Evidence for the association of peroxidases with the antioxidant effect of \textit{p}-coumaric acid in endothelial cells exposed to high glucose plus arachidonic acid

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Although many plant-derived phenolic compounds display antioxidant effects in biological systems, their mechanism of action remains controversial. In this study, the mechanism by which \textit{p}-coumaric acid (\textit{p}-CA) performs its antioxidant action was investigated in bovine aortic endothelial cells under oxidative stress due to high levels of glucose (HG) and arachidonic acid (AA), a free fatty acid. \textit{p}-CA prevented lipid peroxidation and cell death due to HG+AA without affecting the production of reactive oxygen species. The antioxidant effect of \textit{p}-CA was not decreased by buthionine-(5,R)-sulfoximine, an inhibitor of cellular GSH synthesis. In contrast, pretreatment with \textit{p}-CA caused the induction of peroxidases that decomposed \textit{p}-butyl hydroperoxide in a \textit{p}-CA-dependent manner. Furthermore, the antioxidant effect of \textit{p}-CA was significantly mitigated by methimazole, which was shown to inhibit the catalytic activity of \textit{p}-CA peroxidases' in vitro. Therefore, it is suggested that the induction of these previously unidentified \textit{p}-CA peroxidases' is responsible for the antioxidant effect of \textit{p}-CA. [BMB reports 2009; 42(9): 561-567]

\textbf{INTRODUCTION}

The so-called French paradox is the observation that French people suffer an unpredictably low incidence of cardiovascular disease despite having a diet rich in calories and fat (1). The reason for this discrepancy has been attributed to the co-consumption of wine containing high amounts of phenolic compounds, which possess potent antioxidant activity (2, 3). The vascular protection provided by phenolic compounds has been supported by numerous experimental and epidemiological studies (4-6), but the underlying mechanism for their antioxidant effects still remains controversial. One view is that many phenolic compounds exhibit antioxidant effects by chemically scavenging reactive oxygen species and free radicals in biological systems (7, 8). Alternatively, phenolic compounds have been suggested to enhance endogenous defense mechanisms, including antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase (9). In addition, phenolic compounds are known to stimulate antioxidant response element (ARE) that regulates the gene expression of enzymes involved in phase II metabolism of xenobiotics and antioxidant defense (10, 11).

Oxidative stress induced by metabolic overload appears to be a common mechanism of diabetes and cardiovascular disease (12, 13). Free fatty acids are generally kept at low micromolar concentrations in plasma (14), but their concentration increases to the milimolar range in diabetic patients (15). When glycolysis and β-oxidation of free fatty acids produce an excessive amount of acetyl CoA, its influx into the citric acid cycle can generate an amount of mitochondrial NADH in excess of that used during electron transport (12, 16). This condition leads to the overproduction of reactive oxygen species (ROS) at the mitochondrial level, which in turn stimulates NADPH oxidase and other enzymes associated with oxidative stress and inflammation (17, 18). In support of this, high levels of glucose (HG) and arachidonic acid (AA), similar to the serum conditions of diabetic patients, appeared to synergistically induce oxidative stress in cultured endothelial cells (19). This study demonstrated that laminar shear stress, a hemodynamic force generated by blood flow, enhances the antioxidant capacity of endothelial cells, thereby attenuating oxidative stress caused by cardiovascular risk factors (19). Therefore it was suggested that a certain pharmacological approach mimicking the effects of laminar shear stress may improve endothelial function and vascular health.

The aim of this study was therefore to evaluate a pharmacological approach that uses natural antioxidants to attenuate oxidative stress in bovine aortic endothelial cells (BAECs) exposed to HG+AA. Cinnamic acid derivatives were chosen as representative phenolic compounds due to their natural abundance and chemical simplicity. Among these, \textit{p}-coumaric acid (\textit{p}-CA) was used for the investigation of action mechanism as it showed the strongest antioxidant activity. The results found the induction...
of peroxidases that use \( p \)-CA as a co-substrate may be associated with the antioxidant effect of \( p \)-CA, providing a novel clue for the vascular protective effects of dietary phenolic antioxidants.

**RESULTS**

In the first experiment, cultured BAECs were exposed to different concentrations of glucose and AA in combination for 48 h, simulating serum conditions in diabetic or obese patients. Treatment of cells with HG and AA induced lipid peroxidation in a synergistic manner, as monitored by 2-thiobarbituric acid-reactive substances (TBARS) (Fig. 1A). Cell viability was also decreased following HG and AA treatment (Fig. 1B). Following this, numerous cinnamic acid derivatives were com-

**Fig. 1.** \( p \)-CA attenuates oxidative stress induced by HG+AA in BAECs. In (A, B) BAECs were treated for 48 h in normal glucose (NG, 5.0 mM glucose) or HG medium (30 mM glucose) containing varied concentrations of AA. In (C, D) cells were pretreated with varied concentrations of \( p \)-CA for 30 min and then maintained for 48 h in normal growth medium or medium containing HG + 40 \( \mu \)M AA. Lipid peroxidation was measured by TBARS assay (A, C) and cell viability was determined by MTT assay (B, D). Data represent mean ± SEM (n = 3). Bars not sharing the same letter are significantly different from each other (P < 0.05).

**Fig. 2.** Evidence against the antioxidant effects of \( p \)-CA in endothelial cells under HG+AA conditions being associated with direct scavenging of ROS or enhancement of cellular GSH synthesis. In (A) ROS formation was detected using DHR 123, which fluoresces upon oxidation to rhodamine 123. The newly formed rhodamine 123 was extracted from cells and quantified fluorophotometrically. Representative fluorescent images of cells are shown. In (B-D), cells were pretreated with 100 \( \mu \)M BSO for 30 min and 80 \( \mu \)M \( p \)-CA for 30 min in a sequential manner. The cells were maintained in normal growth medium or HG+AA medium for 48 h. Lipid peroxidation was measured by TBARS assay (B). GSH and/or GSSG contents were quantified using an enzymatic cycling assay method (C, D). Data represent mean ± SEM (n = 3). Bars not sharing the same letter are significantly different from each other (P < 0.05).
pared for their potential preventive effects on lipid peroxidation and cell death caused by HG+AA treatment (Online supplement 1). Among the tested compounds, p-CA was most effective at preventing lipid peroxidation and loss of cell viability after HG+AA treatment. As shown in Fig. 1C and D, p-CA inhibited lipid peroxidation and rescued cell viability in a dose-dependent manner.

To elucidate the action mechanism of p-CA, its potential effects on prooxidant and antioxidant levels were examined in cells. HG+AA treatment increased intracellular ROS formation as detected by dihydrorhodamine 123 (DHR123), which fluoresces upon oxidation to rhodamine 123 (Fig. 2A). However, p-CA did not have a significant effect on the level of ROS in cells treated with HG+AA (Fig. 2A). Therefore, inhibition of ROS production or direct chemical scavenging of ROS is not considered to be the major antioxidant mechanism of p-CA.

Given this result, an experiment was conducted to determine if the antioxidant effect of p-CA depends on the intracellular pool of GSH, a primary antioxidant in cells. DL-buthionine-(S, R)-sulfoximine (BSO) was used to inhibit glutamylcysteine ligase, the rate-limiting enzyme in the GSH biosynthetic pathway. BSO depleted intracellular GSH (+GSSG) (Fig. 2C) and enhanced HG+AA-induced lipid peroxidation (Fig. 2B). However, BSO treatment had no significant effect on the antioxidant activity of p-CA (Fig. 2B). Therefore, p-CA is considered capable of inhibiting lipid peroxidation independent of its effect on GSH metabolism. Noteworthy is that p-CA enhanced the oxidation of GSH to GSSG upon HG+AA treatment (Fig. 2D), in contrast to its inhibition of lipid peroxidation.

Removal of lipid hydroperoxide is another plausible mechanism through which antioxidants could exert their effects. Additional experiments were therefore conducted to examine if any peroxidases are involved in the antioxidant mechanism of p-CA in cells. Firstly, the antioxidant activity of p-CA against HG+AA was examined in the absence or presence of the peroxidase inhibitor, methimazole. As shown in Fig. 3A and B, methimazole appeared to weaken the antioxidant and cytoprotective effects of p-CA in a dose-dependent manner. Therefore, the results may indicate that certain peroxidases are involved in the antioxidant mechanism of p-CA. Although methimazole itself tended to inhibit lipid peroxidation caused by HG+AA, here it showed rather cytotoxic effects.

Further experiments were performed to examine if endothelial cells express peroxidases capable of using p-CA as a co-substrate. Cell lysates were incubated with t-butyl hydroperoxide (t-BOOH) in the absence or presence of p-CA. As shown in Fig. 3C, lysates from cells treated with p-CA showed a significant rate of p-CA-dependent t-BOOH breakdown, compared to

Fig. 3. Evidence for the association of peroxidases with the antioxidant effects of p-CA in endothelial cells under HG+AA conditions. In (A) and (B), BAECs were pretreated with 100 μM methimazole, a peroxidase inhibitor, for 30 min and then 80 μM p-CA for 30 min. The cells were maintained in normal growth medium or HG+AA medium (30 mM glucose + 40 μM AA) for 48 h. Lipid peroxidation was measured by TBARS assay (A) and cell viability was determined by MTT assay (B). In C and D, BAECs were pretreated with 40 or 80 μM p-CA for 30 min and then maintained in normal growth medium or HG+AA medium for 48 h. Lysates from these cells were used to assay for peroxidase activity using 200 μM t-BOOH as the substrate and in the absence or presence of 100 μM p-CA (C) or 10 μM GSH (D) as the co-substrate. Data represent mean ± SEM (n=3). Bars not sharing the same letter are significantly different from each other (P < 0.05).
control cells which did not. The results indicate that peroxidases capable of metabolizing p-CA are not normally expressed in endothelial cells, but are inducible when p-CA is available as a substrate. In contrast, the activities of GSH-dependent peroxidases were not affected by p-CA treatment (Fig. 3D).

Using lysates of p-CA-treated cells, in vitro experiments were performed to characterize the peroxidases. As shown in Fig. 4, the catalytic activity of ‘p-CA-dependent peroxidases’ increased linearly with the concentration of p-CA, in the range of 0.1-0.6 mM. Moreover, its catalytic activity in vitro was inhibited by methimazole in a dose-dependent manner (Fig. 4).

**DISCUSSION**

The present study demonstrated a pharmacological approach using p-CA or a similar phenolic compound can potentially attenuate oxidative stress due to metabolic imbalance in endothelial cells. p-CA exhibited potent antioxidant activity against lipid peroxidation caused by HG+AA, which was not surprising since previous studies have shown its antioxidant effects in various in vitro and in vivo models (4, 7, 8). However, that the mechanism by which p-CA performs its antioxidant activity involves peroxidases was hardly expected.

The prevention of HG+AA-induced lipid peroxidation and loss of endothelial cell viability by p-CA (Fig. 1C, D) could not be attributed to direct scavenging of ROS (Fig. 2A) or to enhancement of cellular GSH synthesis (Fig. 2B). An association of GSH peroxidases was also excluded because their enzyme activity remains unaffected by treatment with p-CA or HG+AA (Fig. 3D). Instead, the induction of ‘p-CA peroxidases’ that use p-CA as a co-substrate was suggested as a potential mechanism through which the antioxidant effects of p-CA are mediated (Fig. 3C). In support of this, the antioxidant activity of p-CA was significantly mitigated by methimazole, a specific peroxidase inhibitor (Fig. 3A, B, Fig. 4).

Peroxidases are the enzymes that catalyze reduction of hydrogen peroxide or organic hydroperoxide at the expense of co-substrates. Until now, the phenols/peroxidase reaction has been generally regarded as a mechanism by which the prooxidant activity of natural polyphenols is mediated in animal cells (20). Indeed, phenoxyl radical formed by myeloperoxidase induces lipid peroxidation and protein cross-linking (21, 22). However, the current study suggests that peroxidases might be involved in the antioxidant, not prooxidant, activity of phenolic compounds in endothelial cells under oxidative stress. This notion is consistent with previous in vitro observations that peroxidases mediate the prooxidant or antioxidant activity of phenolic compounds, depending on the chemical environment (23, 24).

As a peroxidase inhibitor, the effects of methimazole on lipid peroxidation are of interest. Methimazole increased and decreased lipid peroxidation caused by HG+AA treatment in the presence and absence of p-CA, respectively (Fig. 3A). Methimazole may have inhibited lipid peroxidation in the presence of p-CA by inhibiting the action of ‘p-CA-peroxidases’, that are induced by p-CA and decrease lipid peroxidation, as mentioned above. In contrast, methimazole may have decreased lipid peroxidation in the absence of p-CA by inhibiting other constitutively expressed peroxidases that increase lipid peroxidation. In this regard, it can be assumed that different peroxidases with either antioxidant or prooxidant effects, both of which can be inhibited by methimazole, are present.

One may argue that if ‘p-CA peroxidases’ indeed contributed to antioxidant protection, they must have lowered the level of ROS upon HG+AA treatment. This would be the case if hydrogen peroxide or other ROS were the optimal substrates for ‘p-CA peroxidases’. However, there are many peroxidases whose optimal substrates are not ROS but organic hydroperoxides like lipid peroxides. At this moment, ‘p-CA peroxidases’ are assumed to have lipid peroxides as their optimal substrate and remove them without affecting ROS levels.

It would be questionable if p-CA prevented cell death by simply inhibiting lipid peroxidation because many other factors, such as oxidation of proteins and nucleic acids, are presumed to be involved in cell death. Indeed, the loss of cell viability due to HG+AA was fully rescued by p-CA even when lipid peroxidation was only partly inhibited (Fig. 1C, D). Furthermore, inhibition of lipid peroxidation by methimazole in the absence of p-CA did not increase of cell viability (Fig. 4).
Cell viability was assayed using MTT (25, 26). In conclusion, the present study demonstrated that induction of p-CA peroxidases underlies the antioxidant activity of p-CA. The identity of the responsible p-CA peroxidases remains to be addressed. ‘Animal peroxidases superfamily’ includes myeloperoxidase (EC 1.11.1.7), lactoperoxidase (EC 1.11.1.7), eosinophil peroxidase (EC 1.11.1.7) and thyroid peroxidase (EC 1.11.1.8). Further studies should test if these known peroxidases or other unknown peroxidases mediate the antioxidant effects of phenolic compounds like p-CA. The purification of p-CA-dependent peroxidases is under way in this laboratory. Once these enzymes are purified and identified, more decisive experiments using specific gene silencing and over-expression should be possible. Identification of the enzyme would provide insights into the mechanism associated with the French paradox, and provide a novel target for the pharmacological approach in preventing cardiovascular disease.

MATERIALS AND METHODS

Reagents
Glucose, cinnamic acid, α-coumaric acid, m-coumaric acid, p-CA, p-methoxycinnamic acid, caffeic acid, ferulic acid, GSH, GSSG, BSO, 2-thiobarbituric acid, 1,1,3,3-tetramethoxypropane, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), 5,5’-dithiobis[2-nitrobenzoic acid], 1-methyl-2-vinylpyridium trifluoromethane sulfonate, NADPH, GSH reductase, DHR 123, methimazole, t-BOOH, ferrous ammonium sulfate and potassium thiocyanate were purchased from Sigma-Aldrich-Fluka (St. Louis, MO, USA). AA was purchased from Cayman chemical (Ann Arbor, MI, USA).

Cell culture and treatments
BAECs purchased from Clonetics Cambrex (Rockland, ME, USA) were cultured (37°C, 5% CO2) in DMEM (Dulbecco’s modified Eagle’s medium) containing 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA). The growth media contained a normal level of glucose (5.0 mM glucose, NG). Typically, BAECs were plated on a 6-well plate at a density of 5 × 104 cells·cm−2 and grown in medium for 48 h to an ~80% confluency. Cells were then pretreated with test materials for 30 min followed by treatments with an additional 25 mM glucose (total 30 mM glucose, HG) and/or 20-60 μM AA for 48 h. Cell viability was assayed using MTT (25, 26).

Analysis of lipid peroxidation
The analysis of TBARS as a marker of lipid peroxidation was performed as previously described (19). Briefly, cells were treated with lysis buffer (20 mM Tris-Cl, 2.5 mM EDTA, 1.0% SDS, pH 7.5). One hundred μl of cell lysate was mixed with 900 μl of 1.0% phosphoric acid and 1.0 ml of 0.9% 2-thiobarbituric acid, followed by heating in a boiling water bath for 45 min. After cooling, 1.5 ml of 1-butanol was added and the mixture was centrifuged at 13,000 rpm for 15 min into two layers. TBARS content of the 1-butanol layer was determined spectrophotometrically at 532 nm with a Shimadzu UV-1650 PC spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

Detection of ROS
The production of ROS was determined using an oxidant-sensitive probe DHR 123 as previously described (19). Cells were pretreated with 100 μM p-CA for 30 min and then loaded with 1.0 μM DHR 123 for 30 min before treatment with HG and AA. Following incubation of the treated cells for 5 h, images were obtained of cells fluorescing due to the oxidation of DHR 123 to rhodamine 123 using a Nikon Eclipse TE2000-U microscope. The newly formed rhodamine 123 was extracted from cells with ice-cold 70% ethanol containing 0.1 M HCl. Precipitated proteins were removed by centrifugation at 13,000 rpm for 15 min. The supernatants were neutralized with NaHCO3 and centrifuged again to remove precipitates. Aliquots of the clear supernatants were measured for their fluorescence intensity at an emission wavelength of 580 nm (excitation at 485 nm) in a FLUO-star OPTIMA multi detection microplate reader (BMG Labtechnologies Inc., Offenburg, Germany).

Analysis of GSH and GSSG
Cells were ruptured in 5% m-phosphoric acid and centrifuged at 13,000 rpm for 15 min. The acid-insoluble precipitates were used in a protein assay while the supernatants were used in an enzymatic cycling assay performed as previously described (19) to determine the content of GSH and GSSG. Data are presented as GSH equivalents in nmol GSH·mg protein−1. One nmol of GSSG is therefore expressed as 2 nmol GSH equivalents. Protein content was determined by dissolving the acid-insoluble precipitates in 1.0 N NaOH.

Assay of peroxidases
GSH and p-CA-dependent peroxidase activity was determined by monitoring decomposition of t-BOOH using the ferrithiocyanate method (27). Cell lysates were prepared in lysis buffer containing 10 mM Tris-Cl, pH 7.4, 120 mM NaCl, 25 mM KCl, 2 mM EDTA, 1 mM EDTA, 0.5% Triton X-100 and protease inhibitor cocktail. Reaction mixtures (100 μl) containing cell lysates (25 μg protein), 200 μM t-BOOH as the substrate, and 10 mM GSH or 100 μM p-CA (or up to 600 μM in some experiments) as the co-substrate were incubated at 37°C for 20-60 min. Reaction mixtures without co-substrate were also run to correct for catalase activity. The reaction was stopped by the addition of 20 μl of 40% (v/v) TCA and the resulting protein precipitates were removed by centrifugation at 13,000 rpm for 15 min. Clear supernatants (100 μl) were taken and mixed with 20 μl of 10 mM ferrous ammonium sulfate and 10 μl of 2.5 M potassium thiocyanate, followed by determination of absorbance at 490 nm with a microplate reader. The amounts
of t-BOOH were determined from a calibration curve constructed using known amounts of t-BOOH. GSH and p-CA-dependent peroxidase activities were determined from the differences in t-BOOH decomposition when the co-substrates were either present or absent.

Statistical analysis
Data were presented as means ± SEM of three or more independent experiments. The statistical analyses were performed using the Sigma Stat 3.1 software program. Significant differences among the groups were determined by a one-way ANOVA. Duncan’s multiple-range test was performed if differences were identified between the groups at P < 0.05.

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