

# Potential Therapeutic Effects of Natural Heme Oxygenase-1 Inducers in Cardiovascular Diseases

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## Abstract

**Significance:** Many physiological effects of natural antioxidants, their extracts or their major active components, have been reported in recent decades. Most of these compounds are characterized by a phenolic structure, similar to that of  $\alpha$ -tocopherol, and present antioxidant properties that have been demonstrated both *in vitro* and *in vivo*. Polyphenols may increase the capacity of endogenous antioxidant defenses and modulate the cellular redox state. Such effects may have wide-ranging consequences for cellular growth and differentiation. **Critical Issues:** The majority of *in vitro* and *in vivo* studies conducted so far have attributed the protective effect of bioactive polyphenols to their chemical reactivity toward free radicals and their capacity to prevent the oxidation of important intracellular components. One possible protective molecular mechanism of polyphenols is nuclear factor erythroid 2-related factor (Nrf2) activation, which in turn regulates a number of detoxification enzymes. **Recent Advances:** Among the latter, the heme oxygenase-1 (HO-1) pathway is likely to contribute to the established and powerful antioxidant/anti-inflammatory properties of polyphenols. In this context, it is interesting to note that induction of HO-1 expression by means of natural compounds contributes to prevention of cardiovascular diseases in various experimental models. **Future Directions:** The focus of this review is on the role of natural HO-1 inducers as a potential therapeutic strategy to protect the cardiovascular system against various stressors in several pathological conditions. *Antioxid. Redox Signal.* 18, 000–000.

## Introduction

**O**XIDATIVE STRESS, the result of an imbalance between antioxidants and pro-oxidants (121), is associated with the aging process as well as over 100 human diseases (122). Under physiological conditions, cells maintain the redox balance through generation and elimination of reactive oxygen species (ROS) by scavenging free radicals and up-regulating antioxidant enzymes. At low levels, ROS act as signaling molecules to promote cell survival, while accelerated production of ROS without concomitant increases in antioxidant enzyme capacity can induce damage and cause cell death (98). Cancer (70), diabetes (107), cardiovascular

diseases (CVD) (34), pulmonary diseases (78), and neurodegenerative diseases, including Alzheimer's and Parkinson's disease (21), have all been associated with increased ROS, demonstrating the role of oxidative stress in a wide array of pathological processes.

In an effort to counteract the detrimental effects of oxidative stress, investigators have studied antioxidant supplements, including Vitamins C and E and  $\beta$ -carotene. Recent clinical trials have been equivocal, with antioxidant vitamins failing to improve markers of oxidative disease (120), and in some cases, even increasing pro-oxidant concentrations (151). Current research efforts have subsequently turned to novel compounds that increase endogenous antioxidant enzyme

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activity, providing the potential for more profound antioxidant protection than that is achieved with supplemental antioxidant vitamins.

Phytochemicals have recently been suggested to be compounds capable of increasing endogenous antioxidants (74, 85, 100) such as heme oxygenase (HO). This is the first and rate-limiting enzyme in the catabolism of heme (85) to yield equimolar amounts of biliverdin, carbon monoxide (CO), and free iron (Fig. 1). To date, two isoforms of HO, designated HO-1 and HO-2, have been identified in mammals (93). HO-1 is also known as heat shock protein-32. Its human form is composed of 288 amino acids with a molecular mass of 32,800 Da and shares about 80% amino acid sequence identity with rat HO-1. On the other hand, human HO-2 is a 36-kDa protein that consists of 316 amino acids with three cysteine residues. HO-1 is highly inducible by hemin and other chemical and physical agents such as ultraviolet rays, hydrogen peroxides, heavy metals, hypoxia, and nitric oxide (NO) (93). HO-1 has been shown to exert cytoprotective properties in various cells, including neurons (88), pancreatic  $\beta$ -cells (51), and cardiomyocytes (138). Under conditions of oxidative stress, hypoxia or hyperthermia, the induction of HO-1 would account for the majority of heme breakdown, leading to the formation of biliverdin and CO. Since HO-1 is induced as a protective mechanism in response to various stimuli, targeted induction of this stress-response enzyme may be considered an important therapeutic strategy for protection against inflammatory processes and oxidative tissue damage. Several original articles and reviews have been published so far regarding the putative role of HO-1 in CVD (13, 33, 39, 73). However, most deal with HO-1 inducers, which are far from being used in everyday clinical

practice, such as CoPP, Hemin,  $\text{SnCl}_2$ , L4F, and adeno- or retroviral vectors (Table 1). Therefore, the present review will focus on the effects of natural antioxidants, which are commercially available and ready to be used in a clinical setting (*i.e.*, supplements).

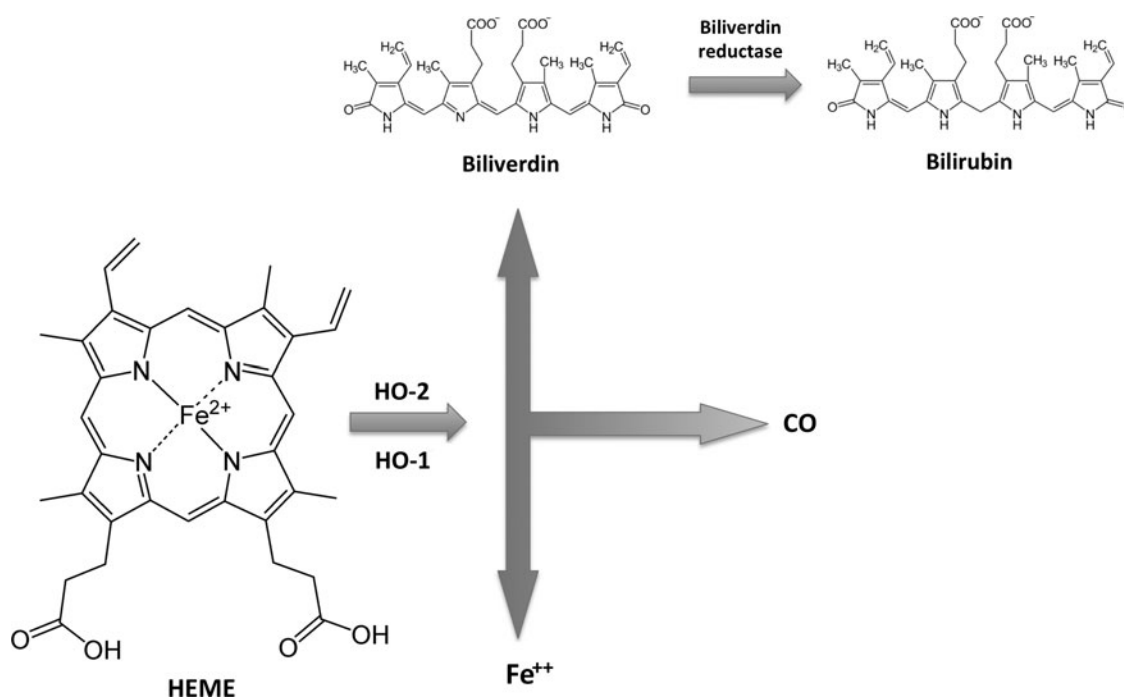
In this review, recent findings on the implications of HO-1 induction in cellular adaptive cytoprotective response to various insults and inflammatory conditions are considered, with particular emphasis placed on targeted HO-1 induction by natural compounds and their potential for cardiovascular protection.

## CVD and Antioxidants

### CVD and oxidative stress

CVD affect more than 80 million people in the United States and are the leading cause of death and disability in the Western World (149). Recent studies have implicated increased production of ROS in the initiation and progression of CVD (2), specifically in the etiology of hypertension (46), congestive heart failure (131), and stroke (21). These studies suggest an important role for ROS in the development of CVD, and highlight the need for therapeutic methods to counteract the changes in the redox status observed in patients with developing heart disease.

Several antioxidant compounds have been tested for prevention of CVD. These antioxidant compounds include probucol, coenzyme Q-10, Vitamin C, Vitamin E, *N*-acetylcysteine, superoxide dismutase (SOD) mimetics, as well as red-wine polyphenols (49). Administration of some exogenous antioxidant compounds has been used for preventive and/or therapeutic intervention in oxidative cardiovascular disorders in



**FIG. 1. Schematic representation of the heme-degradative pathway.** HO-1/HO-2 degrades heme, which is oxidatively cleaved at the methylene bridge to produce equimolar amounts of CO, biliverdin, and iron. Biliverdin is converted to bilirubin in a stereospecific manner by the cytosolic enzyme, biliverdin reductase. Both CO and bilirubin are bioactive molecules, and the iron generated by HO-1 and HO-2 is immediately sequestered by associated increases in ferritin. CO, carbon monoxide; HO-1, heme oxygenase-1.

TABLE 1. COMMONLY USED HEME OXYGENASE-1 INDUCERS

Name of inducer	Tested models	References
Hemin	Hypertensive rat model, VSMC, cardiac ischemia and reperfusion, endothelial cells, animal model of vascular thrombosis.	(4, 30, 55, 58, 143, 148)
CoPP	SHR, cardiomyocyte, endothelial cells, cardiac ischemia and reperfusion.	(15, 36, 61, 62)
Hemearginate	Mineralocorticoid-induced hypertensive rats, SHR, vascular endothelial cell.	(63, 83, 99)
SnCl <sub>2</sub>	SHR	(35, 117)
L-4F	Arterioles isolated from hypercholesterolemic Ldlr <sup>-/-</sup> mice, endothelial cells, cardiac ischemia/reperfusion.	(101, 102, 136)
Adeno/retro/ lentivirus	MSC-treated hearts, rat aortic transplant model, vascular endothelial cells.	(14, 32, 77, 146, 152)

SHR, spontaneously hypertensive rats; VSMCs, vascular smooth muscle cells; MSC, mesenchymal stem cell.

animal models (60). Another strategy for protecting against oxidative cardiac injury may be *via* chemically mediated up-regulation of endogenous antioxidants and Phase II enzymes in the cardiac tissue. Such a strategy relies on a profound understanding of the chemical inducibility of cardiac antioxidant and Phase II enzymes, as well as the underlying signaling mechanisms (155). In general, the antioxidant defense mechanism includes enzymes such as SOD, which removes superoxide; glutathione peroxidase (GPx), which converts hydrogen peroxide into water and various hydroperoxides into less-harmful hydroxides; catalase (CAT), which can also break down hydrogen peroxide; and HO. Phenolic acids are a group of phenolic compounds that are widely distributed in foodstuffs, mostly in whole grains, fruits, vegetables, and beverages. Epidemiological studies have suggested an association between the consumption of phenolic acid-rich foods or beverages and the prevention of many diseases (130). These phenolic compounds exhibit good *in vitro* antioxidant and chemoprotective properties, which may have beneficial effects *in vivo* (24).

Several mechanisms have been suggested to explain a direct or indirect action of antioxidants.

**Direct antioxidants.** Antioxidants, defined as any substance that decreases the severity of oxidative stress by forming less-active radicals or by quenching damage created by free-radical chain reactions, broadly include any substances that delay or prevent the oxidation of a substrate (72). Antioxidant effects of a compound may act by two mechanisms: the compound itself may exhibit direct antioxidant effects through scavenging ROS or inhibiting their formation, or the compound may indirectly upregulate endogenous antioxidant defenses. Direct exogenous antioxidants include Vitamin C, which reacts stoichiometrically with ROS to scavenge aqueous-state free radicals,  $\beta$ -carotene, and Vitamin E, a membrane-bound antioxidant scavenger.

Although supplementation of direct antioxidants is a highly researched topic, the compounds are still only presumed effective (1). Studies of supplementation with a single antioxidant vitamin have shown that this intervention either has no effect or results in increased levels of all-cause mortality (1).

**Indirect antioxidants.** As a result of the apparent ineffectiveness of supplemental antioxidant vitamins in decreasing oxidative stress, recent research has focused on novel ways to induce an endogenous antioxidant response. Current research

efforts have turned to compounds that can be used to increase endogenous antioxidant enzyme activity, providing the potential for more profound antioxidant protection than the traditional approach of antioxidant vitamin supplementation. Phytochemicals, chemical compounds derived from plants, have been examined as a class of these novel inducers of antioxidant enzymes. Also described as indirect antioxidants due to their role in activating Phase II cytoprotective enzymes, phytochemicals stimulate a battery of antioxidant responses in addition to directly scavenging ROS (Fig. 2). Indirect antioxidant compounds act catalytically and are therefore not consumed in the reaction. Unlike direct antioxidants, they have long half-lives, and are unlikely to evoke pro-oxidant effects (132), suggesting the ability to promote a response to oxidative stress, which is both more efficient and longer lasting.

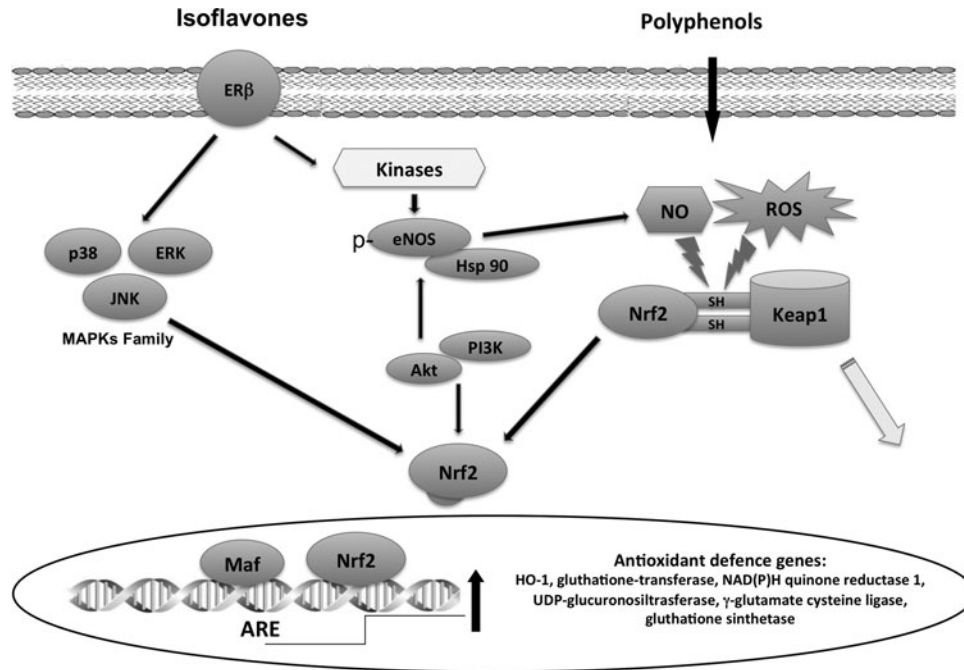
Additionally, studies on polyphenols support the ability of these compounds to activate the nuclear factor erythroid 2-related factor (Nrf2) (7), a critical step in the induction of antioxidant-response mechanisms. By coordinating the expression of cytoprotective proteins, indirect antioxidants provide the potential for greater and more profound upregulation of antioxidant properties and cell protection.

Induction of these cytoprotective proteins is regulated at transcriptional level and is mediated by a specific enhancer, the antioxidant-response element (ARE), found in the promoter of the enzyme's gene.

**The ARE.** The first experimental evidence for the existence of ARE was found in the late 1980's. Indeed, during studies of xenobiotic metabolism, a group of compounds was found to induce Phase I and II xenobiotic metabolizing enzymes (Fig. 2). Many natural and synthetic phenols and thiol-containing compounds can increase transcription of the genes regulated by the ARE, as well as heavy metal atoms, thiol-containing compounds, hydroperoxides, and heme complexes. Although all activators differ structurally, they all share the property of electrophilicity (42).

Located in the 5'-flanking regulatory region of Phase II target antioxidant genes, the *cis*-acting ARE is a DNA site containing the nucleotide sequence 5'-AGTGACTnnnGCAG-3' (38). This site binds nuclear transcription factor Nrf2, resulting in transcription in a number of xenobiotic and antioxidant enzymes (Fig. 2).

**Nrf2: the master regulator of the antioxidant cellular defense system.** Nrf2 is a member of the basic leucine-zipper (bZip) transcription factor family (128). Under normal



**FIG. 2. Transcriptional activation of antioxidant genes by phytochemicals via the Nrf2-Keap1 pathway.** Isoflavones and other polyphenols activate intracellular kinase cascades, leading to acute activation of eNOS, MAPKs, and NO and/or ROS generation. Increased NO, ROS will modify cysteine residues on Keap1 leading to nuclear translocation of the redox-sensitive transcription factor Nrf2. After translocation, Nrf2 forms a heterodimer with Maf and Jun bZip transcription factors, which bind to the ARE and induce transcription of Phase II antioxidant enzymes and HO-1. ARE, antioxidant-response element; bZip, basic leucine zipper; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal kinase; Keap1, Kelch-like ECH-associated protein 1; MAPKs, mitogen-activated protein kinases; NO, nitric oxide; Nrf2, nuclear factor erythroid 2-related factor; PI-3K, phosphatidylinositol-3 kinase; ROS, reactive oxygen species.

conditions, Nrf2 is sequestered in the cytoplasm by its involvement in an inactive complex with Kelch-like ECH-associated protein 1 (Keap1) (128). Initially thought to passively sequester Nrf2 in the cytoplasm, it is now known that Keap1 plays an active role in targeting Nrf2 for ubiquitination and proteasomal degradation by functioning as a component of the Cul3 E3 ubiquitin ligase complex (84).

#### Regulation of Nrf2

Nrf2 can be induced injuriously by ROS (41) or non-injuriously by phytochemicals such as curcumin and sulfurophane (64, 145) (Fig. 2). Upon exposure to oxidants or chemoprotective compounds, cysteine residues on the Keap1/Nrf2 complex sense cellular redox changes, resulting in an alteration in the structure of Keap1. As shown in Figure 1, modification of the Keap1 cysteine residues stabilizes Nrf2, facilitating its translocation into and accumulation in the nucleus. After translocation, Nrf2 forms a heterodimer with Maf and Jun bZip transcription factors, which bind to the 5'-upstream *cis*-acting regulatory sequence known as the ARE (45) and induce transcription of Phase II antioxidant enzymes.

#### Natural Inducers of HO-1

A number of natural antioxidant compounds contained in foods and plants have been demonstrated to be effective nonstressful and noncytotoxic inducers of the response protein HO-1 in various cellular models (Table 2). Most of these compounds are contained in plants, which besides having

been widely used as food, spices, or flavoring since time immemorial, also represent locally traditional medicinal plants.

#### Curcumin

Curcumin (diferuloylmethane) (Fig. 3) is the most investigated natural HO-1 inducer. Curcumin is a yellow pigment obtained by populations living in Asian tropical regions by drying and powdering the rhizome of turmeric (*Curcuma longa* Linn). Widely used as food flavoring, it also plays an important role in traditional medicine due to its anti-inflammatory, anticarcinogenic, and antioxidant properties. The major components of turmeric are the curcuminoids that include curcumin, demethoxycurcumin (DMC), and bisdemethoxycurcumin (BDMC) (16). Their chemical structures are illustrated in Figure 3. Curcumin has been demonstrated to be a potent HO-1 inducer in several cellular models (90). At a cellular level, curcumin has been shown to inhibit expression of adhesion molecules (11), possibly by inhibition of stress transcription factors (89). Ongoing experimental and clinical studies suggest that curcumin and its curcuminoids exhibit unique cytoprotective (114), anti-inflammatory (118), and anticancer properties (19). In recent years, it has also been reported that curcumin acts as a nonstressful and non-cytotoxic inducer of the cytoprotective HO-1 and can maximize the intrinsic antioxidant potential of cells (114) (Fig. 4).

In particular, some authors tested various concentrations of curcumin (0–30  $\mu$ M) on endothelial HO activity and HO-1 protein expression (7). Exposure of endothelial cells to curcumin (1–15  $\mu$ M) for 18 h resulted in a concentration-

TABLE 2. NATURAL INDUCERS OF HEME OXYGENASE 1, ACTIVE METABOLITES AND BIOLOGICAL EFFECTS IN VARIOUS EXPERIMENTAL MODELS

Compound	Active metabolites	Experimental models	Biological effects	References
Caffeic acid phenethyl ester	Caffeic acid phenethyl ester glutathione conjugate	Ischemia/reperfusion	Protective effect on atherosclerosis and ischemia/reperfusion injury	(47, 75)
Cyanidin-3-O-glucoside	Protocatechuic acid	Endothelial cells	Upregulation pro-survival genes.	(125)
Curcumin	Diferuloylmethane	Endothelial cells	Antioxidant properties	(89)
Delphinidin	4'-O-methyl-delphinidin	HUVEC	Antiproliferative effect	(52)
Enterolactone	(3R,4R)-3,4-bis[(3-hydroxyphenyl)methyl]oxolan-2-one	HUVEC	Inhibits LDL peroxidation and coronary heart disease.	(68)
Epigallocatechin-3-gallate	3', 4', 5'-trihydroxyphenyl- $\gamma$ -valerolactone	Aortic endothelial cells, cardiomyocytes	Improves endothelial function and induces anti-inflammatory vascular events.	(116)
Esculetin	6,7-dihydroxycoumarin	VMSC	Inhibition of vascular smooth muscle cells proliferation.	(50)
Fraxetin	Unknown	VMSC	Inhibits vascular proliferation, atherosclerosis, and LDL oxidation.	(142)
Magnesium lithospermate B	Unknown	Otsuka long-evans Fatty rats	Endothelium vasodilation.	(66)
Quercetin	Quercetin 3-O-sulfate Quercetin 3-O-glucoside	Apo E (-/-) mice	Attenuates atherosclerosis and endothelial dysfunction.	(75)
Resveratrol	Piceatannolo, DH-digluconide DH-sulfoglucuronide and DH-disulfate	Vascular smooth muscle cells, ischemia/reperfusion	Antiatherogenic activity, general protective effects in coronary artery disease and others vascular disease	(6, 37)

DH, dihydroresveratrol; HUVEC, human umbilical vein endothelial cells; LDL, low density lipoprotein.

dependent increase in HO activity, showing a maximal effect at 15  $\mu$ M. In the same set of experiments, the authors showed that curcumin attenuates oxidative stress after hypoxia in endothelial cells, and this effect is dependent on increased HO activity (56).

Successive experiments tested whether other derivatives of curcumin present in turmeric, such as DMC and BDMC (Fig. 3), would also stimulate this enzyme induction. The authors found that pure curcumin, DMC, and BDMC all significantly increased HO-1 expression after 6-h incubation. However, despite displaying a similar basic chemical structure, the three compounds affected the pattern of HO-1 protein inducibility in a different fashion. For example, removal of one methoxy group from the molecule of curcumin, as in DMC, affected HO-1 expression slightly. Removal of both methoxy groups, as in BDMC, significantly decreased HO-1 expression. Consistent with these results, HO activity also differed for each curcuminoid tested in this study, the order being curcumin > DMC > BDMC (56). Generally, HO-1 expression is induced by stimuli that activate the mitogen-activated protein kinases (MAPKs) (20, 53) (Fig. 4). Three major subgroups of the MAPK family have been identified to include extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun NH<sub>2</sub>-terminal kinase (JNK), and p38 MAPK. Depending on the stimuli specificity, contradictory results on the regulatory role of different MAPK pathways for HO-1 expression were observed (53). In the case of curcumin, the activation of the p38 MAPK pathway was found to be involved in HO-1 expression (7, 92). To investigate the signal transduction pathways involved in regulating HO-1 expression in response to curcuminoids, some authors examined the effects of three pharmacological inhibitors of signaling intermediates on HO-1 protein levels. Treatment of endothelial cells with the p38 MAPK inhibitor (SB203580) reduced curcuminoid-induced HO-1 expression. Neither JNK inhibitor (SP600125) nor MAPK/ERK kinase inhibitor (U0126) had a significant effect. However, these results clearly show that the pattern of HO-1 protein inducibility differed for each curcuminoid tested in the study, indicating that even subtle changes in the chemical structure can significantly affect the potency of curcuminoids to enhance endothelial HO-1 expression and HO activity (7).

### Resveratrol

Resveratrol (*trans*-3,4,5-trihydroxystilbene) is a natural polyphenolic stilbene that is frequently found in grapes and other food products (103) (Fig. 5). It is present in *cis*- and *trans*-isomers, with the latter being the biologically active form. Resveratrol has been identified in more than 70 species of plants, including grapevines (*Vitis vinifera*), mulberries (*Morus rubra*), *Vaccinium* species, and peanuts (*Arachis hypogea*), and it is thought to have diverse antiatherogenic activities (80, 123), such as the inhibition of low density lipoprotein (LDL) oxidation (9) and platelet aggregation (147) and regulation of vascular smooth muscle proliferation (153). Epidemiological studies have shown that in southern France and other Mediterranean territories, the morbidity and mortality rate of coronary artery disease is low, despite a diet rich in saturated fats and smoking habits (10). This unexpected epidemiological finding was termed the French paradox (137). Exactly how resveratrol exerts its cardioprotective effects is not completely understood, but they have been ascribed to its ability to block

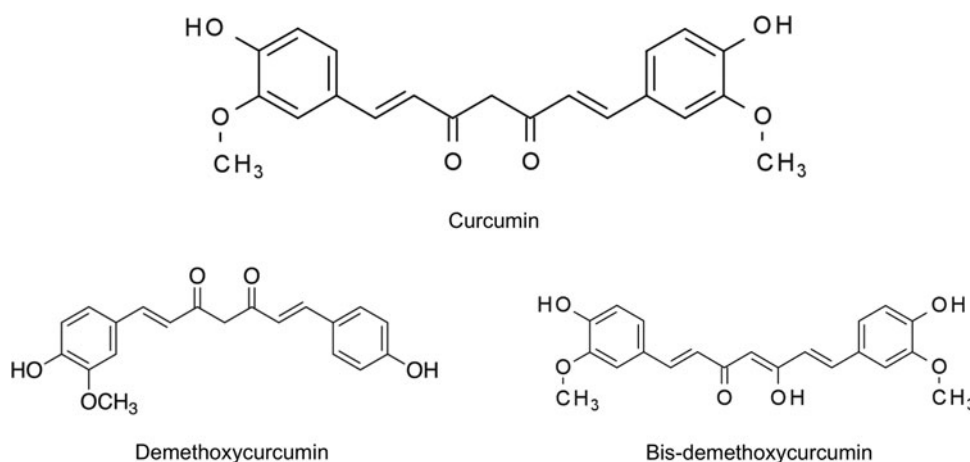


FIG. 3. Chemical structures of curcumin, demethoxycurcumin, and bis-demethoxycurcumin.

platelet aggregation, inhibit oxidation of low-density lipoprotein, and induce NO production. Several studies within the last few years have shown that resveratrol protects against coronary heart disease due to its significant antioxidant properties (22, 108). Additionally, several studies have reported that resveratrol at high concentrations possesses anti-inflammatory activity attributed to blockage of NF- $\kappa$ B activation by inhibiting phosphorylation and degradation of I $\kappa$ B $\alpha$ , thereby preventing nuclear translocation of p65 and p50 (Fig. 6) (94, 104).

Resveratrol-mediated HO-1 induction has been reported in neuronal cultures and has been considered to have potential neuroprotective action (8, 112). The compound is the principal active component of red wine, and its intake is inversely correlated with the incidence of chronic CVD such as atherosclerosis and vascular thrombosis (12). Many studies have found that *trans*-resveratrol prevents the progression of CVD. *Trans*-resveratrol attenuates cardiac hypertrophy in spontaneously hypertensive rats *via* AMP kinase activation (17) and decreases blood pressure in hypertensive rats (97). The compound also suppresses development of myocarditis (150) and

atherogenic lesion formation (31). Resveratrol has also been shown to modulate diverse cell cycle regulatory genes (*e.g.*, *p53*, *Rb*, and cyclins) and these are related to its anticancer or antiproliferation effect (44).

Substantial evidence indicates that resveratrol pharmacologically preconditions the heart to resist ischemia/reperfusion insults. Both NO-dependent pathways (140) and the activation of adenosine receptors (27) are mechanisms that could be mediating the heart preconditioning by resveratrol. In cardiomyocytes, the NO-mediated regulation of cardioprotective enzymes by NO-mediated mechanisms is crucial for cell survival. Resveratrol (*trans*-3,5,4'-trihydroxystilbene), a polyphenolic compound and a naturally occurring phytoalexin, has been designated the active agent (48). The beneficial effect of resveratrol on coronary disease may be attributable, in part, to its ability to retard the progression of early atherosclerotic lesions (111). It also possesses many other biologic activities, including an estrogenic property (28), antiplatelet activity (119), an anti-inflammatory function (25), and a cancer chemopreventive property that has the ability to inhibit angiogenesis and induce apoptosis (29).

Juan *et al.* (59) assessed the induction of HO-1 by resveratrol in human aortic smooth muscle cells at both the mRNA and protein levels (Fig. 6). Northern blot analysis showed that resveratrol at concentrations of 1 and 10  $\mu$ M significantly induced HO-1 induction, but not at concentrations of 20 and 40  $\mu$ M. In particular, induction of HO-1 mRNA by resveratrol was observed at 4 h and increased with time up to 24 h of treatment. Consistently, western blot analysis showed that HO-1 was highly expressed in cells exposed to resveratrol at the concentrations of 1 and 10  $\mu$ M, but not at the concentrations of 20 and 40  $\mu$ M. To reveal the molecular mechanism of resveratrol-mediated HO-1 induction, MAPK and NF- $\kappa$ B inhibitors were employed (Fig. 6). The level of resveratrol-induced HO-1 expression was attenuated by TPCK (a protease inhibitor that blocks activation of NF- $\kappa$ B) and BAY 11-7082 (an inhibitor of I $\kappa$ B $\alpha$  phosphorylation), but not by MAPK inhibitors, including U0126, curcumin, and SB202190, which are inhibitors of Erk1/2, JNK, and p38 MAPK, respectively. Similarly, previous reports also showed that rats receiving resveratrol (gavage, 2.5 mg/kg) exhibited a significant cardioprotection as evidenced by superior postischemic ventricular recovery, reduced myocardial infarct size, and decreased number of apoptotic cardiomyocytes. Resveratrol induced the

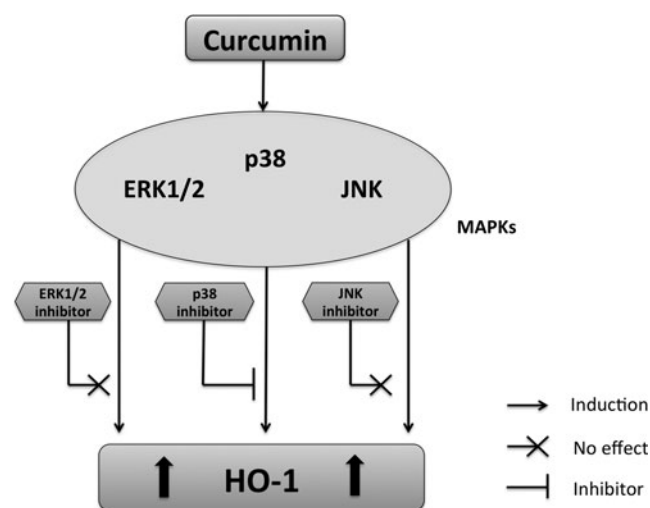


FIG. 4. Induction of heme oxygenase by curcumin. HO-1 expression is induced by stimuli that activate the MAPKs. In the case of curcumin, the activation of the p38 MAPK pathway was found to be involved in HO-1 expression.



resulted in a significant decrease in Cav-1/eNOS association, thus demonstrating that HO-1 regulates Cav-1/eNOS conversely (106). Recently, resveratrol has also been shown to prevent doxorubicin toxicity and apoptosis by an HO-1-dependent pathway (43).

The role of HO-1 in mediating the beneficial effect of resveratrol is further substantiated by a number of studies with pharmacological inhibitors of HO, HO-1<sup>-/-</sup> animals, and siRNA. Furthermore, in a wire-injured femoral artery mouse model, oral administration of *trans*-resveratrol significantly suppressed intimal hyperplasia. The same authors demonstrated that this effect was reversed by an HO activity inhibitor ZnPPIX (65).

### Flavonoids

Flavonoids are naturally occurring antioxidants belonging to the large family of polyphenols. They are widely distributed in plants used as food, as well as traditional medicines, due to their particular variety of clinically relevant properties, such as antitumor, antiplatelet, anti-ischemic, and anti-inflammatory activities.

Quercetin (Fig. 7) is one of the most common flavonoid, and probably, overall the most investigated. In an experimental model of atherosclerosis, quercetin (1.3 mg/day), but not (-)-epicatechin, significantly increased the expression of HO-1 protein in lesions *versus* ApoE(-/-) controls (91).

Anthocyanins are water-soluble plant pigments responsible for the blue, purple, and red color of many plant tissues. They occur primarily as glycosides of their respective aglycone anthocyanidin chromophores (Fig. 7), with the sugar moiety mainly attached at the 3-position on the C-ring or the 5,7-position on the A-ring. Anthocyanins have been shown to be strong antioxidants, and may exert a wide range of health benefits through antioxidant or other mechanisms (71). It has been suggested that anthocyanins play an important role in

the prevention of human diseases associated with oxidative stress, for example, coronary heart disease and cancer (95). The antioxidant properties of anthocyanins have been demonstrated by both *in vitro* and *in vivo* experiments (54, 95, 110, 113, 115).

In this regard, previous studies showed that delphinidin (50 and 100  $\mu$ M) (Fig. 7) significantly induced HO-1 (1.5-fold increase), whereas cyanidin exhibited this effect only at 100  $\mu$ M concentration (1.2-fold increase) (79).

Examples of flavonoids include flavonols, isoflavones, flavonones, and flavan-3-ols (*e.g.*, catechins). Epidemiologic studies have shown that green tea rich in catechins may be protective against coronary atherosclerosis (76). In fact, green tea consumption is usually higher in healthy subjects compared with those with coronary artery disease (76), suggesting that green tea and its polyphenols, for example, catechins, can attenuate risk factors associated with the pathology of atherosclerosis (76). The majority of catechins in green tea include epigallocatechin-3-gallate (EGCG) (Fig. 7), which has been shown to improve endothelial function and to induce anti-inflammatory vascular events. Zheng *et al.* (154) showed that pretreatment with EGCG inhibited the secretion of MCP-1 and the activation of activator protein-1 in porcine aortic endothelial cells stimulated with TNF- $\alpha$ . Moreover, EGCG up-regulated the expression of HO-1 and further induced the secretion of bilirubin. The observed anti-inflammatory effects of EGCG were mimicked by the HO-1 inducer cobalt protoporphyrin and abolished by HO-1 gene silencing.

These results are consistent with previous data showing that while mRNA levels of GPx3, SOD1, and CAT were not influenced by EGCG and theaflavin-3,3'-digallate (TF3), HO-1 was selectively upregulated by EGCG, but not by TF3. However, inhibition of HO-1 did not diminish polyphenol-mediated cardioprotection. While EGCG and TF3 activated Akt, ERK1/2, and p38 MAPK, inhibition of these kinases did not attenuate polyphenol-mediated protection. In this regard, previous studies showed that EGCG-induced phosphorylation of Erk and Akt occurs *via* activation of the mitogen-activated protein kinase-kinase (MEK) and phosphatidylinositol-3 kinase (PI-3K) pathways, respectively (23). Loading of cardiomyocytes with dichlorofluorescein revealed that intracellular levels of ROS were significantly reduced after treatment with EGCG or TF3 as early as 30 min after induction of oxidative stress. In conclusion, activation of prosurvival signaling kinases and upregulation of antioxidant enzymes do not play a major role in tea polyphenol-mediated cardioprotection. In addition, EGCG inhibits STAT-1 activation and reduces cell death after cardiac ischemia/reperfusion injury (129).

### Other compounds and plant extracts

Plant lignans are a group of phenolic compounds that can be found in diets rich in fiber. Enterolactone (Fig. 8) is a breakdown product of plant lignans. The production of mammalian lignans from dietary precursors by intestinal bacteria occurs mainly in the large intestine. After removal of methyl and hydroxyl groups in precursors, enterolactone is absorbed from the gut into the circulation and then excreted in urine, where enterolactone primarily exists as glucuronides (5). Recent studies have shown that high serum enterolactone levels reduce LDL peroxidation *in vivo*, assessed by serum

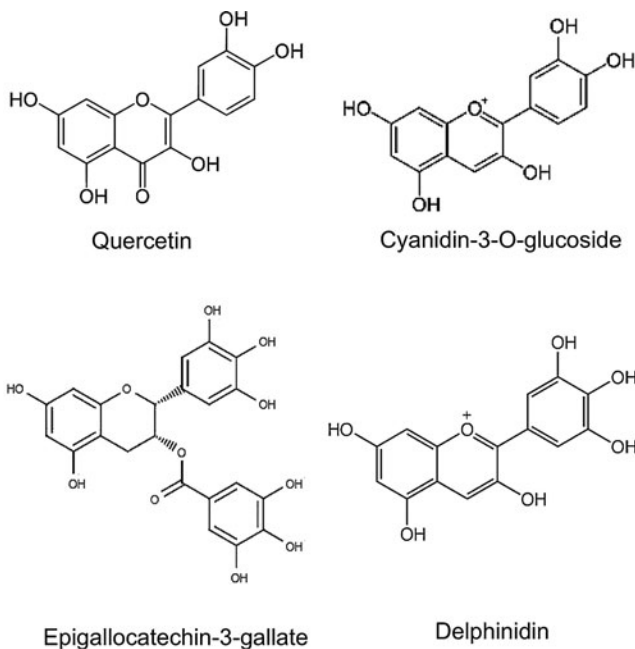


FIG. 7. Chemical structures of quercetin, cyanidin-3-O-glucoside, epigallocatechin-3-gallate, and delphinidin.



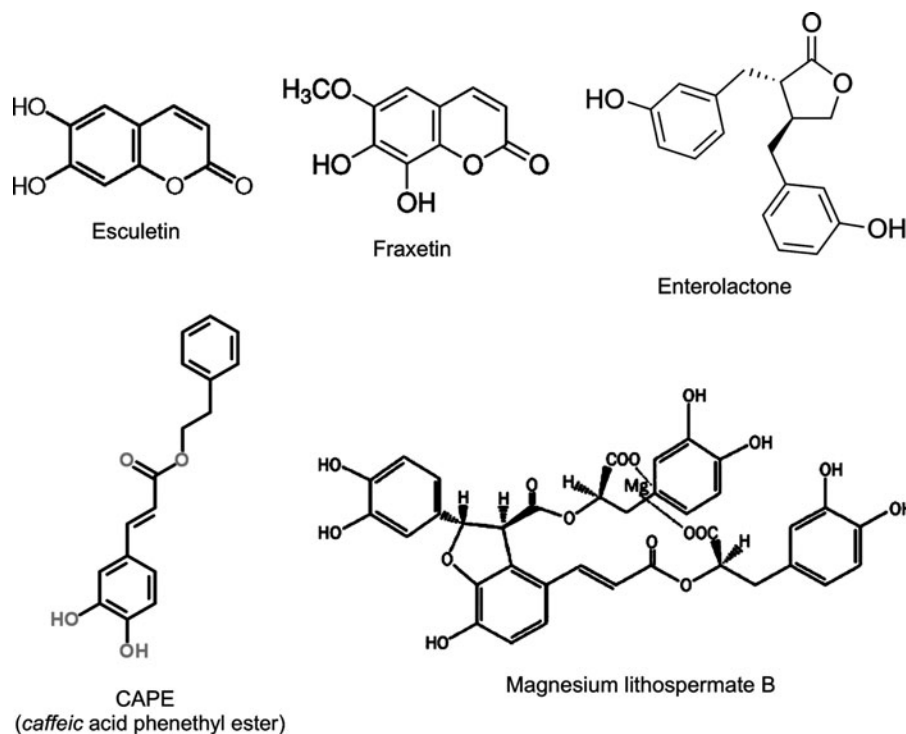


FIG. 8. Chemical structures of esuletin, fraxetin, enterolactone, caffeic acid phenethyl ester (CAPE), and magnesium lithospermate B.

isoprostane levels (135). Enterolactone also reduced lipid peroxidation *in vitro* via direct scavenging of a hydroxyl radical (68). This association implies a protective role of enterolactone against oxidative injury. In addition, estrogen-like biological effects of enterolactone have been reported, which may also result in protection against coronary heart disease (105, 139). Kivela *et al.* showed that enterolactone induced HO-1 in human umbilical vein endothelial cells (HUVEC) in a time- and concentration-dependent manner (50–150  $\mu$ M for 16 h) (69). Induction appeared to be mediated *via* the transcription factor Nrf2, as Nrf2 siRNA abolished HO-1 induction by enterolactone. The authors also showed that exposure to enterolactone increased the binding of Nrf2 to the promoter region of HO-1 (69).

An attractive candidate antioxidant to treat diabetes is magnesium lithospermate B (MLB) (Fig. 8). MLB is the active component of the water-soluble fraction of the Chinese medicine Danshen, a root preparation of Red Sage (*Salvia mitorrhizae*) (67). MLB is an antioxidant worth further study because of its interesting secondary effects in cells. MLB inhibits the enzyme aldose reductase, which is a key component in the polyol biochemical pathway involved in the pathogenesis of diabetic complications (67). Previous research has shown that MLB has antifibrotic, myocardial salvage, and neuroprotective effects (133). MLB prevents hepatitis, uremia, and improves blood circulation, arrhythmia, and renal function (18, 40). Recent work in our laboratory showed that MLB could prevent the development of neointimal hyperplasia in animal models of diabetes and after balloon-induced injury. Starting at 12 weeks, 20-week MLB treatment attenuated the decrease in endothelium-dependent vasodilation in rats. MLB treatment also increased the serum nitrite level and reduced serum concentration of advanced glycation end products. The effect of MLB was greater than an equivalent dose of  $\alpha$ -lipoic acid, a popular antioxidant treatment. MLB rescued the inhibition of eNOS activity and eNOS phosphorylation in en-

dothelial cells cultured in hyperglycemia. This effect was dependent on Akt phosphorylation and associated with decreased O-linked N-acetylglucosamine protein modification of eNOS. MLB also increased nuclear factor erythroid 2-related factor (Nrf-2) activation in a phosphoinositide 3-kinase/Akt pathway-dependent manner. MLB treatment induced the expression of HO-1, and previous studies demonstrated that HO-1 silencing abolished the protective effect of MLB (81).

*Fraxinus rhynchophylla* DENCE (Oleaceae) is a traditional medicinal plant from East Asia (144). Diverse compounds have been isolated from the plant. Among them, ferulaldehyde and scopoletin have inhibitory activity against induction of inducible NO synthase (66), and antitoxoplasmosis effect of oleuropein was reported (57). During the course of characterizing biologically active compounds from natural products, two major coumarins were isolated, esuletin and fraxetin (Fig. 8). Despite numerous studies on the inhibitory activities of natural antioxidants against LDL oxidation, reports on the effects of coumarinoids are still scarce. Lee *et al.* have shown that esuletin inhibits LDL oxidation and Apo-B fragmentation (82). Low concentrations (1–5 mM) of fraxetin potently inhibited LDL oxidation induced by metal and free radicals. Moreover, treatment of vascular smooth muscle cells with higher concentrations (above 30 mM) of fraxetin significantly increased the protein level of HO-1, a key enzyme that inhibits vascular proliferation and atherosclerosis. Subcellular fractionation and reporter gene analysis using an ARE construct revealed that fraxetin increased the level of Nrf2 and reporter activity, and these were associated with the induction of antioxidant enzymes, such as HO-1 and glutathione S-transferase-a.

Caffeic acid phenethyl ester (CAPE) (Fig. 8), a polyphenolic compound concentrated in honeybee propolis, has been reported to exhibit numerous bioactive properties, including

antioxidant (124) and anti-inflammatory activities (96), which may contribute to its protective effects in various pathophysiological processes such as ischemia/reperfusion injury (126, 142) and atherosclerosis (47). To ascertain the involvement of HO-1 induction in the cytoprotective effects of CAPE analogs, their ability to induce HO-1 at 20  $\mu\text{M}$  was determined by reverse transcriptase–polymerase chain reaction, western blotting, and the use of HO-1 inhibitor tin protoporphyrin IX (10–40  $\mu\text{M}$ ) (141). There was significant induction of HO-1 by CAPE derivatives. Inhibition of HO-1 enzymatic activity resulted in reduced cytoprotection. Modification of the catechol ring of CAPE by introduction of fluorine at various positions resulted in dramatic changes in cytoprotective activity. The maintenance of at least one hydroxyl group on the CAPE catechol ring and the phenethyl ester portion was required for HO-1 induction. CAPE and its derivatives were screened for their ability to scavenge intracellular ROS generated in HUVECs by measuring 5-(and-6)-chlormethyl-2', 7'-dichlorodihydrofluorescein diacetate oxidation. The maintenance of 3, 4-dihydroxyl groups on the catechol ring was required for antioxidant activity, but antioxidant activity did not guarantee cytoprotection. Methylation or replacement of one hydroxyl group on the catechol ring of CAPE however provided both pro-oxidant and cytoprotective activities. These results indicate that the induction of HO-1 plays a more important role in the cytoprotective activity of CAPE derivatives than their direct antioxidant activity.

**Critical considerations and future studies.** The amount of experimental data evidencing important properties of many ingredients and/or bioactive substances from plants and food plants is vast and continues to increase rapidly. The use of terms such as nutraceuticals, functional foods, herbal extracts, bioactive dietary constituents, phytochemicals, and similar is becoming copious. In many cases, marketing strategies abuse these terms and health properties are claimed while far from scientifically demonstrated. Thus, researchers require severe scientific objectivity in evaluating the health properties of food ingredients. It is possible to maintain that diverse bioactive substances from plants and food plants are promising candidates as natural HO-1 inducers to be used in CVD. However, some critical evaluations of the literature data are necessary. It is important to note that the majority of studies were conducted in cellular models, whereas few studies were conducted on rats. Thus, the reproduction of natural HO-1 cardiovascular inducers in more relevant *in vivo* models is certainly necessary. With regard to the inductive mechanism of natural HO-1 inducers, although other pathways cannot be excluded, it seems quite clear that the prevalent mechanism is an ARE-mediated HO-1 gene transcription through the Nrf2/ARE signaling pathway.

Other uncertainties derive from the fact that the referred studies report data on natural HO-1 inducers considered both as single chemicals and food extracts. In some cases, little or no information was provided regarding (i) the quantitative measurements of the proposed active compound; (ii) methods of analysis, and (iii) extraction procedures. Obviously, these details are essential for other researchers to reproduce the experiments and to obtain comparable data.

When considering a possible therapeutic use of future natural HO-1 inducer-based drugs, the amount of work yet to be performed is even more significant. Indeed, there is largely

insufficient exhaustive information on absorption, distribution, metabolism, and excretion by main possible routes (oral, intraperitoneal, intravenous, and intratecal). One possible limitation of HO-1 inducers that should be taken into due account is the concomitant reduction of the amounts of intracellular heme, necessary for the assembly of many proteins, including cytochromes (109), cyclooxygenase (86), and NO synthase (87), and the production of ferrous iron, which can trigger oxidative stress through to the Fenton and Haber–Weiss reactions (3).

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#### Abbreviations Used

ARE = antioxidant-response element  
 BDMC = bis-demethoxycurcumin  
 bZip = basic leucine zipper  
 CAPE = caffeic acid phenethyl ester  
 CAT = catalase  
 CO = carbon monoxide  
 CVD = cardiovascular diseases  
 DMC = demethoxycurcumin  
 EGCG = epigallocatechin-3-gallate  
 eNOS = endothelial nitric oxide synthase  
 ERK1/2 = extracellular signal-regulated kinase 1/2  
 GPx = glutathione peroxidase  
 HO-1 = heme oxygenase-1  
 JNK = c-Jun NH2-terminal kinase  
 Keap1 = Kelch-like ECH-associated protein 1  
 MAPKs = mitogen-activated protein kinases  
 MLB = magnesium lithospermate B  
 NO = nitric oxide  
 Nrf2 = nuclear factor erythroid 2-related factor  
 ROS = reactive oxygen species  
 SOD = superoxide dismutase  
 TF3 = theaflavin-3,39-digallate  
 VSMCs = vascular smooth muscle cells