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Inhibition of ICE-family cysteine proteases rescues murine lymphocytes from lipoxygenase inhibitor-induced apoptosis

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Abstract Two lipophilic derivatives of caffeic acid which inhibit lipoxygenase, caffeic acid phenethyl ester (CAPE) and *N,N'*-dicyclohexyl-*O*-(3,4-dihydroxycinnamoyl)-isourea (DCHCU), reduced the proliferative response of murine splenocytes to concanavalin A in vitro. Both CAPE and DCHCU induced apoptosis in murine thymocyte cultures as verified by flow cytometry and by visualisation of DNA with acridine orange staining. CAPE-induced apoptosis was inhibited by z-VAD-*fmk*, an inhibitor of the interleukin-1β-converting enzyme family of cysteine proteases. We suggest that the lipoxygenase pathway of arachidonic acid metabolism plays a role in regulating lymphocyte responses such as proliferation and apoptosis.

Key words: Apoptosis; Lipoxygenase; Caffeic acid derivative; ICE-family cysteine protease

1. Introduction

Arachidonate metabolism via the lipoxygenase (LOX) pathway leads to the formation of hydroxy fatty acids, leukotrienes, lipoxins and other products. The importance of these metabolites in normal physiological responses as well as in various pathophysiological conditions such as hypersensitivity and inflammation is widely recognised [1,2]. LOX-derived eicosanoids have been shown to regulate the production and action of some cytokines including interferons [3], to regulate natural killer cell activity [4], to induce growth-related signals and to regulate cell proliferation [5,6]. Eicosanoids are involved in thymocyte maturation and/or differentiation and they modulate the activity of thymus-dependent lymphocytes [7]. Many pharmacological agents have been developed that inhibit the generation of eicosanoids with different potency and selectivity [8,9]. Screening of natural products has revealed a wide variety of phenolic compounds that inhibit LOX, mainly through redox mechanisms [10].

Our previous investigations have shown that two lipophilic derivatives of caffeic acid, caffeic acid phenethyl ester (CAPE) and an intermediate in its chemical synthesis, *N,N'*-dicyclohexyl-*O*-(3,4-dihydroxycinnamoyl)-isourea (DCHCU) (see Scheme 1), are antioxidants which inhibit plant 5- and 15-lipoxygenases [11,12]. CAPE is a biologically active ingredient of honeybee propolis, a natural product which has been widely used as a folk medicine. We have found that both CAPE and DCHCU inhibit mammalian lipoxygenases: they potently suppress eicosanoid production by human neutrophils and mouse macrophages in vitro and during acute peritoneal inflammation in vivo [13]. In addition, it has been reported that CAPE induces differential growth inhibition of cancer cells compared with normal cells [14], and modulates oxidative stress in several systems [15,16]. To evaluate the effect of CAPE and DCHCU on lymphocyte functions, their action together with that of propolis and quercetin (a flavonoid component of propolis) on two alternative responses was investigated – cell proliferation and apoptosis. We found that both CAPE and DCHCU, used at concentrations selective for mammalian lipoxygenases, cause time-dependent inhibition of the proliferative response and induce apoptotic cell death in murine lymphocytes. CAPE-induced apoptosis was prevented by an inhibitor of the ICE-family of cysteine proteases, Z-Val-Ala-Asp-fluoromethylketone (zVAD-*fmk*).

2. Materials and methods

2.1. Reagents

Zymosan, RPMI-1640, concanavalin A (ConA), glutamine, fetal calf serum (FCS) and antibiotics were obtained from Sigma Chemical Co. (Poole, Dorset, UK). CAPE and DCHCU were synthesised in the A.N. Belozersky Institute of Physico-Chemical Biology (Moscow, Russia) by Drs. G.A. Korshunova and N.V Sumbatyan by the method described elsewhere [12]. The ethanol extract of propolis (EEP) was obtained from Bee Health Ltd. (Scarborough, Yorks, UK). Benzyloxycarbonyl-valinyl-alaninyl-aspartyl(*O*-methyl)-fluoromethylketone (zVAD-*fmk*) was supplied by Enzyme Systems Products Inc. (Dublin, CA, USA). The stock solution of zVAD-*fmk* (50 mM in DMSO) was kept at −20°C and final dilutions were made immediately prior to use. Colorimetric cytotoxicity assay kits were from Proteins International Inc. (Rochester Hills, MI, USA)

2.2. Animals

Male C57BL6 mice, aged 8–10 weeks, were bred and housed in the Department of Biochemistry, University of Oxford. Mice were allowed access ad libitum to standard laboratory chow (SDS No. 1; Special Diet Services, Witham, Essex, UK) and water.

2.3. Proliferation and viability assay

Mouse splenocytes were isolated and cultured as described elsewhere [17]. Briefly, cells (2.5×10^6 cells/ml) were cultured in RPMI-1640 medium supplemented with 10% (v/v) FCS, 2 mM glutamine and antibiotics and incubated in a 5% CO_2 atmosphere at 37°C. Cells were activated by incubation with 5 μg/ml of ConA in the presence of the inhibitors (added 5 min before the stimulation) or of the equivalent amount of ethanol for 6–48 h. [^{3}H]Thymidine (0.2 μCi/well) was added during the last 18 h of the culture and the cells were harvested into glass fibre filters, processed and counted in a β-counter. The cultures were established in triplicate and the results are expressed as % of thymidine incorporation into control cells. Cell viability was assessed by measuring lactate dehydrogenase (LDH) activity released into the medium, using the colorimetric cytotoxicity assay kits according to the instructions of the manufacturer.

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Abbreviations: LOX, lipoxygenase; NDGA, nordihydroguaiaretic acid; ETI, 5,8,11-eicosatrienoic acid; BHPP, *N*-benzyl-*N*-hydroxy-5-phenylpentanamide; CDC, cinnamyl-3,4-dihydroxy-α-cyanocinnamide; ROS, reactive oxygen species; EEP, ethanol extract of propolis; ICE, interleukin-1β-converting enzyme

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2.4. Detection of apoptosis

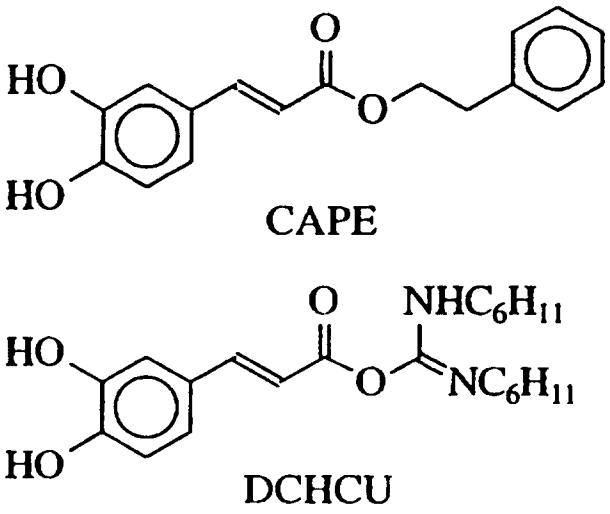
Apoptosis of murine thymocytes was quantified by flow cytometry using a Becton Dickinson FACScan analyser as described elsewhere [18]. Apoptotic thymocytes were identified by their lower forward light scatter (due to cell shrinkage) and higher side scatter (due to increased granularity of the cell) than their viable counterparts [19]. Briefly, mouse thymocytes (2.5×10^6 cells/ml) were cultured in 24-well plates for different times in the presence or absence of ConA and the inhibitors. They were then washed twice with phosphate-buffered saline (PBS), fixed in PBS containing 1% formaldehyde, 0.5% BSA and 0.03% NaN_3 and kept at 4°C until analysis. The percentage of apoptotic and viable cells was quantified by FACScan; for a viable control cell population freshly prepared, non-cultured cells were used.

Confirmation that thymocyte cell death was by apoptosis was provided by visualisation of DNA with acridine orange staining as described elsewhere [20].

3. Results

3.1. Assay of lymphocyte proliferation and viability

The effects of CAPE, DCHCU, quercetin and EEP on ConA-induced proliferation of murine spleen lymphocytes, as assessed by [^{3}H]thymidine incorporation, were investigated (Fig. 1). All four agents inhibited the proliferative response in a dose-dependent fashion (Fig. 1). CAPE had the most potent antiproliferative effect: it caused 50% inhibition of [^{3}H]thymidine incorporation at a concentration of about 2.5 μM after 24 h of incubation. The same effect of DCHCU and quercetin was displayed at concentrations 5 and 15 μM, respectively. EEP completely abolished [^{3}H]thymidine incor-



Scheme 1. Chemical formulae of CAPE and DCHCU.

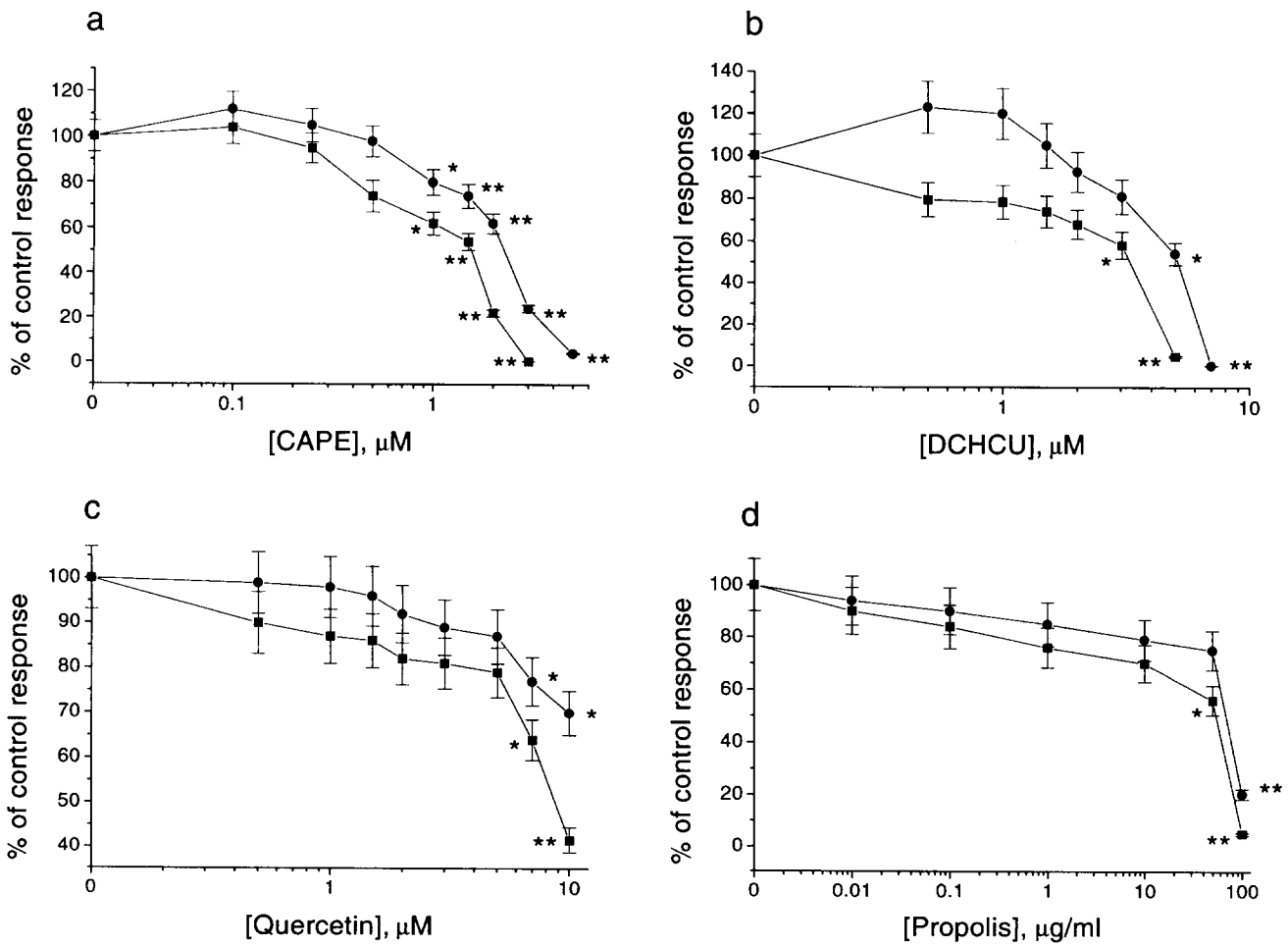


Fig. 1. Inhibition of splenocyte proliferation as determined by [^{3}H]thymidine incorporation. Spleen cells were activated with ConA and cultured in the absence or presence of CAPE (a), DCHCU (b), quercetin (c) or propolis (d) for 24 (●) or 48 (■) h. The percentage inhibition of [^{3}H]thymidine incorporation was determined as the mean cpm of treated cells divided by that of untreated (control) cells. The results are the mean ± S.E.M. of 3–5 independent experiments; *significant difference from untreated cells $P < 0.05$, **$P < 0.01$ (one-way ANOVA).

poration at a concentration of 100 μg/ml but at concentrations less than 50 μg/ml did not significantly affect proliferation. The effect of all agents was greater after 48 h of incubation than after 24 h (Fig. 1). The analysis of cell viability revealed that the culture of splenocytes with 3 μM CAPE or 5 μM DCHCU for 48 h resulted in a substantial cell lysis (up to 50% of the cells were lysed). At concentrations of more than 1.5 μM CAPE was toxic, causing lysis of 10–20% of the cells in a dose-dependent manner after 24 h. DCHCU did not induce cell lysis after 24 h. Neither quercetin nor propolis caused cell lysis.

3.2. *Apoptosis assay*

Apoptotic cell death was observed in murine lymphocytes cultured in the presence of CAPE and DCHCU. The apoptotic effect of all agents was similar upon thymocytes and splenocytes (data not shown); further data for thymocytes only are shown. When thymocytes were incubated with CAPE or DCHCU, the proportion of apoptotic cells increased with time (Fig. 2; DCHCU data not shown). A difference between spontaneous and CAPE-induced apoptosis was apparent after 12 h and was most significant at 24 h of incubation (Fig. 2). After a 36-h culture period the proportion of apoptotic cells did not differ between CAPE-treated and -untreated cultures (Fig. 2). Both CAPE and DCHCU induced apoptosis of thymocytes in a concentration-dependent manner (Fig. 3). The minimal concentration of CAPE significantly affecting apoptosis was 1 μM and that of DCHCU was 10 μM after 12 h of treatment with the compounds. After 24 h of incubation with 1 μM CAPE or 3 μM DCHCU about 50% of thymocytes were apoptotic; at higher concentrations of both compounds no further increase in the percentage of apoptotic cells was observed (Fig. 3). Quercetin did not induce apoptosis at the concentrations at which it affected proliferation (7–10 μM; data not shown). In the presence of 50–100 μg/ml of EEP 50% of thymocytes in culture underwent apoptosis (data not shown). Since EEP is a complex mixture of many biologically

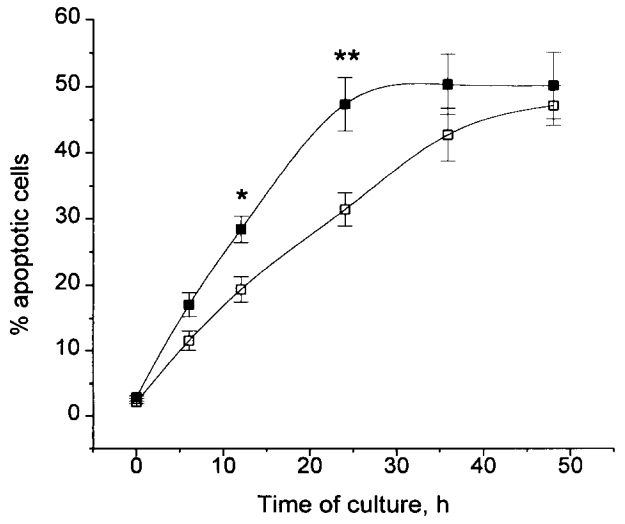


Fig. 2. The time-course of spontaneous (□) or CAPE-induced apoptosis (■) (2.5 μM CAPE) of murine thymocytes. The results are the mean ± S.E.M. from triplicate cultures and 2 independent experiments; *significant difference from spontaneous apoptosis, $P < 0.05$, **$P < 0.01$ (one-way ANOVA).

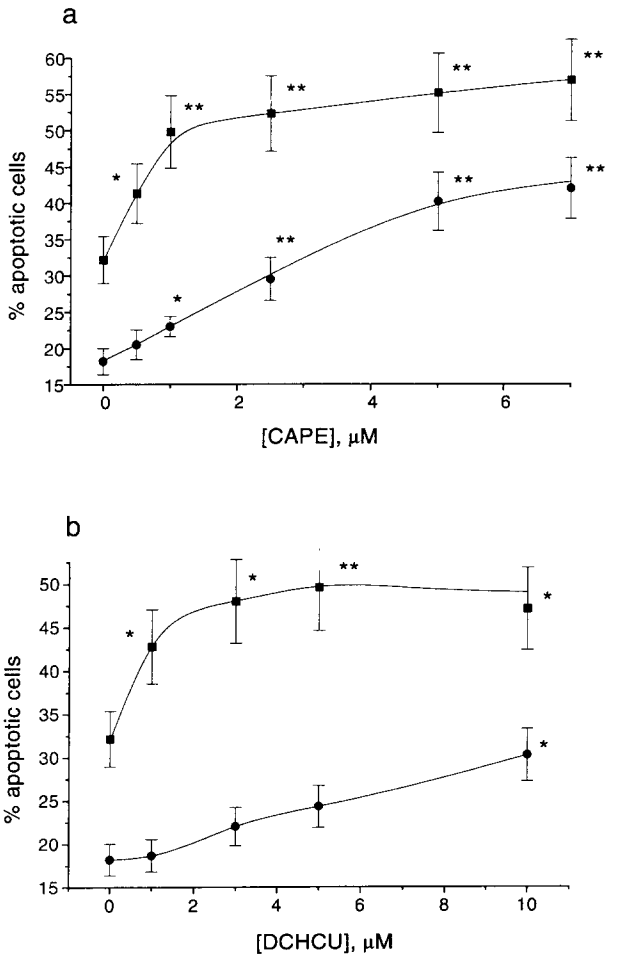


Fig. 3. Dose-dependent effect of CAPE (a) and DCHCU (b) on apoptosis of thymocytes cultured for 12 (●) or 24 (■) h. *Significant difference from spontaneous apoptosis, $P < 0.05$, **$P < 0.01$ (one-way ANOVA).

active compounds it is not clear whether this apoptotic effect was due to the presence of CAPE and/or other cytotoxic agents. We examined cell cultures which were non-activated or activated with ConA. We did not observe a significant difference in the proportion of apoptotic cells in the presence or in the absence of ConA, although the proportion of viable cells was less in ConA-activated cultures. Therefore, we present here the results obtained in the absence of ConA since this allows clearer interpretation of the quantitative apoptosis rate.

3.3. *Effect of ICE-cysteine protease inhibition on CAPE-induced apoptosis*

The new and growing family of interleukin-1β-converting enzyme (ICE) cysteine proteases are now recognised to be major effectors of cellular death by apoptosis [21]. We examined the effect of zVAD-*fmk*, an irreversible, cell-permeable inhibitor of ICE family cysteine proteases [22], on CAPE-triggered apoptosis. At a concentration of 100 μM zVAD-*fmk* reduced CAPE-induced apoptosis such that the level of apoptosis was the same as that of the control cells (Fig. 4). The same treatment of control cells with zVAD-*fmk* did not result in significant inhibition of spontaneous apoptosis (data not shown). These data suggest that CAPE-triggered apoptosis

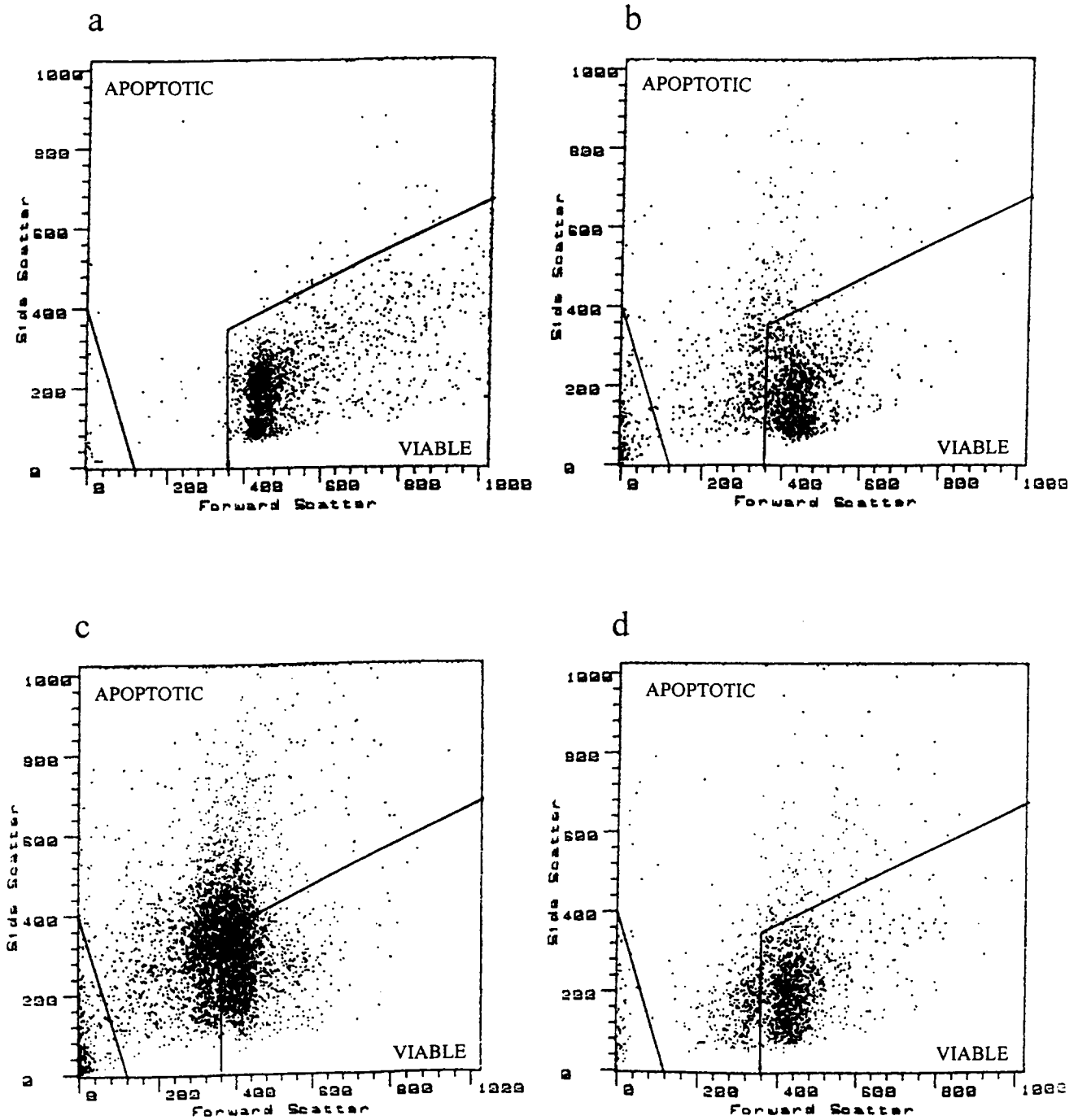


Fig. 4. FACScan dot-plot light scatter profiles of mouse thymocytes at a concentration of 2.5×10^6 cells/ml. Data are shown for non-cultured (a), cultured (b), cultured with 2.5 μM CAPE (c) or cultured with 2.5 μM CAPE and 100 μM z-VAD-*fmk* (d) cells after 24 h. The viable population contains cells with relatively high forward-scatter and low side-scatter properties. Cells undergoing apoptosis appear in the low-forward-scatter/high-side-scatter zone. The results of one experiment, representative of four, are shown.

is dependent on the activity of members of the ICE family of cysteine proteases.

4. Discussion

We have shown here that two caffeic acid derivatives which are LOX inhibitors, DCHCU and CAPE (a natural component of propolis), have antiproliferative action on normal murine lymphocytes. Flow cytometry revealed that one component of the reduction in the proliferative response was an augmentation of apoptosis. CAPE-induced apoptosis was blocked by an inhibitor of ICE-proteases, zVAD-*fmk*, although spontaneous apoptosis was resistant to this inhibitor. These data provide evidence that an ICE-related protease(s) are required for CAPE-triggered apoptosis: zVAD-*fmk* has been shown to inhibit processing and thus activation of the ICE-family member CPP32/Yama [23,24].

The results of our study are in agreement with the work of

Chiao et al. [15], reporting that CAPE causes growth arrest in non-tumorigenic rat embryo fibroblasts (CREF) and apoptotic death in the virally transformed CREF cells (Wt3A). It was suggested that CAPE altered the redox state of the cells and that CAPE-triggered apoptosis in Wt3A cells was associated with their reduced oxidant defences [15]. However, as we have shown previously, CAPE and DCHCU are antioxidants: they inhibit the production of reactive oxygen species (ROS) by human neutrophils and in a cell-free xanthine-xanthine oxidase system to at least as great an extent as the other antioxidant LOX inhibitors, such as NDGA and caffeic acid [11,12]. It seems unlikely therefore that CAPE- and DCHCU-induced apoptosis can be explained simply as a result of the redox imbalance causing increased ROS production. The concentrations of CAPE and DCHCU which induced antiproliferative and apoptotic effects were similar to the concentrations which inhibit LOX in mouse macrophages (the IC_{50}s of CAPE and DCHCU were about 0.5 and 0.8 μM, respectively) [13]. There is now increasing evidence of the possible involvement of lipoxygenases and their products in the regulation of cell growth and death. For example, inhibition of 5-LOX metabolism resulted in significant reduction in the growth of a number of lung cancer cell lines [25] and 5-LOX inhibitors such as 5,8,11,14-eicosatetraynoic acid, A63162 and SC41661A reduced the proliferation of chronic myelogenous leukaemia blast cells, induced their differentiation and promoted apoptosis in promyelocytic cells [26]. It was recently reported that the 12-LOX and probably the 15- and/or 5-LOX arachidonate pathways may function as critical regulators of cell survival and apoptosis in rat Walker 256 (W256) carcinosarcoma cells [27]. Selective 12-LOX inhibitors (BHPP, baicalein and CDC) and general LOX inhibitors (ETI and NDGA) induced W256 cell apoptosis [27].

Thus, the current results along with those of previous studies indicate that CAPE and DCHCU modulate cell proliferation and apoptosis not due to their redox properties but rather due to their anti-lipoxygenase activity. It is not certain, however, whether their effects resulted from specific 5-LOX inhibition or from inhibition of other LOX enzymes, or whether some other mechanisms may be involved.

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References

[1] Samuelsson, B. (1983) Science 220, 568–575.
[2] Feuerstein, G. and Hallenbeck, J.M. (1987) FASEB J. 1, 186–192.
[3] Mayer, M. (1990) Acta Virol. 34, 99–107.
[4] Rola-Pleszczynski, M. (1985) Immunol. Today 6, 302–307.
[5] Tang, D.G., Renaude, C., Stojakovic, S., Dilgio, C.A., Porter, A. and Honn, K.V. (1995) Biochem. Biophys. Res. Commun. 211, 462–468.
[6] Bortuzzo, C., Hanif, R., Kashfi, K., Staiano-Coico, L., Shiff, S.J. and Rigas, B. (1996) Biochim. Biophys. Acta 1300, 240–246.
[7] Juzan, M., Hostein, I. and Gualde, N. (1992) Prostaglandins Leukotrienes Ess. Fatty Acids 46, 247–255.
[8] Goetzl, E.J., An, S. and Smith, W.L. (1995) FASEB J. 9, 1051–1058.
[9] McMillan, R.M. and Walker, E.R. (1992) Trends Pharmacol. Sci. 13, 323–330.
[10] Ford-Hutchinson, A.W., Gresser, M. and Young, R.N. (1994) Annu. Rev. Biochem. 63, 383–417.
[11] Sud'ina, G.F., Mirzoeva, O.K., Pushkareva, M.A., Korshunova, G.A., Sumbatyan, N.V. and Varfolomeev, S.D. (1993) FEBS Lett. 329, 21–24.
[12] Mirzoeva, O.K., Sud'ina, G.F., Pushkareva, M.A., Korshunova, G.A., Sumbatyan, N.V. and Varfolomeev, S.D. (1995) Bioorg. Khim. 21, 143–151.
[13] Mirzoeva, O.K. and Calder, P.C. (1996) Prostaglandins Leukotrienes Ess. Fatty Acids (in press).
[14] Grunberger, D., Banerjee, R., Eisinger, K., Oltz, E.M., Efros, L., Caldwell, M., Estevez, V. and Nakanishi, K. (1988) Experientia 44, 230–232.
[15] Chiao, C., Carothers, A.M., Grunberger, D., Solomon, G., Preston, G.A. and Barrett, J.C. (1995) Cancer Res. 55, 3576–3583.
[16] Bhimani, R.S., Troll, W., Grunberger, D. and Frenkel, K. (1993) Cancer Res. 53, 4528–4533.
[17] Yaqoob, P., Newsholme, E.A. and Calder, P.C. (1994) Immunology 82, 603–610.
[18] Knox, K.A., Finney, M., Milner, A.E., Gregory, C.D., Wakelam, M.J.O., Michell, R.H. and Gordon, J. (1992). Int. J. Cancer 52, 959–266.
[19] Dive, C., Gregory, C.D., Phipps, D.J., Evans, D.L., Milner, A.E. and Wyllie, A.H. (1992) Biochim. Biophys. Acta 1133, 275–285.
[20] Wyllie, A.H., Morris, R.G., Smith, A.L. and Dunlop, D. (1984) J. Pathol. 142, 67–77.
[21] Enari, M., Hug, H. and Nagata, S. (1995) Nature 375, 78–80.
[22] Pronk, G.S., Ramer, K., Amiri, P. and Williams, L.T. (1996) Science 271, 808–810.
[23] An, S. and Knox, K.A. (1996) FEBS Lett. 386, 115–122.
[24] Jacobson, M.D., Weil, M. and Raff, M.C. (1996) J. Cell Biol. 133, 1041–1051.
[25] Avis, I.M., Jett, M., Boyle, T., Vos, M.D., Moody, T., Treston, A.M., Martinez, A. and Mulshine, J.L. (1996) J. Clin. Invest. 97, 806–813.
[26] Anderson, K.M., Seed, T., Plate, J.M.D., Jajeh, A., Meng, J. and Harris, J.E. (1995) Leukemia Res. 19, 789–801.
[27] Tang, D.G., Chen, Y.Q. and Honn, K.V. (1996) Proc. Natl. Acad. Sci. USA 93, 5241–5246.