has been implicated in neurotoxicity (*21*, *22*). Thus, the selective inhibition of nNOS activity has been suggested as one of the therapeutic strategies in neurological diseases and brain injury (*21*, *23*).

Proanthocyanidins from various natural sources were shown to inhibit nitric oxide synthase (NOS) activity and/or nitric oxide (NO) production in cultured cells (*10*, *24*−*26*). For example, Pycnogenol inhibits inducible NOS (iNOS) activity of cytokine-activated macrophage (RAW 264.7) cells at concentrations of 50 μg/mL or greater (*25*). Gallotannins (esters of gallic acid and D-glucose), which belong to a different class of tannins known as hydrolyzable tannins, have been shown to inhibit the nNOS, with an IC50 of 30 μM (*27*). It is possible that the protein-binding action of procyanidins may explain the inhibitory effect of these compounds on NOS activity as in the case of the inhibition of xanthine oxidase activity by Pycnogenol (*28*, *29*).

In the present study, proanthocyanidins were isolated from hops and their in vitro capacity as inhibitors of rat neuronal NOS activity was evaluated. In addition, their ability to scavenge reactive nitrogen/oxygen species was investigated by observing the decrease of artificial LDL oxidation in the presence of hop proanthocyanidins. This study shows that some representatives of proanthocyanidins from hops inhibit nNOS activity in the low micromolar range while others inhibit LDL oxidation even stronger than well-known antioxidants such as α-tocopherol or ascorbic acid. The observed *in vitro* effects suggest a health-promoting impact of dietary proanthocyanidins on NO-related disorders.

MATERIALS AND METHODS

General Experimental Procedures. ^1H NMR experiments (1D and 2D long-range $^1H-^1H$ COSY) were performed on a Bruker DRX 600 spectrometer. Electrospray and atmospheric pressure chemical ionization (ESI and APCI, respectively) mass spectra were recorded on a PE Sciex API III triple-quadrupole mass spectrometer in the positive ion mode. MS−MS experiments were carried out with argon−10% nitrogen as the target gas at a thickness of ca. 1.9×10^{14} atoms per cm^2 using a collision energy of 10 V. The APCI source was equipped with a heated nebulizer interface kept at 500 °C. Samples were introduced into the mass spectrometer by loop injection (ESI) or by HPLC (APCI). Analytical HPLC separations of proanthocyanidin dimers were achieved on a 5-μm LiChrospher RP-18 (4 × 250 mm) with a linear gradient from 5% to 55% MeOH in 1% aqueous HCOOH over 50 min at 1.0 mL/min. Acid-catalyzed degradation reactions were monitored by HPLC on a 5-μm Spherex RP-8 column (4.6 × 250 mm) with a linear gradient from 0 to 40% MeOH in 1% aqueous HCOOH over 50 min at 1.0 mL/min. Preparative HPLC was run on a 10-μm Econosil C_{18} column (250 × 10 mm).

Preparation of Proanthocyanidins from Hops. Air-dried hop cones (*H. lupulus* cv. Saazer, 438 g) were briefly immersed in $CHCl_3$ to extract the resins. The $CHCl_3$ extract was decanted, and the hop cones were dried in a stream of air in a fume hood and then ground using a Wiley mill (sieve no. 40). The ground plant material (408 g) was extracted with 2.5 L of Me_2CO/H_2O (3:1) for 24 h at room temperature with occasional stirring. This extraction step was repeated twice with 1.5 L of Me_2CO/H_2O (3:1). The combined Me_2CO/H_2O extracts were separated from the plant material by filtration and then concentrated on a rotary evaporator. The resulting aqueous extract (1.1 L) was washed with an equal volume of hexane (discarded) and subsequently passed through a column of Sephadex LH-20 pre-swollen in H_2O. The Sephadex LH-20 column was successively eluted with H_2O (500 mL), H_2O/MeOH (3:1, 500 mL), H_2O/MeOH (1:1, 500 mL), H_2O/MeOH (1:3, 500 mL), MeOH (500 mL), MeOH/Me_2CO (1:1, 500 mL), and finally with Me_2CO (500 mL) at a flow rate of 150 mL/hr. Fractions (500 mL) were collected and monitored by HPLC−UV and TLC. The MeOH, MeOH/Me_2CO, and Me_2CO fractions, which contained mainly oligomeric proanthocyanidins (see **Figure 1** for general structure), were

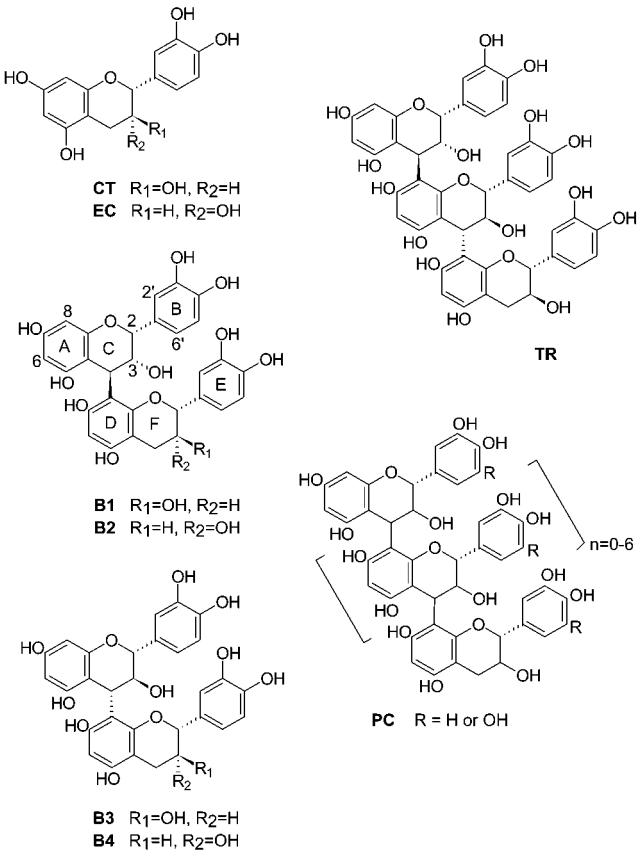

Figure 1. Chemical structures of catechin (**CT**), epicatechin (**EC**), procyanidin B1 (**B1**), procyanidin B2 (**B2**), procyanidin B3 (**B3**), procyanidin B4 (**B4**), a trimer, epicatechin-(4β→8)-catechin-(4α→8)-catechin (**TR**), and the general structure of oligomeric procyanidins (**PC**) from hops, *Humulus lupulus*.

combined and concentrated by rotary evaporation and lyophilization (yield 1.4 g). The H_2O/MeOH (1:3) fraction was further chromatographed on Toyopearl TSK HW-40 S gel using MeOH as the eluent at a flow rate of 1 mL/min. Fractions of 12 mL were collected and examined by HPLC−UV (280 nm). Catechin (24 mg) and epicatechin (4 mg) were isolated from fractions 6−10 by preparative HPLC using a gradient from 5% B (MeCN) to 55% B in A (1% aqueous HCOOH) over 50 min at 5 mL/min. Toyopearl fractions 11−16 yielded procyanidins B3 (4.4 mg), B1 (8.5 mg), B4 (1.6 mg), and B2 (1.0 mg) by preparative HPLC using a linear solvent gradient from 0% B (10% aqueous HOAc) to 100% B in A (2.5% aqueous HCOOH) over 60 min at a flow rate of 5 mL/min. The UV trace was recorded at 280 nm. Peak fractions were collected manually and taken to dryness by lyophilization.

Acetylation of Procyanidins for ^{1}H NMR Analysis. Procyanidin samples (1−4 mg) were dissolved in a mixture of 0.2 mL of pyridine and 0.2 mL of acetic anhydride. After the mixture was stirred for 48 h at room temperature, the peracetate derivatives were purified by preparative HPLC using isocratic elution with 65% MeCN in 1% aqueous HCOOH at 5 mL/min. The UV trace was recorded at 280 nm. Peak fractions were collected manually and evaporated by lyophilization.

Acid-Catalyzed Degradation of Proanthocyanidins in the Presence of Toluene-α-thiol. The sequence of the monomer units in proanthocyanidin dimers and trimers cannot be determined by MS methods, and therefore acid-catalyzed degradation was also carried out. This method takes advantage of the ready cleavage of the interflavanoid C−C linkage in mild acid solution, which yields a flavanol (terminal unit) and a quinone methide (upper unit). The quinonoid intermediate can be captured with a nucleophile such as phenylmethanethiol ($PhCH_2$-SH, toluene-α-thiol) or phloroglucinol (see below). Because catechin, epicatechin, and their adducts (thiobenzyl ethers) can be separated by HPLC (*34*), LC−MS offers a convenient method for identification of the degradation products and thus a method for determination of the monomer sequence in proanthocyanidin dimers and trimers. The procedure was as follows. In a 100-μL reaction vial, 25 μL of an aqueous proanthocyanidin solution (1 mg/mL) was mixed with 25 μL of a 5% solution of $PhCH_2SH$ in EtOH. After addition of 1.0 μL of HOAc, the vial was flushed with N_2, closed, and kept at 96 °C for 24 h. The degradation products were analyzed by LC−APCI−MS.

Partial Acid-Catalyzed Degradation of Proanthocyanidins in the Presence of Phloroglucinol. Because of the obnoxious odor of toluene-α-thiol, phloroglucinol was used as the trapping nucleophile in a later stage of the phytochemical investigations. In a 100-μL reaction vial, 50 μL of an aqueous proanthocyanidin solution (1 mg/mL) was mixed with 50 μL of a phloroglucinol solution (80 mg dissolved in 2 mL of EtOH/H_2O, 1:3). After addition of 2 μL of HOAc, the vial was flushed with N_2, closed, and heated to 100 °C for 6 h. Aliquots of the reaction mixture were taken (t = 0, 0.5, 1, 3, and 6 h) and directly analyzed by LC−APCI−MS.

Procyanidin B1 (epicatechin-(4β→8)-catechin). ESI-MS (positive ion mode) m/z 579 $[MH]^+$ (100),^{1}H NMR ($CDCl_3$, 600 MHz) of peracetate derivative, δ 5.99 (d, J = 2.3 Hz, H-6 ring A), 6.29 (d, J = 2.3 Hz, H-8 ring A), 7.29 (d, J = 1.9 Hz, H-2 ring B), 7.16 (d, J = 8.4 Hz, H-5 ring B), 7.24 (dd, J = 8.4, 1.9 Hz, H-6 ring B), 5.44 (brs, H-2 ring C), 5.15 (t, J = 1.8 Hz, H-3 ring C), 4.42 (d, J = 2.0 Hz, H-4 ring C), 6.68 (s, H-6 ring D), 6.88 (d, J = 2.0 Hz, H-2 ring E), 7.08 (d, J = 8.4 Hz, H-5 ring E), 6.94 (dd, J = 8.4, 2.0 Hz, H-6 ring E), 4.33 (d, J = 9.7 Hz, H-2 ring F), 5.05 (m, H-3 ring F), 2.56 (dd, J = 16.7, 9.2 Hz, H-4_{ax} ring F), 3.21 (dd, J = 16.7, 6.6 Hz, H-4_{eq} ring F).

Procyanidin B3 (catechin-(4α→8)-catechin). ESI-MS (positive ion mode) m/z 579 $[MH]^+$ (100),^{1}H NMR ($CDCl_3$, 600 MHz) of peracetate derivative, δ 6.48 (d, J = 2.3 Hz, H-6 ring A), 6.51 (d, J = 2.3 Hz, H-8 ring A), 7.02 (d, J = 2.0 Hz, H-2 ring B), 7.14 (d, J = 8.4 Hz, H-5 ring B), 6.99 (dd, J = 8.4, 2.0 Hz, H-6 ring B), 4.76 (d, J = 9.9 Hz, H-2 ring C), 5.63 (t, J = 9.8 Hz, H-3 ring C), 4.48 (d, J = 9.3 Hz, H-4 ring C), 6.64 (s, H-6 ring D), 6.93 (d, J = 2.0 Hz, H-2 ring E), 7.13 (d, J = 8.4 Hz, H-5 ring E), 6.73 (dd, J = 8.4, 2.0 Hz, H-6 ring E), 4.95 (d, J = 8.0 Hz, H-2 ring F), 5.03 (m, H-3 ring F), 2.66 (dd, J = 16.7, 7.9 Hz, H-4_{ax} ring F), 2.93 (dd, J = 16.7, 5.7 Hz, H-4_{eq} ring F).

Expression and Purification of Rat Neuronal NOS. cDNA was kindly provided by Dr. T. M. Dawson, Johns Hopkins University) (*30*). The pCWori vector was a gift of Dr. F. W. Dalhquist, University of Oregon (*31*). The rat nNOS was expressed in BL21(DE3) (Novagen) *E. coli* cells. All cells were grown in Terrific Broth media at 37 °C to an OD_{600} = 1.2 at which time 0.1 mM of IPTG and 4 μg/mL of hemin chloride were added to the cultures. The cells were then grown for an additional 16−20 h at 23 °C at 250 rpm before harvesting. Cells were washed in a solution of 50 mM TRIS (pH 7.8), 10% sucrose, and 150 mM NaCl, and then pelleted by centrifugation and frozen at −70 °C. The enzyme was purified according to the protocol published by Gerber et al. (*32*) with slight modification. Cells were thawed and resuspended in Buffer A which contained the following: 50 mM Hepes (pH 7.5), 10% glycerol, 100 μM DTT, 10 μM tetrahydrobiopterin, and the protease inhibitors (1 mM EDTA, 1 μg/mL of trypsin inhibitor, 1 μg/mL pepstatin A, 17 μg/mL of PMSF, 1 μg/mL benzamidine, 10 μg/mL bacitracin, 2.5 μg/mL leupeptin, 2.5 μg/mL aprotinin, and 1.0 μg/mL E-64). The cells were incubated on ice after the addition of 20 mg of lysozyme and brief sonication. The cells were centrifuged, and the lysate was poured over a 10-mL column of 2′,5′-ADP−Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) preequilibrated with buffer A. The column was washed with 100 mL of buffer A followed by 50 mL of buffer A with 0.5 M NaCl. The protein was eluted with 50 mL of buffer A plus 0.5 M NaCl and 20 mM mixture of 2′ and 3′ AMP. Both 2 mM $MgCl_2$ and 1 mM $CaCl_2$ were added to the ADP-eluate, and then the solution was poured over a 10-mL calmodulin-Sepharose column (a generous gift of Dr. S. Anderson, Oregon State University) preequilibrated with buffer A with 2 mM $MgCl_2$ and 1 mM $CaCl_2$. The column was washed with 50 mL of buffer A plus 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.3 M NaCl. Finally, the protein was eluted with buffer A plus 0.3 M NaCl and 3 mM EDTA. Protein concentration was determined with the Lowry assay using bovine serum

albumin as a standard (*33*). A given batch of recombinant purified protein had a specific activity of 150 nmol NO min^{-1} mg^{-1} at 25 °C and was more than 85% pure as judged by SDS-polyacrylamide gel electrophoresis.

Measurement of Enzyme Activity of cDNA-Expressed nNOS. The initial rate of NO production was determined from the NO-mediated conversion of oxyhemoglobin to methemoglobin, monitored at 401 nm using a methemoglobin minus oxyhemoglobin extinction coefficient of 49 mM^{-1} cm^{-1}. This hemoglobin assay was employed in our study instead of the Kobuchi method using radiolabeled arginine (*25*) because of its simplicity and high sensitivity, and it avoided the use of the expensive radioactive substrate and the costly disposal of radioactive wastes. The reaction mixture contained 10 μM oxyhemoglobin, 20 μM L-arginine, 10 μM $CaCl_2$, 100 nM calmodulin, 10 μM tetrahydrobiopterin (Cayman Chemical, Ann Arbor, MI), 100 μM dithiothreitol, 10 μM NADPH, and 50 mM Hepes (pH 7.5), in a final volume of 1 mL. The test chemicals [proanthocyanidin mixture, PC, or as monomers, dimers, and trimer) were dissolved in methanol and added to the incubation mixture in 2-μL volumes. All assays were initiated with 1−2 μg of nNOS and performed at 25 °C.

Antioxidant Activity of Hop Proanthocyanidins. The antioxidant activity of hop procyanidins was evaluated by their ability to inhibit LDL oxidation induced by the peroxynitrite generator, SIN-1, as described by Thomas et al. (*34*). LDL (0.1 mg/mL) was incubated with proanthocyanidins and SIN-1 (100 μM) in 50 mM phosphate buffer, pH 7.4, in a total volume of 200 μL. The proanthocyanidins were added in methanol (0.5%). Vehicle controls contained methanol only. After a 20-hr incubation at room temperature, the amount of thiobarbituric acid-reactive substances (TBARS) formed was measured as described by Miranda et al. (*35*). A 20-μL aliquot of 20% of ice-cold 50% trichloroacetic acid was added to each reaction mixture. The tubes were vortexed, and then 100 μL of 1% thiobarbituric acid in 0.28% NaOH was added to each tube. The samples were acidified by the addition of 20 μL of 1 N HCl. The tubes were heated at 90 °C for 20 min and centrifuged at 16 000*g* for 10 min. An aliquot (200 μL) of the supernatant was transferred to a 96-well plate and the absorbance at 535 nm was measured using a microplate reader (SpectraMax 250).

Statistical Analysis. Using a computerized statistical software (StatView), the data obtained from the nNOS and antioxidant studies were analyzed by either a Student's *t*-test (for comparison between control and treated groups) or a one-way analysis of variance followed by LSD for multiple comparison test. The level of significance was set at $p < 0.05$.

RESULTS

Identification of Procyanidins. Catechin, epicatechin, procyanidins B1−B4, and the procyanidin trimer, epicatechin-(4β→8)-catechin-(4α→8)-catechin, were isolated from the Me_2CO/H_2O (3:1) extract of the female inflorescences of the hop plant by successive column chromatography on Sephadex LH-20, Toyopearl TSK HW-40 gel, and octadecyl silica. The chemical structures of these compounds are shown in **Figure 1**. Catechin (**CT**) and epicatechin (**EC**) were identified by MS and HPLC comparison with authentic markers. Compounds **B1** and **B3** (cf. **Figure 1**) both showed molecular ions with *m/z* 579 $[MH]^+$ in their ESI mass spectra, indicating they represented procyanidin dimers. Their monomer sequence and the linkage positions were established by comparison of ^1H NMR data of their peracetates with literature data published for peracetylated procyanidins of the B-series (*36*). Thus, the structures of compounds **B1** and **B3** were assigned as procyandins B1 [epicatechin-(4β→8)-catechin] and B3 [catechin-(4α→8)-catechin], respectively. Both compounds were subjected to acid-catalyzed degradation in the presence of phenylmethanethiol ($PhCH_2SH$), and the products were analyzed by LC−MS. Compound **B1** yielded catechin ($[MH]^+$ at *m/z* 291, Rt 16.3 min) and epicatechin-4-thiobenzyl ether ($[MH]^+$ at *m/z* 413, R_t 32.8 min), which identifies epicatechin as the upper unit and catechin as the terminal unit. Compound **B3** released catechin ($[MH]^+$ at *m/z* 291, R_t 16.3 min) and two catechin-4-thiobenzyl ethers (both $[MH]^+$ at *m/z* 413, minor and major derivative eluted at R_t 30.0 and 31.4 min, respectively), thus confirming the sequence being catechin→catechin in **B3**. It was previously shown that procyanidin dimers with catechin as the upper unit give two catechin thiobenzyl ethers (3,4-*trans* and 3,4-*cis*), whereas dimers with epicatechin as the upper unit produce only one epicatechin thiobenzyl derivative (3,4-*trans*) upon thiolytic cleavage of the interflavanoid linkage (*37*). The thiolysis data obtained with compounds **B1** and **B3** were used to determine the monomer sequence in the procyanidin dimers **B2** and **B4** ($[MH]^+$ at *m/z* 579). Compound **B2** released epicatechin ($[MH]^+$ at *m/z* 291, R_t 20.0 min) and epicatechin-4-thiobenzyl ether, and **B2** was thus characterized as an epicatechin→epicatechin dimer. Likewise, the monomer sequence in compound **B4** was determined to be catechin→epicatechin. Plotting of the HPLC retention times of compounds **B3**, **B1**, **B4**, and **B2** (21.6, 23.0, 26.0, and 29.6 min, respectively) against those reported for procyanidins B3, B1, B4, and B2 (18.5, 20.4, 23.8, and 26.6 min, respectively, see *34*, similar gradient) resulted in a correlation coefficient of $r^2 = 0.9861$. When the retention time reported for procyanidin B2 [epicatechin-(4→8)-epicatechin] was substituted with the retention time reported for procyanidin B5 (38.3 min.) [epicatechin-(4→6)-epicatechin], the r^2 value dropped to 0.9139, indicating that the retention time of compound **B2** was much more in line with that of procyanidin B2 than with its 4→6 analogue. Likewise, substitution of the retention time reported for procyanidin B4 [catechin- (4→8)-epicatechin] with that reported for its 4→6 analogue, procyanidin B8 (22.2 min.), resulted also in a decrease of the r^2 value (0.9805). Thus, comparison of the HPLC retention times on reversed-phase C-18 suggested that the interflavanoid linkages in compounds **B2** and **B4** are both 4→8. Compound **B2** was therefore identified as procyanidin B2, and compound **B4** was identified as procyanidin B4. The amounts of **B2** and **B4** isolated were not sufficient for peracetylation and ^1H NMR analysis. Only four procyanidin dimers were detected in the Toyopearl fraction containing the proanthocyanidin dimers by LC−MS, indicating that 4→6 linked procyanidin dimers (B5−B8) were absent in the hop extract.

A procyanidin trimer (**TR**, $[MH]^+$ at *m/z* 867) was obtained from a later fraction eluting from the Toyopearl TSK HW-40 column. The monomer sequence was established by acid-catalyzed degradation in the presence of phloroglucinol as the nucleophile (*37*). The reaction was first performed with compounds **B1** and **B3** with the aim to generate epicatechin-(4→2)-phloroglucinol and catechin-(4→2)-phloroglucinol for LC−MS comparison. Sampling of the reaction with the trimer **TR** at various stages (0, 1, 3, and 6 h) revealed the early release of catechin and epicatechin-(4→2)-phloroglucinol. After 3 h, the reaction yielded significant amounts of catechin-(4→2)-phloroglucinol and a trace of catechin-(4α→8)-catechin (**B3**). A fifth product with a molecular ion $[MH]^+$ at *m/z* 703 was assigned to epicatechin-(4β→8)-catechin-(4→2)-phloroglucinol. From the pattern of the reaction products it was concluded that trimer **TR** contained catechin as the terminal unit, while the early release of epicatechin-(4→2)-phloroglucinol indicated that epicatechin is the upper unit. The appearance of catechin-(4→2)-phloroglucinol during the later stages was attributed to release of catechin-(4→2)-phloroglucinol from the intermediate dimer, catechin-(4α→8)-catechin. Catechin was thus identified as the middle unit, connected to the terminal catechin unit through a 4α→8 linkage. The linkage type between the upper epicatechin

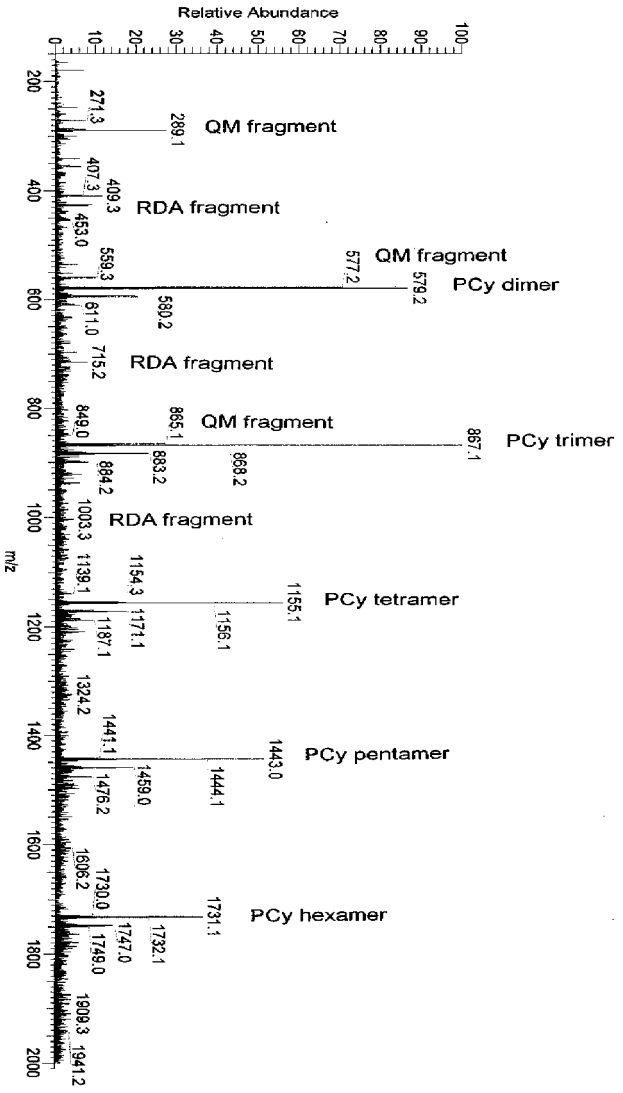

Figure 2. Electrospray mass spectrum of a mixture of proanthocyanidin oligomers from hops. The sample (0.2 mg/mL MeCN/1% aq. HCOOH, 1:1) was introduced into a Finnigan LCQ ion-trap mass spectrometer by injection through a 50-μm fused silica capillary at a flow rate of 5 μL/min: PCy = procyanidin, QM = quinone methide, RDA = retro Diels–Alder (for structures of fragment ions, see ref. *39*).

and the middle catechin unit could not be established, but was assumed to be 4→8 because none of the procyanidin dimers detected in the hop extract contained 4→6 linkages. Trimer **TR** was therefore tentatively identified as epicatechin-(4β→8)-catechin-(4α→8)-catechin (*38*).

Characterization of Oligomeric Proanthocyandins by Electrospray Mass Spectrometry. The Me_2CO/H_2O extract of the hop cones was fractionated on Sephadex LH-20 by eluting with H_2O/MeOH/Me_2CO mixtures. The MeOH/Me_2CO fractions contained a complex mixture of oligomeric proanthocyanidins (designated as PC) which was examined by ESI-MS using continuous flow injection of a sample solution. The ESI mass spectrum (**Figure 2**) showed the presence of procyanidin dimers (*m*/*z* 579, 24%), trimers (*m*/*z* 867, 27%), tetramers (*m*/*z* 1155, 15%), pentamers (*m*/*z* 1443, 14%), hexamers (*m*/*z* 1731, 10%), heptamers (*m*/*z* 2019, 6.5%), and octamers (*m*/*z* 2307, 3.5%) (percentages indicate the approximate composition of the procyanidin fraction). Oligomers containing one or two gallocatechin units (with 3′,4′,5′-trihydroxy substitution on the B-ring) appeared as minor peaks at 16 and 32 mass units higher relative

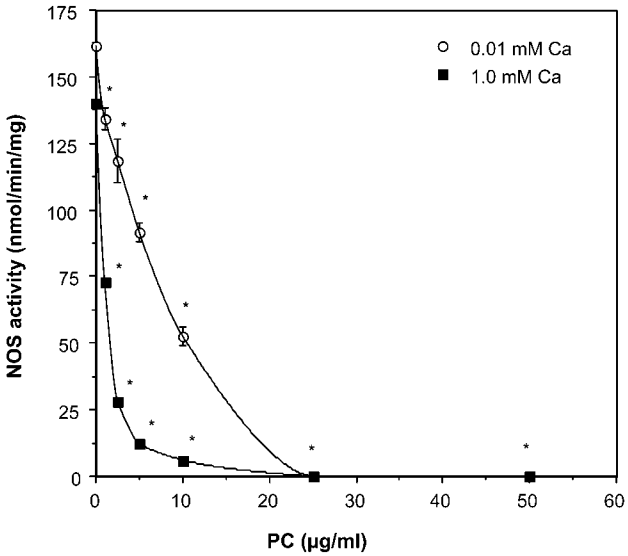

Figure 3. Effect of oligomeric proanthocyanidins (PC) from hops, *Humulus lupulus*, on the enzyme activity of cDNA-expressed neuronal nitric oxide synthase derived from rat. The NOS assays were done in the presence of 10 μM $CaCl_2$ (—○—) or 1 mM $CaCl_2$ (–■–). Each data point represents the mean ± SE of three determinations. Asterisks indicate $p < 0.05$ versus control values.

to the $[MH]^+$ peaks representing the all-catechin proanthocyanidins; collectively their abundance was estimated at 23−31% of the total of proanthocyanidins. Although ESI-MS analysis provides a convenient way to describe the qualitative and semiquantitative composition of the oligomeric fraction, an accurate quantitative analysis of the data is not possible because of in-source fragmentation and the lower apparent MS response of the higher oligomers. For instance, the abundance of in-source fragment ions, notably quinone methide fragments (*m*/*z* 289, 577, and 865) and retro Diels−Alder fragments (*m*/*z* 409, 715, and 1003) (*39*), indicates that a significant proportion of molecular ions suffer in-source dissociation.

Inhibition of nNOS activity. Experiments were performed to assess the influence of PC isolated from hops on the catalytic activity of cDNA-expressed rat nNOS (**Figure 3**). At 1 and 10 μg/mL, PC inhibited nNOS activity (in the presence of 0.01 mM $CaCl_2$) to 23% and 67%, respectively. Complete inhibition of nNOS activity was observed at 25 μg/mL of PC. PC became a stronger inhibitor of NOS when excess calcium (1.0 mM $CaCl_2$) was used in the incubation. The approximate IC50 values of PC in NOS assays containing 0.01 and 1.0 mM $CaCl_2$ were 7.15 μg/mL and 2.4 μg/mL, respectively. Thus, the inhibitory effect of PC is not apparently related to the binding of calcium to hop procyanidins.

To help understand the mechanisms of inhibitory action of PC on rat nNOS activity, it is important to examine the role of the individual monomeric and oligomeric components of hops on nNOS activity. The effect of monomeric and oligomeric procyanidins on rat nNOS activity is shown in **Figure 4**. Among the procyanidin dimers tested, B1, B2, and B4 were found to inhibit nNOS activity. Procyanidin B2 was the most active inhibitor, causing more than 50% inhibition of nNOS activity at 20 μg/mL (equivalent to 34.5 μM). The trimer, epicatechin-(4β→8)-catechin-(4α→8)-catechin (TR) slightly inhibited nNOS activity. However, procyanidin B3 and the monomers, catechin and epicatechin, did not inhibit rat nNOS activity. The positive control, EG, was a strong inhibitor of nNOS, reducing the activity more than 50% at 20 μg/mL. EG was used as a positive control in view of recent findings that this compound (at 50 to

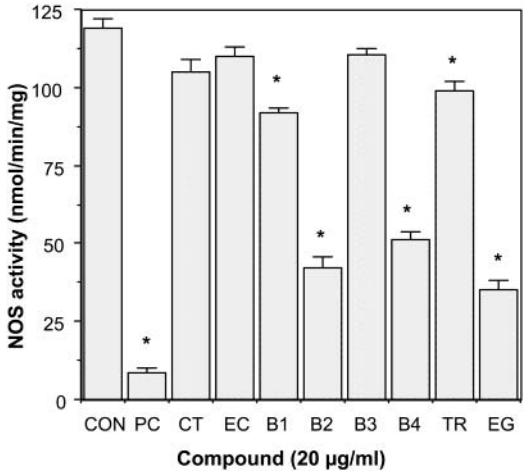

Figure 4. Effect of procyanidin monomers, (CT, catechin; EC, epicatechin), dimers (B1, B2, B3, and B4), trimer [TR, epicatechin-(4β→8)-catechin-(4α→8)-catechin], EG (epigallocatechin gallate), and oligomeric proanthocyanidins (PC) on the enzyme activity of cDNA-expressed rat neuronal nitric oxide synthase (nNOS). The nNOS assays were done in the presence of 10 μM $CaCl_2$. The compounds were tested at a final concentration of 20 μg/mL. Results represent the mean ± SE of three determinations. Asterisk indicates $p < 0.05$ versus control (vehicle control).

750 μM) directly inhibits the activity of nNOS (56% to 93% inhibition) (*24*). PC was more potent than EG or the individual oligomers as an inhibitor of rat nNOS activity.

Antioxidant Activity. To further assess the biological activity of the hop procyanidins, as a mixture or as individual oligomeric units, in terms of their potential benefits in the prevention of NO-related disorders, experiments were performed to evaluate their antioxidant activity against artificial oxidation of LDL, induced by exposure of LDL to 3-morpholinosydnonimine (SIN-1) which liberates $NO^{\bullet}$ radicals and superoxide anions ($O_2^{-\bullet}$) in oxygenated solutions. Both radical species may combine to form peroxynitrite ($ONOO^-$) which, in turn, may give rise to $ONO^{\bullet}$ and $^{\bullet}OH$ radicals. Since peroxynitrite itself does not induce LDL oxidation measured by the formation of TBARS after 3 h of incubation (*40*), it was necessary to first establish the effectiveness of SIN-1 in causing LDL oxidation in our test system using TBARS as an endpoint (**Figure 5A**). Thomas et al. (*34*) have demonstrated that both peroxynitrite and SIN-1 can induce LDL oxidation as measured by lipid hydroperoxide formation but TBARS formation was not determined in their study. In the present study, SIN-1 was found to induce oxidation of LDL (at a final concentration of 0.1 mg/mL) as shown by the dose-dependent increase in TBARS formation after a 20-hr incubation. Increasing the amount of SIN-1 from 100 μM to 200 μM did not produce a further increase in TBARS. Thus, in all succeeding experiments, the concentration of SIN-1 in the reaction mixtures was set at 100 μM. An LDL concentration of 0.1 mg/mL was also used in all of our experiments, because this concentration fell within the ascending portion of the dose−response curve (**Figure 5B**). A time−response study showed that a 20-hr incubation of LDL and SIN-1 at room temperature was optimal for the formation of TBARS (data not shown).

To determine whether PC or the individual oligomeric procyanidins are capable of inhibiting SIN-1-induced oxidation of LDL, experiments were performed to determine TBARS in LDL exposed to SIN-1 (100 μM) and test compounds for 20 h (**Figure 6**). At a concentration of 1 μg/mL, catechin and epicatechin were as potent as the procyanidin dimers (B1, B2, B3, and B4), trimer (TR), and PC in inhibiting LDL oxidation

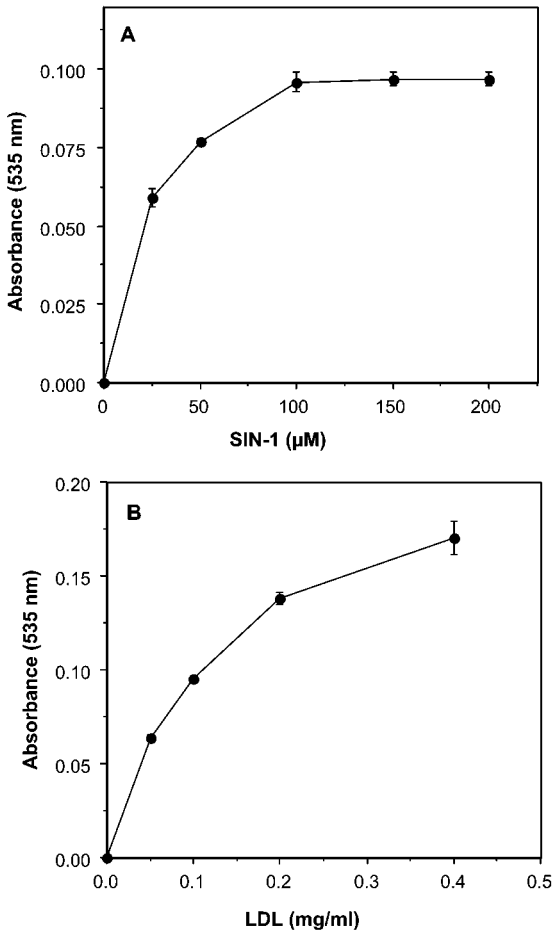

Figure 5. LDL oxidation measured as thiobarbituric acid-reactive substances (TBARS) with absorbance at 535 nm as a function of LDL and SIN-1 concentrations. Results represent the mean ± SE (indicated by error bars in each point) of four determinations.

induced by SIN-1. However, the positive control, EG, showed much lower inhibitory activity against SIN-1-induced oxidation of LDL. At 0.1 μg/mL, all the test compounds significantly inhibited TBARS formation, with procyanidin B3 causing the greatest inhibition. EG was noninhibitory at 0.1 μg/mL.

The antioxidant activity of both PC and TR was further evaluated by determining their ability to inhibit TBARS formation in LDL subjected to oxidation by SIN-1 using a wide range of concentrations of the test compounds (**Figure 7**). Both substances significantly inhibited TBARS formation at concentrations as low as 0.05 μg/mL, with similar IC50 values of 0.33 μg/mL. The antioxidant activities of both PC and TR were much greater than that of α-tocopherol (TOC, IC50 = 3.23 μg/mL) or ascorbic acid (AA, IC50 = 0.99 μg/mL) (**Figure 7**). On a molar basis, the IC50 of TR is 0.38 μM which is more than 1 order of magnitude smaller than the IC50 of α-tocopherol (TOC, 7.5 μM) or ascorbic acid (AA, 5.6 μM).

DISCUSSION

Identification and Characterization of Hop Proanthocyanidins. The discovery of phytochemicals that are potentially useful in the prevention of NO-related diseases has led us to investigate the composition, chemistry, and biological activity of proanthocyanidins present in hops. Hops are known to contain condensed tannins (*3*) but the detailed chemical structures and biological activity of the individual flavanol oligomers have not been completely elucidated. Our present study has revealed that

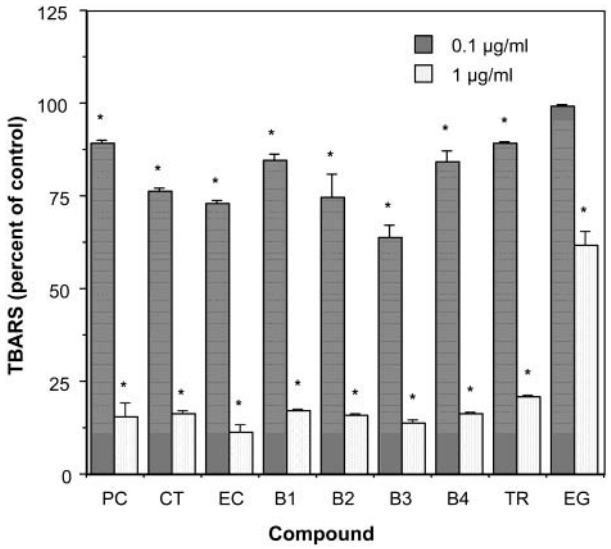

Figure 6. Effect of oligomeric proanthocyanidins (PC), catechin (CT), epicatechin (EC), procyanidin dimers (B1, B2, B3, and B4), procyanidin trimer, TR [epicatechin-(4β→8)-catechin-(4α→8)-catechin], and EG (epigallocatechin gallate) on SIN-1-induced oxidation of LDL measured as TBARS. Results (as percent of control) represent the mean ± SE of four determinations. Bars topped with asterisks are significantly different from control values (vehicle control), $p < 0.05$.

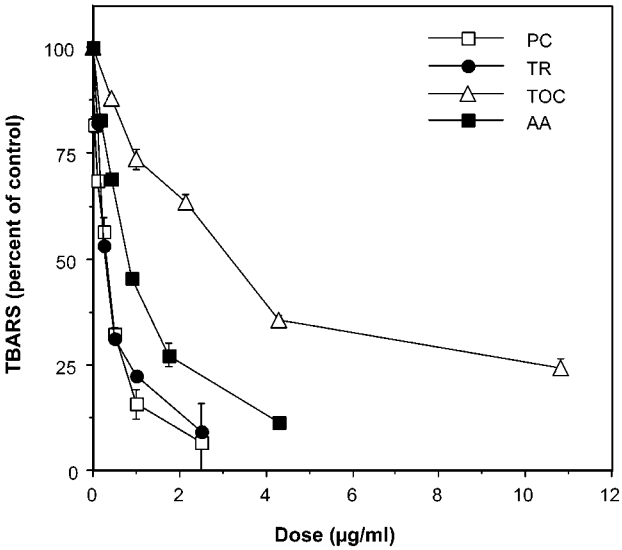

Figure 7. Comparison of the antioxidant activities of oligomeric proanthocyanidins (PC) and procyanidin trimer, TR [epicatechin-(4β→8)-catechin-(4α→8)-catechin], α-tocopherol (TOC), and ascorbic acid (AA) against SIN-1-induced oxidation of LDL. Results (as percent of vehicle control) are expressed as the mean ± SE of four determinations.

hop proanthocyanidins contain mainly subunits of catechin and epicatechin. By using HPLC, MS, NMR, and chemical degradation methods, four procyanidin dimers with 4→8 linkages were identified as B1, B2, B3, and B4. Procyanidin dimers with 4→6 linkages, as those found in grape seed extracts (*37*) and blueberries (*41*), were not detected in the hop extract. The hop extract also contained procyanidin trimers, one of which (TR) was obtained as a homogeneous substance and characterized by chemical degradation. The oligomeric fraction (PC) was examined by electrospray MS which showed the presence of trimers, tetramers, pentamers, hexamers, heptamers, and octamers composed of "catechin" units only or with one or two "gallocatechin" units. The chemical structures of many of the components of the oligomeric proanthocyanidin fraction remain to be established.

Inhibitory Effects on nNOS Activity. Catechin, epicatechin, procyanidins B1−B4, TR, and PC were isolated from the hop extract in quantities that allowed us to perform limited characterization of their biological activities. These compounds, particularly the monomers and dimers, which are found also in edible fruits and many food products and supplements, may have possible beneficial implications in human health. Specifically, the oligomeric procyanidins, individually or as a mixture, may be useful in preventing NO-related neurological disorders by suppressing the overproduction of NO through inhibition of NOS activity. The proanthocyanidin mixture, PC, was a potent inhibitor of nNOS activity, with an IC50 of 2.4 μg/mL (in the presence of 1 mM $CaCl_2$) or 7.15 μg/mL (in the presence of 0.01 mM $CaCl_2$). On a weight basis (at 20 μg/mL), PC was a more effective inhibitor of rat nNOS than the individual monomers (catechin and epicatechin), dimers (B1, B2, B3, and B4), or the procyanidin trimer, TR. Procyanidins B2 and B4 were significantly more active than the other two dimers, B1 and B3. Procyanidin B3, and the monomers, catechin and epicatechin, were noninhibitory. The positive control, EG, was less inhibitory than PC but more inhibitory than the oligomers. These findings indicate that dimeric proanthocyanidins having epicatechin as the terminal flavanol unit (as in B2 and B4) are stronger inhibitors of nNOS activity than dimers in which catechin represents the terminal unit (B1 and B3, and also TR). Although the differences in nNOS inhibition by the individual procyanidin dimers suggest a specific mode of interaction, the stronger inhibition observed for the mixture of oligomers (PC) is most likely due to nonspecific binding to the enzyme. These observations are in line with an interaction study by Moini et al. (*28*) who demonstrated that procyanidins up to trimers did not change the electrophoretic mobility of milk xanthine oxidase (XO) on a PAGE gel using nondenaturing conditions, whereas interaction between procyanidins with a higher degree of polymerization and XO gave rise to a band with low electrophoretic mobility on the gel. These authors also showed that the electrophoretic mobility of XO could be restored by addition of Triton X-100, but not by urea or NaCl, indicating that the procyanidin oligomer−XO interaction is primarily of a hydrophobic nature. Alternatively, the potent activity of PC could be due to synergy among the various oligomeric units that constitute the mixture. Further studies, using bioassay-guided systematic fractionation, are needed to identify the most active inhibitory component of PC and to test the hypothesis that synergy occurs among different oligomeric units in the inhibition of nNOS.

A limited number of studies have been reported on the effect of proanthocyanidins on NO production in intact cells (*42*, *43*). Therefore, we have measured NO production in lipopolysaccharide (LPS)-activated mouse macrophage RAW 264.7 cells using the method of Kim et al. (*44*). RAW 264.7 cells were grown in 96-well plates and exposed to LPS (0.5 μg/mL; Sigma L3139) in the presence and absence of PC and the dimers, B1, B2, B3, and B4 (all at concentrations of 10, 20, or 50 μg/mL). The cells were incubated for 16 h at 37 °C before the culture media was analyzed for nitrite (the stable conversion product of NO) using the Griess reagent. Our results showed that PC and the dimers did not inhibit NO production in LPS-activated RAW cells (data not shown), suggesting that these compounds were not inhibitors of iNOS activity or iNOS expression in these cells. Unlike iNOS, nNOS has unique structural features such as the PDZ domain, an autoinhibitory element in the FMN domain (also in eNOS), and has a higher requirement for Ca^{2+} (*45*) that may help explain the inhibitory effect of the oligomeric

proanthocyanidins on nNOS. The effect of PC and the individual procyanidins from hops on eNOS activity has not been determined.

Scavenging of Reactive Nitrogen/Oxygen Species. There have been a number of studies on the antioxidant and free radical scavenging activities of oligomeric proanthocyanidins (*4*−*6*, *8*, *10*, *12*). The majority of these studies report the activities of oligomers from dimers to nonamers, without identifying the individual chemical structures that make up these oligomeric components. In the present study, the antioxidant activity of four different dimers and one trimer with known structures was compared with the monomers catechin and epicatechin. At a concentration of 1.0 μg/mL, all the test compounds were extremely inhibitory and no marked differences were noted in their individual antioxidant activities against SIN-1-induced oxidation of LDL. However, at 0.1 μg/mL, procyanidin B3 appeared to exhibit the greatest antioxidant activity in this system. The trimer, TR, and the oligomeric proanthocyanidin mixture, PC, had comparable antioxidant activities with the same IC50 value of 0.33 μg/mL. These results suggest that the potent antioxidant activity of PC may be attributed, in part, to residual dimers and trimers present in the oligomeric fraction that were not removed by chromatographic fractionation.

On a molar basis, TR was 1−2 orders of magnitude more active than α-tocopherol or ascorbic acid as an antioxidant against SIN-1-induced oxidation of LDL. Both TOC (*46*) and AA (*34*) have been reported to be potent inhibitors of LDL lipid peroxidation induced by SIN-1 using cholesteryl ester hydroperoxide formation as an endpoint. The monomeric and oligomeric procyanidins were also more potent antioxidants than EG in LDL exposed to SIN-1 (**Figure 6**). The latter finding is not in agreement with previous studies which showed that EG is a more effective antioxidant than catechin or epicatechin in an aqueous-based system of oxidation (*47*).

Proanthocyanidins have been reported to scavenge peroxynitrite (*13*), nitric oxide (*48*), superoxide (*49*), or other reactive oxygen species (*10*). In our LDL system, SIN-1 was used as the oxidizing system. As LDL oxidation by SIN-1 seems to be mediated by peroxynitrite (*50*), it is suggested that the antioxidant activity of the monomeric and oligomeric procyanidins may be due to scavenging of peroxynitrite. However, the antioxidant activity of the procyanidins can also be explained by the scavenging of the initial products released by SIN-1, such as NO and superoxide anion. Another mechanism may involve the scavenging of lipid peroxyl radicals formed during the process of LDL lipid peroxidation initiated by SIN-1. In the case of ascorbic acid, the repair function rather than the scavenging of peroxynitrite was demonstrated to be the decisive antioxidant mechanism (*51*). Therefore, further experiments are needed to explain the actual antioxidative activity of the oligomeric procyanidins.

CONCLUSION

The procyanidin dimers B1−B4, the procyanidin trimer, and the mixture of oligomers investigated in this study are potent inhibitors of nNOS activity and SIN-induced oxidation of LDL, suggesting a potential for dietary procyanidins in preventing diseases associated with reactive nitrogen or oxygen species. Procyanidin dimers and (epi)catechin are of interest because they represent the end-products of acid-catalyzed cleavage of oligomers in the gastric milieu (*52*). However, before the oligomeric proanthocyanidins can be considered important for the prevention of NO-related disorders such as Alzheimer's disease, Parkinson's disease, stroke, and atherosclerosis, their bioavailability in the intact animal or in humans has to be demonstrated. Further studies are also needed to show that dimers and trimers, once absorbed, can cross the blood−brain barrier to protect the central nervous system from nitrogen-reactive species.

ABBREVIATIONS USED

CT, catechin; EC, epicatechin; EG, epigallocatechin gallate; LDL, low-density lipoprotein; nNOS, neuronal nitric oxide synthase; PC, a mixture of oligomeric procyanidins; SIN, 1,3-morpholinosydnonimine; TR, epicatechin-(4β→8)-catechin-(4α→8)-catechin; TBARS, thiobarbituric acid-reactive substances.

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