

**DIFFERENTIAL ACTIVATION OF HEME OXYGENASE-1
BY CHALCONES AND ROSOLIC ACID IN ENDOTHELIAL CELLS**

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Abbreviations: HO-1, heme oxygenase-1; NO, nitric oxide; CAPE, caffeic acid phenethyl ester; RA, rosolic acid; CUR, curcumin; ATA, aurointricarboxylic acid; 2'-OH-CAL, 2'-hydroxychalcone.

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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 anti-carcinogenic 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 μ M) 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 MAPK inhibitors, we determined that the ERK, p38 and JNK 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.

INTRODUCTION

An 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 NF- κ B, a transcriptional factor controlling the expression of pro-inflammatory 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. susceptible 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), signalling the up-regulation of phase 2 enzymes (Dinkova-Kostova et al., 2001). This hypothesis is strongly sustained by recent findings showing 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 (ARE) 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 protein (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 (Prester et al., 1995) inasmuch as: 1) it is induced by several agents that also evoke the phase 2 response (Prester et al., 1995; Motterlini et al., 2000b; Balogun et al., 2003b; Hill-Kapturczak et al., 2001); 2) its inducibility by phytochemicals is linked to the ARE element and the redox-sensitive Nrf2 (Prester 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; Motterlini et al., 2000a; Clark et al., 2000a; Clark et al., 2000b; Jeney et al., 2002; Choi and Otterbein, 2002; Morse and Choi, 2002; Foresti et al., 2004; Clark et al., 2003). We have already reported on the effect of curcumin and caffeic acid phenethyl ester to induce HO-1 in endothelial cells (Motterlini et al., 2000b), astrocytes (Scapagnini et al., 2002) and renal cells (Balogun et al., 2003b; Hill-Kapturczak et al., 2001; Balogun et al., 2003a). 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. (Somerville, NJ, USA). Rosolic acid (RA), aurintricarboxylic acid, *para*-rosaniline chloride, curcumin (CUR), caffeic acid phenethyl ester (CAPE) and all other reagents were from Sigma-Aldrich Company Ltd. (Poole, Dorset) 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 previously described (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, USA) 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, penicillin (100 U/ml) and streptomycin (0.1 mg/ml). Cells were kept at 37 °C in a humidified atmosphere of air and 5% CO₂. 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 sulphhydryl 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 was assessed using PD098059 (ERK inhibitor, 10 μM), SB203580 (p38 inhibitor, 10 μM) or SP600125 (JNK inhibitor, 10 μM). 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 pre-treated with polyphenols for 6 h followed by 2 h exposure to hydrogen peroxide (3 mM).

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, New Jersey, USA). 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 was terminated by the addition of 1 ml of chloroform. After vigorous vortex and centrifugation, the extracted bilirubin in the chloroform layer was measured by the difference in absorbance between 464 and 530 nm ($\epsilon=40 \text{ mM}^{-1} \text{ cm}^{-1}$).

Western blot analysis for heme oxygenase-1 (HO-1). Samples of endothelial cells treated for the heme oxygenase activity assay were also analyzed by Western immunoblot technique as previously described (Foresti et al., 1997). Briefly, an equal amount of protein (30 μg /well) from each sample was separated by SDS-polyacrylamide gel electrophoresis, transferred overnight to nitrocellulose membranes and the non-specific binding of antibodies was blocked with 3% non-fat dried milk in PBS. Membranes were then probed with a polyclonal rabbit anti-HO-1 antibody (Stressgen, Victoria, 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 amplified alkaline phosphatase kit from Sigma (Extra-3A).

Cell viability assay. Cell viability was performed by an Alamar Blue assay according to manufacturers' instructions (Serotec, UK) (Motterlini et al., 2000b). The assay is based on detection of metabolic activity of living cells using a redox indicator which changes from oxidized (blue) to reduced (red) form. The intensity of the red colour is proportional to the

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viability of cells which is calculated as difference in absorbance between 570 nm and 600 nm and expressed as percentage of control.

Statistical analysis. Differences among the groups were analyzed using the t test or one-way ANOVA combined with the Bonferroni test whereas. Values were expressed as a mean \pm 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; Scapagnini et al., 2002; Balogun et al., 2003b; Hill-Kapturczak et al., 2001), 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 incubation (Fig. 1). However, despite displaying 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 at concentrations of 5 or 10 μM had little effect on heme oxygenase activity, but concentrations of 20 or 30 μM produced a several-fold increase (Fig. 1A). In contrast, 2,2'-hydroxychalcone altered endothelial heme oxygenase 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 behaviour inasmuch as 5, 10 and 30 μM caused a similar three-fold increase in heme oxygenase activity, while only at 20 μM the activity was approximately eight-fold higher than control (Fig. 1C). It has to be noted that at 50 μM all chalcones exhibited increased cytotoxicity (data not shown).

Effect of rosolic acid (RA), aurintricarboxylic acid and para-rosaniline chloride on heme oxygenase activity and HO-1 protein expression in vascular endothelial cells. Next, we tested

another series of compounds which still contain phenyl groups but are quite different from chalcones. Fig. 2 shows the chemical structure of RA, aurintricarboxylic acid and *para*-rosaniline chloride. Rosolic acid is a chemical constituent derived from the rhizome of *Plantago 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 chains, which are hydroxyl groups for RA, carboxylic groups for aurintricarboxylic acid and amines for *para*-rosaniline 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 μ M RA, whereas 15 μ M aurintricarboxylic acid or *para*-rosaniline 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 was the consequence of HO-1 induction. For comparison, the expression of HO-1 was also analysed following treatment of cells with 15 μ M curcumin, caffeic acid phenethyl ester (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, while 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 examined whether both endogenous and exogenous thiols were involved in the induction of HO-1 mediated by RA. We found that pre-incubation 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 μ M RA (Fig. 5A). Note that after the pre-treatment, N-acetylcysteine was washed out prior to exposure to RA. Similarly, co-incubation 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 cells viability after exposure to rosolic acid (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. 6A and B). In contrast, 15 μM 2'-hydroxychalcone or RA did not produce any evident cytotoxicity (Fig. 6A and B). This finding is of particular relevance for RA, since the compound continued to 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 appear 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 pre-treated 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 Figure 7, exposure of cells to

hydrogen peroxide resulted in a substantial loss in cell viability and pre-treatment with either RA or 2'-hydroxychalcone significantly attenuated H₂O₂-mediated cytotoxicity. Interestingly, ATA failed to exert any cytoprotective effect against H₂O₂ 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 1, 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, while a 15 % decrease was observed for 2'-hydroxychalcone. Lastly, the increase in heme oxygenase activity elicited by RA was reduced by 18 % when the JNK pathway was blocked, while 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 signalling mechanisms to induce HO-1. In addition, they also suggest that these specific plant constituents depend only marginally on the MAPK pathway in order to up-regulate HO-1 and that other mechanisms are probably involved.

DISCUSSION

Mammalian cells have developed several defence 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 (Talalay and Fahey, 2001; Dinkova-Kostova et al., 2001) and that 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 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; Foresti et al., 2004; Motterlini et al., 2003), was recently shown to be up-regulated by plant constituents including curcumin (Motterlini et al., 2000b; Scapagnini et al., 2002; Balogun et al., 2003b; Balogun et al., 2003a; Hill-Kapturczak et al., 2001), 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 the 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, 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 enhance noticeably 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 μ M 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 *para*-rosaniline 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: *para*-rosaniline chloride > aurintricarboxylic acid > RA. As a consequence, one would expect that of the three triphenylmethanes, *para*-rosaniline would be the stronger inducer, followed by aurintricarboxylic acid and RA. To reconcile our results with the reactivity of these compounds and to gain insights into the mechanisms implicated, it helps to consider the

chemical behaviour 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. Thiyl radical could also interact with other radicals and/or sulphhydryl residues of 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, while stereoelectronic constraints would prevent this event in the case of aurintricarboxylic acid or *para*-rosaniline 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 co-incubation 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 and co-workers 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 (Martin et al., 2004). 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 appeared to be well tolerated. Moreover, cells pre-treated 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 as 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 by potentially activate 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 (Foresti et al., 2004; Otterbein et al., 2003; Abraham et al., 1995; Panahian et al., 1999), we do not exclude that these compounds will stimulate the expression of other defensive enzymes, as 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, and 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.

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FOOTNOTES

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NOTE ADDED IN PROOF. While our manuscript was under the reviewing process, Alcaraz and co-workers reported that the ant-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 (Alcaraz et al., 2004).

FIGURE LEGENDS

Figure 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 μ M) 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 \pm S.E.M. of 5-6 independent experiments. * $P < 0.05$ vs. control.

Figure 2. Chemical structure of triphenylmethanes. Rosolic acid (RA), aurintricarboxylic acid and *para*-rosaniline 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.

Figure 3. Effect of short or prolonged incubation with rosolic acid on endothelial heme oxygenase activity. Endothelial cells were exposed to 15 or 30 μ M rosolic acid (RA) for 6 (**A**) or 18 h (**B**) and analyzed for heme oxygenase activity. Data are expressed as the mean \pm SEM of 5 independent experiments. * $P < 0.05$ vs. control.

Figure 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 μ M) for 6 (**A**) or 18 h (**B**) and HO-1 protein expression evaluated using appropriate antibodies as described in Materials and Methods. Western blot is representative of

three independent experiments. CON = control; CUR = curcumin; CAPE = caffeic acid phenethyl ester; 2'OH-CAL = 2'-hydroxychalcone; RA = rosolic acid.

Figure 5. Effect of increased intra- and extracellular thiols on the stimulation of heme oxygenase activity by rosolic acid. (A) Cells were pre-treated with 2.5 mM N-acetylcysteine (18 h) to increase intracellular thiols content prior to exposure to 15 μ M rosolic acid (RA) for 6 h. Heme oxygenase activity was measured at the end of the incubation as described in Materials and Methods. (B) Cells were co-incubated with N-acetylcysteine and rosolic acid for 18 h for heme oxygenase activity measurements. Data are expressed as the mean \pm SEM of 4 independent experiments. * $P < 0.05$ vs. control. $\dagger P < 0.05$ vs. RA alone.

Figure 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 μ M) and cell viability was measured spectrophotometrically using an Alamar Blue assay according to manufacturers' instructions. Data are expressed as the mean \pm SEM of 5 independent experiments. * $P < 0.05$ vs. control (CON). RA = rosolic acid, CUR = curcumin, CAPE = caffeic acid phenethyl ester and 2'OH-CAL = 2'-hydroxychalcone.

Figure 7. Protective effects of rosolic acid and 2'-hydroxychalcone against oxidative stress. Endothelial cells were initially pre-incubated for 6 hours in the presence of 15 μ M rosolic acid (RA) or 2'-hydroxychalcone (2'OH-CAL); aurintricarboxylic acid (ATA), which does not induce HO-1, was used as a negative control for RA. Then the medium was removed and cells were exposed for 2 h to 3 mM hydrogen peroxide (H_2O_2). Cell viability was measured spectrophotometrically using an Alamar Blue assay according to manufacturers' instructions.

Data are expressed as the mean \pm SEM of 5 independent experiments. * $P < 0.05$ vs. control (CON); † $P < 0.05$ vs. H₂O₂ alone.

Figure 8. Schematic diagram showing a possible mechanism leading to the formation of the thiyl radical and induction of HO-1. (A) Rosolic acid (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 give then rise to a thiyl radical (RS[•]). The thiyl radical would then propagate oxidative stress leading to HO-1 induction. (B) The Michael addition reaction occurs also 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 the consequent thiyl radical formation difficult. More information about the diagram can be found in the text.

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 μ M rosolic acid (RA), aurintricarboxylic acid or *para*-rosaniline chloride. Cell viability was also measured after incubation of the triphenylmethanes for 6h and no significant cytotoxicity was observed. Data are expressed as the mean \pm SEM of 5 independent experiments. * $P < 0.05$ vs. control.

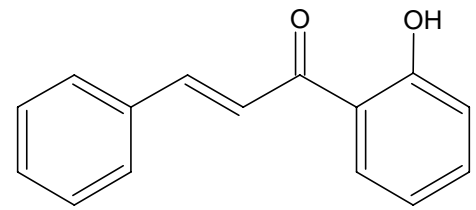
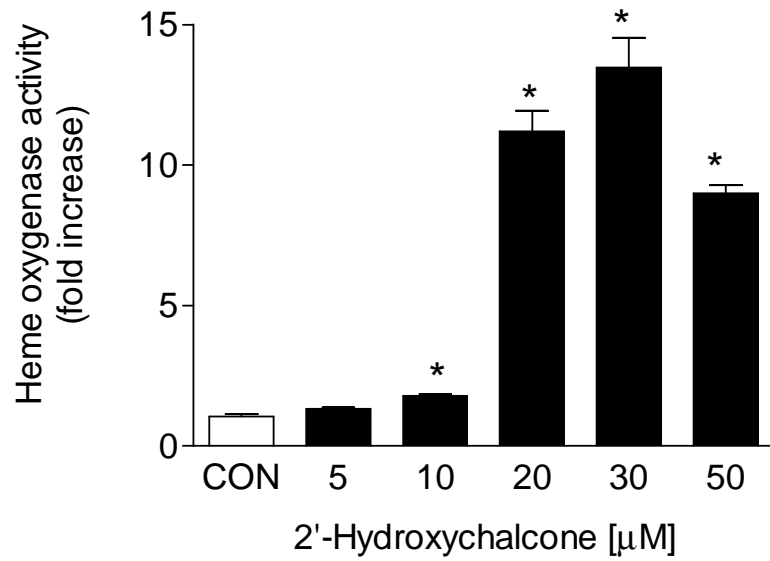
	Heme oxygenase activity (pmol bilirubin/mg prot/h)	Cell viability (% control)
Control (6 h)	453 \pm 43	100 \pm 2.9
Rosolic acid (6 h)	2676 \pm 117*	94 \pm 1.7
Aurin tricarboxylic acid (6 h)	332 \pm 70	98 \pm 2.3
Para-rosaniline chloride (6 h)	375 \pm 64	96 \pm 3.1

Table 2. Effect of MAPK inhibitors on the increase of heme oxygenase activity mediated by rosolic acid and 2'-hydroxychalcone. Endothelial cells were pre-incubated for 15 min with PD098059 (ERK inhibitor, 10 μ M), SB203580 (p38 inhibitor, 10 μ M) or SP600125 (JNK inhibitor, 10 μ M) prior to exposure for 6 h to rosolic acid (RA, 15 μ M) or 2'-hydroxychalcone (2'-OH-CAL, 15 μ M). Heme oxygenase activity was determined as described in 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 prot/h)			
	-	+ PD098,059	+ SB203580	+ SP600125
RA	100 %	86 \pm 0.01 %	100 \pm 0.01 %	72 \pm 0.17 %
2'-OH-CAL	100 %	80 \pm 0.02 %	85 \pm 0.02 %	127 \pm 0.05 %

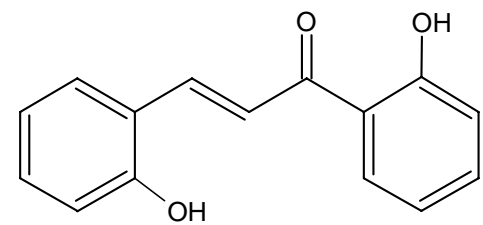
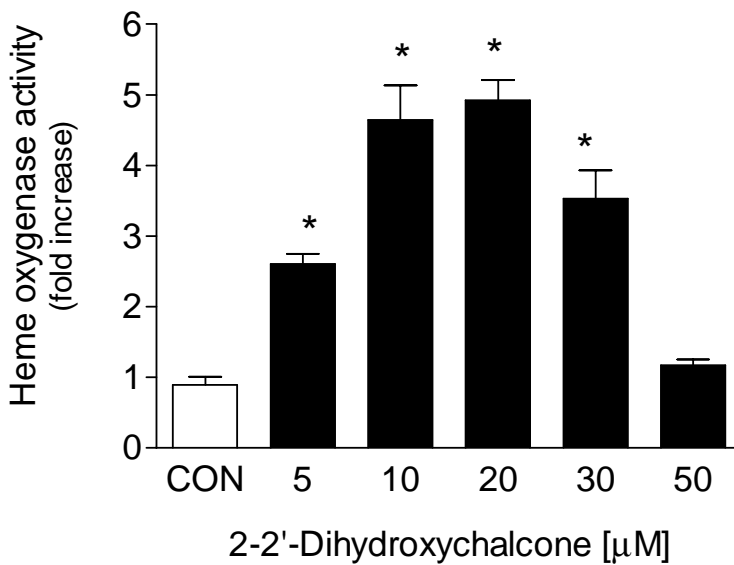
Fig. 1

A



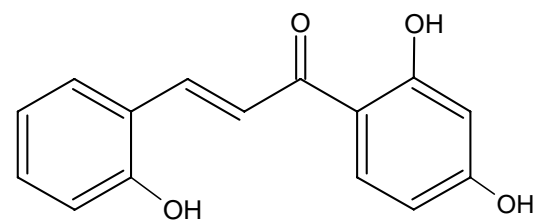
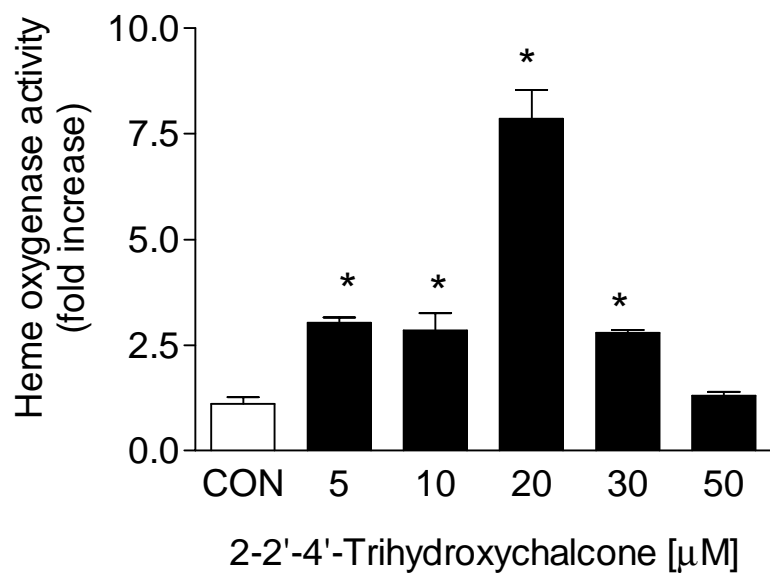
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B

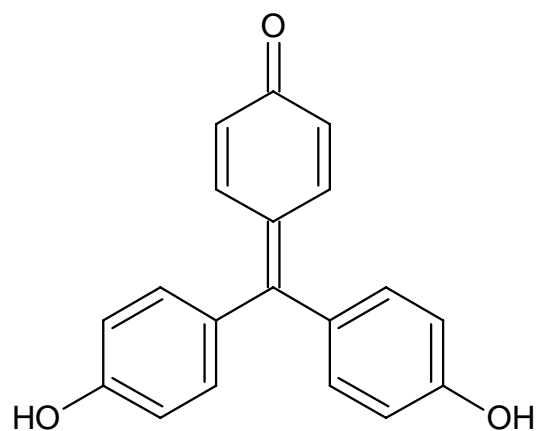


2,2'-Dihydroxychalcone

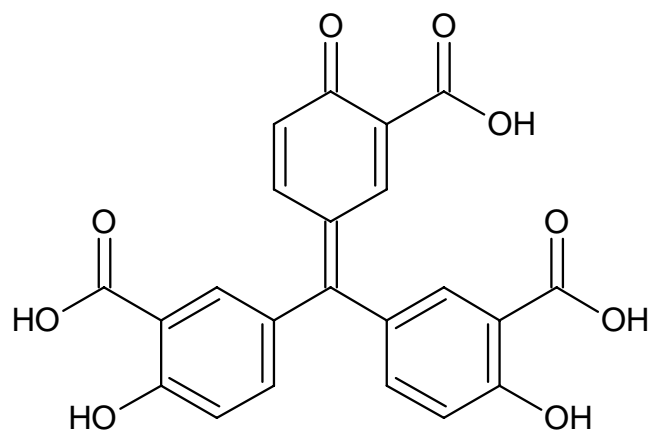
C



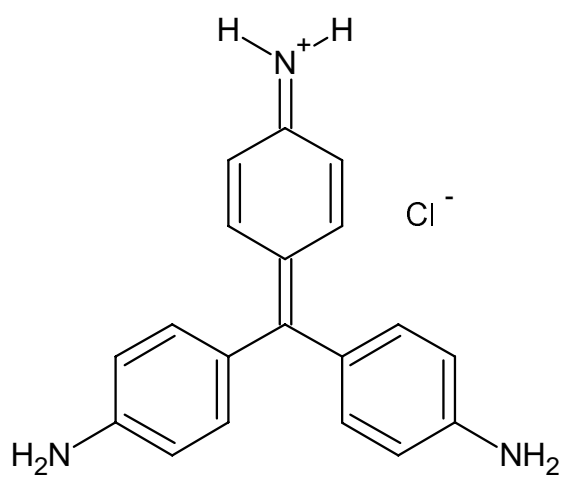
2,2',4'-Trihydroxychalcone



ROSOLIC ACID



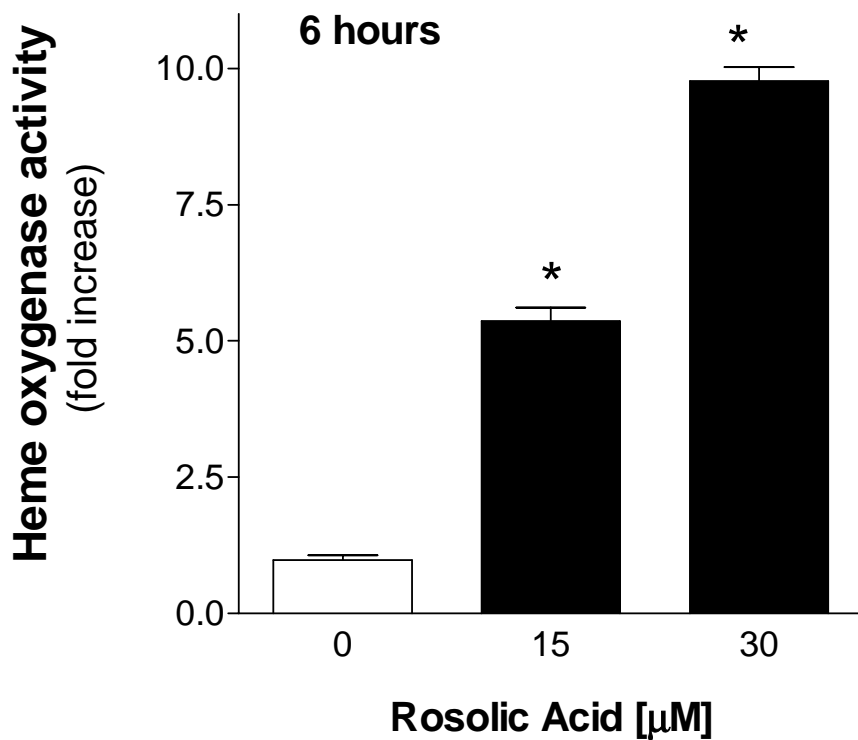
AURINTRICARBOXYLIC ACID



PARA-ROSANILINE CHLORIDE

Fig. 3

A



B

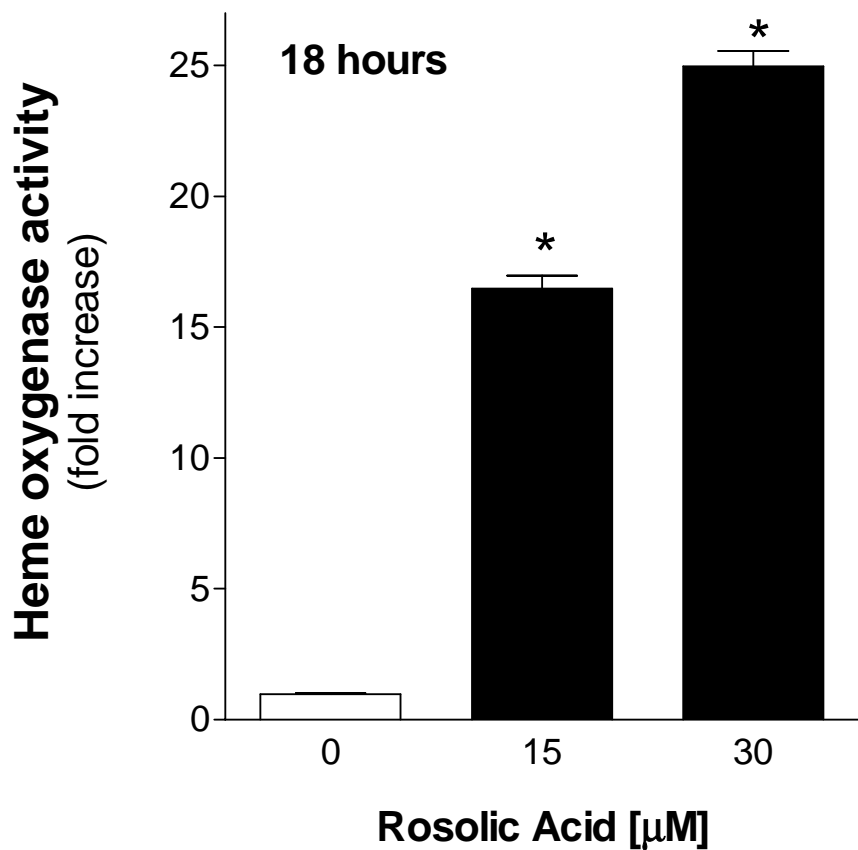


Fig. 4

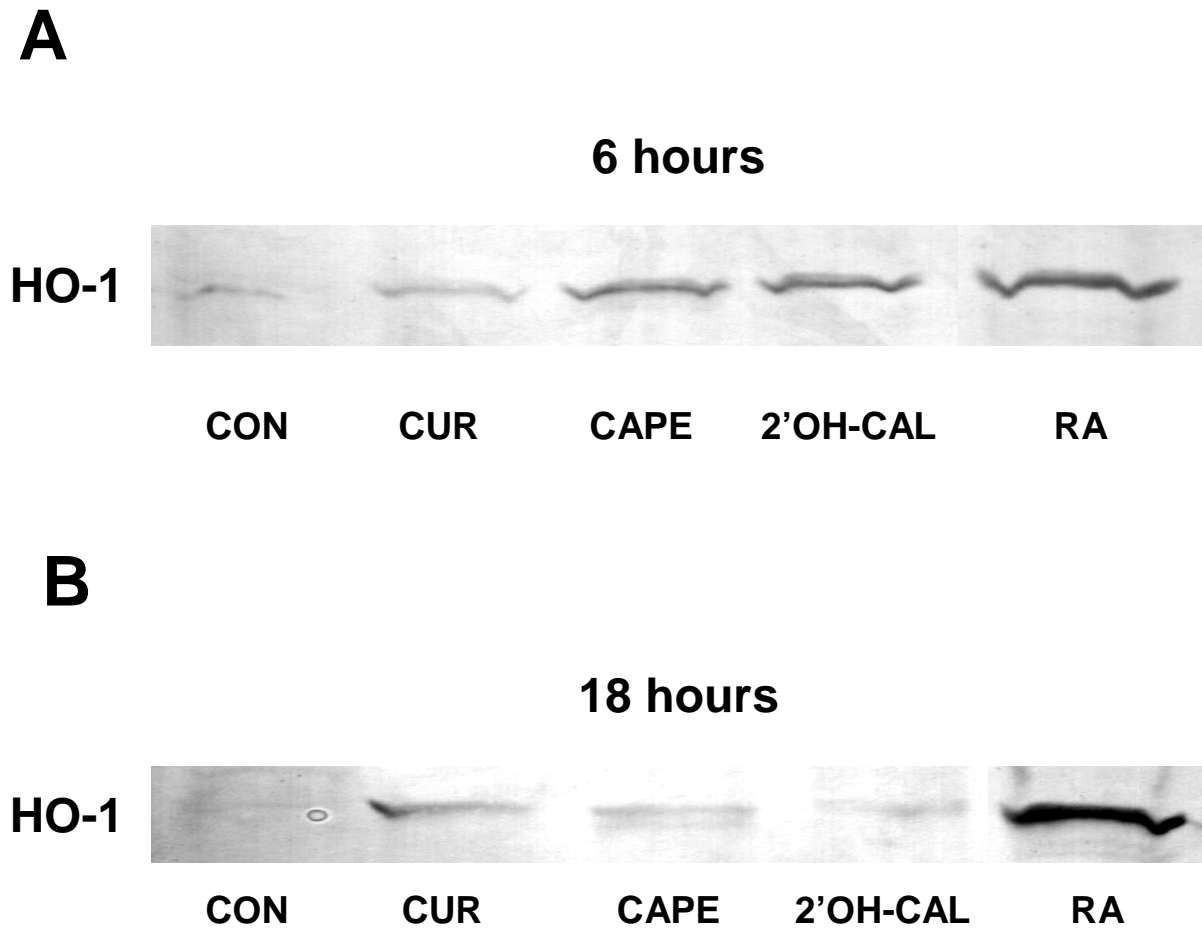


Fig. 5

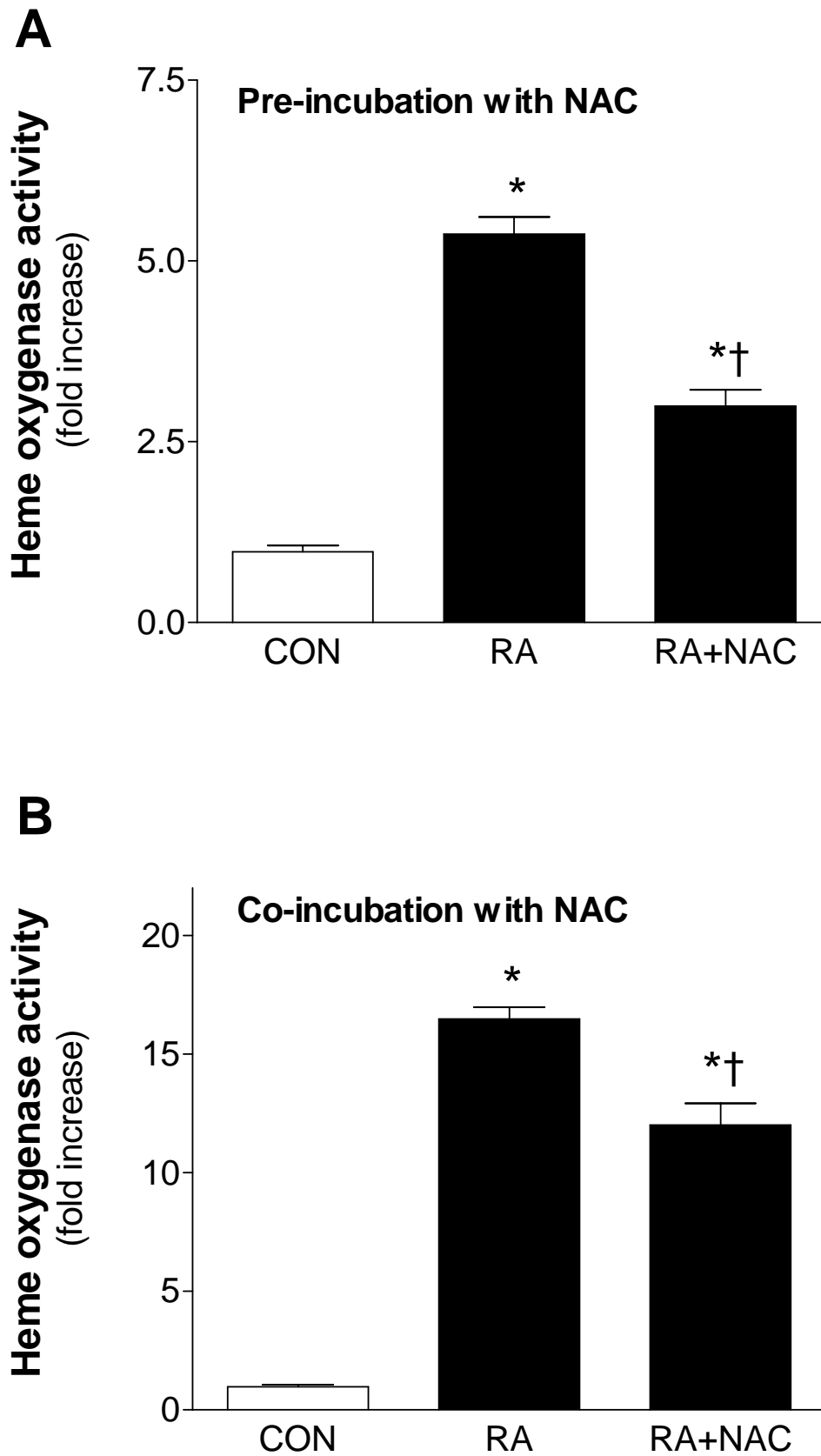
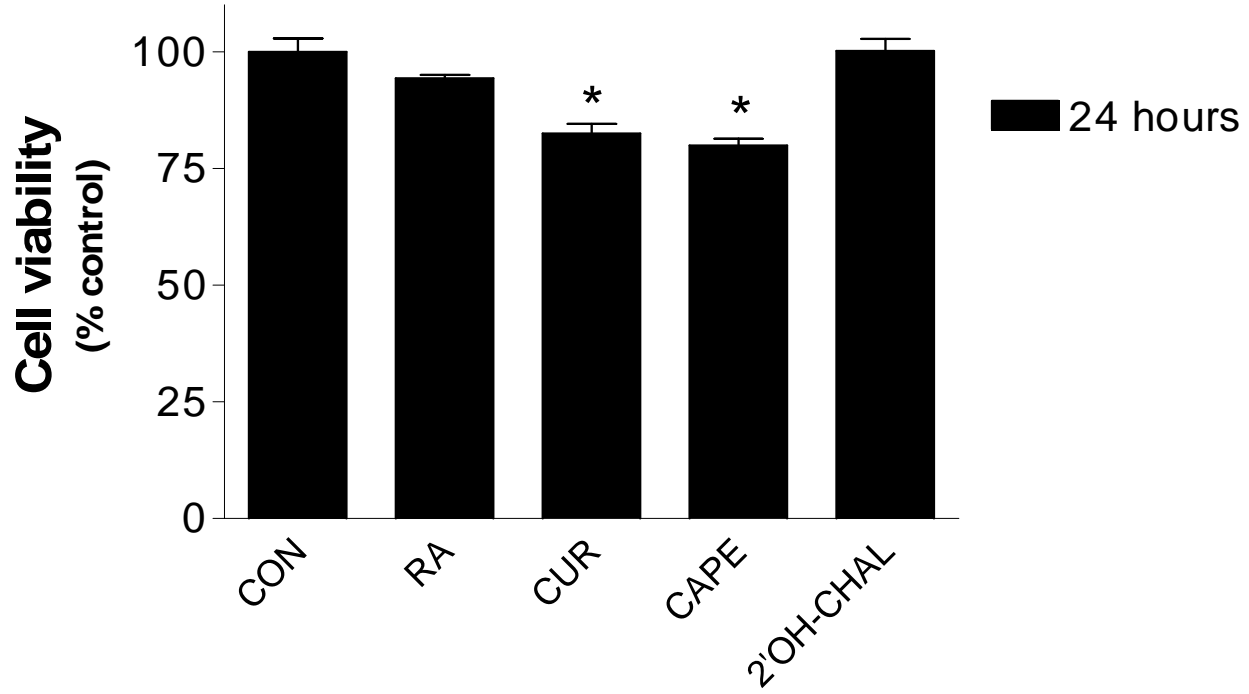


Fig. 6

A



B

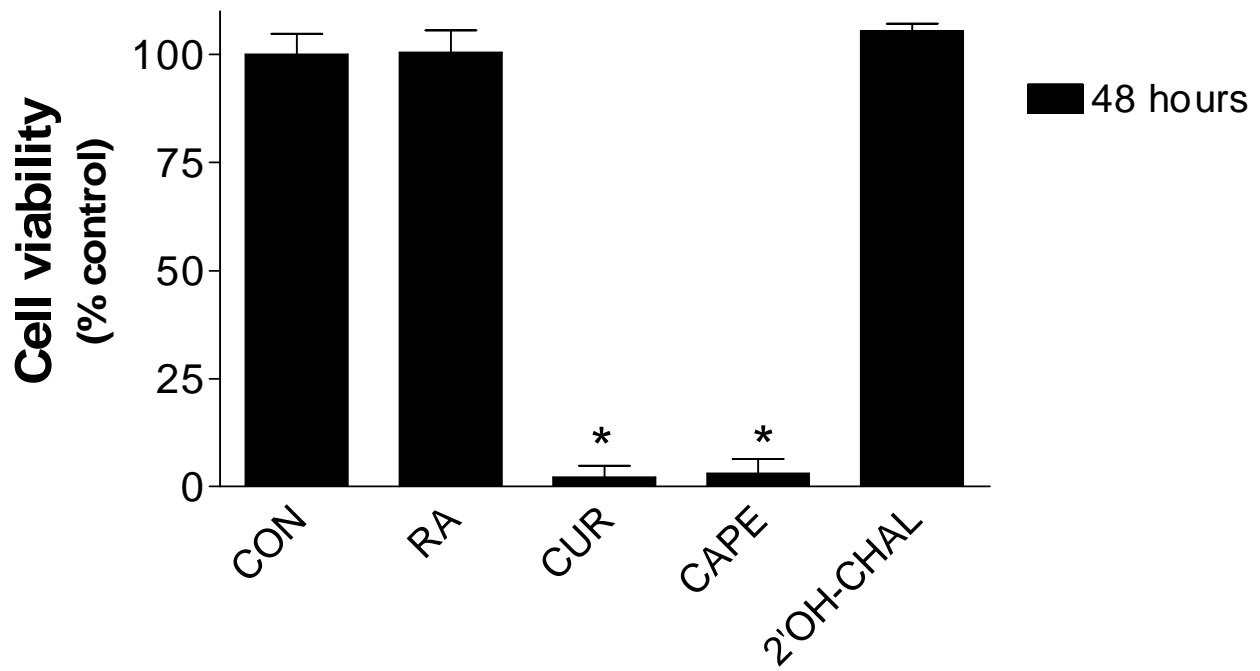


Fig. 7

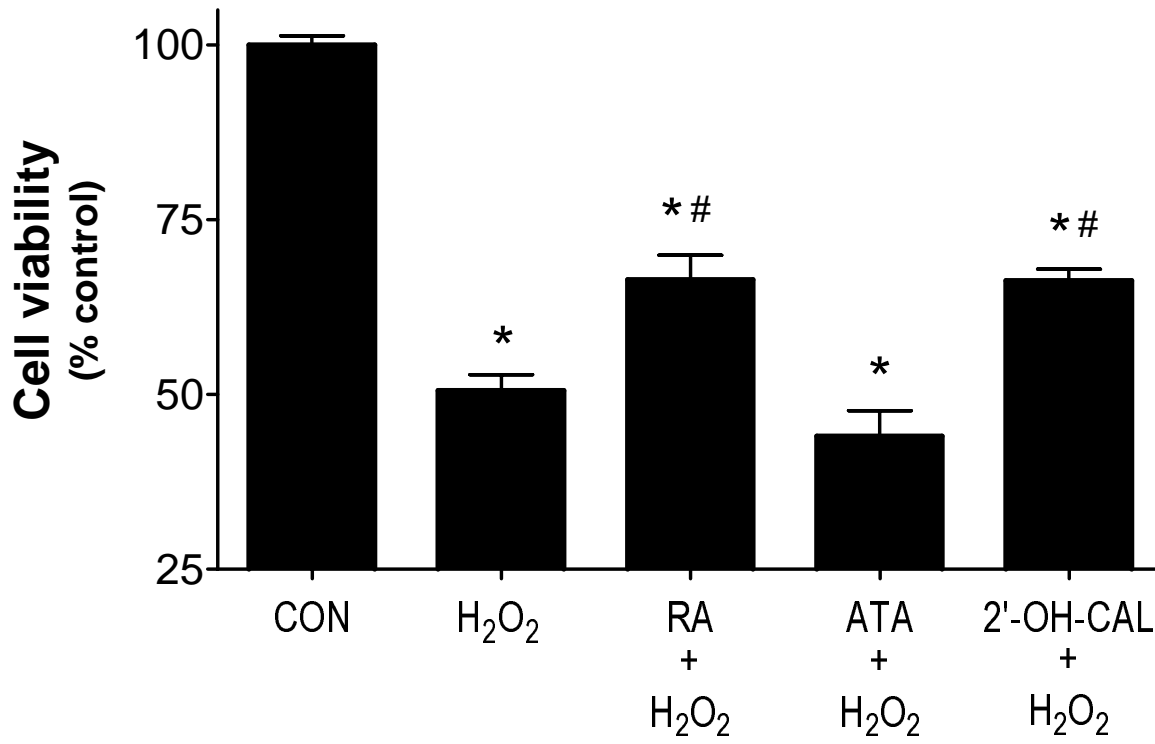
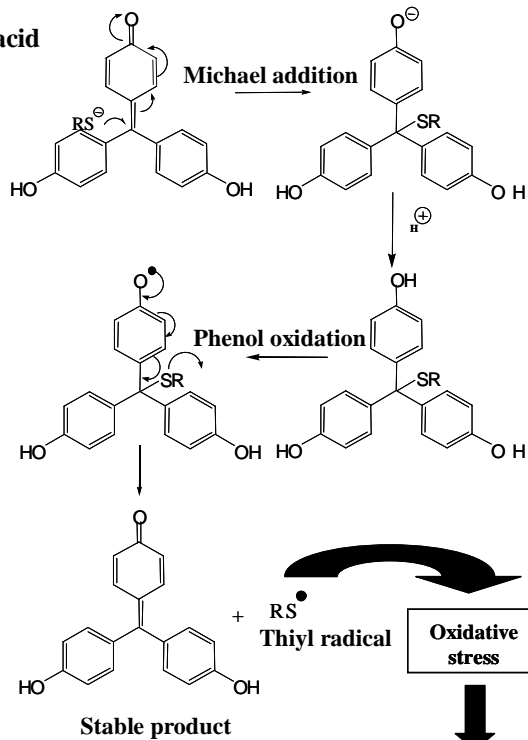


Fig. 8

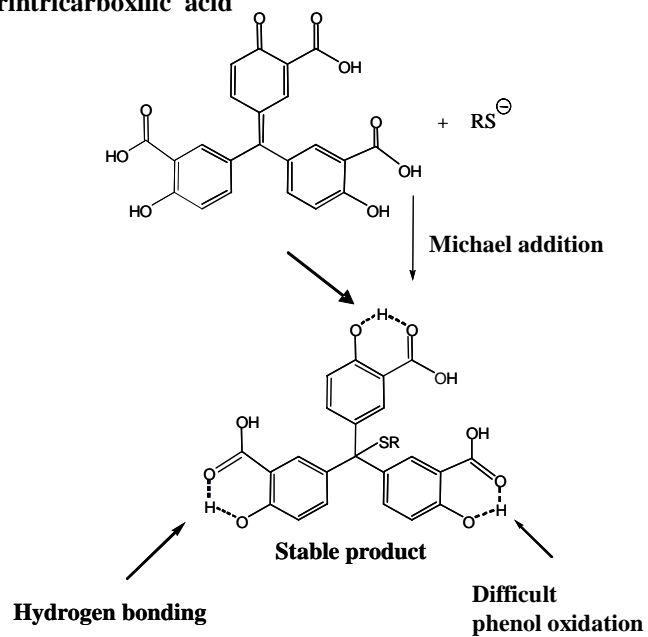
A

Rosolic acid



B

Aurintricarboxylic acid



HO-1