Journal of Natural Products
Vol. 50, No. 3, pp. 392-399, May-Jun 1987

STUDIES ON THE ACTIVITIES OF TANNINS AND RELATED COMPOUNDS, X. EFFECTS OF CAFFEETANNINS AND RELATED COMPOUNDS ON ARACHIDONATE METABOLISM IN HUMAN POLYMORPHONUCLEAR LEUKOCYTES

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ABSTRACT.—As part of a series of biological examinations of various tannins and related compounds, the present paper reports the effects of caffeetannins and related compounds isolated from medicinal plants on arachidonate metabolism in human peripheral polymorphonuclear leukocytes (PMN-L).

The formation of leukotriene B_4 (LTB_4) induced by calcium ionophore A 23187 (A 23187) in human PMN-L was inhibited by 3,5-, 4,5-, and 3,4-di-*O*-caffeoylquinic acid, caffeoylmalic acid, caffeoyltartric acid, rosmarinic acid, and caffeic acid. Rosmarinic acid strongly inhibited the formation of 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) and LTB_4 (5-lipoxygenase products) at concentrations of 10^{-5}-10^{-3}M. On the other hand, the formation of prostaglandin E_2 (PGE_2) was enhanced in a concentration-dependent fashion by caffeic acid, caffeoylmalic acid, caffeoyltartaric acid, and 3,4-di-*O*-caffeoylquinic acid. On the basis of experimental results, it seems likely that caffeoyl derivatives could be developed as therapeutic drugs for treatment of allergic inflammation such as asthma.

Leukotrienes participate in immunoregulation and in a variety of diseases, including asthma, inflammation, and various allergic conditions. In the presence of 5-lipoxygenase, free arachidonic acid is converted into 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE). The metabolite is further reduced into 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) or dehydrated to an unstable intermediate, leukotriene A_4 (LTA_4) (1-2). This intermediate can be converted enzymatically to leukotrienes including LTB_4, LTC_4, LTD_4, and LTE_4 which are referred to as "the slow reacting substance of anaphylaxis" (SRS-A) (3-5). Among these metabolites, 5-HPETE and 5-HETE enhance histamine release induced by antigen from human basophilic leukocytes (6), and the latter increases the activities of lysosomal enzymes such as β-glucuronidase and their release of lysozyme induced by platelet activating factor in human PMN-L (7). Another metabolite, LTB_4, also enhances the activities of lysosomal enzymes (8) and induces chemotaxis (9). Therefore, specific inhibitors of 5-lipoxygenase should be useful not only in therapy of allergic diseases such as asthma and inflammation but also in the studies on the biosynthesis and functions of leukotriene.

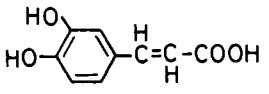
On the other hand, it has been reported that cyclooxygenase products, thromboxane A_2 (TXA_2) and prostaglandin E_2 (PGE_2), are formed from human PMN-L in the presence of calcium ionophore A 23187 (A 23187) (10). Of these, TXA_2, which is readily converted into a stable compound, TXB_2, causes platelet aggregation (11), and PGE_2 causes uterine contraction (12) and increases intracellular cyclic AMP levels in human PMN-L (13).

As a part of a series of biological examinations of various tannins and related compounds, this laboratory has previously reported that caffeoylquinic acids such as 3,5-, 4,5-, and 3,4-di-*O*-caffeoylquinic acid and chlorogenic acid, isolated from the leaves of *Artemisia* species, and caffeic acid prevent liver injury and hyperlipemia induced by oral

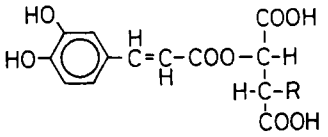
administration of peroxidized corn oil (14) and inhibit the lipid peroxidation in rat liver microsomes and mitochondria (15). The present paper reports the effects of caffeetannins and related compounds on arachidonate metabolism in human PMN-L.

MATERIALS AND METHODS

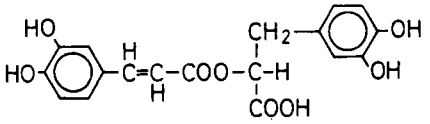
MATERIALS.—(1-^{14}C) Arachidonic acid (specific activity: 54.5 mCi/mmol), (^{3}H) PGE_2, and (^{3}H) TXB_2 were purchased from New England Nuclear. LTB_4 and methylester of 5-HETE were purchased from Paesel Gmbh & Co. Precoated Si gel 60 tlc plastic sheets were obtained from Merck Co. Calcium ionophore A 23187 (A 23187) was purchased from Boehringer Mannheim. Caffeic acid was purchased from Sigma Co. Other chemicals were reagent grade. Caffeoylquinic acids were isolated from the leaves of *Artemisia* species by methods described previously (14-16). Caffeoylmalic acid, caffeoyltartaric acid, and rosmarinic acid were isolated from the leaves of *Acalypha australis* L., *Rabdosia japonica* Hara, and *Perilla frutescens* Britton var. *crispa* Decne., respectively, using the methods of Okuda *et al.* (17,18). The structures of these compounds are shown in Figure 1. Test compounds were dissolved in Hepes/saline (25mM Hepes in 135 mM NaCl, pH 7.4) buffer before use.



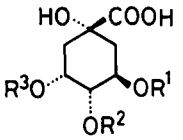
caffeic acid



caffeoylmalic acid: R=H
caffeoyltartaric acid: R=OH



rosmarinic acid



chlorogenic acid: $R^2=R^3=H$, R^1=caffeoyl

3,5-di-*O*-caffeoylquinic acid: $R^2=H$, $R^1=R^3$=caffeoyl

4,5-di-*O*-caffeoylquinic acid: $R^1=H$, $R^2=R^3$=caffeoyl

3,4-di-*O*-caffeoylquinic acid: $R^3=H$, $R^1=R^2$=caffeoyl

FIGURE 1. Caffeetannins and related compounds

ISOLATION OF HUMAN PMN-L.—PMN-L, isolated from healthy human venous blood by sedimentation using 6% dextran and Ficoll/hypaque, were suspended in Hepes/saline buffer (pH 7.4) containing

2mM $CaCl_2$. These cells, identified as more than 97% PMN-L by Giemsa staining test and light microscopy, were more than 95% viable as judged by the trypan blue exclusion test.

MEASUREMENT OF (1-^{14}C) ARACHIDONIC ACID METABOLISM INDUCED BY A 23187 IN HUMAN PMN-L.—Human PMN-L (2.5×10^6 cells) were preincubated with test compounds for 10 min at 37°. Then, (1-^{14}C) arachidonic acid (0.1 μCi; 560 ng) and 10 μM A 23187 were added and incubated for 10 min at 37°. The reaction was stopped by adding 0.5 N HCOOH, and the mixture was extracted with 8 volumes of EtOAc. The EtOAc phase was evaporated under N_2, and the residue was dissolved in a small amount of EtOAc (40 μl), applied to precoated Si gel tlc plastic sheets, and developed with two solvents: EtOAc-2,2,4-trimethylpentane-HOAc-H_2O (100:50:20:100, v/v, upper phase) and $CHCl_3$-MeOH-HOAc-H_2O (13:12:1.5:1.2, v/v). Metabolites were identified by comparison of their mobilities on tlc with those of authentic samples and by gc-ms as described previously (19,20). Spots of radioactivity were detected by autoradiography, cut out with scissors, and counted in a liquid scintillation counter. The PMN-L isolated from 35 to 36-year-old men and 35-, 47-, and 60-year-old women were used for the effects of caffeic acid, caffeoylmalic acid, caffeoyltartaric acid, and 3,4-di-*O*-caffeoylquinic acid, and those of rosmarinic acid, 3,5-di-*O*-caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid on arachidonate metabolism, respectively.

CYTOTOXICITY.—Caffeetannins and related compounds used in this experiment did not cause human PMN-L suspension or release of more than 6% of their lactate dehydrogenase at concentrations of 10^{-3}-10^{-6} M and, therefore, were not toxic to the cells (data not shown).

ANALYSIS OF DATA.—The calculation of the 50% inhibitory concentration (IC_{50}) for the formation of 15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE), 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT), 5-HETE, and LTB_4, respectively, were determined by regression methods, and the results are expressed as IC_{50} and 95% confidence limits (C.L.) for each IC_{50} as a range.

RESULTS

When arachidonic acid was incubated with human PMN-L in the presence of calcium ionophore, it was converted into the 15-lipoxygenase product (15-HETE), the 5-lipoxygnease products (5-HETE and LTB_4), the cyclooxygenase products (HHT and PGE_2), and unidentified metabolites corresponding to a mixture of TXB_2 and lipoxygenase products (Figure 2). The formation of 15-HETE, 5-HETE, LTB_4, HHT, and PGE_2 from arachidonic acid induced by A 23187 and arachidonic acid (control) was 4.18 ± 0.16, 3.86 ± 0.19, 9.58 ± 0.35, 1.10 ± 0.11, and 0.54 ± 0.07 ng/10^6 cells (means±standard errors for 21 experiments), respectively. Furthermore, the amounts of 15-HETE, 5-HETE, LTB_4, HHT, and PGE_2 from arachidonic acid in PMN-L isolated from 35-36 year old men were 4.43 ± 0.16, 4.38 ± 0.10, 9.53 ± 0.38, 1.20 ± 0.05, and 0.54 ± 0.02 ng/10^6 cells (means±standard errors for 12 experiments) in the control. The amounts of 15-HETE, 5-HETE, LTB_4, HHT, and PGE_2 from arachidonic acid in PMN-L isolated from women were 3.85 ± 0.29, 3.17 ± 0.29, 9.66 ± 0.62, 0.97 ± 0.08, and 0.65 ± 0.05 ng/10^6 cells (means±standard errors for 9 experiments) in the control. The formation of 15-HETE, 5-HETE, and LTB_4 from arachidonic acid is increased time dependently during 10 min incubation after the addition of A 23187 and arachidonic acid, while HHT and PGE_2 formation from arachidonic acid is terminated for about 5 min (data not shown). Moreover, the number of the formations of 15-HETE, 5-HETE, LTB_4, HHT, and PGE_2 after 10 min incubation was found to be proportional to the amount of cell present (data not shown).

As shown in Figure 2B, indomethacin inhibited the formation of cyclooxygenase products HHT and PGE_2 at concentrations of 10^{-4}-10^{-6}M.

Figures 2A and 3 show the concentration dependence of the effects of rosmarinic acid on the formation of the lipoxygenase products (15-HETE, 5-HETE, and LTB_4), and the cyclooxygenase products (HHT and PGE_2). Rosmarinic acid inhibited the formation of 5-HETE and LTB_4 at concentrations of 10^{-5}-10^{-3}M, concentration dependently, and the formation of 15-HETE and HHT was inhibited at concentrations of 10^{-4}-10^{-3}M, but at a concentration of 10^{-4}M, rosmarinic acid stimulated the formation of PGE_2. Caffeic acid at concentrations of 10^{-6}-10^{-3}M inhibited the formation of

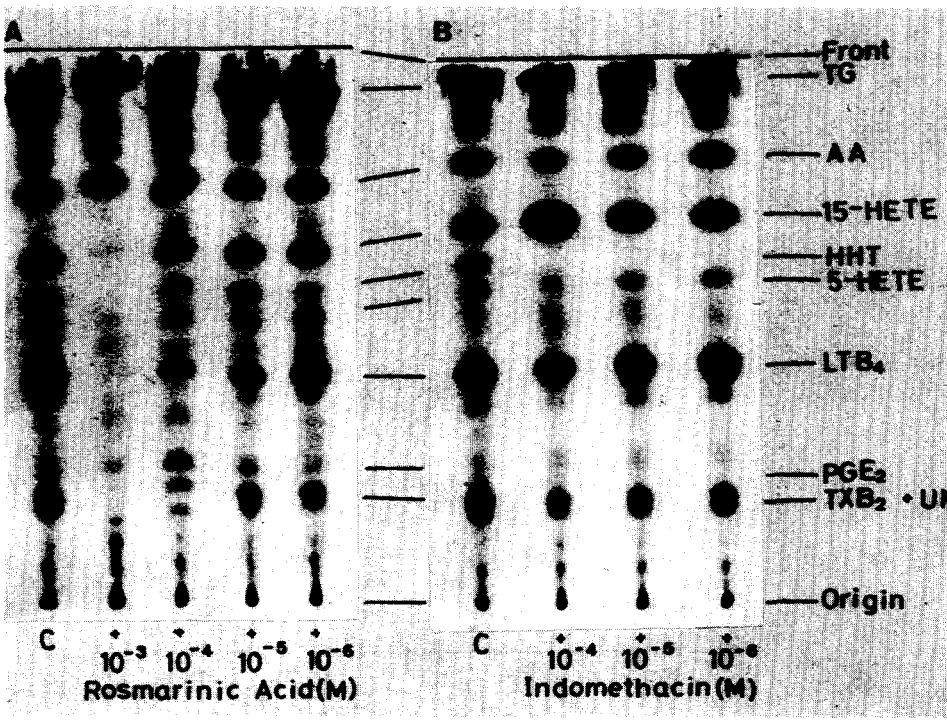


FIGURE 2. Autoradiograph of tlc plates of (1-^{14}C) arachidonic acid metabolites induced by A 23187 in human PMN-L TG, triacylglycerol; C, A 23187 (10 μM); AA, arachidonic acid, 15-HETE, 15-hydroxy-5,8,11,13-eicosatetraenoic acid; HHT, 12-hydroxy-5,8,10-heptadecatrienoic acid; 5-HETE, 5-hydroxy-6,8,11,14-eicosatetraenoic acid; LTB_4, leukotriene B_4; PGE_2, prostaglandin E_2; TXB_2, thromboxane B_2; UM, unidentified material

5-HETE, and the formation of LTB_4 was also inhibited concentration dependently at concentrations of 10^{-5}-10^{-3}M, but caffeic acid stimulated the formation of 15-HETE and PGE_2 at concentrations of 10^{-5}-10^{-4}M. Moreover, it inhibited the formation of HHT at a concentration of 10^{-3}M, while it stimulated the formation of PGE_2. Caffeoylmalic acid and caffeoyltartaric acid at concentrations of 10^{-4}-10^{-3} stimulated the formation of PGE_2 but inhibited 5-HETE and LTB_4, the 5-lipoxygenase products. Caffeoylquinic acids (3,5-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, and 3,4-di-*O*-caffeoylquinic acid) at concentrations of 10^{-4}-10^{-3}M inhibited the formation of 5-HETE and LTB_4, the 5-lipoxygenase products. The formation of PGE_2 were stimulated by these compounds at a concentration of 10^{-4}M. Chlorogenic acid had no effect (data not shown).

The molar IC_{50} values of caffeic acid, caffeoylmalic acid, caffeoyltartaric acid, rosmarinic acid, 3,5-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, and 3,4-di-*O*-caffeoylquinic acid for the formation of 15-HETE, 5-HETE, LTB_4, HHT, and PGE_2 are summarized in Table 1.

DISCUSSION

In this experiment we detected the formation of 15-HETE (15-lipoxygenase product), 5-HETE and LTB_4 (5-lipoxygenase products), HHT and PGE_2 (cyclooxygenase products), a mixture of unidentified materials (lipoxygenase product), and thromboxane B_2 (cyclooxygenase product) induced by the A 23187 in human PMN-L. Unidentified materials were not inhibited by indomethacin (cyclooxygenase inhibitor). The structures of unidentified materials are now under investigation. It was found that 15-HETE and 5-HETE values from arachidonic acid in PMN-L isolated from 35 to 36-

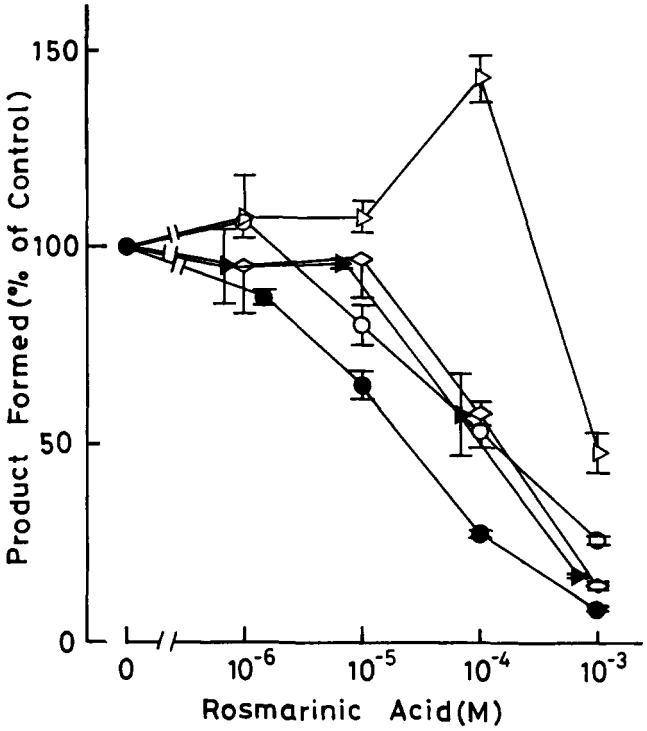


FIGURE 3. Effects of rosmarinic acid on the formation of 15-HETE, HHT, 5-HETE, LTB_4, and PGE_2 from arachidonic acid in human PMN-L. Values are means ± standard errors for 3 experiments. ◇, 15-HETE; ▶, HHT; ▷, PGE_2; ○, 5-HETE; ●, LTB_4.

year-old men were consistent in the control, while the above products in PMN-L isolated from 35-, 47-, and 60-year-old women were not consistent in the control. These findings suggest that the sensitivity of PMN-L may be different by the age or sex. Therefore, the above results are in further need of clarification.

We previously found that baicalein, esculetin, daphnetin, and fraxetin are potent and selective inhibitors of the formation of both 12-HETE (12-lipoxygenase product) in a rat platelet homogenate and 5-HETE (5-lipoxygenase product) in a rat peritoneal PMN-L homogenate (19-21).

The present experiments showed that caffeetannins and related compounds, except for chlorogenic acid, inhibit concentration dependently the formation of the 5-lipoxygenase products, 5-HETE and LTB_4, induced by A 23187 in human peripheral PMN-L. These findings suggest that the presence of two phenolic hydroxyl groups *ortho* to each other and a $-CH=CH-$ group are essential for inhibition of the formation of 5-HETE and LTB_4 by caffeoylquinic acids, since chlorogenic acid had no effect on the formation of 5-HETE and LTB_4. However, monocaffeoyl derivatives such as caffeic acid, caffeoylmalic acid, caffeoyltartaric acid, and rosmarinic acid inhibited the formation of 5-HETE and LTB_4. The degree of inhibition for 5-HETE and LTB_4 formation was in the order rosmarinic acid>caffeic acid>caffeoyltartaric acid>caffeoylmalic acid>chlorogenic acid. The effects of rosmarinic acid and caffeic acid, which are stronger than those of the other monocaffeoyl derivatives, may reflect their relative hydrophobic character that probably affects their penetration into cells. The test compounds, except for chlorogenic acid, stimulated the formation of PGE_2 via the cyclooxygenase route in human PMN-L at a concentration of 10^{-4}M. The synthesis of

TABLE 1. Effects of Caffeetannins and Related Compounds on Arachidonate Metabolism in Human PMN-L[a]

Compounds	IC_{50}(x 10^{-5}M, 95% C.L.)				
	15-HETE[b]	5-HETE	LTB_4	HHT	PGE_2
3,4-DCQ	N.E.[b]	74.9 (46.6−103.2)	9.25 (8.48−10.0)	8.00 (6.15−9.85)	E.E.
3,5-DCQ	*[c]	66.6 (50.0−83.2)	8.59 (7.69−9.49)	90.2 (48.4−132.0)	[d]
4,5-DCQ	*[e]	60.1 (48.7−71.5)	44.3 (31.9−56.7)	74.0 (37.4−110.6)	*[f]
Caffeic Acid	[g]	27.6 (N.S.)	41.1 (25.5−56.7)	N.E.	E.E.
Caffeoylmalic Acid	[h]	91.8 (66.4−117.2)	54.8 (41.1−68.5)	79.6 (54.1−105.1)	E.E.
Caffeoyltartaric Acid	*[i]	87.3 (66.0−108.6)	43.3 (26.9−59.7)	*[i]	N.E.
Rosmarinic Acid	45.5 (27.5−63.5)	21.4 (11.1−31.8)	5.56 (5.39−5.73)	45.9 (24.9−66.9)	*[j]
Chlorogenic Acid	N.E.	N.E.	N.E.	N.E.	N.E.

[a]Values are means for three experiments.

[b]Abbreviations: 15-HETE=15-hydroxy-5,8,11,13-eicosatetraenoic acid; 5-HETE=5-hydroxy-6,8,11,14-eicosatetraenoic acid; LTB_4=leukotriene B_4; HHT=12-hydroxy-5,8,10-heptadecatrienoic acid; PGE_2=prostaglandin E_2; C.L.=Confidence Limits; DCQ=di-*O*-caffeoylquinic acid; E.E.=enhancing effect; N.E=no effect; N.S.=not significance; *=>100.

[c]The percent activities of 3,5-DCQ on the formation of 15-HETE are 50.9±10.1 and 121.1±4.08 % at concentrations of 10^{-3}M and 10^{-4}M, respectively, as compared to control values.

[d]The percent activities of 3,5-DCQ on the formation of PGE_2 are 97.5±23.8 and 160.8±16.0 % at concentrations of 10^{-3}M and 10^{-4}M, respectively, as compared to control values.

[e]The percent activities of 4,5-DCQ on the formation of 15-HETE are 56.9±3.48 % at a concentration of 10^{-3}M, as compared to control values.

[f]The percent activities of 4,5-DCQ on the formation of PGE_2 are 79.5±3.53, 155.0±5.72 and 126.0±2.87 % at concentrations of 10^{-3}M, 10^{-4}M and 10^{-5}M, respectively, as compared to control values.

[g]The percent activities of caffeic acid on the formation of 15-HETE are 96.5±2.85, 153.7±10.7 and 136.7±2.05 % at concentrations of 10^{-3}M, 10^{-4}M and 10^{-5}M, respectively, as compared to control values.

[h]The percent activities of caffeoylmalic acid on the formation of 15-HETE are 114.8±4.81, 141.5±10.5 and 106.3±4.23 % at concentrations of 10^{-3}M, 10^{-4}M and 10^{-5}M, respectively, as compared to control values.

[i]The percent activities of caffeoyltartaric acid on the formation of 15-HETE and HHT are 55.0±16.2 and 57.9±2.11 %, respectively, at a concentration of 10^{-3}M as compared to control values.

[j]The percent activities of rosmarinic acid on the formation of PGE_2 are 48.0±4.78 and 143.4±5.80 % at concentrations of 10^{-3}M and 10^{-4}M, respectively, as compared to control values. Not tested at concentrations higher than 10^{-2}M.

PGE_2 was reported to be increased by some radical scavengers, probably by preventing inactivation of the enzyme by oxygen radicals (22,23). It is well known that superoxide-derived free oxygen radicals are formed by the addition of Fe^{2+} plus ADP, ADP plus ascorbic acid, Fe^{2+} plus ascorbic acid, and NADPH plus ADP in mitochondria and microsomes; lipid peroxides in mitochondria and microsomes are formed in consequence (24,25). We previously reported that caffeetannins such as 3,5-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, and 3,4-di-*O*-caffeoylquinic acid and caffeic acid inhibited the lipid peroxidation induced by ADP plus ascorbic acid and ADP plus NADPH in liver mitochondria and microsomes (16). Therefore, their effects in stimulating the formation of the cyclooxygenase product PGE_2 can be explained by their effects in scavenging free oxygen radicals. It was also reported that PGE_2 increases the intracellular cyclic AMP level in human PMN-L. In this study PGE_2 concentrations induced by caffeoyl derivatives might not be sufficient to induce cyclic AMP formation. Thus, the inhibition of 5-lipoxygenase products 5-HETE and LTB_4 by caffeoyl derivatives could not be explained by PGE_2 formation. Therefore, the inhibition of 5-lipoxygenase products 5-HETE and LTB_4 by caffeoyl derivatives may be modulated by other arachidonate metabolites except for PGE_2

The formation of 15-HETE was stimulated by caffeic acid, caffeoylmalic acid, and caffeoyltartaric acid at a concentration of 10^{-4}M. It has been reported that the 15-lipoxygenase product 15-HETE selectively inhibits both 12- and 5-lipoxygenase enzyme (26,27). Therefore, it seems likely that the inhibitory effects on the formation of 5-HETE and LTB_4 by the above caffeoyl derivatives may be partly inhibited by 15-HETE.

Medicinal plants containing caffeetannins and related compounds used in this study have traditionally been used in the treatment of inflammatory diseases and allergic diseases in Japan and Korea. On the basis of the experimental results, it seems likely that caffeoyl derivatives could be developed as therapeutic drugs for treatment of allergic inflammation such as asthma. Further work is needed to clarify the mechanisms of inhibition of LTB_4 and 5-HETE formation in human PMN-L.

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Received 17 September 1986