Modulation of Nicotinamide Adenine Dinucleotide Phosphate Oxidase Expression and Function by 3',4'-Dihydroxyflavonol in Phagocytic and Vascular Cells

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ABSTRACT

Previously we have demonstrated that 3',4'-dihydroxyflavonol (DiOHF), a novel synthetic flavonol, protects against ischemia reperfusion injury in both heart and brain. In this study, we characterized the pharmacological effects of DiOHF on phagocytic and vascular NADPH oxidase. Superoxide release (lucigenin-enhanced chemiluminescence or cytochrome c reduction), NADPH oxidase activation (membrane translocation of p47phox), and subunit expression (real-time polymerase chain reaction and Western blot) were examined in differentiated HL-60 cells, human neutrophils, vascular endothelial and smooth muscle cells, and mouse aorta. DiOHF concentration dependently suppressed superoxide accumulation (EC50 = 8.4 ± 1.7 μM) in vascular smooth muscle cells, which appears to be attributable to its superoxide scavenging activity (EC50 = 6.1 ± 1.1 μM measured in a cell-free system). DiOHF had similar effects in HL-60 cells and isolated aortic rings. In HL-60 cells, but not endothelial or smooth muscle cells, DiOHF and quercetin (10 and 30 μM) significantly reduced the protein expression of p47phox, whereas p67phox was not altered. DiOHF did not affect phorbol ester-induced membrane translocation of either p47phox or protein kinase C in leukocytes. Our results suggest that suppression of NADPH oxidase-dependent superoxide accumulation may contribute to the cytoprotective actions of DiOHF during ischemia-reperfusion injury.

Increased production of reactive oxygen species (ROS), including superoxide, hydrogen peroxide (H2O2), and hydroxyl radicals (HO•), has been implicated in many cardiovascular diseases such as hypertension, heart failure, atherosclerosis, and acute myocardial infarction (Wattanapitayakul and Bauer, 2001). Mounting evidence suggests that reduced NADPH oxidase is an important enzymatic source of endogenous superoxide anion generation (Griendling et al., 2000). Originally identified in phagocytic leukocytes, this enzyme is a multicomponent complex consisting of the cytosolic p47phox and p67phox subunits, the membrane-bound Nox2 (also called gp91phox) and p22phox subunits, and a small G protein Rac (Babior, 2004). In phagocytic cells, activation of the oxidase generates large amount of superoxide by one-electron reduction of oxygen using NADPH as the electron donor, resulting in bactericidal respiratory bursts in these cells (Babior, 2004). All of the aforementioned subunits, including other isoforms of Nox such as Nox1 and Nox4, are also expressed in nonphagocytic cells, including vascular endothelial and smooth muscle cells (Griendling et al., 2000), whereas the mechanisms of activation and the biological functions of the enzyme are less clear. Evidence has suggested that activation of this enzyme and subsequent ROS generation in vascular cells may be involved in intracellular signaling, thereby modulating cell physiology, such as cellular proliferation, apoptosis, hypertrophy, and expression of inflammatory molecules (Ray and Shah, 2005). Excessive ROS production from both phagocytic (Cathcart, 2004) and nonphagocytic NADPH oxidases (Jiang et al., 2004) and the resultant oxidative stress have been implicated in a variety of cardiovascular disorders.

Flavonols are a group of polyphenolic compounds (see Fig. 1) commonly found in vegetables, fruits, and green tea (Bravo, 1998), which exhibit potential cardiovascular protec-
active actions in humans (Geleijnse et al., 2002). These effects are thought to be mediated by their antioxidant (Rice-Evans et al., 1996) and antiinflammatory actions (Jiang and Dustin, 2003). For example, flavonoids such as fisetin and quercetin inhibited macrophage-induced low-density lipoprotein oxidation in vitro (de Whalley et al., 1990). In a very recent study, it has been demonstrated that various natural flavonol compounds suppressed cytokine-induced inflammatory adhesion molecule expression in human aortic endothelial cells, which is relevant to atherosclerosis (Lotito and Frei, 2006). In our previous studies, we have identified a synthetic flavonol, 3',4'-dihydroxyflavonol (DiOHF; Fig. 1), which demonstrated remarkable cytoprotective effects in ischemia reperfusion injury in both heart (Wang et al., 2004) and brain (C. Roulston and G. J. Dustin, unpublished data). In both cases, DiOHF has been found to have potent inhibitory effects against NADPH oxidase-dependent superoxide generation. Given these observations and the critical role of NADPH oxidase in cardiovascular disease (Jiang et al., 2004), we were prompted to investigate the interactions of DiOHF with NADPH oxidase in both phagocytic and nonphagocytic cells. We also compared the pharmacological effects of DiOHF with those of a naturally occurring counterpart, quercetin, and an unrelated flavone, chrysin.

**Materials and Methods**

**Materials.** Rabbit anti-Nox2, p47phox, p67phox, and mouse anti-Na-K ATPase (clone C464.6) antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit anti-p22phox (FL-195) and mouse anti-p47phox (D-10) were from Santa Cruz Biochemicals (Santa Cruz, CA). Mouse anti-protein kinase C (PKC) (clone MC5) was from Sigma-Aldrich (St. Louis, MO). The following compounds were used: allopurinol, ascorbic acid, chrysin (Aldrich Chemical Co., Milwaukee, WI), cytochrome c (from horse heart), diethyldithiocarbamic acid (DETCP), diphenylethyleniodide chloride (DPI), indomethacin, lucigenin, N-nitro-l-arginine methyl ester (l-NAME), NADPH (MP Biomedical, Irvine, CA), nitroblue tetracosium (NBT; Bio-Rad, Hercules, CA), 17-octadecynoic acid (17-ODYA), phorbol 12-myristate 13-acetate (PMA), quercetin (MP Biomedical), superoxide dismutase (SOD), xanthine, and xanthine oxidase. Drugs unspecified of source were from Sigma-Aldrich. Stock solutions of allopurinol, chrysin, DiOHF, DPI, 17-ODYA, and quercetin were dissolved in dimethylsulfoxide (DMSO). Indomethacin was dissolved in 100% ethanol. All other drugs were dissolved in Krebs-HEPES buffer. Krebs-HEPES buffer contains 98.0 mM NaCl, 4.7 mM KCl, 25.0 mM NaHCO3, 1.2 mM MgSO4, 1.2 mM KH2PO4, 2.5 mM CaCl2, 11.1 mM d-glucose, and 20.0 mM HEPES-Na.

**Cell Culture.** Rat aortic smooth muscle cells (RASMCs) were isolated from the aorta. All experiments were carried out in accordance with the guidelines of Institutional Animal Ethics Committee and National Health and Medical Research Council of Australia. The thoracic aorta of male Sprague-Dawley rats was isolated into cold phosphate-buffered saline (PBS) and cleaned of fat. The whole aorta was incubated with a digestion mixture containing collagenase I (1 mg/ml), elastase (0.5 mg/ml), and trypsin (1.25 mg/ml) (all from Sigma-Aldrich) in serum-free Dulbecco’s modified Eagle’s medium (DME) (Invitrogen, Carlsbad, CA) at 37°C for 10 min, then the adventitia was pooled off with forceps. This procedure also assisted to remove the endothelial cells. The vessel was chopped into small blocks, rinsed in the mixture, and transferred to a vial containing 1.5% DMSO for 3 to 4 days.

**Immunoprecipitation and Western Blot Analysis.** Confluent smooth muscle cells cultured in DMEM supplemented with 10% FCS in a six-well plate were treated with quercetin or chrysin for 24 h. Cells in each well were lysed with 0.3 ml of cold lysis buffer containing 25 mM Tris, pH 7.5, 2 mM EDTA, 100 mM NaCl, 50 mM NaF, 1 mM Na2VO4, 1 mM dithiothreitol, 1% Triton X-100, 0.25% deoxycholate, and the complete proteinase inhibitor cocktail (Roche Applied Science, Indianapolis, IN) and collected by scraping. Total protein was extracted by incubating the sample at 4°C for 1 h. After centrifugation at 14,000 rpm for 25 min, the protein concentration in the supernatant was determined with the Bio-Rad protein assay reagent (catalog no. 500-0006). Equal amounts of protein (200–300 μg) from each sample were diluted to a final concentration of 1 mg/ml with lysis buffer. The samples were precleared with 20 μl of 50% slurry of protein G agarose beads (Sigma-Aldrich, catalog no. P-4691) and incubated with 5 μg of rabbit anti-p22phox or mouse anti-p47phox antibodies overnight. The immunocomplex was precipitated with 50% protein G agarose beads (20 μl/sample) by continuous mixing for 1 h. The beads were collected by centrifugation, washed with PBS and resuspended in 30 μl (2×) of SDS-polyacrylamide gel
electrophoresis sample buffer, and boiled for 5 min. All procedures were performed at 4°C.

For Western blot, the protein samples prepared in 1× Laemmli buffer were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk powder in Tris-buffered saline, pH 7.5, and hybridized overnight with primary antibodies. The primary antibody was detected with horseradish peroxidase-conjugated anti-IgG and visualized with an ECL kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Cell Membrane Preparation. PMN or HL-60 cells were resuspended in the above-mentioned lysis buffer without Triton and deoxycholate, sonicated for 3 × 5 s bursts at the lowest power on ice, and centrifuged at 500 g for 10 min. The supernatant was layered on top of a 15% sucrose cushion and subject to ultracentrifugation at 100,000g for 1 h. The resultant membrane pellet was then dissolved in 1× Laemmli buffer and boiled for 4 min, and the supernatant was used as the cytosolic fraction.

Cytochrome c Reduction Assay. Differentiated HL-60 cells were resuspended in Hanks’ balanced salt solution containing 200 μM cytochrome c and loaded into a 96-well assay plate (−2 × 10⁸ cells in 100-μl volume per well). All samples were loaded in duplicate, with one well serving as the background by including SOD (300 U/ml). The NADPH oxidase was activated by adding PMA (100 ng/ml final concentration), and absorbance at 550 nm was monitored continuously over 1 h. The SOD-inhibitable cytochrome c reduction was calculated from the ΔA₅₅₀ between the test and background wells, with an extinction coefficient of 21.1 mM⁻¹ cm⁻¹ (Clark and Nauseef, 1996).

Mouse Aortic Ring Preparation. Male apoe⁻/⁻ mice were purchased from the Animal Resource Centre (Western Australia) and fed with normal mouse chow and water ad libitum. Animals were killed by decapitation under general anesthesia with ketamine (100 mg/kg) and loaded into a 96-well plate (−2 × 10⁸ cells in 100-μl volume per well). All samples were loaded in duplicate, with one well serving as the background by including SOD (300 U/ml). The NADPH oxidase was activated by adding PMA (100 ng/ml final concentration), and absorbance at 550 nm was monitored continuously over 1 h. The SOD-inhibitable cytochrome c reduction was calculated from the ΔA₅₅₀ between the test and background wells, with an extinction coefficient of 21.1 mM⁻¹ cm⁻¹ (Clark and Nauseef, 1996).

Lucigenin-Enhanced Chemiluminescence. Lucigenin-enhanced chemiluminescence was used to measure the level of superoxide in both cells and aortic rings. To measure superoxide in aortic rings, tissue suspensions were prepared with DETCA (3 mM) in Krebs-HEPES buffer for 1 h to inactivate the endogenous SOD activity. Various enzyme inhibitors and flavonoids were added to the preinhibusion solution 40 min before assay. Tissue segments were transferred into a 96-well OptiPlate (PerkinElmer Life and Analytical Sciences, Boston, MA) with 300 μl of assay solution in each well, which contains 5 μM lucigenin and 100 μM NADPH. The chemiluminescence was detected with a microplate scintillation counter (Topcount model.9912; PerkinElmer Life and Analytical Sciences) running in Single-Photon-Count mode with a 1-min interval for each sample. A buffer blank background was subtracted from each respective reading. To measure superoxide in cultured cells, 300 μl of cell suspensions (−10⁶ cells per well) was loaded with lucigenin (final concentration, 5 μM) and transferred into an OptiPlate. NADPH (100 μM) or PMA (100 ng/ml) was used to stimulate the NADPH oxidase in RASMCs or HL-60 cells, respectively.

The superoxide scavenging effects of flavonoids were also examined in a cell-free system comprising xanthine (100 μM) plus xanthine oxidase (0.03 U/ml). The assay was carried out in 300 μl of reaction mixture containing xanthine, xanthine oxidase, and lucigenin (5 μM) in PBS, pH 7.4, with or without the test compound. Because the superoxide signal produced by xanthine/xanthine oxidase was transient, the reaction was initiated by addition of xanthine oxidase into the mixture, and the count was initiated 1 min after xanthine oxidase addition.

NBT Reduction Assay. NBT is reduced by superoxide to formazan, which can be quantified colorimetrically at a wavelength of 550 nm. To measure superoxide in aortic rings, tissues were pretreated with DETCA (3 mM) for 1 h in Krebs-HEPES buffer, washed, and then incubated with NBT (100 μM) with or without NADPH for 3 h. After incubation, the tissues were homogenized in 0.6 ml of 1% SDS solution with a glass pestle tissue grinder. The homogenate was centrifuged at 10,000g for 10 min, and the pellet was washed with 1 ml of 70% ethanol, air dried, and dispersed in 0.3 ml of pyridine. The formazan was extracted by heating pyridine to 80°C for 2 h and quantified by measuring the absorbance at 550 nm of 250 μl of supernatant in a 96-well plate. Superoxide production was expressed as picomoles of formazan per milligram of wet tissue, using an extinction coefficient of 0.72 mM·mm⁻¹ (Shi et al., 2001). To eliminate the influence of nonspecific diaphorase activity of the tissue, readings with the tissues that were coincubated with a synthetic SOD mimetic M40403 (1 mM) (kindly provided by Dr. Daniela Salvemini, MetaPhore Pharmaceuticals, St. Louis, MO) (Jiang et al., 2003) were used as blanks.

H₂O₂ Measurement. Ferrithiocyanate assay (Thurman et al., 1972) was used to determine the concentration of H₂O₂ generated by xanthine (100 μM) and xanthine oxidase (0.01 U/ml) in Krebs-HEPES. After incubation at 37°C for 1 h, 1 ml of sample was mixed with 50 μl of 50% (v/v) trichloroacetic acid, 0.2 ml of 10 mM ferrous ammonium sulfate, and 0.1 ml of 2.5 M potassium thiocyanate. The absorption at 490 nm was determined with a microplate reader (Molecular Devices, Sunnyvale, CA) using 0.3 ml of the mixture. The standard curve was generated using serial dilutions of H₂O₂.

Real-Time PCR. RASMCs grown in a six-well plate were incubated with 0.5 ml well RNAwiz reagent (Ambion, Austin, TX) at 4°C for 1 h, and the total RNA was extracted according to the manufacturer’s instruction. Total RNA was reverse-transcribed to cDNA using random hexamer and random reverse transcription reagents (Applied Biosystems, Foster City, CA) at 48°C for 30 min followed by 95°C for 5 min. The real-time PCR reactions were performed in the ABI Prism 7700 system (Applied Biosystems) using the cDNA as template. The 18s RNA was used as housekeeping gene. The sequences of the primers were: 18s forward, CGGCTACCATC-CAAGGA; 18s reverse, GCTGGAATTACCCGGGCTT; Nox1 forward, GCGGCCCTCGCCATTT; Nox1 reverse, TTAAGTAGTAATTAC-CTCTCTAGGA; p22phox forward, CAAAATTCGACGAGAT- GAG; and p22phox reverse, GGGAGGACACCTGGGAAAC. The probes were labeled at the 5’ end with the reporter dye of either 5-carboxylfluorescein (for target genes) or VIC (for housekeeping gene) and at the 3’ end with the quencher molecule 5-carboxytetramethylrhodamine. The sequences of the probes are: 18s, TCTGGCGACACA-GACTTGGCTC; Nox1, AAAAACGGCTCGGCAAATGCTGCG; and p22phox, TCTGTCGACCTCGTGCTCGGAG. Amplification of both target and housekeeping genes were performed in separate tubes using the Taqman Universal PCR master mix kit (Applied Biosystems) and 20 ng of cDNA sample. Thermal cycler parameters were 2 min at 50°C, 10 min at 95°C, and 40 cycles of 95°C × 30 s and 60°C × 1 min. The results of real time PCR were expressed as 2⁻ΔΔC₅ value.

Data and Statistical Analysis. Chemiluminescence intensity was expressed as counts per second. Results were normalized to per milligram of dry tissue (aorta) or to cell number. Western blot results were analyzed with a densitometer (Bio-Rad GS-710). Some of the data were converted to percentage of control. The EC₅₀ values were calculated using GraphPad Prism (GraphPad Software Inc., San Diego, CA). Data were presented as mean ± S.E.M. The data were analyzed with unpaired Student’s t test or one-way analysis of variance followed by Tukey’s test as appropriate. A value of P < 0.05 was regarded as statistically significant.

Results

DiOHF Is a Potent Superoxide Scavenger. In a cell-free system comprising xanthine (100 μM) plus xanthine oxidase (0.03 U/ml), the superoxide generation rate detected by lucigenin was 975 ± 185 cps (n = 24). Consistent with our previous observations (Jiang et al., 2003), this chemiluminescence signal was almost abolished by Cu/Zn-SOD (100 U/ml),
indicating that downstream metabolites of superoxide, such as H$_2$O$_2$ (Faulkner and Fridovich, 1993), are unlikely to be involved in the reaction with lucigenin. Both DiOHF and quercetin (0.1–100 μM) reduced the superoxide level in a concentration-dependent manner (Fig. 2a). The EC$_{50}$ values of these two compounds did not differ significantly (6.1 ± 1.1 μM for DiOHF and 4.4 ± 0.8 μM for quercetin, P = 0.2). The superoxide scavenging potency of DiOHF is similar to that of a synthetic manganese-based SOD mimetic, M40403 (EC$_{50}$ = 6.3 μM) (Jiang et al., 2003). It appears that at higher concentrations quercetin is more potent than DiOHF, but the difference in the maximum suppression by 100 μM of the two compounds is not statistically significant. Chrysin at 10 μM suppressed the superoxide signal by 60%. In contrast to DiOHF and quercetin, increased concentrations of chrysin did not further reduce the superoxide level. The maximal suppression of superoxide by chrysin at 30 and 100 μM is significantly less than those of DiOHF and quercetin (P < 0.05).

Because superoxide scavenging compounds are often indiscriminately called SOD mimetics, we examined whether DiOHF and quercetin dismutate superoxide. In aqueous solution (Krebs-HEPES buffer) containing xanthine-xanthine oxidase, there was a spontaneous dismutation of superoxide as determined by the accumulation of H$_2$O$_2$ without exogenous SOD (Fig. 2b) measured with the ferrithiocyanate assay. The H$_2$O$_2$ generation was significantly enhanced by native SOD (100 U/ml). In contrast to SOD, both DiOHF and quercetin significantly decreased H$_2$O$_2$ formation, with quercetin being more potent than DiOHF (Fig. 2b). The ferrithiocyanate assay had a good linearity over the range of H$_2$O$_2$ concentrations as shown by the standard curve ($r^2 = 0.99$, Fig. 2c).

**DiOHF Suppresses Superoxide Generation from Vascular NADPH Oxidase.** To validate the use of lucigenin to measure superoxide in vascular tissues (Skatchkov et al., 1999), we measured superoxide generation in rat aortic rings using the SOD-inhibitable NBT reduction assay. As shown in Fig. 3a, lucigenin at 5 μM did not significantly change the NADPH-stimulated superoxide, which excludes the possibility that lucigenin may artificially increase superoxide production through a redox cycling (Liochev and Fridovich, 1997).

In RASMCs, NADPH (10–3000 μM) concentration-dependently stimulated superoxide production. In the following experiments, 100 μM NADPH was used. The absolute chemiluminescence signal generated from RASMCs by adding NADPH to the common antioxidant ascorbic acid, which suppressed the NADPH-stimulated superoxide, which excludes the possibility of lucigenin may artificially increase superoxide production only at millimolar concentrations (Fig. 3b). The EC$_{50}$ values of DiOHF and quercetin are not significantly different (8.4 ± 1.7 μM for DiOHF and 16.8 ± 3.9 μM for quercetin, P > 0.05). In contrast to DHF and quercetin, chrysin in the same concentration range had no significant effect on superoxide production (Fig. 3b).

Effects of flavonoids on superoxide accumulation from vascular NADPH oxidase were also examined in isolated aortic rings from apoE$^\text{−/−}$ mice, in which the NADPH oxidase activity was approximately 2-fold higher than that in wild-type controls (931 ± 96 versus 581 ± 141 cps/mg tissue, respectively, n = 6). Similar to RASMCs, the NADPH-stimulated superoxide production was inhibited by DPI, but not affected by allopurinol, L-NAME, indomethacin, or 17-ODYA (data not shown). Both DiOHF and quercetin concentration-dependently reduced the superoxide level in RASMCs (Fig. 3b). The EC$_{50}$ values of DiOHF and quercetin are not significantly different (8.4 ± 1.7 μM for DiOHF and 16.8 ± 3.9 μM for quercetin, P > 0.05). In contrast to DHF and quercetin, chrysin in the same concentration range had no significant effect on superoxide production (Fig. 3b).

**DiOHF Suppresses Superoxide Generation from Phagocytic NADPH Oxidase.** In HL-60 cells, PMA-induced NADPH oxidase activation and a burst of superoxide release as determined by SOD-inhibitable cytochrome c reduction over a period of up to 1 h (Fig. 4a). Cotreatment with DiOHF or quercetin inhibited the PMA-induced superoxide accumulation in a concentration-dependent manner (Fig. 4b). In contrast, the effect of chrysin was weak. These inhibitory effects of DiOHF or quercetin on phagocytic NADPH oxidase were also confirmed with chemiluminescence assays (Fig. 4, c and d). To exclude the possibility that DiOHF or quercetin may reoxidize the prereduced cytochrome c, we tested the effects of these compounds on cytochrome c prereduced by PMA-stimulated HL-60 cells. As shown in Supplemental Fig.
the cytosolic subunits with the membrane-bound cytochrome hibit NADPH oxidase by interfering with the assembly of Phagocytic Cells.

Effects of various enzyme inhibitors on superoxide production in aortic rings from apoE0 mice

**TABLE 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Superoxide Production</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1215 ± 201</td>
</tr>
<tr>
<td>DPI (5 μM)</td>
<td>129 ± 41*</td>
</tr>
<tr>
<td>Control</td>
<td>1712 ± 491</td>
</tr>
<tr>
<td>Allopurinol (100 μM)</td>
<td>1916 ± 395</td>
</tr>
<tr>
<td>t-NAME (200 μM)</td>
<td>2031 ± 767</td>
</tr>
<tr>
<td>Control</td>
<td>962 ± 135</td>
</tr>
<tr>
<td>Indomethacin (30 μM)</td>
<td>822 ± 57</td>
</tr>
<tr>
<td>17-ODYA (10 μM)</td>
<td>1016 ± 304</td>
</tr>
</tbody>
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* P < 0.005 vs. control, unpaired Student's t test.

1, addition of DiOHF or quercetin at 30 μM did not oxidize the reduced cytochrome c.

**DiOHF Did Not Affect NADPH Oxidase Assembly in Phagocytic Cells.** To investigate whether flavonoids can inhibit NADPH oxidase by interfering with the assembly of the cytosolic subunits with the membrane-bound cytochrome b558, we examined p47phox translocation from the cytosol to membrane in PMA-stimulated HL-60 cells. Unexpectedly, we could not observe accumulation of p47phox in the particulate fraction as reported in previous studies (Korchak et al., 1998) (Fig. 5a). Functional experiments demonstrated that PMA at the same concentration is sufficient to induce superoxide release in these cells (Fig. 4a). The distribution of p47phox was not affected by DiOHF, quercetin, or chrysin in HL-60 cells either before or after PMA stimulation (Fig. 5b). To confirm the purity of the cytosolic and particulate fractions, we used antibodies against Na-K ATPase (a plasma membrane marker) and GAPDH (a cytosolic marker). As shown in Fig. 5b, Na-K ATPase could only be detected in the membrane sample, whereas GAPDH was predominantly present in the cytosolic sample. In contrast, under the same experimental conditions, PMA-stimulated membrane translocation of PKC could be readily detected in HL-60 cells (Fig. 5c).

**Effects of Flavonoids on NADPH Oxidase Expression.** We finally investigated whether flavonoids, apart from their acute antioxidant activity, can inhibit NADPH oxidase expression in both HL-60 and vascular cells. Consistent with the observations reported by others (Inoue et al., 2001), differentiation of HL-60 cells with DMSO for 3 days significantly increased expressions of both p47phox and p67phox, whereas the level of Nox2 remained low (Supplemental Fig. 2). Treatment of the differentiated HL-60 cells with DiOHF or quercetin at 10 and 30 μM for 24 h significantly reduced the level of p47phox, whereas p67phox was not changed (Fig. 6, a and b). No cytotoxic effect was observed after 24-h treatment with either DiOHF or quercetin at 30 μM.

Then, we examined whether DiOHF has similar effects on p47phox expression in vascular cells. As shown in Fig. 7a, the protein level of p47phox in HMECs was not significantly changed by DiOHF (30 μM for 24 h). Unexpectedly, we found that treatment with DiOHF at 30 μM significantly increased the mRNA level of p47phox in HMECs (Fig. 7c, right), whereas quercetin had no effect. Moreover, neither DiOHF nor quercetin had any effects on the expressions of Nox2 (Fig. 7b) or Nox4 (Fig. 7c, left). Finally, we measured the expres-
sion of p47phox in cultured RASMCs. Because the protein level of p47phox was too low to be detected by direct Western blot, we concentrated the protein by immunoprecipitation before Western blot analysis. Figure 7d shows that treatment with DiOHF, quercetin, or chrysin for 24 h had no effect on p47phox expression in RASMCs. Moreover, DiOHF did not significantly change the mRNA level of p47phox in vascular smooth muscle cells (Fig. 7e).

Discussion

In this study, we characterized the effects of one synthetic (DiOHF) and two naturally occurring (quercetin and chrysin) flavonoid compounds on phagocytic and vascular NADPH oxidases, which are thought to be important in the pathogenesis of cardiovascular disease (Griendling et al., 2000; Cathcart, 2004). We found that both of the flavonols DiOHF and quercetin potently inhibited superoxide accumulation from
Both phagocytic and vascular NADPH oxidases, as assessed in HL-60 and vascular smooth muscle cells and mouse aortic tissues. The efficacies of DiOHF and quercetin in suppressing superoxide accumulation in vascular smooth muscle cells are much higher than that of ascorbic acid, a commonly used antioxidant, which is only effective in the millimolar range. In contrast, the flavone compound chrysin lacks any effect in all of these circumstances. This is in line with the observations that many members of the flavonol family are potent antioxidants, which may prevent free radical-induced lipid peroxidation (de Whalley et al., 1990; Dambros et al., 2005). The antioxidant properties of flavonols are thought to be attributable to their superoxide free radical scavenging activities (Chen et al., 1990). Using a cell-free superoxide generating system, we confirmed that both DiOHF and quercetin are potent superoxide scavengers, with EC₅₀ values in the micromolar range. Similar to the results in living cells, we found that the superoxide scavenging activity of chrysin is lower than DiOHF and quercetin. Our results suggest that the functional inhibition of NADPH oxidase activity by flavonols is mainly a result of scavenging of superoxide by these compounds, and this is supported by the similar EC₅₀ values obtained in smooth muscle cells.

The difference in potency between DiOHF or quercetin and chrysin can be explained by structure-effect relations. Previous studies have suggested that a 3-hydroxy group in ring C in conjunction with a 2,3-double bond and a 4-carbonyl moiety is important for radical scavenging activity of flavonoids (Bors et al., 1990); lack of 3-OH or glycosylation of this group in flavonoids decreases their antioxidant efficacy (Rice-Evans et al., 1996). Because the 3-OH group is present in DiOHF and quercetin, but not in chrysin, our results further highlight the significance of this structure for the antioxidant activity of flavonoids. In addition, the catechol (3',4'-orthodihydroxy) structure in ring B of quercetin may also contribute to the higher activity of radical scavenging compared with chrysin (Rice-Evans et al., 1996). On the other hand, it has been suggested that a 5,7-dihydroxy arrangement in ring A might partly compensate for the absence of the essential active structures in ring C (as in chrysin) and therefore be responsible for the radical scavenging activities observed in certain flavonoids lacking the active moieties in ring C. This effect may explain the weak antioxidant activity of chrysin observed in isolated aortic rings without NADPH stimulation (data not shown).

Although it has been widely reported that flavonoids have superoxide-scapenging activities, it is not known whether this reaction is accompanied by generation of H₂O₂ (i.e., dismutase activity). In a cell-free superoxide generating system comprising xanthine plus xanthine oxidase, we found that most superoxide is spontaneously converted to H₂O₂ (as evidenced by the small increase seen with exogenous SOD), which may represent an autodismutation reaction in this system. In contrast to native SOD, DiOHF and quercetin significantly decreased H₂O₂ accumulation. The strong superoxide scavenging activity of DiOHF and quercetin without enhanced H₂O₂ accumulation suggests that these flavonols may be superior to authentic dismutase mimetics because H₂O₂ can be converted to hypochlorous acid in the presence of myeloperoxidase, which has been implicated in many pathophysiological situations. For example, hypochlorous acid may activate matrix metalloproteinase in vitro, and it has been suggested that increased H₂O₂ accumulation may contribute to proteolytic processes in atherosclerotic plaques and subsequent plaque rupture in coronary arteries (Fu et al., 2001).

There is little information about the effects of flavonoids on the activation of NADPH oxidase, which depends on the translocation of the cytosolic subunits p47phox and p67phox to the membrane (Babior, 2004). Tauber et al. (1984) studied the effects of four flavonoids, including quercetin, on NADPH oxidase function in freshly isolated neutrophils. They found that quercetin suppressed zymosan-induced oxygen consumption by neutrophils with an IC₅₀ of approximately 100 μM, indicating that flavonoids may directly inhibit the enzymatic activity of phagocytic NADPH oxidase in addition to superoxide scavenging. However, they also reported that quercetin suppressed NADPH oxidation by the membrane preparation from zymosan-activated neutrophils, suggesting that the effects of quercetin may be independent of interference with the assembly of the enzyme. In the present study, we directly measured translocation of the cytosolic subunits to the plasma membrane. Unexpectedly, using the HL-60 cell line, we could not detect PMA-stimulated membrane translocation of p47phox as reported by others (Korchak et al., 1998); the reasons for this discrepancy are not clear. Proper subcellular fractioning was confirmed by the enrichment of GAPDH and Na-K ATPase in the cytosolic and particulate preparations, respectively. Moreover, we have shown that PMA-stimulated membrane translocation of PKC was readily detected under the same experimental conditions. We also showed that neither DiOHF nor quercetin had any effect on PMA-induced PKC activation in HL-60 cells. Moreover, NADPH oxidase assembly was assured in fresh peripheral neutrophils, where PMA stimulation consistently induced mobilization of p47phox, but not p67phox or Nox2, to the membrane.
membrane. Here again, we failed to find any evidence that flavonoids had an impact on this process, suggesting that the observed suppression of superoxide accumulation by these flavonoids was unlikely to involve direct inhibition of NADPH oxidase assembly in phagocytic cells.

Finally, we studied the effects of DiOHF on the expression of NADPH oxidase subunits because some recent studies have reported that flavonoids may modulate NADPH oxidase expression both in vitro and in vivo (Al-Awwadi et al., 2005; Sanchez et al., 2006, 2007). Interestingly, we found that DiOHF concentration dependently reduced the protein level of p47phox in differentiated HL-60 cells. This effect is specific because p67phox was not changed. Moreover, quercetin had a similar effect. This effect of DiOHF was restricted to the phagocytic NADPH oxidase because this was not observed in either vascular endothelial or smooth muscle cells. Paradoxically, DiOHF (but not quercetin) increased the mRNA level of p47phox by approximately 13-fold in endothelial cells at an intermediate concentration. In addition, the expression of other subunits including Nox2 and Nox4 were not affected by DiOHF or quercetin in the vascular cells. Given the increased p47phox mRNA in vascular cells, we tested whether there is rebound oxidative stress after withdrawal of DiOHF treatment. We examined the effect of removal of DiOHF from the cell culture after a 24-h treatment but did not find any increase in the superoxide level compared with untreated cells (data not shown), which is consistent with the unchanged protein levels of p47phox.

In summary, DiOHF is a potent superoxide scavenger that markedly suppresses superoxide accumulation from both vascular and phagocytic NADPH oxidase. DiOHF also significantly reduced NADPH oxidase subunit expression in phagocytic cells. These antioxidant effects may contribute to the observed cytoprotective actions during ischemia reperfusion injury in the heart and brain and potentially other organs. Of note, the biological effects of flavonoids are complex; therefore, other mechanisms may also be important in preventing oxidative injury, even at concentrations that do not directly scavenge superoxide.

Acknowledgments
We thank Gary S. H. Yip for technical assistance in chemiluminescence assays.

References


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