

Modulation by flavonoids of PAF and related phospholipids in endothelial cells during oxidative stress

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Abstract PAF-dependent transacetylase (TA) modifies the functions of platelet-activating factor (PAF), a potent inflammatory lipid, either by transferring the acetyl group from PAF to lysophospholipids (TA_L activity), or to sphingosine (TA_S activity) or by hydrolyzing PAF (acetylhydrolase activity). In stimulated endothelial cells (EC), TA_L activity contributes to the synthesis of acyl-PAF, an acyl analog of PAF, that antagonizes PAF functions and is regulated by the cellular redox state. In this study, we investigated the possible involvement of TA in the flavonoid antioxidant mechanism(s) during oxidative stress in EC induced by hydrogen peroxide. The treatment of EC with H₂O₂ resulted in 4-fold increase of the acetyl-CoA acetyltransferase activity (AT), that is responsible for PAF biosynthesis, while the TA_L activity increased only by 53%. However, the preincubation of H₂O₂-treated EC with the flavonoids hesperedin, naringin, and quercetin strongly inhibited AT activity and activated TA_L by 290%, 340%, and 250%, respectively. The induction of TA_L activity resulted in enhanced biosynthesis of 1-acyl-2-[³H]acetyl-PAF in intact EC and was related to the flavonoid structure. These findings suggest that TA_L is involved in the flavonoid anti-inflammatory action by enhancing the production of acyl-PAF.—Balestrieri, M. L., D. Castaldo, C. Balestrieri, L. Quagliuolo, A. Giovane, and L. Servillo. Modulation by flavonoids of PAF and related phospholipids in endothelial cells during oxidative stress. *J. Lipid Res.* 2003. 44: 380–387.

Supplementary key words atherosclerosis • inflammation • transacetylase lysophospholipids • platelet-activating factor

Platelet-activating factor (PAF) is a potent lipid mediator involved in inflammation, allergic reactions, and reproduction (1, 2). Its biological functions can be modified by the transacetylase (TA), an enzyme that shows three catalytic activities [transacetylase lysophospholipids (TA_L), transacetylase sphingosine (TA_S), and acetylhydrolase (AH)] differentially regulated (3). TA transfers the acetyl

group of PAF to synthesize either acyl-analogs of PAF or [C₂]ceramide, or hydrolyses PAF to lyso-PAF. TA was purified to apparent homogeneity from rat kidney membranes and cytosol (4). PAF is synthesized by different cell types as a response to specific stimuli. In activated endothelial cells (EC), acyl-PAF, an acyl analog of PAF, is predominantly produced instead of PAF. Acyl-PAF acts as a naturally occurring specific noncompetitive inhibitor of PAF-induced activation of human neutrophils (5) and leukotriene C₄ release from human leucocytes (6). It is known that PAF-like lipids are responsible for downregulating growth factors and inhibiting proliferation in EC exposed to oxidized LDL (7). Moreover, acyl-PAF decreases the susceptibility of the LDL particle to oxidative modifications (8). In activated ECs, TA_L contributes to the biosynthesis of acyl-PAF (9, 10), and its activity is regulated by the thiol cellular levels [i.e., glutathione (GSH)] (11). In particular, the enzyme activity increases in the presence of thiol-oxidant agents and is decreased by thiol-antioxidants (11). The cellular redox homeostasis represents the antioxidant defense mechanism that protects cells from the increased levels of reactive oxygen species (ROS) in the vasculature, including superoxide anion, H₂O₂, and hydroxyl radical. Because of their location at the interface of the vascular system, ECs in the blood vessel wall are from time to time exposed to peroxide, as during local inflammatory reaction or because of the contact with oxidized lipoproteins. At the site of the inflammation, H₂O₂ generated by activated neutrophils (12) modulates the inflammatory process by upregulating the expression of adhesion molecules, (13, 14) controlling cell proliferation or apoptosis (15), and modulating platelet aggregation

Abbreviations: AH, acetylhydrolase; AT, acetyltransferase activity; CP, creatine phosphate; CPK, creatine phosphokinase; EC, endothelial cell; LPE, lyso-glycerophosphoethanolamine; PAF, platelet-activating factor; PLA₂, phospholipase A₂; PLC, phospholipase C; TA, transacetylase; TA_L, transacetylase lysophospholipids; TA_S, transacetylase sphingosine.

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Manuscript received 24 July 2002 and in revised form 9 October 2002.

Published, JLR Papers in Press, November 4, 2002.

DOI 10.1194/jlr.M200292.JLR200

(16). In addition, the H_2O_2 production plays a key role in the pathogenesis of atherosclerosis by modifying LDL and increasing their atherogenicity (17, 18). Flavonoids are a group of antioxidants present in plants comprising flavones, flavonols, and flavonones as major members. Flavones, such as quercetin, are present in onions, lettuce, and olives, whereas flavanones (naringin and hesperetin) are mostly contained in citrus fruit and citrus peel (19).

They scavenge superoxide radical and other radical anions, which in part contribute to their antioxidant properties. In vitro studies have shown that flavonoids act on enzyme systems critically involved in the initiation and maintenance of the inflammatory response; they are inhibitors of the acetyltransferase activity (AT) (20, 21) and phospholipase A_2 (PLA $_2$) (22), inhibit platelet function (23) and EC adhesion protein gene expression (24), and protect against the development of atherosclerosis (25, 26). Moreover, addition of flavonoids to EC incubated with oxidized LDL may attenuate such a cytotoxic effect of the modified lipoprotein (27). These data led us to hypothesize that flavonoids may regulate PAF metabolism in EC. Consequently, the present study was designed to evaluate the role of flavonoids in modulating TA activities during induced oxidative stress in EC.

METHODS

Materials

PAF, GSH, 5-sulfosalicylic acid, glutathione reductase, naringin, naringenin, hesperidin, hesperetin, quercetin, hydrogen peroxide, acetyl-CoA, phospholipase C (PLC) type XI from *Bacillus cereus*, benzoic anhydride, DMSO, indometacin, creatine phosphate (CP), creatine phosphokinase (CPK), and 4-dimethylamino-pyridine were obtained from Sigma. Alkenyl-lyso-glycerophosphoethanolamine (LPE) was a product from Serdary Research Lab. [3H]acetyl-PAF (13.5 Ci/mmol) and [3H]acetyl-CoA (1.54 Ci/mmol) were purchased from NEN Life Science Products. All culture reagents were from Life Technologies, Inc. Phospholipids were tested for purity by TLC and only >95% pure phospholipids were used in the experiments.

Cell culture

EC from calf pulmonary artery endothelium were obtained from the American Type Culture Collection (CPAE, CCL 209). Monolayers of cells between passages 19–25 were grown in MEM

with 20% FBS. Cells were cultured in 75 cm 2 flasks, and only subconfluent monolayers between passages 19 and 25 were used for the experiments to avoid the cellular changes which EC undergo at higher passages. The subconfluent monolayers were prepared the day before the experiment by seeding EC in 100 mm Petri dishes at the nonsaturating density of 5×10^6 cells/dish.

Homogenates were prepared in the homogenization buffer (0.25 M sucrose, 100 mM Tris-HCl, pH 7.3, 1 μ g/ml leupeptin, 1 mM dithiothreitol) by sonication, and the protein content of the cell homogenates was determined as previously described (9).

Cell treatments

The H_2O_2 used in the present work was a 30% stable solution, and appropriate dilutions were made immediately prior to use in serum-free media to avoid rapid degradation by antioxidants present in the serum. Subconfluent monolayers of ECs were washed twice with 5 ml of HBSS-10 mM HEPES and incubated for the indicated time with H_2O_2 in serum-free media. When flavonoids were tested before H_2O_2 treatment, cells were preincubated at 37°C for 1 h with flavonoids prepared in DMSO. At the end of the preincubation, the media were removed and the cells were washed twice with 5 ml of HBSS-10 mM HEPES, thus eliminating the possibility of a direct interaction between H_2O_2 and flavonoids outside the cellular environment. The final DMSO concentration in the media was less than 0.1% and the cell viability was >95%, as assessed by trypan blue dye exclusion.

AT assay

The AT assay system consisted of 500 μ M [3H]acetyl-CoA (0.2 μ Ci), 50 μ M lysoPAF suspended in 3.3% BSA-saline, 100 mM Tris-HCl (pH 7.2), and 100 μ g of homogenate in a final volume of 0.5 ml. Incubations were carried out at 37°C for 15 min, and the lipids were extracted by the method of Bligh and Dyer (28). The extracted lipids were separated by TLC using a solvent system of $CHCl_3$ - CH_3OH - NH_4OH - H_2O (60:35:8:2.3, v/v/v/v). The radioactivity of the areas corresponding to PAF was determined by liquid scintillation counting.

TA assays

TA $_L$, TA $_S$, and AH activities were determined according to the methods we previously described (9, 3). TA $_L$ and TA $_S$ activity was determined using [3H]acetyl-PAF as acetyl donor and LPE or sphingosine as substrate acceptors, respectively. The extracted lipids (28) were separated by TLC, and the radioactivity of the areas corresponding to [3H]acetyl-PAF, [3H]acetyl-PE, and [3H]acetyl-sphingosine were determined by liquid scintillation counting.

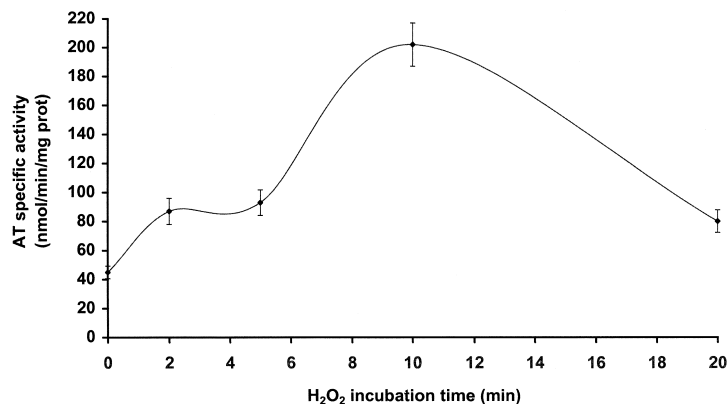


Fig. 1. Time-dependent activation of acetyltransferase activity (AT) in H_2O_2 -treated endothelial cells (EC). Monolayers of subconfluent EC were stimulated with 1 mM H_2O_2 at the indicated times. At the end of the incubations, the total cell homogenates were prepared and the acetyltransferase (AT) activity was determined as described in Methods. Data are mean \pm SEM of three separate experiments in duplicate ($n = 6$).

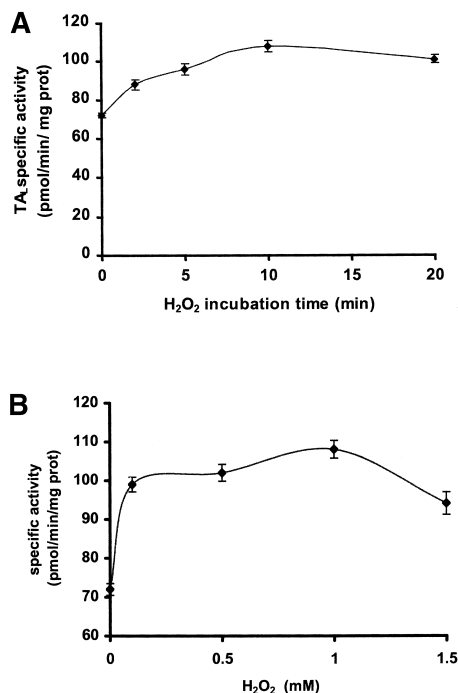


Fig. 2. Activation of transacetylase lysophospholipids (TA_L) in H₂O₂-treated EC. A: Monolayers of subconfluent EC were stimulated with (1 mM) H₂O₂ at the indicated times. B: EC were stimulated for 10 min with different H₂O₂ concentrations. At the end of the incubations, the TA_L activity was determined on the total cell homogenates as described in Methods. Data are mean \pm SEM of three separate experiments in duplicate ($n = 6$).

Measurement of [³H]arachidonic acid release

EC grown in six wells-cell culture cluster dishes were prelabeled with [³H]arachidonic acid (0.25 μ Ci/ml) for 40 h at 37°C. Prelabeled cells were then washed with 2 ml of HBSS-10 mM HEPES and treated with flavonoids (25 μ M) and H₂O₂ (1 mM) as described. The [³H]arachidonic acid release in the media was determined as previously described (10).

Purification and measurement of PAF

PAF released and associated to EC after treatment with flavonoids and H₂O₂ was extracted (28) and purified by TLC using a solvent system of CHCl₃-CH₃OH-H₂O (60:35:6, v/v/v).

PAF was detected by its ability to induce platelet aggregation by a pathway independent of both adenosine diphosphate and cyclooxygenase-derived metabolites as described (29). Platelets (2 to 5×10^7) were stirred at 900 rpm in 300 μ l of Tris-buffered Tyrode's (2.6 mM KCl, 1 mM MgCl₂, 137 mM NaCl, 1.3 mM CaCl₂, 0.1% glucose, and 1 mM Tris) supplemented with 0.25% gelatine in presence of indometacin (10 μ M) and of CP-CPK enzymatic system (312.5 μ g/ml of CP and 152.5 μ g/ml of CPK). The amount of PAF was expressed in nanograms per milliliter and calculated over a calibration curve of synthetic PAF. The standard curve was constructed by adding 10 μ l of solution of synthetic PAF at different concentrations (1 ng/ml to 15 ng/ml) to washed rabbit platelets.

Determination of the rate of transfer of the [³H]acetyl group from [³H]acetyl-PAF to 1-acyl-2-lysoPAF

Monolayers of EC cells were preincubated at 37°C for 1 h with the indicated flavonoids (25 μ M) as described before, followed by treatment with H₂O₂ (1 mM) for 10 min in serum-free media in presence of [³H]acetyl-PAF (2.5 μ Ci) in 0.1% BSA. At the end

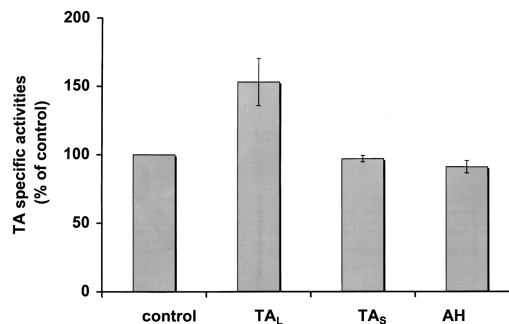


Fig. 3. Effect of H₂O₂ on the three transacetylase (TA) activities. Subconfluent EC were stimulated with 1 mM H₂O₂ for 10 min and the total cell homogenates were used for the determination of the TA_L, transacetylase sphingosine (TA_S), and acetylhydrolase (AH) activities. Data were represented as means \pm SEM of three separate experiments in duplicates ($n = 6$). The P values between cells treated with H₂O₂ (1 mM) and control cells were <0.05 .

of the incubations, the media were removed and the cells were rinsed twice with 5 ml of HBSS-10 mM HEPES before scraping into 3 ml of methanol. The cellular lipids were extracted (28) and the amounts of [³H]acetyl groups transferred from [³H]acetyl PAF to 1-acyl-2-lysoPAF were measured after PLC hydrolysis, benzoylation, and TLC analysis as previously described (9).

Determination of total GSH level

After the incubation with H₂O₂ in presence or absence of flavonoid pretreatment as described above, EC were scraped into 0.5 ml of sulfosalicylic acid 10% (w/v) and centrifugated at 15,000 g for 10 min. The supernatant was used for the determination of total GSH according to the method described (30).

Statistical analysis

Data are expressed as mean \pm SEM from at least three independent experiments in duplicate. Statistical analysis was performed by Student's t -test. Probability values were considered significant at $P < 0.05$.

RESULTS

H₂O₂ modifies PAF metabolism

To assess the effect of H₂O₂ on the biosynthesis of PAF and its analogs, EC were incubated for different times with 1 mM H₂O₂, then AT and TA activities were measured. This relatively high concentration of H₂O₂ was found not to be cytotoxic (13). On the other hand, it has been speculated that under certain conditions oxidants are released into relatively sequestered microenvironments, thus reaching high local concentrations (31). As shown in **Fig. 1**, H₂O₂ induced a time-dependent activation of AT. The increase of AT activity reached the maximum at 10 min (4-fold over the basal level) and returned to near basal level at 20 min. **Figure 2A** indicates that the time-dependent increase of TA_L activity was minimal and, specifically, at 10 min was only 1.5 more than in the untreated cells. Also, the concentration-dependence curve shows that the maximal H₂O₂-induced TA_L activity occurred just at about 1 mM H₂O₂ (108 vs. 72 pmol/min/mg prot in control cells) (**Fig. 2B**). Since the TA_L strongly

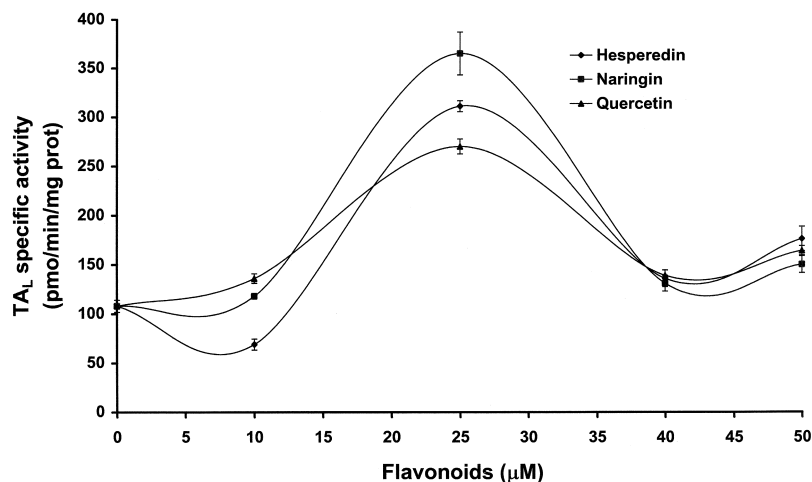


Fig. 4. Dose-dependent activation of TA_L in flavonoid-pretreated EC. Various concentrations of hesperedin, naringin, and quercetin were preincubated with EC for 1 h at 37°C. Subsequently, ECs were treated with 1 mM H_2O_2 for 10 min and the TA_L activity was determined. Data are expressed as means \pm SEM ($n = 4$) and are representative of four experiments in duplicate. The P values between cells treated with H_2O_2 (1 mM) and cells treated with flavonoids (25 μ M) plus H_2O_2 (1 mM) were <0.001 .

contributes to the acyl-PAF biosynthesis in activated EC (9), these data indicated that during induced oxidative stress, the activation of AT is predominant and that TA_L scarcely contributes to the acyl-PAF biosynthesis.

H_2O_2 effect on TA activities

It is known that PAF-AH (II) protects MDBK cells against oxidative stress-induced death. This enzyme translocates from cytosol to membrane in response to H_2O_2 treatment and hydrolyzes the oxidized phospholipids

(32). To determine whether the AH activity of TA functions as an antioxidant phospholipase during induced oxidative stress, EC were incubated for 10 min with 1 mM H_2O_2 and then AH activity was determined. The TA_S activity was also determined in order to assess the $[C_2]$ ceramide production. As shown in **Fig. 3**, AH and TA_S activities were not influenced by H_2O_2 treatment (91% and 97% of the control, respectively). Therefore, the activation of TA by H_2O_2 is selective for the TA_L portion of the TA activities.

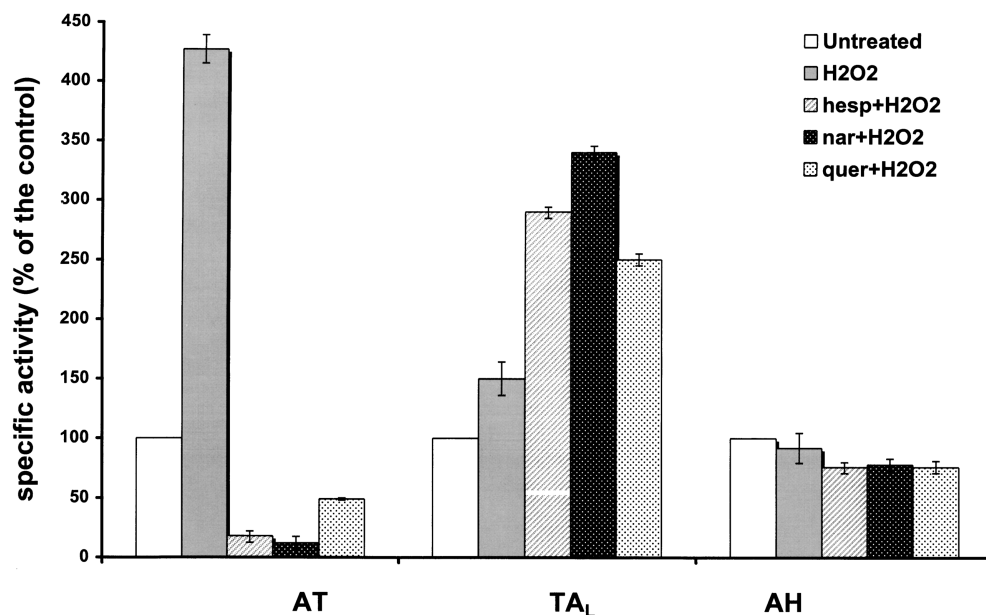


Fig. 5. Effect of flavonoids on AT and TA activities. AT and TA activities were determined on the total cell homogenates from untreated EC, and EC pretreated for 1 h with or without hesperedin (hesp), naringin (nar), and quercetin (quer) (25 μ M) followed by stimulation with H_2O_2 (1 mM) for 10 min, as described in Methods. Data are expressed as means \pm SEM ($n = 4$) with $P < 0.001$ for the AT activity (H_2O_2 vs. untreated), and TA_L activity (flavonoids plus H_2O_2 vs. H_2O_2). The differences between untreated vs H_2O_2 and flavonoids plus H_2O_2 versus H_2O_2 were not significant for AH and TA_S activities ($P > 0.1$).

TABLE 1. Measurement of platelet-activating factor

	PAF
	ng/ml
Untreated	9.5 ± 1.6
H ₂ O ₂	34.2 ± 5.2
Hesperedin + H ₂ O ₂	12.7 ± 1.2
Naringin + H ₂ O ₂	11.4 ± 2.4
Quercetin + H ₂ O ₂	13.4 ± 1.8

The platelet-activating factor (PAF) produced by endothelial cells (EC) treated with H₂O₂ (1 mM) and H₂O₂ + flavonoids (25 μM) was quantified as described in Methods. Data are expressed as means ± SEM (n = 5).

Induction of TA_L activity by flavonoids

To test the hypothesis that TA could contribute to the anti-inflammatory actions of flavonoids, EC were preincubated for 1 h with quercetin (5,7,3',4', tetrahydroxyflavanol), naringin (4',5,7, trihydroxyflavanone-7-rhamnoglucoside), and hesperedin (3',5,7, trihydroxy-4-methoxyflavanone-7-rhamnoglucoside), and then with 1 mM H₂O₂ for 10 min. Flavonoids significantly activated the TA_L activity in a dose-dependent manner (Fig. 4), with a maximal activation at 25 μM. Citrus flavanones, hesperedin and naringin, resulted in the most potent compounds (290% and 340% more than in H₂O₂-treated EC, respectively) compared with the flavanol quercetin (250% more than in H₂O₂-treated EC). Flavonoids alone did not present any effect on the TA_L activity in H₂O₂-untreated cells (data not shown). These results indicate that the TA_L is involved in the flavonoid anti-inflammatory actions in EC during oxidative stress, most probably by increasing the acyl-PAF biosynthesis.

Flavonoid effects on PAF metabolism

To determine whether flavonoids exert the anti-inflammatory action also influencing the AH portion of the TA with consequent PAF hydrolysis, the AH activity on the total homogenates from EC preincubated with flavonoids and then treated with H₂O₂, was assayed. Furthermore, since quercetin it is known to inhibit AT (20) and PLA₂ (22) activities, we tested the effect of naringin, hesperedin, and quercetin on the AT and PLA₂ activities as well. As illustrated in Fig. 5, naringin, hesperedin, and quercetin induced only the TA_L but not the AH activity of TA. There-

fore, AH activity does not mediate the flavonoid action during induced oxidative stress; besides, the AT activity was strongly inhibited not only by quercetin, as previously shown (20), but also by naringin and hesperedin.

We measured the [³H]arachidonic acid release as an index of the PLA₂ activity and found that flavonoids also inhibited the lysoPAF production by PLA₂. In fact, the [³H]arachidonic acid released in the media was 1,500 cpm/well in EC pretreated with flavonoids compared with 3,800 cpm/well in EC treated only with H₂O₂.

Taken together, these data indicate that flavonoids protect EC during oxidative stress by inhibiting PAF biosynthesis and, interestingly, by directing the utilization of the produced PAF as an acetyl donor for the synthesis of the less-active PAF analogs (TA_L activity) instead of its hydrolysis (AH activity).

PAF measurement

To confirm that flavonoids inhibit PAF synthesis, the amount of PAF produced by EC stimulated with H₂O₂ and preincubated with or without flavonoids was determined. As shown in Table 1, EC produce PAF in response to H₂O₂ treatment (34.2 ng/ml) when compared with untreated cells (9.5 ng/ml). However, preincubation with hesperedin, naringin, and quercetin inhibit PAF synthesis, which return to near basal level (12.7 ng/ml, 11.4 ng/ml, and 13.4 ng/ml, respectively).

Effect of flavonoids on the transfer of [³H]acetyl group from [³H]acetyl-PAF to 1-acyl-2-lysoPAF

The transfer of the [³H]acetyl group from [³H]acetyl-PAF to 1-acyl-2-lysoPAF was used as an index of the TA_L activity in intact EC. As shown in Fig. 6, we found that the amounts of transferred [³H]acetyl group from [³H]acetyl-PAF to 1-acyl-2-lysoPAF increased from 315 × 10³ cpm/flask in H₂O₂-treated cells to about 650 × 10³ (3) cpm/flask in flavonoids-pretreated EC. These results confirm the flavonoid induced TA_L activation and indicate that the enzyme induction is indeed reflected in the enhanced biosynthesis of acyl-PAF.

Comparison of structurally related flavonoids

The structure-activity relationship of two types of flavonoids were examined. Hesperetin 25 μM (3',5,7,tri-

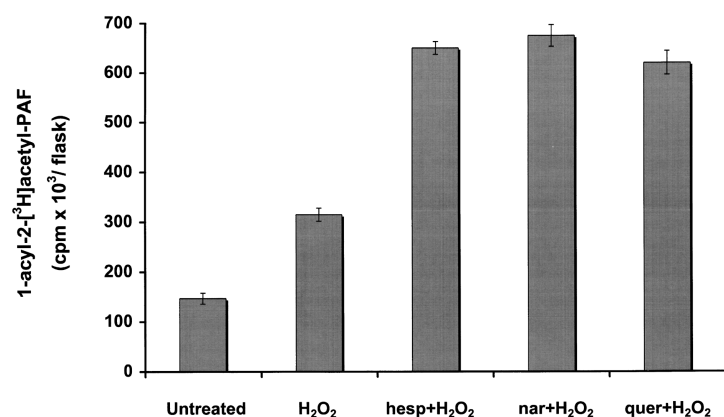


Fig. 6. Effect of flavonoids on the transfer of the [³H]acetyl group from [³H]acetyl-platelet-activating factor (PAF) to 1-acyl-2-lysoPAF. EC were preincubated with hesp, nar, and quer (25 μM), and then stimulated with H₂O₂ (1 mM) in the presence of [³H]acetyl-PAF (2.5 μCi) in 0.1% BSA. The cellular lipids were extracted and the rate of transfer of [³H]acetyl group from PAF to 1-acyl-2-lysoPAF was measured as described in Methods. Data are expressed as means ± SEM (n = 4) from two separate experiments.

TABLE 2. Effect of structurally related flavonoids on TA_L and AT activities

	TA _L	AT
	<i>pmol/min/mg prot</i>	<i>nmol/min/mg prot</i>
Untreated	72 ± 3.6	45 ± 2.3
H ₂ O ₂	108 ± 5.6	202 ± 6.1
Hesperedin + H ₂ O ₂	311 ± 10.2	36 ± 1.8
Hesperetin + H ₂ O ₂	194 ± 5.6	87 ± 1.6
Naringin + H ₂ O ₂	365 ± 11.8	24 ± 0.8
Naringenin + H ₂ O ₂	121 ± 4.1	91 ± 1.1

The transacetylase lysophospholipids (TA_L) and AT activities were determined after EC treatment with a series of structurally related flavonoids (25 μM) followed by H₂O₂ (1 mM) stimulation. Data are expressed as means ± SEM (n = 4).

hydroxy-4-methoxyflavanone) and naringenin 25 μM (4', 5,7-trihydroxyflavanone) were incubated with EC for 1 h before H₂O₂ treatment, and successively the TA_L and AT activities were determined. As shown in **Table 2**, the TA_L-specific activity in hesperetin- and naringenin-treated ECs was 194 and 121 pmol/min/mg prot, respectively, compared with 311 and 365 pmol/min/mg prot measured when EC were pretreated with hesperedin and naringin, respectively. In the EC treated with H₂O₂ only, the TA_L-specific activity was 108 pmol/min/mg protein. On the other hand, Table 2 shows that hesperetin and naringenin are less potent AT inhibitors than hesperedin and naringin. Therefore, glycosylated flavanones (hesperedin and naringin) resulted in more potent TA_L activators and AT inhibitors compared with their aglycone counterparts (hesperetin and naringenin). These observations indicated that the flavonoid biological activity on PAF and acyl-PAF metabolism is related to their structure and point out the importance of their glycidic moiety.

Changes in redox state of EC

Antioxidants protect vascular cells against oxidative stress by scavenging ROS generated from the inflammatory stimuli (33) and by directly modulating redox-sensitive pathways. (34, 35) Moreover, changes in thiol cellular level influence TA_L activity and thiol oxidizing reagents lead to an increased acyl-PAF production by TA_L in EC. (11) In order to elucidate the possible mechanism(s) by which flavonoids regulate TA_L activity, the changes in the

redox state of the EC were monitored by measuring the total intracellular GSH level in the presence or absence of treatment with flavonoids. As shown in **Fig. 7**, a decrease in the total GSH was observed in H₂O₂-treated EC (8.7 GSH equivalents, i.e., nmol of GSH/2.5 × 10⁶ cells versus 14.3 GSH equivalents in untreated samples), reflecting oxidative stress. Conversely, in EC pretreated with hesperedin, naringin, and quercetin, the total GSH levels results were comparable with the untreated cells (12.9, 14.8, and 13.5 GSH equivalents, respectively). These data suggest that the flavonoid-induced TA_L activation we observed does not depend on the oxidative state of the intracellular milieu.

DISCUSSION

Since EC layer the inner walls of blood vessels, they can easily become exposed to peroxides under stress conditions, such as those produced by activated neutrophils at the site of the inflammation (12). Oxidative stress and the production of ROS have been implicated in a variety of diseases, including Alzheimer's disease, cancer, and vascular diseases such as atherosclerosis. In particular, two interacting processes are now believed to initiate atherosclerosis: endothelial dysfunction (i.e., vasoconstriction, platelet activation, leukocyte adhesion, inflammation, thrombogenesis) and lipid accumulation and modification with a consequent production of oxidized LDL. (17, 18) These processes are mediated by a number of agents, including inflammatory mediators. The LDL susceptibility to oxidative modifications can be decreased by acyl-PAF, (8) an acyl analog of PAF (1). Acyl-PAF is the predominant component produced by activated EC, and the TA_L contributes to its biosynthesis (9, 10). The inhibition of LDL oxidation is a well-characterized process that includes effects on the concentration or reactivity of oxidants capable of modifying LDL and on the susceptibility or resistance of LDL to these oxidants. (36) Like oxidants, antioxidants constitute a diverse group of compounds with different properties. They operate by inhibiting oxidant formation, intercepting oxidants once they have formed, and repairing oxidant-induced injury. In the

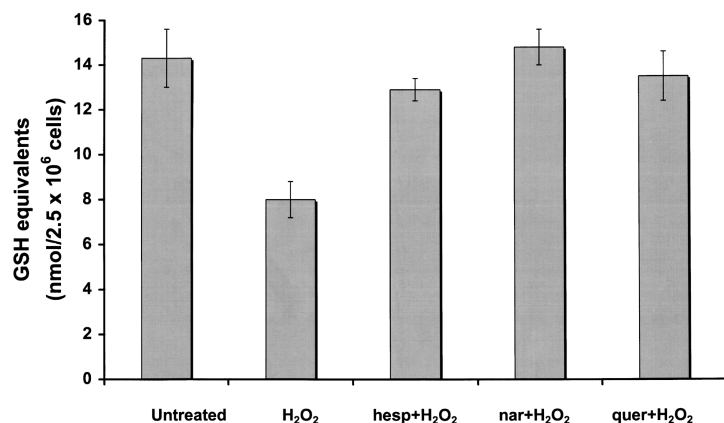


Fig. 7. Changes in redox state of EC. Treatments of the cells with hesp, nar, quer, and H₂O₂ were made under the same conditions as described in Fig. 4. Data are the means ± SEM (n = 4) from two separate experiments.

present study, we provide evidence that TA_L is involved in the flavonoid anti-inflammatory action in EC by transferring the acetyl group from PAF to 1-acyl-2-lyso-PAF, thus synthesizing acyl-PAF. In detail, we showed that the oxidative stress induced a noticeable induction of AT, the key enzyme of the PAF biosynthetic route, while TA_L is only scarcely activated (Figs. 1, 2) compared with other inflammatory stimuli (9). AH activity has been found to protect against oxidative stress (32), most probably by hydrolyzing oxidized phospholipids and, in human LDL, PAF-AH possesses both transacetylase and acetylhydrolase activities that remove PAF and its ether-linked analogs from LDL particles upon LDL oxidation (37). Moreover, thiol cellular level regulates the cytokine-mediated apoptosis and the ceramide production (38). However, our results showed that both AH and TA_S activities are not induced during the oxidative stress, indicating that AH does not protect EC against oxidative stress and that apoptosis-mediators, such as $[C_2]$ ceramide, are not produced by the TA_S . These results are consistent with our previous observations that thiol modifying agents modulate only TA_L activity in EC (11). The most intriguing of our findings is that flavonoids, such as quercetin, hesperedin, and naringin, exert their anti-inflammatory action not only by modifying monocyte adhesion in the inflammatory process of atherosclerosis (26) but also by regulating the enzyme activities responsible for the PAF and acyl-PAF biosynthesis. In particular, we showed for the first time the TA_L involvement in the flavonoid anti-inflammatory action in EC.

These cells synthesize acyl-PAF as a predominant product in response to inflammatory stimuli (39, 40), and TA_L activity contributes to its biosynthesis in EC. (9) Therefore, it was unlikely that the flavonoid-mediated TA_L induction was not correlated to an increase of acyl-PAF production. However, we verified this fact by directly measuring the incorporation of the $[^3H]$ acetyl group into 1-acyl-2-lysoPAF by incubating EC with $[^3H]$ acetyl-PAF. The results indicated that flavonoid action on TA_L is associated with an increased acyl-PAF production (Fig. 6). On the whole, these results suggest that TA_L mediates flavonoid anti-inflammatory action in EC during oxidative stress. This enzyme provides for the PAF degradation with a concomitant biosynthesis of acyl-PAF, which can exert its beneficial role during the initiation and progression of atherosclerosis (8). The mechanism by which flavonoids inhibit AT activity is unclear. (20) Evidence suggests that the biological action of citrus flavonoids is possibly linked to their interaction with key regulatory enzymes involved in cell activation and in receptor binding (21). Flavonoid antioxidant properties are mostly related to their direct scavenging action against free radicals and ROS (33) or to their interaction with intracellular-occurring antioxidative agents such as glutathione peroxidase (34, 35). We showed that, in flavonoid-pretreated EC, the variation of total GSH level is not significant if compared with untreated cells. Therefore, it is conceivable that the mechanism(s) responsible for the TA_L activation during flavonoid treatment of ECs is independent of the reducing state of the intracellular milieu. However, further studies

are required to elucidate the flavonoid-induced pathway leading to TA_L activation in EC.

In summary, in this report we provide for the first time evidence that TA_L activity of TA contributes to the flavonoid anti-inflammatory action in EC. Elucidating the regulatory process(es) involved in the protection against redox-sensitive endothelium dysfunctions could be of clinical relevance for the development of novel therapeutic strategies for the treatment of the atherosclerosis and other inflammatory diseases. ■

This work was supported by the project Ricerche e sperimentazioni nel settore dell'agricoltura Italiana of the Ministero delle Politiche Agricole e Forestali.

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