

Vascular Antioxidant Effects of Flavonoids

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SUMMARY

1. Oxidative stress, *ie.* increased reactive oxygen species (ROS) production, is involved in pathogenesis of various cardiovascular diseases, and the use of antioxidants to target this excess ROS formation thus is of significant scientific and clinical interest. Flavonoids are polyphenolic compounds known to have ROS scavenging activities, however, their effects have not been characterised in vascular tissues. Based on structure-activity relationships, the synthetic flavonoid 3',4'-dihydroxyflavonol (DHF) has been designed specifically for optimal antioxidant activity. The effects of this compound on superoxide ($O_2^{\bullet-}$) production by vascular cells and tissues were examined, and compared with those of two natural flavonoids, quercetin and chrysin.
2. Treatment of cultured rat aortic smooth muscle cells (RASMCs) with NADPH (100 μ M) markedly stimulated $O_2^{\bullet-}$ generation in these cells, and these $O_2^{\bullet-}$ molecules were primarily derived from NADPH oxidase, the enzyme that has been demonstrated to be a major source of ROS in vasculature. Incubation of RASMCs with DHF and quercetin produced a concentration-dependent suppression of NADPH-stimulated $O_2^{\bullet-}$ production (for DHF, $pIC_{50} = 5.1 \pm 0.1$; for quercetin, $pIC_{50} = 4.8 \pm 0.1$), while chrysin had no effect. Structural differences between flavonoid compounds may account for this difference in their efficacy.
3. Aortic segments from apolipoprotein(E)-deficient ($apoE^0$) mice, which had hyperlipidemia and atherosclerosis, were measured for $O_2^{\bullet-}$ production by lucigenin-enhanced chemiluminescence. Vascular $O_2^{\bullet-}$ production at basal conditions was higher in the aorta of $apoE^0$ mice as compared with wild-type controls, indicating that $apoE^0$ mice have increased vascular oxidative stress. In contrast, the substrate (NADPH, 100 μ M)-stimulated NADPH oxidase activity was not significantly different between the two mouse strains.
4. Diphenylene iodonium (5 μ M), an inhibitor of NADPH oxidase, significantly decreased $O_2^{\bullet-}$ production in $apoE^0$ mouse aorta both at basal conditions and following stimulation with NADPH. Pharmacological inhibitors of four other potential $O_2^{\bullet-}$ generating enzymes,

allopurinol (xanthine oxidase), N^o-nitro-L-arginine methyl ester (nitric oxide synthase), indomethacin (cyclooxygenase), and 17-octadecynoic acid (cytochrome P450) did not affect O₂^{•-} production by apoE⁰ mouse aorta, either in the absence or presence of NADPH.

5. Treatment of apoE⁰ mouse aorta with DHF, quercetin and chrysin markedly suppressed O₂^{•-} production at basal conditions (82%, 83%, and 91% inhibition respectively). However, only DHF and quercetin attenuated NADPH-stimulated O₂^{•-} production (94% and 91% inhibition respectively).
6. These data suggest that DHF and quercetin physiologically antagonise the NADPH oxidase activity, while chrysin had minor effect. Given the pivotal role of ROS in the development of cardiovascular disease, designing new flavonoids with higher and more specific antioxidant activity with minimal toxicity may represent a new approach for identifying cardiovascular therapeutics.

1. INTRODUCTION

1.1 Reactive oxygen species and vascular disease

Reactive oxygen species (ROS) are highly reactive molecules that are constantly produced by enzymatic reactions in cells. Members of the ROS family include superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^{\bullet}) (Palmer & Paulson, 1997). In normal physiological conditions, ROS are produced at low levels which are necessary for maintaining normal cell functions, and the endogenous antioxidant defence systems of the body have the capacity to avert any harmful effects. However, several established risk factors for cardiovascular disease have been linked to excessive generation of ROS, known as a state of oxidative stress. For instance, in animal models of hyperlipidemia (Miller *et al.*, 1998; Mugge *et al.*, 1994), hypertension (Morawietz *et al.*, 2001; Zalba *et al.*, 2000; Suzuki *et al.*, 1995), and diabetes (Hink *et al.*, 2001; Sano *et al.*, 1998), elevated levels of vascular $O_2^{\bullet-}$ production have been found. Moreover, clinical studies have demonstrated that hypercholesterolemia and diabetes in humans are also associated with increased vascular $O_2^{\bullet-}$ generation (Guzik *et al.*, 2000). All these data strongly suggest that increased oxidative stress is involved in the pathophysiology of cardiovascular disease.

Several mechanisms have been proposed to explain how excessive production of ROS leads to vascular pathology. Firstly, ROS are able to promote the oxidation of low-density lipoprotein (LDL) (Hiramatsu *et al.*, 1987; Heinecke *et al.*, 1986). Uptake of oxidatively modified lipoproteins by macrophages transforms these cells into foam cells, which are a key component of atherosclerotic plaques (Berliner & Heinecke, 1996; Steinberg, 1997). Secondly, $O_2^{\bullet-}$ rapidly inactivates endothelium-derived nitric oxide (NO), a molecule with intrinsic anti-atherogenic properties, leading to endothelial dysfunction, which is a hallmark of early atherosclerosis (Darley-Usmar *et al.*, 1995). Moreover, the reaction between $O_2^{\bullet-}$ and NO generates peroxynitrite ($ONOO^-$), which has been found to be cytotoxic to endothelial and vascular smooth muscle cells through a broad range of biological actions such as lipid oxidation and mitochondrial DNA damage (Moore *et al.*, 1995; Ballinger *et al.*, 2000). Thirdly, ROS have been shown to be involved in increased expression of certain vascular proinflammatory genes that are pertinent to atherogenesis (Griendling *et al.*, 2000a; Kunsch

& Medford, 1999), such as monocyte chemoattractant protein-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) (Aiello *et al.*, 1999; Dawson *et al.*, 1999; Chen *et al.*, 1999; Boring *et al.*, 1998; Nakashima *et al.*, 1998; Sakai *et al.*, 1997). In addition, evidence has shown that many intracellular signal transduction molecules are sensitive to changes of intracellular redox status induced by ROS formation. For example, intracellular Ca^{2+} mobilisation (Dreher *et al.*, 1995; Roveri *et al.*, 1992), protein phosphorylation via altered balance of protein kinase and phosphatase activity (Sundaresan *et al.*, 1995; Baas & Berk, 1995; Sullivan *et al.*, 1994), and activation of transcription factors such as nuclear factor κB (Schreck *et al.*, 1991; Shono *et al.*, 1996) are all subject to modulation by oxidative stress. These ROS-mediated actions lead to direct regulation of cell function and gene expression.

Formation of ROS begins with one-electron reduction of oxygen molecules, leading to the formation of $\text{O}_2^{\bullet-}$. Endogenous superoxide dismutase (SOD) converts $\text{O}_2^{\bullet-}$ to H_2O_2 which, in turn, is converted to water by catalase or glutathione peroxidase (Griendling *et al.*, 2000a). Alternatively, H_2O_2 may react with $\text{O}_2^{\bullet-}$ to form OH^{\bullet} in the Haber-Weiss reaction, or accepts an electron from a reduced metal ion to form OH^{\bullet} in the Fenton reaction (Kukreja & Hess, 1992).

1.2 NADPH oxidase

Many enzyme systems can generate $\text{O}_2^{\bullet-}$. These include xanthine/xanthine oxidase (Miyamoto *et al.*, 1996; Chambers *et al.*, 1985), nitric oxide synthase (NOS) (Kerr *et al.*, 1999; Xia *et al.*, 1998), the mitochondrial electron transport chain (Nishikawa *et al.*, 2000), cyclooxygenase, lipoxygenase (Kukreja *et al.*, 1986), and cytochrome P450 monooxygenase. All of these enzymes are present in vasculature. However, none of these enzyme systems can totally explain the production of ROS in vascular tissues under either normal or pathophysiological conditions (Munzel *et al.*, 1999).

Recently, a $\text{O}_2^{\bullet-}$ generating NADPH oxidase that is similar to that in leukocytes has been found to be expressed in vascular tissues (Bayraktutan *et al.*, 1998; Meyer *et al.*, 1999). This enzyme comprises two membrane-bound subunits, gp91^{phox} (or an analogue of gp91^{phox}, nox, as expressed in smooth muscle cells) and p22^{phox}, and two cytosolic subunits, p47^{phox}

and p67^{phox} (Sorescu *et al.*, 2002). Upon activation, p47^{phox} and p67^{phox} translocate to the membrane and associate with the membrane-bound subunits (De Leo *et al.*, 1996). gp91^{phox}/nox is the catalytic domain which is responsible for the electron transfer from NADPH to oxygen, leading to O₂^{•-} formation (Griendling *et al.*, 2000b). A cytosolic small G protein, rac, also participates in the assembly of the enzyme complex (Quinn *et al.*, 1993). Evidence suggests that NADPH oxidase is a major source of O₂^{•-} in the vascular wall. Firstly, NADPH oxidase has been shown to be expressed in both endothelial (Jones *et al.*, 1996; Hohler *et al.*, 2000; Bayraktutan *et al.*, 2000) and vascular smooth muscle cells (Lavigne *et al.*, 2001; Fukui *et al.*, 1995). Secondly, blocking of this enzyme by pharmacological inhibitors, such as diphenylene iodonium (DPI) and apocynin, or by transfection of antisense oligonucleotide to p22^{phox} effectively inhibited O₂^{•-} production in vascular tissues or cells (Rajagopalan *et al.*, 1996; Pagano *et al.*, 1995; Li & Shah, 2001; Griendling *et al.*, 1994; Ushio-Fukai *et al.*, 1996).

Evidence has suggested that NADPH oxidase may have an important role in the pathogenesis of vascular disease. It has been found, for instance, that O₂^{•-} production in arteries from hyperlipidemic Watanabe rabbits can be blocked by DPI (Warnholtz *et al.*, 1999). Clinical studies have found that NADPH oxidase-derived O₂^{•-} is increased in blood vessels from patients with risk factors for atherosclerosis (Guzik *et al.*, 2000). Furthermore, atherosclerotic human coronary arteries were shown to have elevated protein expression of p22^{phox} (Azumi *et al.*, 1999). Moreover, disruption of the p47^{phox} gene reduced the size of atherosclerotic lesions in apolipoprotein(E)-deficient (apoE⁰) mice (Barry-Lane *et al.*, 2001). Thus, this enzyme is becoming a potential target for therapeutic interventions in vascular disease.

1.3 Cardiovascular protective effects of antioxidants

The link between ROS and vascular pathogenesis has triggered considerable research into antioxidant nutrients and pharmaceuticals for the prevention and treatment of vascular diseases. The beneficial effects of the naturally occurring antioxidants α -tocopherol (vitamin E) and ascorbate (vitamin C) have been extensively studied. In hypertensive rats and hyperlipidemic monkeys, α -tocopherol has been shown to reduce oxidative stress (Newaz &

Nawal, 1998; Sharma *et al.*, 1999); moreover, α -tocopherol suppresses endothelial expression of VCAM-1 and ICAM-1 and inhibits monocyte adhesion *in vitro* (Wu *et al.*, 1999). In addition, ascorbate has been demonstrated to protect isolated human LDL against oxidative modification induced by endothelial cells or copper (Martin & Frei, 1997; Retsky *et al.*, 1999). Despite these findings, however, the ability of α -tocopherol and ascorbate to moderate the progression of vascular disease is still uncertain. For instance, in a major clinical trial, the Heart Outcomes Prevention Evaluation (HOPE) study, it was reported that the beneficial effects of α -tocopherol found in animals did not translate into clinically significant benefits for patients who were at increased risk of or had vascular disease (Lonn *et al.*, 2001).

Synthetic antioxidant compounds have also been investigated in various animal models of vascular disease. N,N'-diphenyl-1,4-phenylenediamine has been found to impede atherogenesis in apoE⁰ mice and cholesterol-fed rabbits (Tangirala *et al.*, 1995; Sparrow *et al.*, 1992), but this drug is mutagenic and cannot be administered to humans. Probucol has been shown to retard atherogenesis in hyperlipidemic Watanabe rabbits (Oshima *et al.*, 1998; Nagano *et al.*, 1992), but was paradoxically found to be proatherogenic in murine models of atherosclerosis (Moghadasian *et al.*, 1999; Bird *et al.*, 1998). On the other hand, probucol has little effect on human atherosclerosis (Regnstrom *et al.*, 1996; Walldius *et al.*, 1994). Therefore, searching for new antioxidants that have higher efficacy and specificity in cardiovascular system is likely to be fruitful.

1.4 Actions of flavonoids

Flavonoids are a group of plant-originating compounds that are mainly found in vegetables, fruits, seeds, nuts, tea, and red wine (Pietta, 2000). The basic structure of flavonoids is the diphenylpropane skeleton (Cao *et al.*, 1997), consisting of fifteen carbon atoms arranged in three rings (Figure 1). Based on their molecular structure, flavonoids are divided into different classes, *eg.* flavonols, flavones, flavanols, and flavanones (Fuhrman & Aviram, 2001). Research on flavonoids was triggered by the discovery of the “French paradox”, whereby populations with high red wine consumption have a relatively low cardiovascular mortality rate (Renaud & de Lorgeril, 1992). Clinical evidence from the Zutphen Elderly Study (Hertog *et al.*, 1993) and the Rotterdam Study (Geleijnse *et al.*, 1999) has since

indicated that flavonoids have cardiovascular protective effects against coronary heart disease and atherosclerosis. This notion is supported by animal studies. For example, apoE⁰ mice given red wine or ginger flavonoids had diminished atherogenesis (Hayek *et al.*, 1997; Fuhrman *et al.*, 2000). Also, it has been found that quercetin significantly lowered blood pressure in spontaneously hypertensive rats (Duarte *et al.*, 2001b). These data have generated interest in searching for and designing flavonoid compounds as therapeutic agents against cardiovascular diseases.

Flavonoids are known to be strong antioxidants. For example, various flavonoids have been shown to inhibit cell-mediated LDL oxidation *in vitro* (Rosenblat *et al.*, 1999; Yoshida *et al.*, 1999), and dietary flavonoid supplementation has been found to reduce the susceptibility of LDL to oxidation (Aviram *et al.*, 2000; Stein *et al.*, 1999; Ishikawa *et al.*, 1997). Flavonoids have been found to scavenge ROS that are generated enzymatically and non-enzymatically in cell-free systems (Robak & Gryglewski, 1988; Chen *et al.*, 1990; Cimanga *et al.*, 2001). Furthermore, they have been shown to downregulate gene expression of VCAM-1, ICAM-1, and MCP-1 in human endothelial cells and rabbit aorta (Gerritsen *et al.*, 1995; Lee *et al.*, 2001), inhibit the activity of the proinflammatory enzymes cyclooxygenase and lipoxygenase (Hoult *et al.*, 1994; Laughton *et al.*, 1991), reduce the aggregation of platelets (Freedman *et al.*, 2001), and inhibit the activity of xanthine oxidase (Selloum *et al.*, 2001; Moini *et al.*, 2000). However, the antioxidant efficacies of flavonoids in vascular tissues have not been well defined. Moreover, their interactions with vascular NADPH oxidase activity have not yet been studied.

The correlation between antioxidant activity and chemical structure of flavonoids is not fully understood. However, current data suggest that a 2,3-double bond in conjugation with a 4-oxo function in ring C is largely responsible for the antioxidant activity of flavonoid compounds, and the presence of 3- and 5-OH groups further strengthens this activity (Bors *et al.*, 1990). An increasing number of hydroxyl groups in ring B reputedly enhances antioxidant potential (Hu *et al.*, 1995), particularly if a catechol (3',4'-orthodihydroxy) group is present (Burda & Oleszek, 2001). A 5,7-metadihydroxy arrangement in ring A is also thought to make some contribution to antioxidant actions (Rice-Evans *et al.*, 1996). Based on

some of these general principles, a synthetic flavonol, 3',4'-dihydroxyflavonol (DHF), has been specifically designed with the intention of optimising antioxidant activity (Figure 1).

In this study, the antioxidant efficacy of DHF in vascular cells, especially its actions on NADPH oxidase activity, will be examined. The effects of DHF will be compared with those of two natural flavonoids, the flavonol quercetin and the flavone chrysin. Furthermore, the abilities of these three flavonoids to reduce oxidative stress in the aorta of apoE⁰ mice (see below) will be investigated. Previous studies have shown that quercetin, the most abundant dietary flavonol, attenuates atherogenesis in apoE⁰ mice (Hayek *et al.*, 1997). Chrysin and DHF have been shown to have vasorelaxant activities (Duarte *et al.*, 2001a; Chan *et al.*, 2000). In addition, our laboratory has recently found that DHF significantly reduced the infarct size and ischaemic damage after myocardial ischaemia and reperfusion in sheep (Sheng Wang, personal communication).

1.5 Apolipoprotein(E)-deficient mice as a model of atherosclerosis

ApoE⁰ mice were developed using gene targeting to disrupt the apolipoprotein(E) gene in embryonic stem cells (Plump *et al.*, 1992). Apolipoprotein(E) is a constituent of lipoproteins and functions as a ligand for receptors that clear these lipoproteins from the plasma (Zhang *et al.*, 1992). Thus, apoE⁰ mice have deficiency in clearing lipoproteins from plasma and develop severe hyperlipidemia. These animals, when fed a normal chow diet, spontaneously develop atherosclerotic lesions, and the progression of these lesions with age is similar to that of human lesions (Reddick *et al.*, 1994), making apoE⁰ mice the most widely used experimental model of atherosclerosis (Reardon & Getz, 2001).

1.6 Aims

The aims of this study were:

1. to assess the antioxidant efficacy of DHF in vascular smooth muscle cells and, especially, its interaction with NADPH oxidase activity, and compare these effects with those of two natural flavonoids, quercetin and chrysin;
2. to characterise the enzymatic source of O₂^{•-} in apoE⁰ mouse aorta; and
3. to determine whether these flavonoids can reduce the oxidative stress in the aorta of apoE⁰ mice *in vitro*.

FIGURE 1

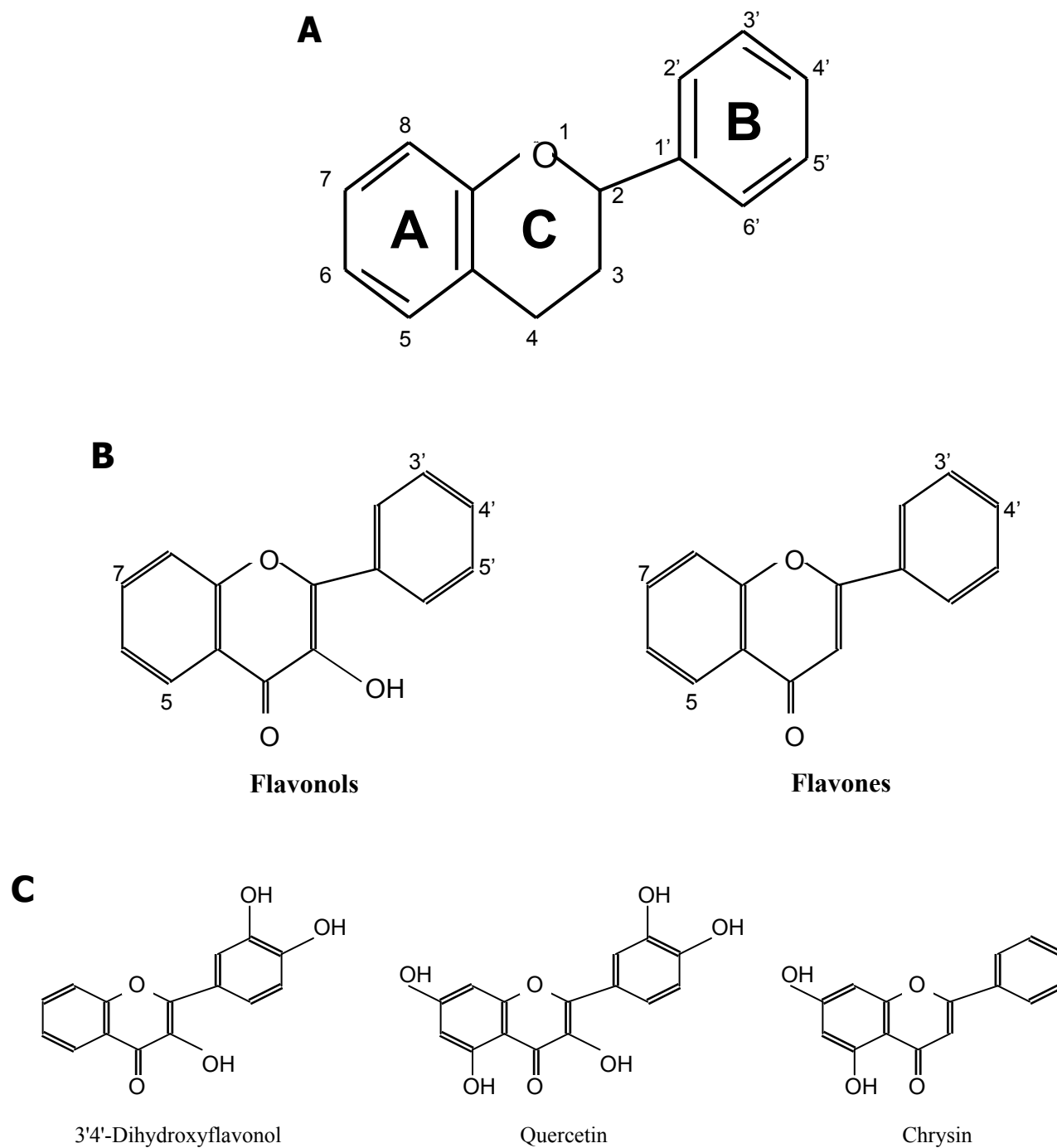


Figure 1. (A) Basic structure of flavonoids; fifteen carbon atoms are arranged in three rings, which are conventionally labelled A, B, and C. (B) General structure of flavonols and flavones. (C) Structure of the compounds being examined in this study.

2. MATERIALS AND METHODS

2.1 Animals

All studies were in accordance with guidelines from the National Health and Medical Research Council, and the Howard Florey Institute Animal Experimentation Ethics Committee (Ethics # 99023). Adult male apoE⁰ mice were purchased from the Animal Resources Centre (Western Australia), and wild-type C57BL/6J mice were supplied by the Howard Florey Institute. Homozygous apoE⁰ mice had >99% genetic similarity to wild-type mice. Animals were maintained on a regular chow diet and water *ad libitum*. The average body weight of the animals used was 33.6±0.5 g.

2.2 Tissue harvesting

After heparinisation (1000 U/kg, ip.) and anaesthetisation by isoflurane inhalation, mice were decapitated. The thoracic aorta was isolated and removed to a Petri dish containing Krebs physiological salt solution (PSS) with the following composition [mM]: NaCl 118.0; KCl 4.7; CaCl₂ 2.5; MgSO₄•7H₂O 1.2; KH₂PO₄ 1.2; NaHCO₃ 25.0; and D-glucose 11.1. Aortas were then cleaned of adipose and periadventitial tissues, and cut into ring segments of 3 mm long.

2.3 Cell culture

Rat aortic smooth muscle cells (RASMCs) used for this study were initially obtained from fresh aorta of Sprague-Dawley rats by enzymatic digestion. RASMC cultures were established and maintained in our laboratory. Cells were grown in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 50 U/mL penicillin, and 50 µg/mL streptomycin, and incubated at 37°C in a humidified atmosphere with 5% CO₂ and 95% O₂. For experimentation, cells were seeded into 6-well plates in 1mL DMEM as described above at a density of 10⁵ cells per well. Experiments were conducted on cells at passages 4 to 12. Confluent cells were quiesced by incubating in the above culture medium containing 0.1% FCS for 24 hrs before use.

2.4 Lucigenin-enhanced chemiluminescence assay

Lucigenin-enhanced chemiluminescence was used to measure the production of O₂^{•-} in both mouse aortic rings and RASMCs. The lucigenin (bis-*N*-methyl acridinium nitrate) cation reacts with O₂^{•-} to form an unstable intermediate which then decomposes, emitting a photon

in the process. Lucigenin was used at 5 μM , a concentration that has been shown to accurately reflect levels of ambient $\text{O}_2^{\bullet-}$ and is not subject to redox cycling and artifactual production of $\text{O}_2^{\bullet-}$ observed with higher concentrations (Li *et al.*, 1998).

Immediately after dissection, tissue rings were transferred to 12-well plates containing 1 mL preincubation solution prepared in Krebs-Hepes buffer (KHB, pH 7.4, composition [mM]: NaCl 98.0; KCl 4.7; CaCl_2 2.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.2; KH_2PO_4 1.2; NaHCO_3 25.0; Na-Hepes 20.0; and D-glucose 11.1), and incubated for 40 mins at 37°C in a humidified atmosphere with 5% CO_2 and 95% O_2 . In some experiments, the preincubation solutions contained diethyldithiocarbamic acid (DETCA, 3 mM), which inactivates endogenous Cu,Zn-SOD. Tissue rings were pretreated with one of the following drugs: DPI (5 μM); allopurinol (100 μM); N^{o} -nitro-L-arginine methyl ester (L-NAME, 200 μM); indomethacin (30 μM); 17-octadecynoic acid (17-ODYA, 10 μM); DHF (100 μM); quercetin (100 μM); chrysin (100 μM), which were added in the preincubation solution. Tissues from the same animal without drug treatment were used as controls. In some experiments, NADPH (100 μM) was added to the preincubation solution to stimulate NADPH oxidase activity. Assay solutions were identical to preincubation solutions, but comprised 5 μM dark-adapted lucigenin and excluded DETCA, which was found by our laboratory to interfere with the chemiluminescence signal. 300 μL assay solution was transferred into each well of a 96-well microplate (Packard), and tissue segments were placed into the appropriate assay solution in the microplate. The microplate was then sealed with a plastic protector sheet and loaded into a Topcount microplate scintillation counter (model 9912, Packard). Chemiluminescence was recorded for 1 min with the counter running in Single-Photon-Count mode. A buffer blank background was subtracted from each respective reading. At the end of the assay, tissues were dried for 48 hours in a 65°C oven to allow normalisation of $\text{O}_2^{\bullet-}$ counts to dry tissue weight (counts/s/mg).

In a subset of experiments, tissue rings were preincubated with either vehicle or apocynin (1 mM) in 6-well plates for 24 hrs in 2 mL DMEM containing 0.1% FCS. The $\text{O}_2^{\bullet-}$ levels were measured as described above.

To measure the $O_2^{\bullet-}$ production in RASMCs, cells were detached by 1 mg/mL collagenase and simultaneously treated with DETCA (3 mM). Cells were transferred into 15-mL tubes and collected by centrifugation (1000 rpm \times 4 mins). The pellet was washed with KHB and centrifuged again, then resuspended in assay solutions comprising 5 μ M lucigenin, NADPH (100 μ M), and the flavonoid compound being tested. 300 μ L cell suspension was transferred into individual wells of a 96-well microplate, and chemiluminescence was recorded as described above. At the end of the assay, cell concentration was counted using a cell counter under microscope to allow normalisation of $O_2^{\bullet-}$ counts to a standard cell concentration (counts/s/million cells/mL). The results were expressed as percentages of control counts.

2.5 Drugs

The following drugs were used: allopurinol, apocynin, chrysin, DETCA, DHF, DPI, indomethacin, L-NAME, NADPH, 17-ODYA, and quercetin. Stock solutions of allopurinol, apocynin, chrysin, DHF, DPI, 17-ODYA, and quercetin were dissolved in dimethylsulfoxide (DMSO). Indomethacin was dissolved in 100% ethanol. All other drugs were dissolved in KHB. DETCA and NADPH were from ICN Pharmaceuticals (Ohio, USA); DHF was from Indofine Chemical (NJ, USA); all other drugs were from Sigma Aldrich (MO, USA). DMEM, FCS, L-glutamine, penicillin and streptomycin were from CSL (Vic, Australia).

2.6 Data analysis

All data are expressed as mean \pm standard error of the mean (SEM). Statistical analysis was performed using SigmaStat (version 2.3). Differences between groups of treatments were assessed by the unpaired Student's *t*-test (for 2 groups) or by one way analysis of variance (ANOVA) followed by the Bonferroni test (for multiple comparisons). pIC_{50} values, which is the negative logarithm molar concentration required to produce 50% inhibition, were calculated by non-linear regression using GraphPad Prism (version 2). A value of $P < 0.05$ was recognised as statistically significant.

3. RESULTS

3.1 Antioxidant efficacies of flavonoids in vascular smooth muscle cells

In RASMCs, $O_2^{\bullet-}$ production could not be detected either at basal conditions or after DETCA treatment. Addition of NADPH in DETCA-treated cells markedly stimulated $O_2^{\bullet-}$ production in these cells. In the following experiments, 100 μ M NADPH was used as a substrate of NADPH oxidase, since this concentration has been found to stimulate about 50% of the maximal enzymatic activity. Also, it has been found by our laboratory that NADPH-stimulated $O_2^{\bullet-}$ production can be blocked by the NADPH oxidase inhibitor DPI, but not affected by the xanthine oxidase inhibitor allopurinol, the NOS inhibitor L-NAME, the cyclooxygenase inhibitor indomethacin, or the cytochrome P450 inhibitor 17-ODYA. Treatment with DHF (0.1-100 μ M) concentration-dependently reduced NADPH-stimulated $O_2^{\bullet-}$ production in RASMCs (Figure 2). The pIC_{50} value of DHF is 5.1 ± 0.1 (n=6). Similarly, quercetin (0.1-100 μ M) also produced concentration-dependent suppression of NADPH-stimulated $O_2^{\bullet-}$ production in RASMCs (Figure 3). The pIC_{50} value of quercetin is 4.8 ± 0.1 (n=4), which is lower than that of DHF, but this difference did not reach a statistical significance ($P=0.07$). In contrast to DHF and quercetin, chrysin at the same concentration range had no effect on NADPH-stimulated $O_2^{\bullet-}$ production in RASMCs (Figure 4).

3.2 Superoxide production in the thoracic aorta of apoE⁰ and wild-type mice

Thoracic aortic rings from both apoE⁰ and wild-type mice produced $O_2^{\bullet-}$ at basal conditions. This ambient $O_2^{\bullet-}$ level in apoE⁰ mouse aorta was significantly higher than that in wild-type mouse aorta (Table 1). Since this basal $O_2^{\bullet-}$ signal as detected by lucigenin-enhanced chemiluminescence assay appeared to be low and variable, the following experiments were carried out in tissue rings that were pretreated with DETCA (3 mM) to inactivate endogenous Cu,Zn-SOD. The level of $O_2^{\bullet-}$ in apoE⁰ mouse aorta was significantly higher than that in wild-type mouse aorta (Table 1). When NADPH (100 μ M) was added to the tissue rings as a substrate of NADPH oxidase, $O_2^{\bullet-}$ production was markedly stimulated. However, there was no significant difference in aortic $O_2^{\bullet-}$ production between the two mouse strains in the presence of NADPH (Table 1).

Table 1. Superoxide production in thoracic aortic rings from apoE⁰ and wild-type mice, as measured by lucigenin-enhanced chemiluminescence. Values (counts/s/mg) represent the mean \pm SEM. **P*<0.05 vs. Wild-type; unpaired Student's *t*-test.

Incubation condition	Mouse strain	
	Wild-type (n=6)	Apo E ⁰ (n=6)
Basal	28 \pm 9	101 \pm 31 *
DETCA	75 \pm 18	199 \pm 51 *
DETCA+NADPH	581 \pm 141	794 \pm 94

3.3 Diphenylene iodonium suppresses superoxide production in apoE⁰ mouse thoracic aorta

DPI (5 μ M), a flavin antagonist which is known to inhibit NADPH oxidase, blocked the production of O₂^{•-} in the thoracic aorta of apoE⁰ mice (Table 2). Similarly, DPI also blocked the O₂^{•-} production stimulated with NADPH (100 μ M) (Table 2). In contrast, 24-hr incubation of tissue rings with apocynin (1 mM) did not affect O₂^{•-} production, either in the absence or presence of NADPH (Table 2).

Table 2. Effects of NADPH oxidase inhibitors on superoxide production in thoracic aortic rings from apoE⁰ mice, as measured by lucigenin-enhanced chemiluminescence. Values (counts/s/mg) represent the mean \pm SEM. **P*<0.05, ***P*<0.005 vs. Control; unpaired Student's *t*-test.

Incubation condition	Tissue treatment			
	Control (n=4)	DPI (n=4)	Control (n=5)	Apocynin (n=5)
DETCA	109 \pm 16	40 \pm 14 *	87 \pm 17	66 \pm 12
DETCA+NADPH	1215 \pm 201	129 \pm 41 **	1557 \pm 347	842 \pm 163

3.4 Enzyme systems besides the NADPH oxidase system do not contribute extensively to superoxide production in apoE⁰ mouse thoracic aorta

To eliminate other potential sources of O₂^{•-} in the thoracic aorta of apoE⁰ mice, the effects of the xanthine oxidase inhibitor allopurinol, the non-specific NOS inhibitor L-NAME, the

cyclooxygenase inhibitor indomethacin, and the cytochrome P450 inhibitor 17-ODYA were examined. None of these inhibitors had any significant effect on $O_2^{\bullet-}$ production either in the absence or presence of NADPH (Figures 5 & 6).

3.5 Effects of flavonoids on the oxidative stress in the thoracic aorta of apoE⁰ mice

Aortic segments were treated with 100 μ M DHF and compared with an untreated tissue segment from the same animal. DHF significantly reduced $O_2^{\bullet-}$ production in tissue rings incubated in the presence of DETCA (3 mM) alone or DETCA plus NADPH (100 μ M) (Figure 7). Quercetin at 100 μ M had similar effects as DHF (Figure 8). The effects of 100 μ M chrysin on aortic segments were also examined. When tissue rings were incubated in the presence of DETCA alone, chrysin significantly decreased $O_2^{\bullet-}$ production (Figure 9). However, when $O_2^{\bullet-}$ production was stimulated with NADPH, chrysin treatment did not change the response as compared to control tissues (Figure 9).

FIGURE 2

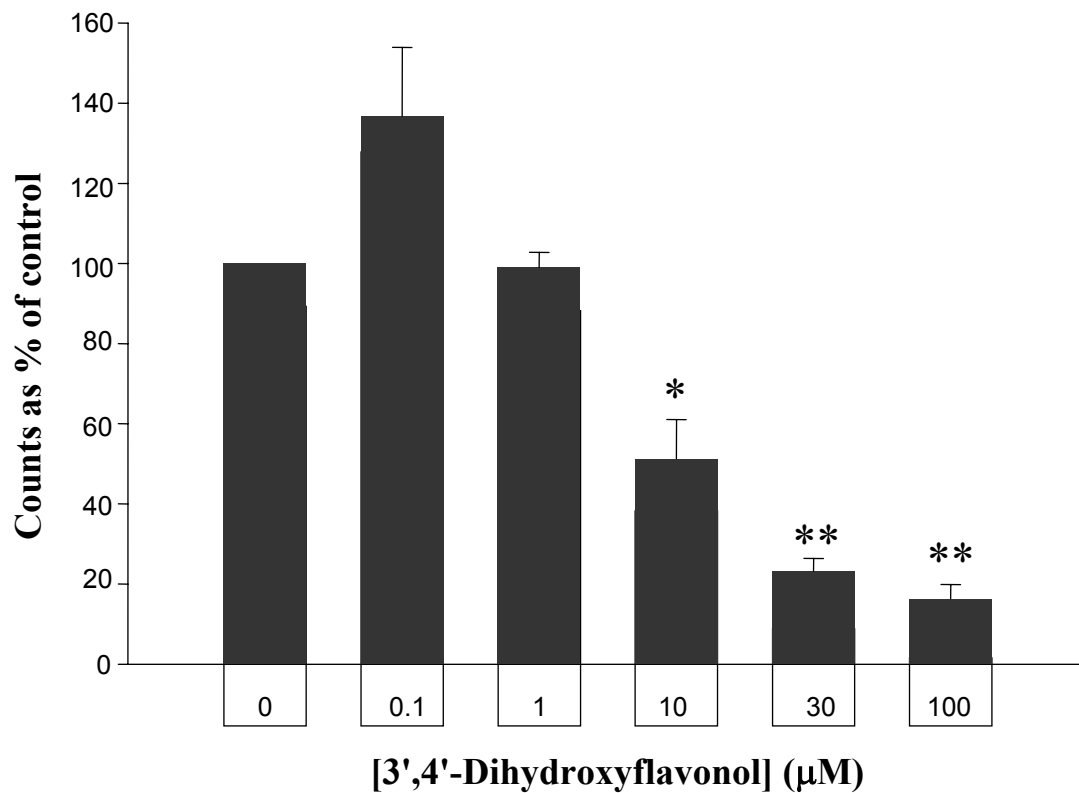


Figure 2. Effects of various concentrations of 3',4'-dihydroxyflavonol on NADPH-dependent O₂^{•-} production in RASMCs preincubated with DETCA (3 mM) and NADPH (100 µM). O₂^{•-} production was measured by lucigenin (5 µM)-enhanced chemiluminescence. Data represent the mean ± SEM from 5 experiments. **P*<0.005, ***P*<0.001 vs. Control; one way ANOVA.

FIGURE 3

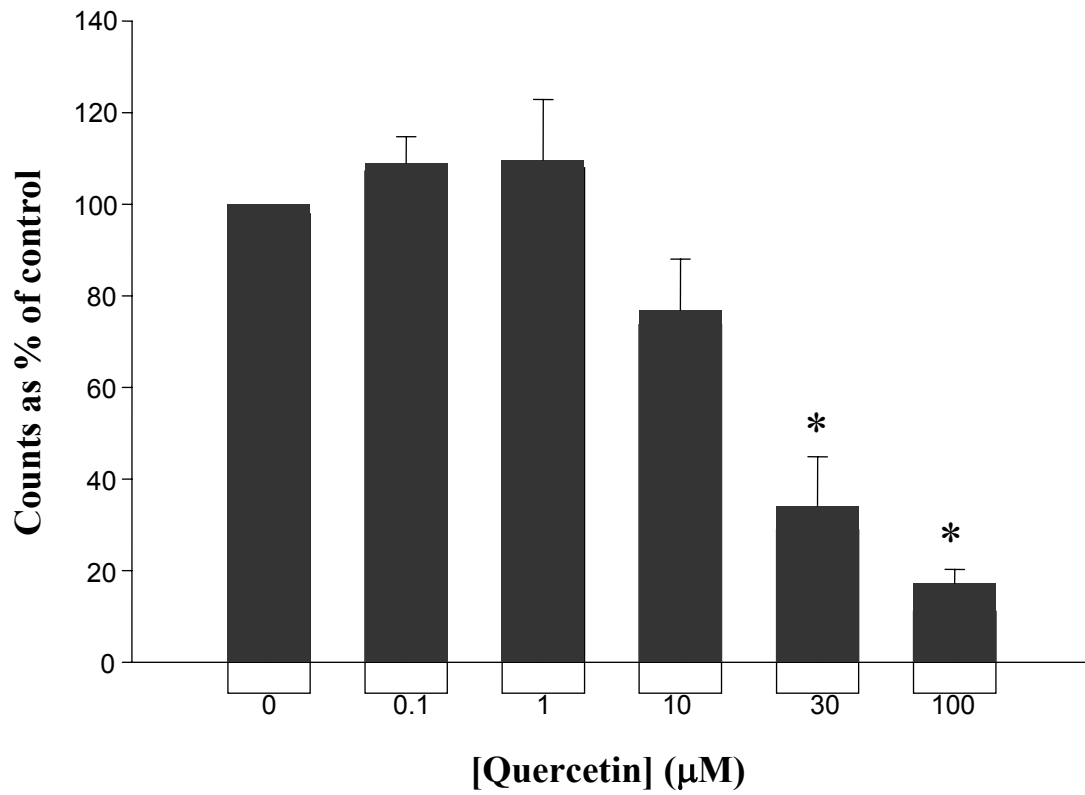


Figure 3. Effects of various concentrations of quercetin on NADPH-dependent O₂^{•-} production in RASMCs preincubated with DETCA (3 mM) and NADPH (100 µM). Data represent the mean ± SEM from 4 experiments. **P*<0.001 vs. Control; one way ANOVA.

FIGURE 4

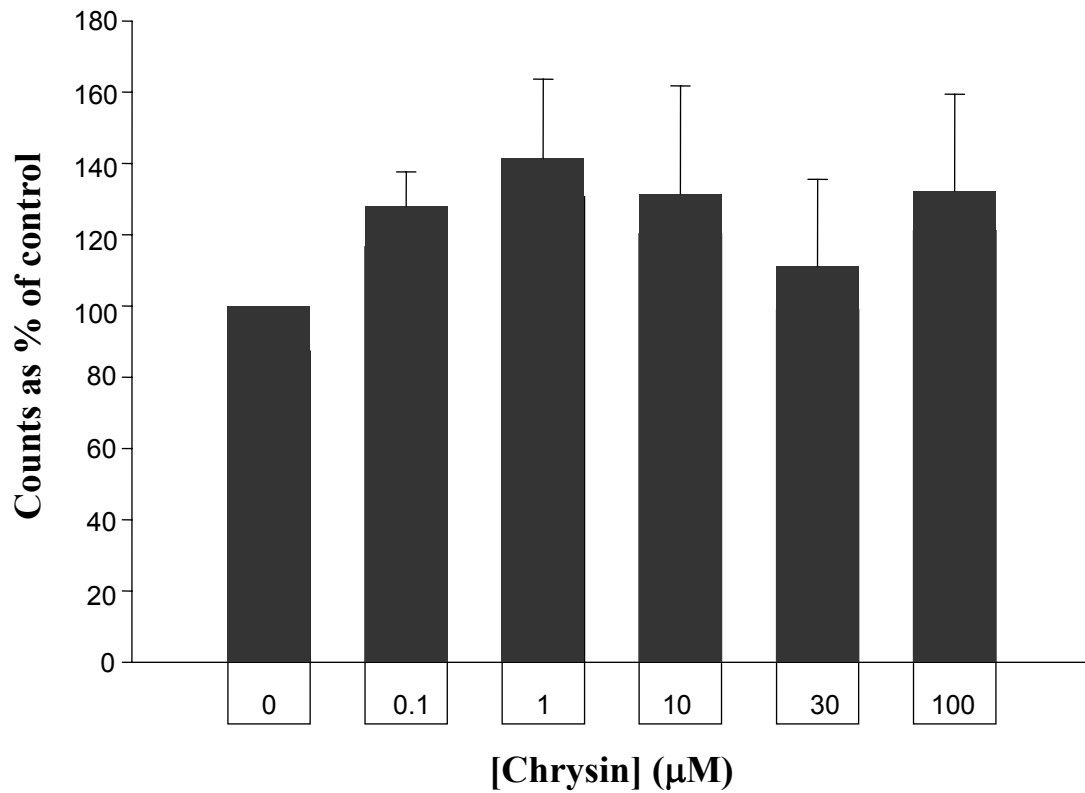


Figure 4. Effects of various concentrations of chrysin on NADPH-dependent O₂^{•-} production in RASMCs preincubated with DETCA (3 mM) and NADPH (100 µM). Data represent the mean ± SEM from 6 experiments. Statistical analysis by one way ANOVA revealed no significant differences in O₂^{•-} production for any of the five concentrations vs. Control.

FIGURE 5

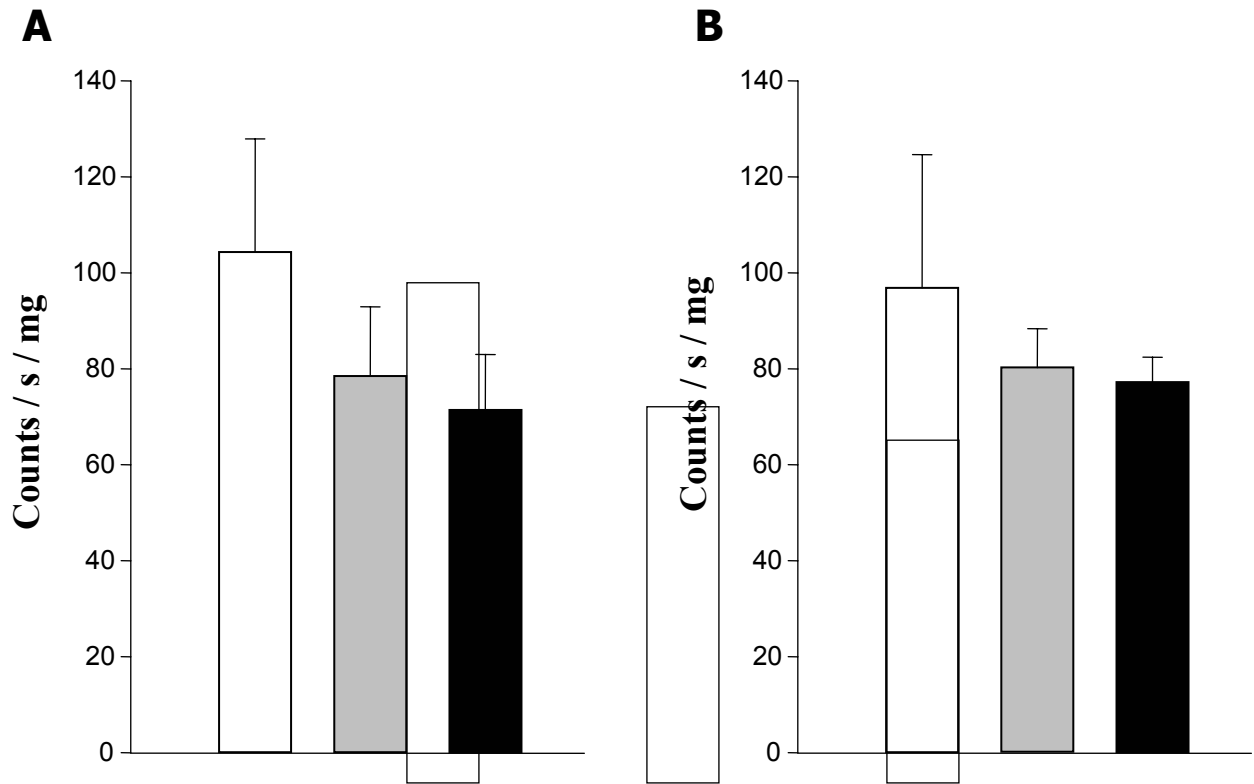


Figure 5. Effects of specific enzyme inhibitors on $O_2^{\bullet-}$ production in thoracic aorta pretreated with DETCA (3 mM) from apoE⁰ mice. Three rings were obtained from a single animal, and were treated with (A) vehicle (□), allopurinol (100 μ M, ▒), or L-NAME (200 μ M, ■) respectively; or (B) vehicle (□), indomethacin (30 μ M, ▒), or 17-ODYA (10 μ M, ■) respectively. Data represent the mean \pm SEM from 4 experiments. Statistical analysis by unpaired Student's *t*-tests of each individual treatment vs. Vehicle revealed no significant differences.

FIGURE 6

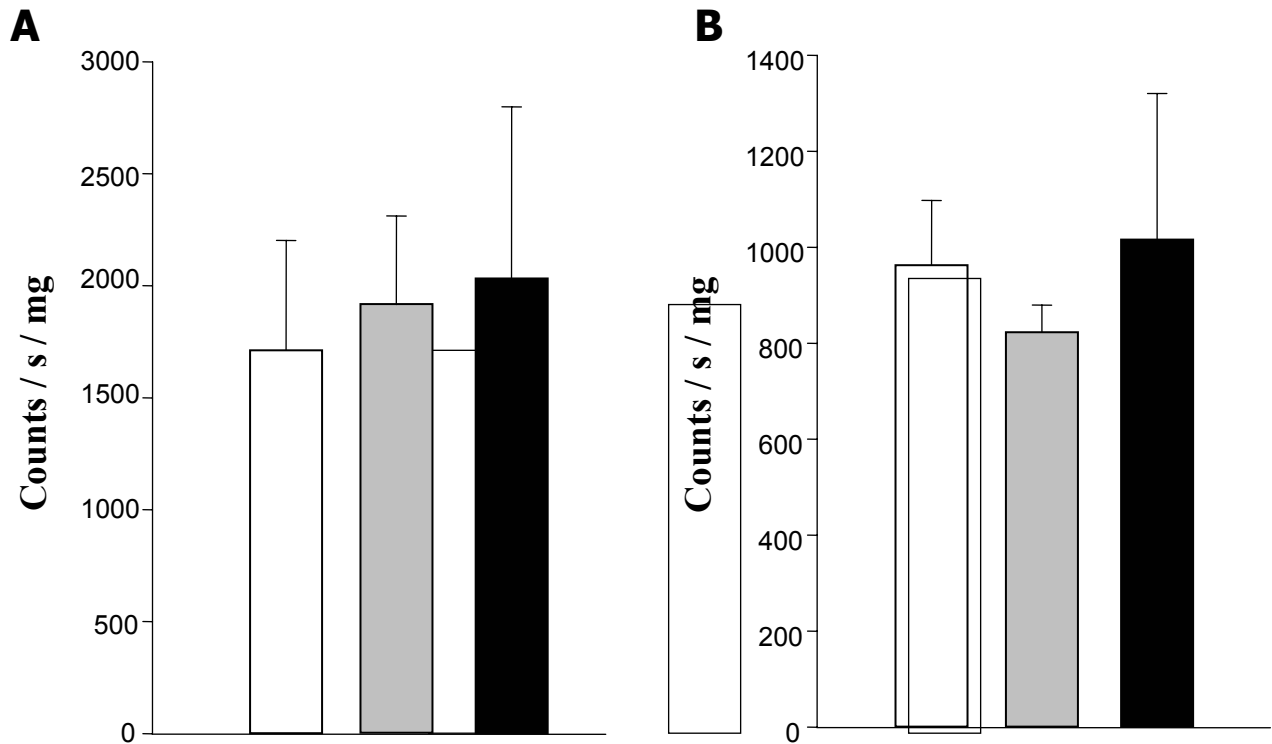


Figure 6. Effects of specific enzyme inhibitors on $O_2^{\bullet-}$ production in thoracic aorta pretreated with DETCA (3 mM) and stimulated with NADPH (100 μ M) from apoE⁰ mice. Three rings were obtained from a single animal, and were treated with (A) vehicle (□), allopurinol (100 μ M, ▒), or L-NAME (200 μ M, ■) respectively; or (B) vehicle (□), indomethacin (30 μ M, ▒), or 17-ODYA (10 μ M, ■) respectively. Data represent the mean \pm SEM from 4 experiments. Statistical analysis by unpaired Student's *t*-tests of each individual treatment *vs.* Vehicle revealed no significant differences.

FIGURE 7

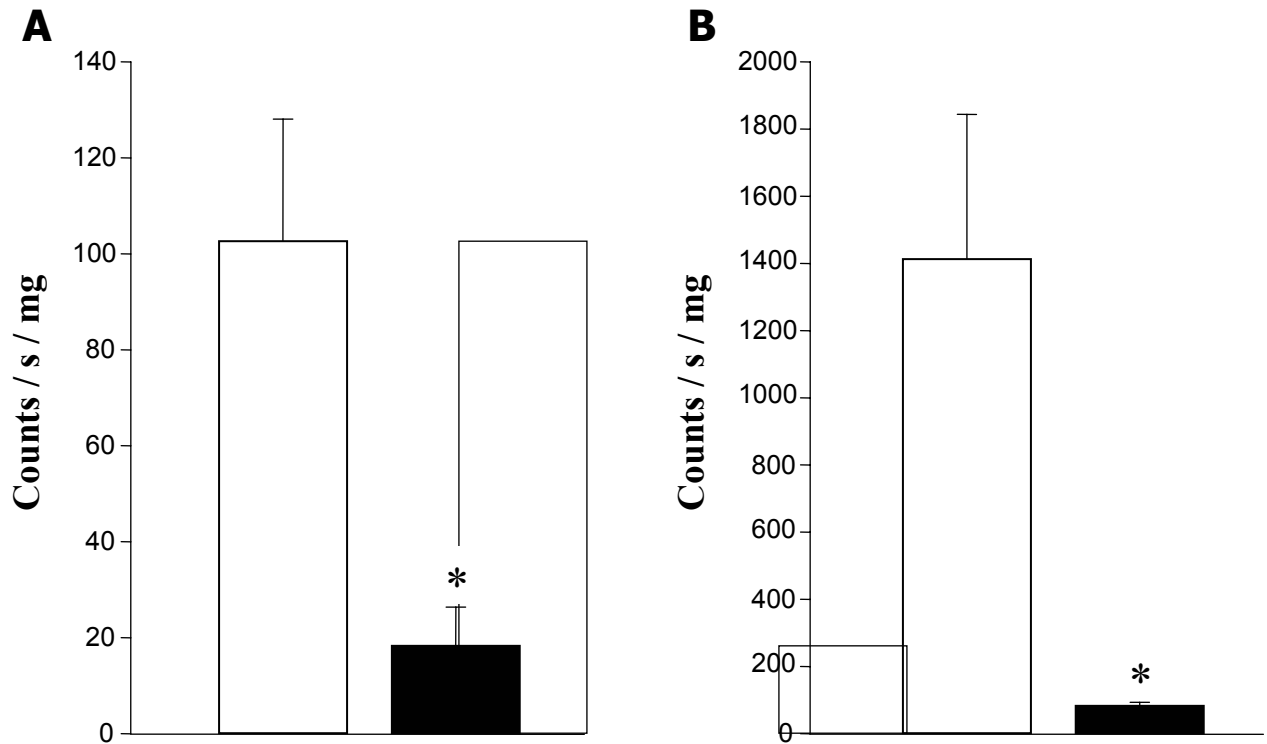


Figure 7. Effects of 3',4'-dihydroxyflavonol on $O_2^{\bullet-}$ production in the absence (A) or presence (B) of NADPH (100 μ M) in thoracic aorta pretreated with DETCA (3 mM) from apoE⁰ mice. Two rings were obtained from a single animal, and were treated with vehicle (□) or 3',4'-dihydroxyflavonol (100 μ M, ■). Data represent the mean \pm SEM from 6 experiments. * P <0.05 vs. Vehicle; unpaired Student's t -test.

FIGURE 8

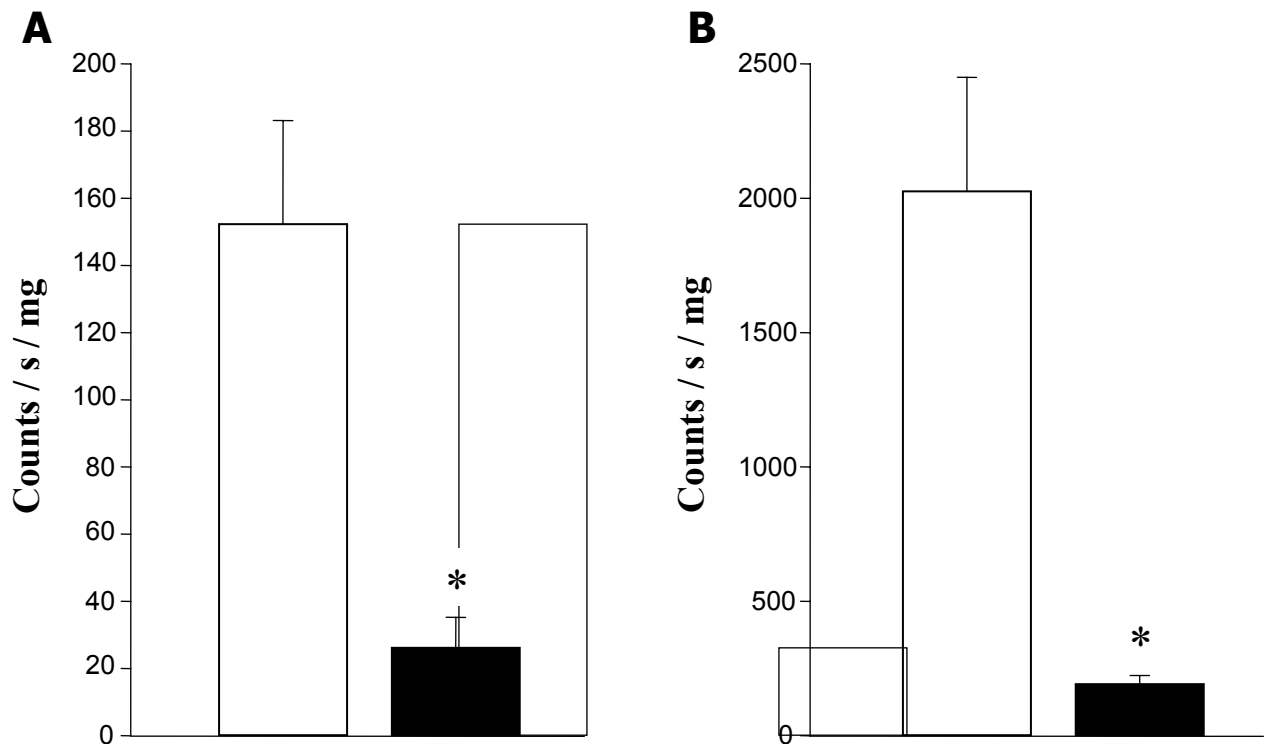


Figure 8. Effects of quercetin on $O_2^{\bullet-}$ production in the absence (A) or presence (B) of NADPH (100 μ M) in thoracic aorta pretreated with DETCA (3 mM) from apoE⁰ mice. Two rings were obtained from a single animal, and were treated with vehicle (□) or quercetin (100 μ M, ■). Data represent the mean \pm SEM from 6 experiments. * P <0.005 vs. Vehicle; unpaired Student's t -test.

FIGURE 9

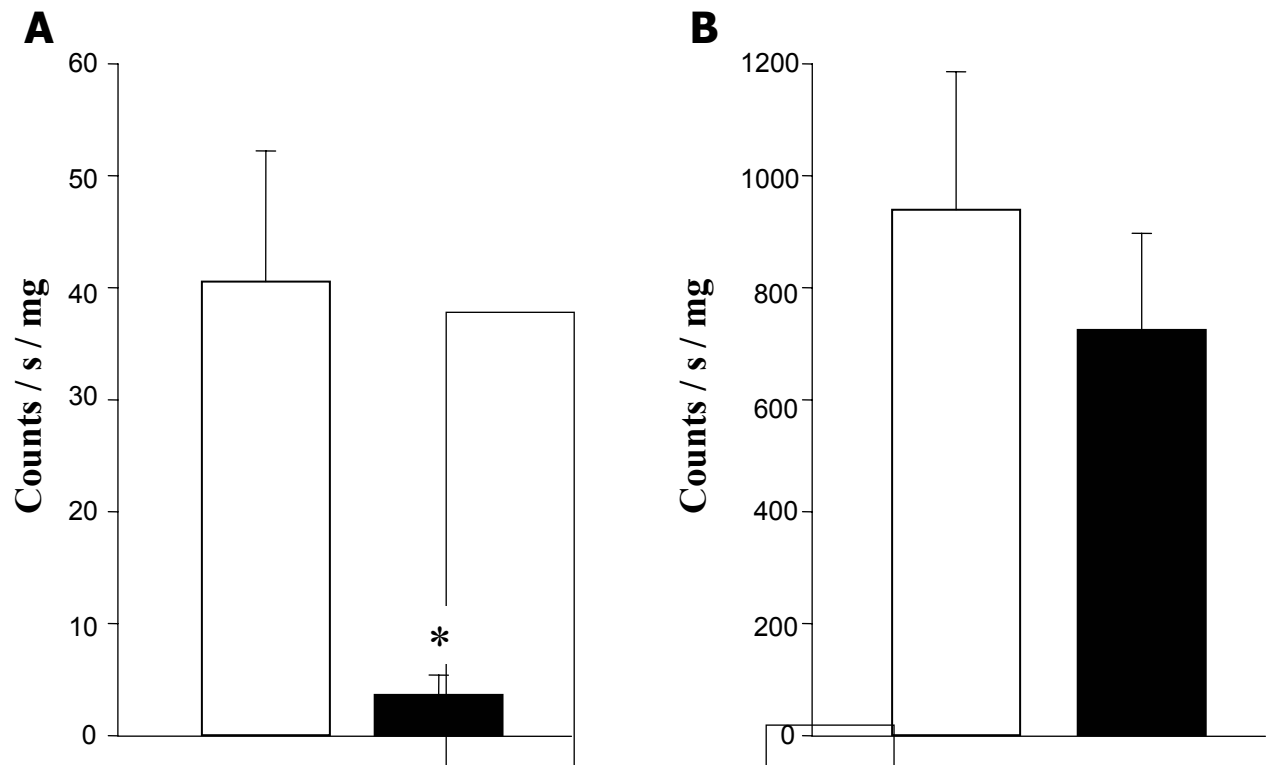


Figure 9. Effects of chrysin on $O_2^{\bullet-}$ production in the absence (A) or presence (B) of NADPH (100 μ M) in thoracic aorta pretreated with DETCA (3 mM) from apoE⁰ mice. Two rings were obtained from a single animal, and were treated with vehicle (□) or chrysin (100 μ M, ■). Data represent the mean \pm SEM from 6 experiments. * P <0.05 vs. Vehicle; unpaired Student's t -test.

4. DISCUSSION

Earlier studies into the effects of flavonoids on $O_2^{\bullet-}$ generation by various cell-free systems, such as the xanthine/xanthine oxidase system and the phenazine methosulfate-NADH system, have demonstrated that compounds of flavonols (see Introduction), such as quercetin, are potent $O_2^{\bullet-}$ scavengers, while some evidence has suggested that they may also have direct inhibitory effects on certain $O_2^{\bullet-}$ producing enzymes (Robak & Gryglewski, 1988). A direct biochemical reaction of flavonols with $O_2^{\bullet-}$ has been reported (Afanas'ev *et al.*, 1989). This reaction is supposed to be attributable to the highly reactive hydroxyl substituents of flavonols, which give these compounds the ability to scavenge $O_2^{\bullet-}$ as well as other free radicals (Korkina & Afanas'ev, 1997). A generalised reaction between flavonoids and $O_2^{\bullet-}$ is given in Equation 1 (de Groot & Rauen, 1998):



While many flavonoid compounds have been shown to have $O_2^{\bullet-}$ scavenging activities, their relative efficacies have not yet been characterised in cardiovascular cells or tissues. This is of particular importance for identifying new cardiovascular therapeutics on the basis of their antioxidant activity, since it has been recognised that flavonoids across different classes, or even within the same class, may have remarkable differences in their antioxidant efficacies and mechanisms of biological actions. This study for the first time investigated the *in vitro* $O_2^{\bullet-}$ scavenging effects of flavonoid compounds in vascular cells and tissues. We found that in RASMCs pretreated with DETCA (3 mM), both DHF and quercetin decreased the $O_2^{\bullet-}$ signal stimulated by NADPH (100 μ M) in a concentration-dependent manner. At the highest concentration used in this study, *ie.* 100 μ M, both compounds inhibited NADPH-driven $O_2^{\bullet-}$ production by about 85%. As mentioned in Introduction, it has been demonstrated that these $O_2^{\bullet-}$ anions are largely generated by NADPH oxidase. Therefore, taken together, these results suggest that DHF and quercetin may physiologically (or functionally) antagonise the activity of smooth muscle NADPH oxidase, which may have a pivotal role in the pathogenesis of increased oxidative stress in the vascular wall found in various cardiovascular diseases (d'Uscio *et al.*, 2001; Rajagopalan *et al.*, 1996).

Our findings confirmed earlier notions for the importance of the C-3 hydroxyl group in ring C and the catechol substitution pattern in ring B for a high $O_2^{\bullet-}$ scavenging activity, since these features are shared by DHF and quercetin. Of the two compounds, the synthetic flavonoid DHF was found to be slightly more potent than the natural flavonoid quercetin (a pIC_{50} value of 5.1 for DHF vs. 4.8 for quercetin), although the difference is marginal ($P=0.07$). The pIC_{50} value obtained for quercetin in this study is also relevant to a pIC_{50} value of ~ 5.8 reported by other authors who used the nitrite method to examine the ability of quercetin to suppress $O_2^{\bullet-}$ generation in a cell-free xanthine/xanthine oxidase system (Cos *et al.*, 1998). The difference between the two pIC_{50} values may be due to an additional direct inhibitory effect of quercetin on xanthine oxidase (Selloum *et al.*, 2001; Moini *et al.*, 2000). Previous studies have shown that the presence of a 5,7-metadihydroxy arrangement in ring A appeared to be essential for antioxidant activity in flavonoids lacking other obvious antioxidant moieties (Rice-Evans *et al.*, 1996). However, our findings indicate that the 5,7-metadihydroxy arrangement in ring A (as in quercetin) does not enhance the $O_2^{\bullet-}$ scavenging activity of quercetin as compared to DHF.

We found that chrysin had no effect on the $O_2^{\bullet-}$ production in NADPH-stimulated RASMCs at the same concentration range (0.1-100 μ M) as DHF and quercetin, suggesting that chrysin, in contrast to DHF and quercetin, has little physiological antagonistic activity against the NADPH oxidase in smooth muscle cells. The reason for the contrast between chrysin and quercetin is not totally clear. It may be due to the structural difference between these compounds in that chrysin lacks a C-3 hydroxyl group in ring C and a catechol moiety in ring B. This further highlights the significance of these two features for the antioxidant activity of flavonoids. Evidence has suggested that one compound lacking a redox-active moiety in ring B might be compensated for the antioxidant activity by substituents in ring A (Arora *et al.*, 1998), but we did not observe this phenomenon in chrysin, in relation to $O_2^{\bullet-}$ scavenging. On the other hand, it has previously been found that a 2,3-double bond in conjugation with a 4-oxo function in ring C, of which a structure exists in chrysin, is essential for radical scavenging properties of flavonoid compounds (Bors *et al.*, 1990). Also, it has been reported that chrysin strongly reduced the phorbol ester-induced $O_2^{\bullet-}$ formation in

smooth muscle cells (Hecker *et al.*, 1996). Together with our finding that chrysin significantly decreased the $O_2^{\bullet-}$ production without NADPH stimulation in aortic tissues from apoE⁰ mice, a $O_2^{\bullet-}$ scavenging property of chrysin cannot be completely ruled out. Although the reasons for these discrepancies about the antioxidant efficacy of chrysin are unclear, these variable results indicate that chrysin, and maybe closely related compounds, are unlikely to be effective antioxidant agents with therapeutic values. Nevertheless, the present results suggest that the antioxidant property of flavonoids is compound-specific, and individual compounds differ in their potencies. Designing new flavonoids with higher and more specific antioxidant activity with minimal toxicity may represent a new approach for identifying cardiovascular therapeutics.

It is well established that vascular diseases are accompanied by increased $O_2^{\bullet-}$ production in the blood vessel wall. Using lucigenin-enhanced chemiluminescence, we observed that the ambient $O_2^{\bullet-}$ level in thoracic aortas from apoE⁰ mice was about 3 fold higher than that in aortas from wild-type control mice. This is consistent with the findings reported by others (Laursen *et al.*, 2001; d'Uscio *et al.*, 2001). The increased $O_2^{\bullet-}$ level could be due to an impairment of the endogenous free radical scavenging mechanisms, such as SOD and glutathione, for evidence has shown that the vascular SOD activity can be substantially altered in pathophysiological states (Fukai *et al.*, 1998; Sharma *et al.*, 1992; Del Boccio *et al.*, 1990). However, our results did not support this hypothesis, because SOD inhibitor DETCA similarly increased the $O_2^{\bullet-}$ level in aortas from both mouse strains, and the difference in $O_2^{\bullet-}$ production between the two strains was preserved after DETCA treatment, suggesting that the increase of *de novo* generation of $O_2^{\bullet-}$, but not the decrease of its removal, is responsible for the increased vascular oxidative stress in apoE⁰ mice. This finding is in line with a previous study which found that the protein expression of Cu,Zn-SOD was not different between the two mouse strains (d'Uscio *et al.*, 2001).

We characterised the enzymatic source of $O_2^{\bullet-}$ in apoE⁰ mouse aorta, by using specific pharmacological inhibitors. Firstly, the NADPH oxidase inhibitor DPI caused 63% and 89% reductions in $O_2^{\bullet-}$ production in the absence and presence of NADPH respectively. DPI

disrupts electron transfer through the flavin-containing subunit gp91^{phox} (or its analogous nox1 or nox4). The attenuation of O₂^{•-} production by DPI under both conditions indicates that NADPH oxidase appears to be a significant source of O₂^{•-} in mouse aorta. Similar observations were also made in rats (Rajagopalan *et al.*, 1996) and rabbits (Pagano *et al.*, 1995). Secondly, aortic O₂^{•-} production either in the absence or presence of NADPH was not altered by the xanthine oxidase inhibitor allopurinol, the NOS inhibitor L-NAME, the cyclooxygenase inhibitor indomethacin, or the cytochrome P450 inhibitor 17-ODYA, suggesting that these enzymes do not have a significant role in mouse vascular O₂^{•-} production. This is also supported by previous studies (Laursen *et al.*, 2001). It is noted that the effect of another NADPH oxidase inhibitor, apocynin, was variable. Apocynin acts by preventing the assembly of NADPH oxidase by interfering with the translocation of the p47^{phox} and p67^{phox} cytosolic subunits (Stolk *et al.*, 1994), while it has little impact on enzymes that are already assembled. In vascular tissues, the half-life of each subunit of NADPH oxidase has not been determined, and the rate of enzyme turnover is unknown. Degradation of existing NADPH oxidase complexes must be allowed to occur before the effects of apocynin become readily detectable. Therefore, the variable effects of apocynin observed in this study might be attributable to the dynamics of the turnover of NADPH oxidase subunits.

It should be noted that the role of xanthine oxidase in the aorta of apoE⁰ mice is uncertain, while some evidence has shown that xanthine oxidase might be a significant source of O₂^{•-} in hypertensive rats (Ohara *et al.*, 1993) and hypercholesterolemic humans (Cardillo *et al.*, 1997). Some authors reported that xanthine oxidase may exist in a form which uses NADH as a substrate and this activity is not inhibited by classical xanthine oxidase inhibitors (Cai & Harrison, 2000). Therefore, if this is true in mouse aorta, our result of the ineffectiveness of allopurinol cannot totally exclude an involvement of xanthine oxidase in the generation of O₂^{•-} radicals.

NADPH, acting as a substrate of NADPH oxidase, stimulates vascular O₂^{•-} production in the aorta of both apoE⁰ and wild-type mice. The two mouse strains were found to produce similar levels of O₂^{•-} after stimulation of aortas with NADPH, suggesting that there was not a

notable difference of the enzymatic activity driven by its substrate. This is somewhat paradoxical to the finding that the resting $O_2^{\bullet-}$ production is higher in apoE⁰ mouse aorta, and that the mRNA expression of two subunits of NADPH oxidase, gp91^{phox} and p47^{phox}, is elevated in apoE⁰ mouse aorta (Grant Drummond, personal communication). The reason for this discrepancy is unknown. Some possible explanations include that firstly, $O_2^{\bullet-}$ generation with different substrate availabilities might involve divergent enzymological mechanisms in mouse aorta; and secondly, there might be a difference of the accessibility of this enzyme to its substrate in physiological and pathophysiological situations.

DHF and quercetin similarly suppressed $O_2^{\bullet-}$ production in aortic segments from apoE⁰ mice by 80% and 90% in the absence and presence of NADPH respectively. This is consistent with the finding in RASMCs that both flavonols can physiologically antagonise the vascular NADPH oxidase activity. When NADPH stimulation was absent, treatment of apoE⁰ mouse aorta with either DHF or quercetin markedly decreased the $O_2^{\bullet-}$ production to a level even lower than that in untreated wild-type mouse aorta, indicating that these compounds can effectively reduce the oxidative stress in diseased vessels. Furthermore, the effects of DHF and quercetin virtually negated the stimulatory effect of NADPH, returning $O_2^{\bullet-}$ production to the level that was found in the absence of NADPH. Similar to the observations in RASMCs, chrysin had no effect on the NADPH-stimulated $O_2^{\bullet-}$ production in apoE⁰ mouse aorta, suggesting that this compound has minor effect on substrate-driven NADPH oxidase activity. However, chrysin significantly blocked $O_2^{\bullet-}$ production at basal conditions. Although the mechanism of this phenomenon is unclear, it appears to be in agreement with the notion that different enzymological mechanisms might be involved in $O_2^{\bullet-}$ generation under different substrate concentrations.

It is not entirely clear why the activity of NADPH oxidase is increased in disease states. Substantial evidence has suggested that the increased action and/or expression of this $O_2^{\bullet-}$ generating enzyme in disease states may be the consequence of the upregulatory effects of a combination of proatherogenic factors, such as angiotensin II (Ang II) and oxidized LDL (Weiss *et al.*, 2001; Schmidt-Ott *et al.*, 2000; Rueckschloss *et al.*, 2001). Given that NADPH

oxidase is a most significant source of $O_2^{\bullet-}$ in the vascular wall, an objective of future studies would be to investigate other mechanisms by which flavonoids interact with the NADPH oxidase system. For instance, it has been found that flavonols have the ability to directly inhibit neutrophil NADPH oxidase activity, as measured by oxygen consumption (Tauber *et al.*, 1984), therefore the potential ability of flavonoids to directly inhibit the activity of NADPH oxidase in vasculature warrants research. A flavonoid compound with the dual actions of NADPH oxidase inhibition and $O_2^{\bullet-}$ scavenging is likely to have higher antioxidant activity than a compound that only scavenges $O_2^{\bullet-}$. Another potential mechanism of action of flavonoids which needs further investigation is the ability to regulate the expression of NADPH oxidase subunits. For example, Ang II has been shown to upregulate NADPH oxidase expression (Mollnau *et al.*, 2002, Cifuentes *et al.*, 2000), and Ang II signaling is, at least in part, dependent on intracellular ROS molecules (Takahashi *et al.*, 2000). Thus, it is possible that flavonoids might block the upregulation of NADPH oxidase induced by Ang II. We performed some preliminary studies to examine the protein expression of two NADPH oxidase subunits, namely p47^{phox} and p67^{phox}, in RASMCs treated with Ang II alone or Ang II plus quercetin. However, these analyses have not yet yielded any conclusive evidence (results not shown). One limitation of this study was that we did not establish a direct link between the anti-radical effects of flavonoids and their cardiovascular protective actions, although some evidence has shown that quercetin may prevent the development of atherosclerosis in apoE⁰ mice (Hayek *et al.*, 1997).

In conclusion, this study demonstrates that flavonoids can physiologically antagonise vascular NADPH oxidase activity, which is primarily responsible for the enhanced vascular oxidative stress in pathological states. Both the natural flavonol quercetin and the synthetic flavonol DHF effectively decreased the oxidative stress in aortic tissues from apoE⁰ mice. Given the overwhelming evidence of the deleterious role of $O_2^{\bullet-}$ in vascular disease, further structural modification of flavonoid compounds to improve their antioxidant potential is likely to be a fruitful approach to discover therapeutic interventions.

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