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Differential Activation of Heme Oxygenase-1 by Chalcones and Rosolic Acid in Endothelial Cells

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ABSTRACT

The induction of heme oxygenase-1 (HO-1) is widely recognized as an effective cellular strategy to counteract a variety of stressful events. We have shown that curcumin and caffeic acid phenethyl ester, two naturally occurring phytochemicals that possess antioxidant, anti-inflammatory, and anticarcinogenic activities, induce HO-1 in many cell types. This suggests that stimulation of HO-1 could partly underlie the beneficial effects exerted by these plant-derived constituents. Here we examined the ability of additional plant constituents to up-regulate heme oxygenase activity and HO-1 in aortic endothelial cells. Incubation of endothelial cells with a series of polyphenolic chalcones (5–50 μM) resulted in increased heme oxygenase activity; interestingly, the chemical structure dictated the pattern of heme oxygenase induction, which was unique to each particular compound employed. We also found that rosolic acid, a constituent isolated from the rhizome of *Plantago asiatica L.* dramatically increased HO-1 in a concentration- and time-dependent manner. Severe cytotoxicity was observed after prolonged exposure (24 or 48 h) of cells to curcumin and caffeic acid phenethyl ester, whereas 2′-hydroxychalcone and rosolic acid did not affect cell viability. By using different mitogen-activated protein kinase inhibitors, we determined that the extracellular signal-regulated kinase, p38, and c-Jun NH_2-terminal protein kinase pathways play only a minor role in the induction of HO-1 by rosolic acid and 2′-hydroxychalcone. On the other hand, increased intra- and extracellular thiols markedly reduced the rise in heme oxygenase activity elicited by rosolic acid. Thus, this study identified novel plant constituents that highly induce HO-1 in endothelial cells and investigated some of the mechanisms involved in this effect.

Impressive and interesting literature has emerged to support the idea that plant-derived chemical substances could be used for therapeutic intervention in several disease states, including inflammatory conditions and cancer. In particular, polyphenols such as curcumin, caffeic acid phenethyl ester, and resveratrol interfere with inflammatory processes by inhibiting the expression of inducible nitric oxide (NO) synthase and NO production, as well as by blocking the activation of nuclear factor-κB, a transcriptional factor that controls the expression of proinflammatory molecules (Calixto et al., 2003). Furthermore, Talalay and his group have intensively examined the ability of plant constituents derived from cruciferous vegetables to reduce susceptibility to cancer (Talalay and Fahey, 2001). In this instance, plant constituents act as chemoprotective agents by enhancing the expression of phase 2 enzymes, a group of proteins that catalyze a series of reactions essential for cellular defense and survival. The ability to induce such a response is based on the peculiar characteristic of different plant-derived compounds to act as Michael reaction acceptors, i.e., susceptibility to attack by nucleophiles. Important nucleophiles that likely mediate the response are highly reactive sulfhydryl groups present on a potential cellular "sensor(s)" that react with the inducers (natural compounds), signaling the up-regulation of phase 2 enzymes (Dinkova-Kostova et al., 2001). This hypothesis is strongly sustained by recent findings that show the effect of inducers on the complex between the transcriptional factor Nrf2 and its cytoplasmic repressor protein Keap1 (Dinkova-Kostova et al., 2002). Apparently, inducers disrupt the complex Keap1-Nrf2 by binding covalently with Keap1, enabling Nrf2 to translocate into the nucleus, where it activates the antioxidant response element of phase 2 genes and accelerates their transcription (Dinkova-Kostova et al., 2002). The covalent modification involves two critical cysteine residues (C273 and C288) of the 25 contained within the Keap1 pro-

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ABBREVIATIONS: NO, nitric oxide; HO-1, heme oxygenase-1; CAPE, caffeic acid phenethyl ester; RA, rosolic acid; ATA, aurintricarboxylic acid; 2′OH-CAL, 2′-hydroxychalcone; MAPK, mitogen-activated protein kinase; PD098059, 2′-amino-3′-methoxyflavone; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; SP600125, anthra[1,9-*cd*]pyrazole-6 (2*H*)-one; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal protein kinase; PBS, phosphate-buffered saline.

tein (Wakabayashi et al., 2004). The fact that *nrf2*-deficient mice do not induce the phase 2 response when treated with inducers and display increased susceptibility to cancer point to the central role played by Nrf2 in the expression of phase 2 enzymes (Ramos-Gomez et al., 2001).

Heme oxygenase-1 (HO-1) can be legitimately considered part of the phase 2 response (Prestera et al., 1995) inasmuch as 1) it is induced by several agents that also evoke the phase 2 response (Prestera et al., 1995; Motterlini et al., 2000b; Hill-Kapturczak et al., 2001; Balogun et al., 2003b); 2) its inducibility by phytochemicals is linked to the antioxidant response element and the redox-sensitive Nrf2 (Prestera et al., 1995; Choi and Alam, 1996; Balogun et al., 2003b; Martin et al., 2004); and 3) it catalyzes reactions (degradation of pro-oxidant heme to form the powerful antioxidant biliverdin/bilirubin and the signaling molecule carbon monoxide) that exert protection against toxic compounds and oxidative stress in a variety of cells and tissues (Maines, 1997; Foresti and Motterlini, 1999; Clark et al., 2000a,b, 2003; Motterlini et al., 2000a; Choi and Otterbein, 2002; Jeney et al., 2002; Morse and Choi, 2002; Foresti et al., 2004). We have already reported on the effect of curcumin and caffeic acid phenethyl ester (CAPE) to induce HO-1 in endothelial cells (Motterlini et al., 2000b), astrocytes (Scapagnini et al., 2002), and renal cells (Hill-Kapturczak et al., 2001; Balogun et al., 2003a,b). Interestingly, in renal cells, curcumin could up-regulate HO-1 also at temperatures below 37°C (Balogun et al., 2003a). In the present study, we further examined a series of plant constituents for their ability to stimulate HO-1 expression in endothelial cells and investigated potential mechanisms involved in this response.

Materials and Methods

Chemicals and Reagents. 2′-Hydroxychalcone, 2,2′-hydroxychalcone, and 2,2′,4′-trihydroxychalcone were purchased from INDOFINE Chemical Company, Inc. (Hillsborough, NJ). Rosolic acid (RA), aurintricarboxylic acid (ATA), pararosaniline chloride, curcumin, CAPE, and all other reagents were obtained from Sigma Chemical (Poole, Dorset, UK) unless specified otherwise. Stock solutions of polyphenolic compounds (5 mM) were prepared freshly on the day of the experiment by dissolving the compounds in ethanol. Aurintricarboxylic acid was prepared in distilled water. As described previously (Motterlini et al., 2000b), the amount of ethanol used in our studies does not induce HO-1.

Cell Culture and Experimental Protocols. Bovine aortic endothelial cells (Coriell Cell Repositories, Camden, NJ) were used in all studies. Cells were grown in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cells were kept at 37°C in a humidified atmosphere of air and 5% CO_2. Confluent cells were exposed to various concentrations of polyphenolic compounds, and heme oxygenase activity and HO-1 protein expression were determined at different times after treatment. *N*-Acetylcysteine (1 and 2.5 mM), a precursor of glutathione synthesis, was also used to examine whether sulfhydryl donors affect the changes in heme oxygenase activity observed with rosolic acid. The participation of the MAPK pathway in the increase of heme oxygenase activity by rosolic acid and 2′-hydroxychalcone (2′OH-CAL) was assessed using 10 μM PD098059 (ERK inhibitor), 10 μM SB203580 (p38 inhibitor), or 10 μM SP600125 (JNK inhibitor). Cell viability was determined in cells treated with various polyphenolic compounds at 24 or 48 h. To assess the protection against oxidative stress, cells were initially pretreated with polyphenols for 6 h followed by a 2-h exposure to 3 mM hydrogen peroxide.

Assay for Endothelial Heme Oxygenase Activity. Heme oxygenase activity was determined at the end of each treatment as described previously by our group (Motterlini et al., 2000b). Briefly, cells were washed and gently scraped in cold PBS using a rubber policeman (Thomas Scientific, Swedesboro, NJ). The cell pellet obtained after centrifugation was added to a reaction mixture containing NADPH, glucose-6-phosphate dehydrogenase, rat liver cytosol as a source of biliverdin reductase, and the substrate hemin. The reaction mixture was incubated in the dark at 37°C for 1 h and terminated by the addition of 1 ml of chloroform. After vigorous vortexing and centrifugation, the extracted bilirubin in the chloroform layer was measured by the difference in absorbance between 464 and 530 nm (ϵ = 40 mM^{-1} cm^{-1}).

Western Blot Analysis for Heme Oxygenase-1. Samples of endothelial cells treated for the heme oxygenase activity assay were also analyzed by Western immunoblot technique as described previously (Foresti et al., 1997). Briefly, an equal amount of protein (30 μg/well) from each sample was separated by SDS-polyacrylamide gel electrophoresis and transferred overnight to nitrocellulose membranes, and the nonspecific binding of antibodies was blocked with 3% nonfat dried milk in PBS. Membranes were then probed with a polyclonal rabbit anti-HO-1 antibody (Stressgen Biotechnologies Corporation, Victoria, BC, Canada) (1:1000 dilution in Tris-buffered saline, pH 7.4). After three washes with PBS containing 0.05% (v/v) Tween 20, blots were visualized using an EXTRA-3A amplified alkaline phosphatase kit from Sigma Chemical.

Cell Viability Assay. Cell viability was performed by an Alamar blue assay according to the manufacturer's instructions (Serotec, Oxford, UK) (Motterlini et al., 2000b). The assay is based on detection of metabolic activity of living cells using a redox indicator that changes from oxidized (blue) to reduced (red) form. The intensity of the red color is proportional to the viability of cells, which is calculated as the difference in absorbance between 570 and 600 nm and expressed as percentage of control.

Statistical Analysis. Differences among the groups were analyzed using the *t* test or one-way analysis of variance combined with the Bonferroni test. Values were expressed as mean ± S.E.M., and differences between groups were considered to be significant at $p <$ 0.05.

Results

Effect of 2′-Hydroxychalcone, 2,2′-Dihydroxychalcone, and 2,2′,4′-Trihydroxychalcone on Heme Oxygenase Activity in Endothelial Cells. Based on the fact that HO-1 is highly inducible in many cell types treated with curcumin and caffeic acid phenethyl ester (Motterlini et al., 2000b; Hill-Kapturczak et al., 2001; Scapagnini et al., 2002; Balogun et al., 2003b), we wondered whether other polyphenolic compounds that are known to induce phase 2 enzymes would also affect the expression of HO-1 in endothelial cells. We tested a series of chalcones, a diverse group of naturally occurring plant metabolites that can be regarded as open-chain flavonoids (Dinkova-Kostova et al., 2001). Interestingly, we found that 2′-hydroxychalcone, 2,2′-hydroxychalcone, and 2,2′,4′-trihydroxychalcone all significantly increased heme oxygenase activity after 6 h of incubation (Fig. 1). However, despite displaying a similar basic chemical structure in which two aromatic rings are bridged by an α,β-unsaturated carbonyl moiety, the three compounds affected heme oxygenase in a different fashion. For example, 2′-hydroxychalcone had little effect on heme oxygenase activity at concentrations of 5 or 10 μM, but concentrations of 20 or 30 μM produced a severalfold increase (Fig. 1A). In contrast, 2,2′-hydroxychalcone altered endothelial heme oxy-

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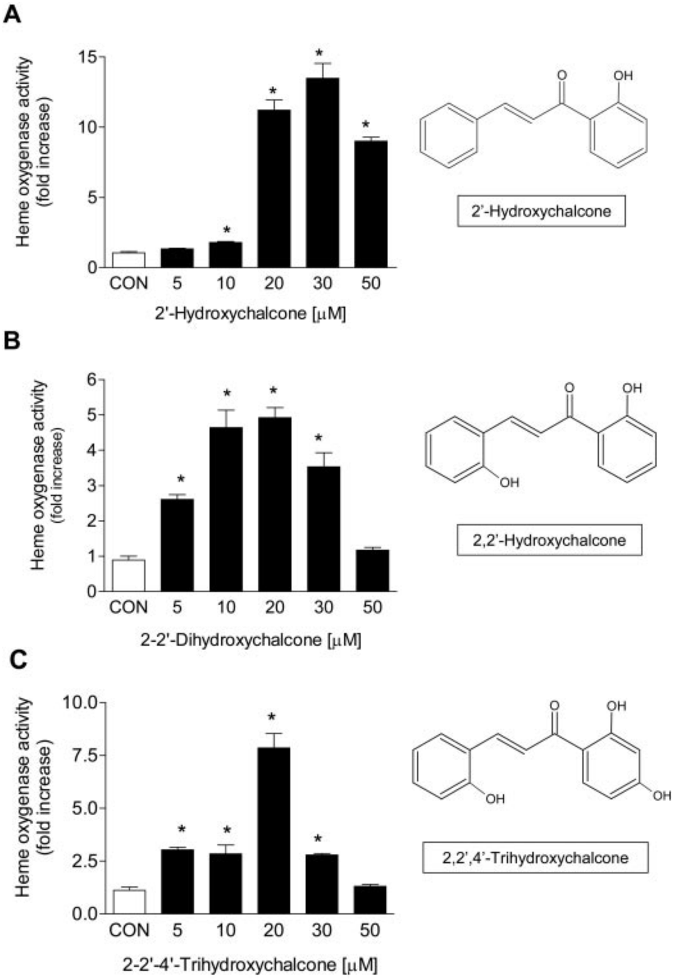

Fig. 1. Effect of chalcones on heme oxygenase activity in vascular endothelial cells. Heme oxygenase activity was measured in endothelial cells 6 h after exposure to various concentrations (0–50 μM) of 2′-hydroxychalcone (A), 2,2′-dihydroxychalcone (B), or 2,2,4′-trihydroxychalcone (C). Each figure is accompanied by the chemical structure of the specific chalcone used in the experiments, highlighting the presence of aromatic rings and the position of the hydroxyl groups on the rings. In the control group, cells were incubated with medium alone. Each bar represents the mean ± S.E.M. of five to six independent experiments. *, $p < 0.05$ versus control.

genase according to a bell-shape effect; the peak of induction occurred at 20 μM, but at 50 μM the activity was decreased to control levels (Fig. 1B). 2,2′,4′-Trihydroxychalcone exhibited a curious behavior inasmuch as 5, 10, and 30 μM caused a similar 3-fold increase in heme oxygenase activity, whereas the activity was approximately 8-fold higher than control at just 20 μM (Fig. 1C). It has to be noted that all chalcones exhibited increased cytotoxicity at 50 μM (data not shown).

Effect of RA, Aurintricarboxylic Acid, and Pararosaniline Chloride on Heme Oxygenase Activity and HO-1 Protein Expression in Vascular Endothelial Cells. Next we tested another series of compounds that still contain phenyl groups but are quite different from chalcones. Figure 2 shows the chemical structure of RA, aurintricarboxylic acid, and pararosaniline chloride. Rosolic acid is a chemical constituent derived from the rhizome of *P. asiatica L.* (Minghong et al., 1995). It is evident that the main structure of one carbon bound to three phenyl groups is common for all three compounds. The major distinction is related to the side

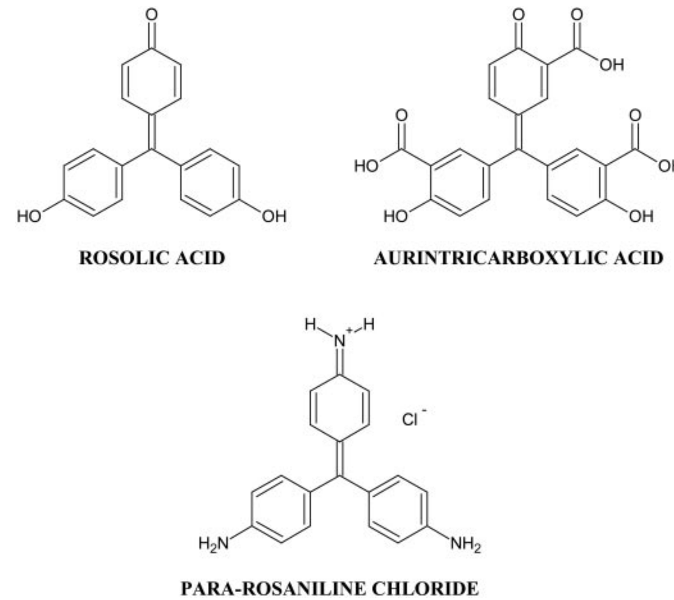

Fig. 2. Chemical structure of triphenylmethanes. RA, aurintricarboxylic acid, and pararosaniline chloride share a common basic structure characterized by one carbon bound to three aromatic rings. The side chains on the aromatic rings diversify the compounds and influence their ability to induce heme oxygenase.

chains, which are hydroxyl groups for RA, carboxylic groups for aurintricarboxylic acid, and amines for pararosaniline chloride. As reported in Table 1, the difference in side chains strongly affected the ability of these compounds to induce endothelial HO-1. In fact, heme oxygenase activity was highly elevated only following incubation of cells with 15 μM RA, whereas 15 μM aurintricarboxylic acid or pararosaniline chloride produced no changes. Based on these preliminary findings, we concentrated more closely on the effect of RA. Exposure of cells to RA resulted in a strong concentration- and time-dependent increase in heme oxygenase activity (Fig. 3). The protein expression of HO-1 was also up-regulated (Fig. 4), confirming that the increase in heme oxygenase activity resulted from HO-1 induction. For comparison, the expression of HO-1 was also analyzed following treatment of cells with 15 μM curcumin, CAPE, 2′-hydroxychalcone, or RA for 6 or 18 h (Fig. 4). Interestingly, we observed that RA was the most potent inducer of HO-1 among the compounds tested at 6 h (Fig. 4A); in addition, although at 18 h HO-1 was gradually decreasing when cells were incubated with curcumin, CAPE, or 2′-hydroxychalcone, its expression was further enhanced with RA (Fig. 4B). We also

TABLE 1

Effect of rosolic acid and its derivatives on endothelial heme oxygenase activity and cell viability

The table shows the activity of heme oxygenase measured 6 h after exposure of endothelial cells to 15 μM RA, aurintricarboxylic acid, or pararosaniline chloride. Cell viability was also measured after incubation of the triphenylmethanes for 6 h, and no significant cytotoxicity was observed. Data are expressed as the mean ± S.E.M. of five independent experiments.

	Heme Oxygenase Activity	Cell Viability
	pmol bilirubin/mg protein/h	*% of control*
Control (6 h)	453 ± 43	100 ± 2.9
Rosolic acid (6 h)	2676 ± 117*	94 ± 1.7
Aurintricarboxylic acid (6 h)	332 ± 70	98 ± 2.3
Pararosaniline chloride (6 h)	375 ± 64	96 ± 3.1

* $p < 0.05$ vs. control.

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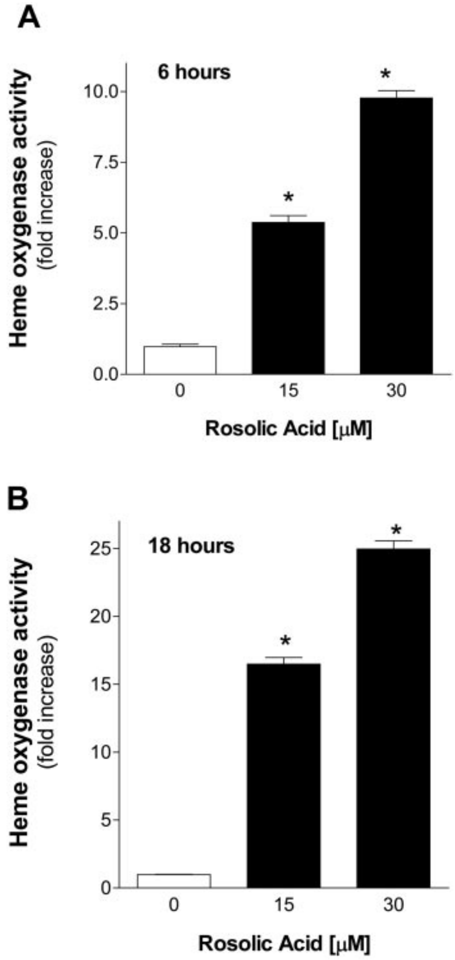

Fig. 3. Effect of short or prolonged incubation with rosolic acid on endothelial heme oxygenase activity. Endothelial cells were exposed to 15 or 30 μM RA for 6 (A) or 18 h (B) and analyzed for heme oxygenase activity. Data are expressed as the mean ± S.E.M. of five independent experiments. $*$, $p < 0.05$ versus control.

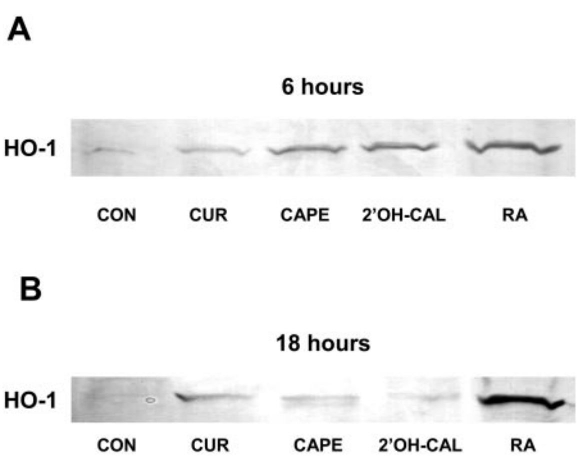

Fig. 4. HO-1 expression in cells exposed to curcumin, caffeic acid phenethyl ester, 2′-hydroxychalcone, and rosolic acid. Endothelial cells were incubated with various plant constituents (15 μM) for 6 (A) or 18 h (B), and HO-1 protein expression was evaluated using appropriate antibodies as described under *Materials and Methods*. Western blot represents three independent experiments. CON, control; CUR, curcumin.

examined whether both endogenous and exogenous thiols were involved in the induction of HO-1 mediated by RA. We found that preincubation of cells for 18 h with 2.5 mM *N*-acetylcysteine, a treatment known to increase intracellular glutathione levels (Foresti et al., 1997), markedly decreased the rise in heme oxygenase activity elicited by 15 μM RA (Fig. 5A). Note that *N*-acetylcysteine was washed out prior to exposure to RA after the pretreatment. Similarly, coincubation of *N*-acetylcysteine with RA resulted in a significant ($p < 0.05$) reduction of heme oxygenase activity (Fig. 5B). To address whether thiyl radicals were mediating HO-1 induction by RA, we also used the thiyl radical scavenger iodoacetamide. However, we found that iodoacetamide was very cytotoxic at 200 μM, whereas at 10 μM it induced HO-1, making it difficult to interpret the results (data not shown).

Endothelial Cell Viability after Exposure to RA, Curcumin, Caffeic Acid Phenethyl Ester, or 2′-Hydroxychalcone. Considering that stressful events such as oxidative or nitrosative reactions are usually responsible for HO-1 up-regulation in cells and tissues (Motterlini et al., 2002), we assessed the viability of endothelial cells after prolonged (24 or 48 h) incubation with various polyphenolic compounds that induce HO-1 in our experimental setting. It was of interest to find that 15 μM curcumin or CAPE caused a time-dependent decrease in cell viability, with >95% damage observed after 48 h (Fig. 6, A and B). In contrast, 15 μM 2′-hydroxychalcone or RA did not produce any evident cytotoxicity (Fig. 6, A and B). This finding is particularly relevant for RA, since the compound continued to

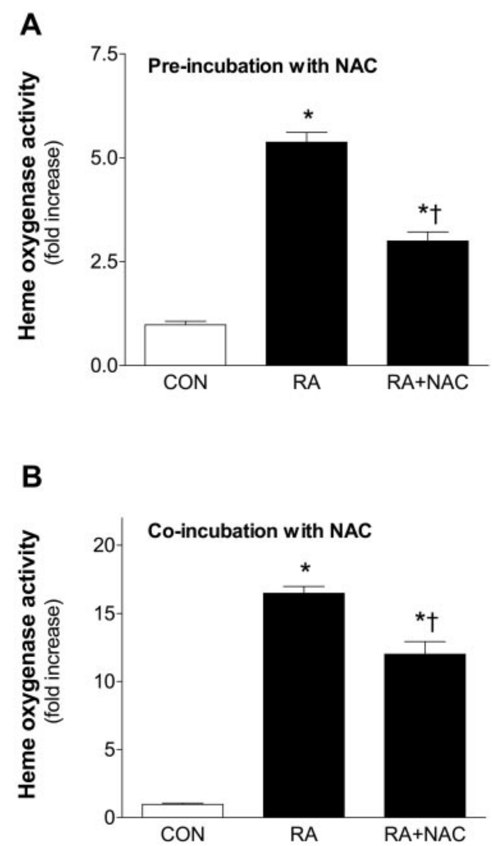

Fig. 5. Effect of increased intra- and extracellular thiols on the stimulation of heme oxygenase activity by rosolic acid. A, cells pretreated with 2.5 mM *N*-acetylcysteine (18 h) to increase intracellular thiols content prior to exposure to 15 μM RA for 6 h. Heme oxygenase activity was measured at the end of the incubation as described under *Materials and Methods*. B, cells coincubated with *N*-acetylcysteine and rosolic acid for 18 h for heme oxygenase activity measurements. Data are expressed as the mean ± S.E.M. of four independent experiments. $*$, $p < 0.05$ versus control and †, $p < 0.05$ versus RA alone.

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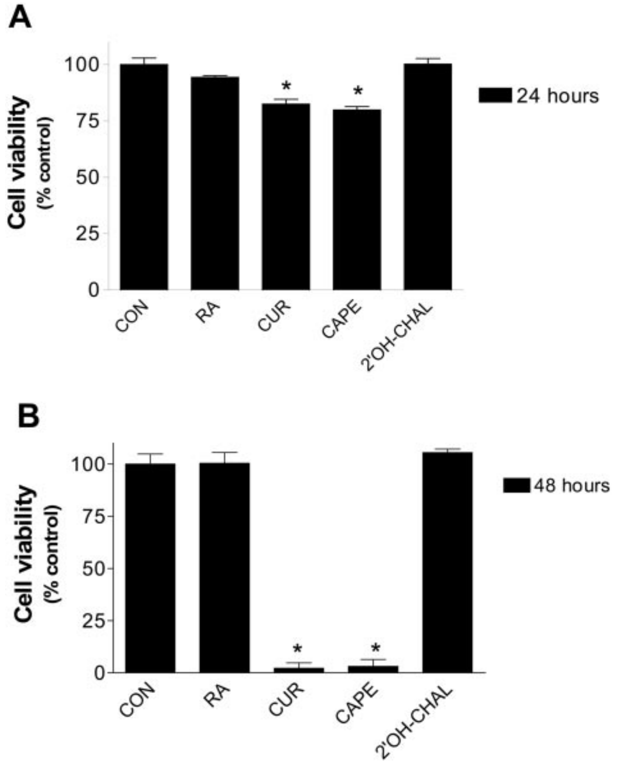

Fig. 6. Viability of cells exposed to rosolic acid, curcumin, caffeic acid phenethyl ester, or 2′-hydroxychalcone. Endothelial cells were incubated for 24 (A) or 48 h (B) with various plant constituents (15 μM), and cell viability was measured spectrophotometrically using an Alamar blue assay according to the manufacturer's instructions. Data are expressed as the mean ± S.E.M. of five independent experiments. *, $p < 0.05$ versus control (CON). CUR, curcumin.

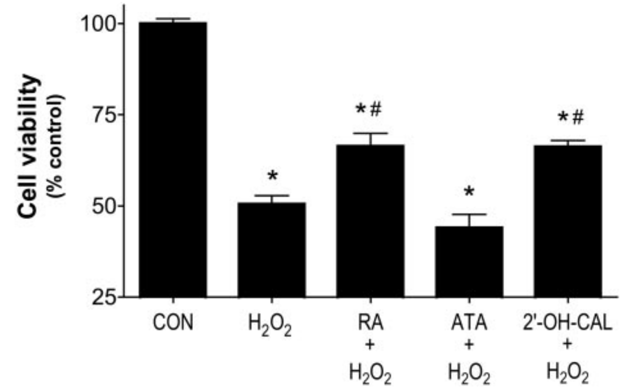

Fig. 7. Protective effects of rosolic acid and 2′-hydroxychalcone against oxidative stress. Endothelial cells were initially preincubated for 6 h in the presence of 15 μM RA or 2′OH-CAL; ATA, which does not induce HO-1, was used as a negative control for RA. Then the medium was removed, and cells were exposed to 3 mM H_2O_2 for 2 h. Cell viability was measured spectrophotometrically using an Alamar blue assay according to the manufacturer's instructions. Data are expressed as the mean ± S.E.M. of five independent experiments. *, $p < 0.05$ versus control (CON) and †, $p < 0.05$ versus H_2O_2 alone.

TABLE 2

Effect of MAPK inhibitors on the increase of heme oxygenase activity mediated by rosolic acid and 2′-hydroxychalcone

Endothelial cells were preincubated for 15 min with 10 μM PD098059 (ERK inhibitor), 10 μM SB203580 (p38 inhibitor), or 10 μM SP600125 (JNK inhibitor) prior to exposure for 6 h to 15 μM RA or 15 μM 2′OH-CAL. Heme oxygenase activity was determined as described under *Materials and Methods*, and results are expressed as a percentage of the activity promoted by RA or 2′OH-CAL, respectively.

	Heme Oxygenase Activity (pmol bilirubin/mg protein/h)			
		+ PD098059	+ SB203580	+ SP600125
			%	
RA	100	86 ± 0.01	100 ± 0.01	72 ± 0.17
2′OH-CAL	100	80 ± 0.02	85 ± 0.02	127 ± 0.05

stimulate HO-1 expression for long periods of time (18 h), whereas HO-1 protein levels were already considerably decreasing at 18 h with curcumin, CAPE, and 2′-hydroxychalcone (Fig. 4B).

Protective Effects of Rosolic Acid and 2′-Hydroxychalcone against Oxidative Stress. From the data obtained so far, RA and 2′-hydroxychalcone seem to be the most potent inducers of HO-1 expression and activity. Therefore, we wanted to test whether these two polyphenolic compounds were able to protect endothelial cells against oxidative stress. For this purpose, cells were initially pretreated with RA or 2′-hydroxychalcone (15 μM) for 6 h to allow HO-1 induction to take place. Aurintricarboxylic acid, which does induce HO-1, was used as a negative control for RA. The medium was then removed, and cells were exposed to hydrogen peroxide for 2 h before assessing cell viability. As shown in Fig. 7, exposure of cells to hydrogen peroxide resulted in a substantial loss in cell viability, and pretreatment with either RA or 2′-hydroxychalcone significantly attenuated H_2O_2-mediated cytotoxicity. Interestingly, ATA failed to exert any cytoprotective effect against H_2O_2, suggesting that induction of HO-1 is required to counteract oxidative stress.

MAPK Pathway Plays a Minor Role in the Increase in Heme Oxygenase Activity Mediated by Rosolic Acid and 2′-Hydroxychalcone. We also wanted to examine whether the MAPK pathway was involved in the induction of heme oxygenase activity by RA or 2′-hydroxychalcone. As shown in Table 2, inhibition of the ERK pathway resulted in a 14 and 20% decrease of heme oxygenase activity with RA and 2′-hydroxychalcone, respectively. When the p38 pathway was inhibited, heme oxygenase activity remained unchanged in the case of RA, whereas a 15% decrease was observed for 2′-hydroxychalcone. Last, the increase in heme oxygenase activity elicited by RA was reduced by 18% when the JNK pathway was blocked, whereas inhibition of the same pathway conversely enhanced the increase in activity observed with 2′-hydroxychalcone. These findings indicate that RA and 2′-hydroxychalcone activate different intracellular signaling mechanisms to induce HO-1. In addition, they also suggest that these specific plant constituents depend only marginally on the MAPK pathway to up-regulate HO-1 and that other mechanisms are probably involved.

Discussion

Mammalian cells have developed several defense strategies to counteract the threat imposed by oxidative and other kinds of stress. A key role in this response (designated phase 2 response; see Introduction) is played by enzymes that perform a variety of protective actions (Dinkova-Kostova et al., 2001; Talalay and Fahey, 2001) and can be highly induced by synthetic and natural chemical agents. In this respect, it is intriguing that many phytochemicals found in vegetables normally present in the human diet can exert such an action (Talalay and Fahey, 2001) and that consumption of fruits and vegetables is associated with a decrease in cancer risk and

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development of cardiovascular disease (Talalay and Fahey, 2001; Wu et al., 2004). HO-1, the inducible enzyme that uses heme as a substrate to produce bilirubin/biliverdin and carbon monoxide (Foresti and Motterlini, 1999; Motterlini et al., 2003; Foresti et al., 2004), was recently shown to be up-regulated by plant constituents, including curcumin (Motterlini et al., 2000b; Hill-Kapturczak et al., 2001; Scapagnini et al., 2002; Balogun et al., 2003a,b), caffeic acid phenethyl ester (Scapagnini et al., 2002), and carnosol (Martin et al., 2004). In the present study, we report on the ability of chalcones to enhance the activity of heme oxygenase and expression of HO-1 protein in vascular endothelial cells. Chalcones are naturally occurring substances ubiquitously present in plants, where they participate in defense strategies as antioxidants and antifungal and antimicrobial agents (Dinkova-Kostova, 2002). Our results show that the pattern of inducibility differed for each chalcone tested, indicating that even subtle changes of the chemical structure can significantly affect the potency and mode of chalcones to up-regulate HO-1. In fact, the main difference among the three chalcones is the number of hydroxyl groups present (one for 2′-hydroxychalcone, two for 2,2′-hydroxychalcone, and three for 2,2′,4′-trihydroxychalcone) and their position on the phenyl rings (*ortho* for 2′-hydroxychalcone and 2,2′-hydroxychalcone and *ortho* and *meta* for 2,2′,4′-trihydroxychalcone; see chemical structure in Fig. 1). The presence of hydroxyl groups in the *ortho* position on the aromatic ring is already known to noticeably enhance the inducer potency of plant constituents (Dinkova-Kostova et al., 2001). Indeed, the three chalcones examined here are able to induce other phase 2 enzymes [e.g., NAD(P)H/quinone reductase] (Dinkova-Kostova et al., 2001), and the novel findings that HO-1 is also up-regulated extend our knowledge on additional protective pathways that chalcones can modulate. It has to be noted that all chalcones tested promoted increased cytotoxicity at 50 μM, suggesting that the decreased heme oxygenase activity observed at this concentration may reflect the increased cell damage.

In this study, we also investigated whether RA, a triphenylmethane of plant origin with Michael reaction acceptor functionality, was capable of affecting HO-1 expression. RA is formed by one carbon bound to three aromatic rings, and each of the rings carries a hydroxyl group as a side chain, albeit in the *para* position (see chemical structure in Fig. 2). Therefore, RA contains the crucial features that influence the ability of Michael reaction acceptors to induce the phase 2 response (Talalay and Fahey, 2001; Dinkova-Kostova, 2002); in support of this notion, the compound indeed highly up-regulated HO-1. It was also interesting to observe that aurintricarboxylic acid and pararosaniline chloride, two triphenylmethanes that share the same basic chemical structure of RA but display carboxylic and amine groups as side chains of the aromatic rings, respectively, did not affect heme oxygenase. These findings further emphasize the importance of hydroxyl groups in the inducer potency of phytochemicals. However, the data were surprising since all three compounds are Michael reaction acceptors, and, based on the stability of the final products, the Michael addition will occur in the following order: pararosaniline chloride > aurintricarboxylic acid > RA. As a result, one would expect that of the three triphenylmethanes, pararosaniline would be the strongest inducer, followed by aurintricarboxylic acid and RA. To reconcile our results with the reactivity of these compounds and gain insights into the mechanisms implicated, it helps to consider the chemical behavior of Michael reaction acceptors (see scheme in Fig. 8). Upon reaction of a Michael acceptor with a nucleophile, a stable product will be formed. This product could undergo phenol oxidation and successively give rise to thiyl radicals, which would propagate the oxidative stress reactions intracellularly. The thiyl radical could also interact with other radicals and/or sulfhydryl residues of

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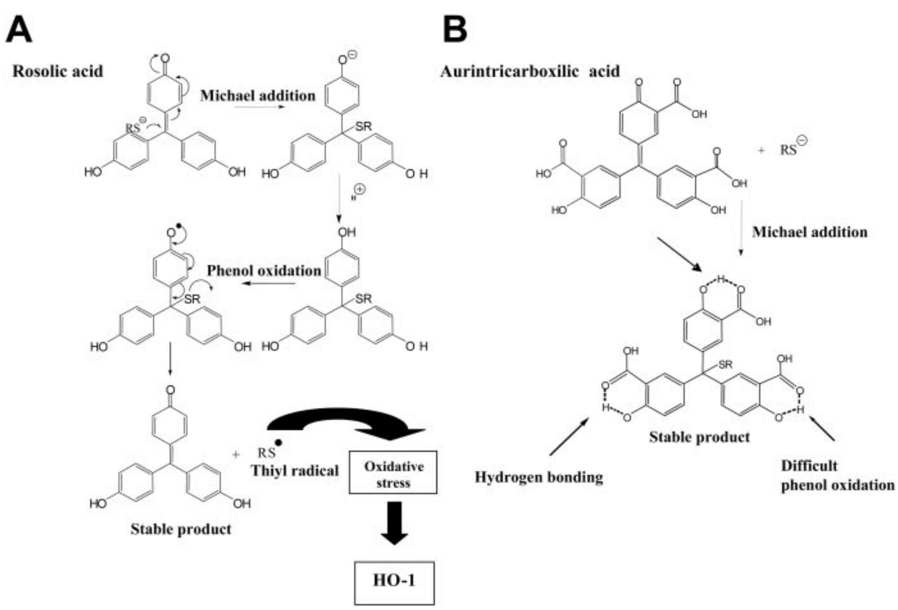

Fig. 8. Schematic diagram showing a possible mechanism leading to the formation of the thiyl radical and induction of HO-1. A, RA reacts with a nucleophile (RS^-) to form the product of the Michael addition. The product can easily undergo phenol oxidation (which can occur at any of the three equivalent positions) and then give rise to a thiyl radical ($RS^\cdot$). The thiyl radical would then propagate oxidative stress, leading to HO-1 induction. B, Michael addition reaction also occurs with aurintricarboxylic acid, but the formation of a hydrogen bonding between the carboxylic group and the phenol in the *ortho* position will confer the product a further stability, rendering the phenol oxidation and resulting thiyl radical formation difficult. More information about the diagram can be found in the text.

proteins, including transcriptional factors involved in the activation of the phase 2 response and, consequently, HO-1 induction. Of the complexes that could be obtained following reaction of nucleophiles with any of the three triphenylmethanes analyzed, it is likely that only the one containing RA would easily form the thiyl radical, whereas stereoelectronic constraints would prevent this event in the case of aurintricarboxylic acid or pararosaniline chloride. Thus, we suggest that the ability of RA to stimulate HO-1 expression is defined by its propensity to release thiyl radicals that react with thiol groups of proteins. Sustaining this hypothesis are the results showing that both coincubation of RA with the thiol donor *N*-acetylcysteine or increasing intracellular thiols prior to exposure to RA significantly reduce the activation of heme oxygenase. Martin et al. (2004) recently reported that carnosol, a phytochemical derived from the herb rosemary, increases HO-1 protein in cell cultures via activation of the MAPK cascade pathways. In our experiments, the use of p38, ERK, and JNK inhibitors did not convincingly support a major role of these signaling pathways in the activation of heme oxygenase by RA and 2′-hydroxychalcone, pointing to the involvement of other mechanisms used by these two natural compounds to induce HO-1.

It was also interesting to observe that RA continues to up-regulate HO-1 with prolonged incubation, whereas curcumin, caffeic acid phenethyl ester, and 2′-hydroxychalcone exerted only a transient effect. This phenomenon may be caused by a different capability of vascular endothelial cells to metabolize natural compounds and our data would indicate that curcumin, caffeic acid phenethyl ester, and 2′-hydroxychalcone are metabolized faster than RA. Since prolonged exposure to curcumin and caffeic acid phenethyl ester dramatically decreased cell viability, we speculate that the products of their metabolism may be toxic to endothelial cells. On the other hand, RA and 2′-hydroxychalcone seemed to be well tolerated. Moreover, cells pretreated with either RA or 2′-hydroxychalcone were more resistant to hydrogen peroxide-mediated cytotoxicity. The fact that aurintricarboxylic acid, which is effectively a negative control for RA because it does not induce HO-1, failed to protect cells against hydrogen peroxide is indicative of the importance of certain polyphenolic compounds to counteract oxidative stress by potently activating the HO-1 pathway.

In conclusion, we report on new natural plant constituents that can induce the antioxidant protein HO-1 and have investigated the pattern of induction as well as some major mechanisms involved in this effect. Despite the fact that up-regulation of HO-1 or manipulation of the HO-1 gene are sufficient to produce many beneficial outcomes in a variety of stressful conditions (Abraham et al., 1995; Panahian et al., 1999; Otterbein et al., 2003; Foresti et al., 2004), we do not exclude the possibility that these compounds will stimulate the expression of other defensive enzymes, as is already known in the case of chalcones (Dinkova-Kostova et al., 2001), and that cellular and tissue protection will be achieved via the concerted action of the multiple pathways being activated. The concept that regular consumption of specific types of food, especially fruits and vegetables, can stimulate the stress response suggests that sophisticated and effective therapies able to provide a constant and adequate barrier against the insurgence of many human diseases already exist and may be further developed for therapeutic purposes.

Note Added in Proof. While our manuscript was under the reviewing process, Alcaraz et al. (2004) reported that the anti-inflammatory effects of 3′,4′,5′,3,4,5-hexamethoxy-chalcone in RAW 264.7 cells are mediated by HO-1 activation, confirming that chalcones have the inherent ability to potently induce HO-1 in different cell types.

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