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COMMUNICATIONS

Characterization of cysteinyl-leukotriene formation in primary astroglial cell cultures

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Abstract—The formation and composition of cysteinyl-leukotrienes (LT) in primary astroglial cell cultures prepared from newborn rat brain has been studied. Small amounts of cysteinyl-LT determined in terms of LTC_4-like material in the supernatants of the cultures, became detectable after stimulation of the cells with 10^{-5} M ionophore A23187. Cysteinyl-LT formation increased with time, reaching about 600 pg (mg protein)$^{-1}$ after 60 min incubation. In contrast, considerable thromboxane (TX) B_2 synthesis was found at 5 min following A23187-stimulation (about 30 ng TXB_2 (mg protein)$^{-1}$). The synthesis of cysteinyl-LT was abolished by 5×10^{-5} M nordihydroguaiaretic acid (NDGA). Irrespective of the duration of incubation, blockage of prostanoid synthesis by 10^{-6} M indomethacin did not result in increased cysteinyl-LT production. Reversed phase HPLC combined with radioimmunological detection showed that, after 60 min incubation in the presence of A23187, LTC_4 and LTD_4 accounted for practically all the LTC_4-like immunoreactive material in the supernatants of cell cultures. No significant amounts of LTE_4 could be detected. The results show that astrocytes may contribute to brain LTC_4 and LTD_4 synthesis. However, the cellular site of cerebral LTE_4 formation seems to be other than the astroglia.

The formation of prostanoids, hydroxy fatty acids, and leukotrienes (LT) by brain tissue is well established (Wolfe 1982; Feuerstein & Hallenbeck 1987; Miyamoto et al 1987). Prostanoids, as growing evidence shows, are produced predominantly by mature astrocytes (Seregi et al 1987; Murphy & Pearce 1988; Hertting & Seregi 1989), except for prostacyclin, which is mainly of vascular origin (Wolfe 1982). The cellular source of lipoxygenase products is less understood, however, such information may help to elucidate the functional significance of these eicosanoids in the brain. Monohydroxy fatty acids can originate from nerve cells (Birkle & Bazan 1987; Piomelli et al 1987) or from vascular elements (Hambrecht et al 1987), while LTB_4 has been shown to be synthesized by neurons (Birkle & Bazan 1987), oligodendrocytes (Shirazi et al 1987) and astrocytes (Hartung et al 1988). The formation of LTC_4-like material by cultured astroglial cells has also been described (Hartung & Toyka 1987). In this study, we have investigated the kinetics of formation of cysteinyl-LT compared with that of TXB_2 in rat primary astroglial cell cultures. Since rodent brain tissue is capable of synthesizing LTC_4, LTD_4 and LTE_4 both in in-vitro (Lindgren et al 1984) and in-vivo conditions (Moskowitz et al 1984; Simmet et al 1988a), the composition of LTC_4-like material produced by rat cultured astrocytes was also studied.

Materials and methods

Primary astroglial cell cultures were prepared from newborn rat brain hemispheres. A detailed description of preparation and culture conditions, as well as the morphological and immunocytochemical characterization of the cultures, has been published previously (Keller et al 1985).

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Fourteen day old cultures were used. For experimental incubations, cells were washed 3 times with Dulbecco's Phosphate-Buffered Saline containing 0·1 g glucose monohydrate L^{-1} (DPBS), and were preincubated for 15 min at 37°C in the above buffer. Incubations were carried out in duplicate or triplicate in 1 mL DPBS at 37°C for 5, 10, 20, 40 or 60 min in the absence or presence of 10^{-5} M ionophore A23187. The inhibitors studied were present both during preincubation and incubation. After incubation, the supernatants of the cultures were decanted and proteins were precipitated by boiling for 4 min with subsequent centrifugation. The concentrations of cysteinyl-LT and of TXB_2 were estimated in the deproteinized supernatants, using radioimmunoassays (Dembinska-Kiec et al 1984; Keller et al 1985). The anti-cysteinyl-LT antiplasma used for the present experiments exhibits 40% relative crossreaction with both LTD_4 and LTE_4 (Aehringhaus et al 1982). Since LTC_4 standard curves were constructed, the amount of cysteinyl-LT was expressed as LTC_4-like material. LTC_4-like material was further characterized by reversed phase HPLC, as described previously (Simmet et al 1988a). Briefly, supernatants from 10 culture dishes were pooled after 60 min incubation in the presence of 10^{-5} M A23187, were extracted by C_{18} SEP-PAK and were eluted with methanol. After evaporation, the residue was redissolved in 30% methanol (v/v) and was injected onto a C_{18}-Nucleosil column (250 × 4 mm, particle size 5 μm, Macherey-Nagel, Düren, FRG), using a solvent system methanol–water–acetic acid (68:32:0·01, v/v/v; pH 5·5). Eluate fractions collected at 1 min intervals were finally tested for inhibition of binding of [^{3}H]LTC_4 to the anti-cysteinyl-LT antiplasma used for the radioimmunoassay. Protein content of cell cultures was determined according to Lowry et al (1951).

Indomethacin, nordihydroguaiaretic acid and TXB_2 were purchased from Sigma (St. Louis, MO, USA), unlabelled LTs were from Paesel (Frankfurt, FRG), whereas tritiated LTC_4 and TXB_2 were from New England Nuclear (Dreieich, FRG). Analytical grade chemicals and HPLC grade solvents from commercial sources were used.

Results and discussion

Fig. 1 shows the time course of cysteinyl-LT and TXB_2 formation by primary astroglial cell cultures without and upon A23187 (10^{-5} M) stimulation, as well as the effect of cyclooxygenase and 5-lipoxygenase blocking agents on the synthesis of these eicosanoids.

Large amounts of TXB_2 (about 30 ng (mg protein)$^{-1}$) could be measured 5 min after ionophore stimulation, as found previously (Keller et al 1985). Interestingly, the synthesis of LTC_4-like material started much more slowly following stimulation. During incubation for 20, 40 or 60 min, A23187-induced cysteinyl-LT synthesis increased gradually with time, but the amount of LTC_4 like material (about 600 pg (mg protein)$^{-1}$ h^{-1}) remained about 100-fold less than the amount of TXB_2 formed.

The 5-lipoxygenase inhibitor NDGA (5×10^{-5} M) abolished

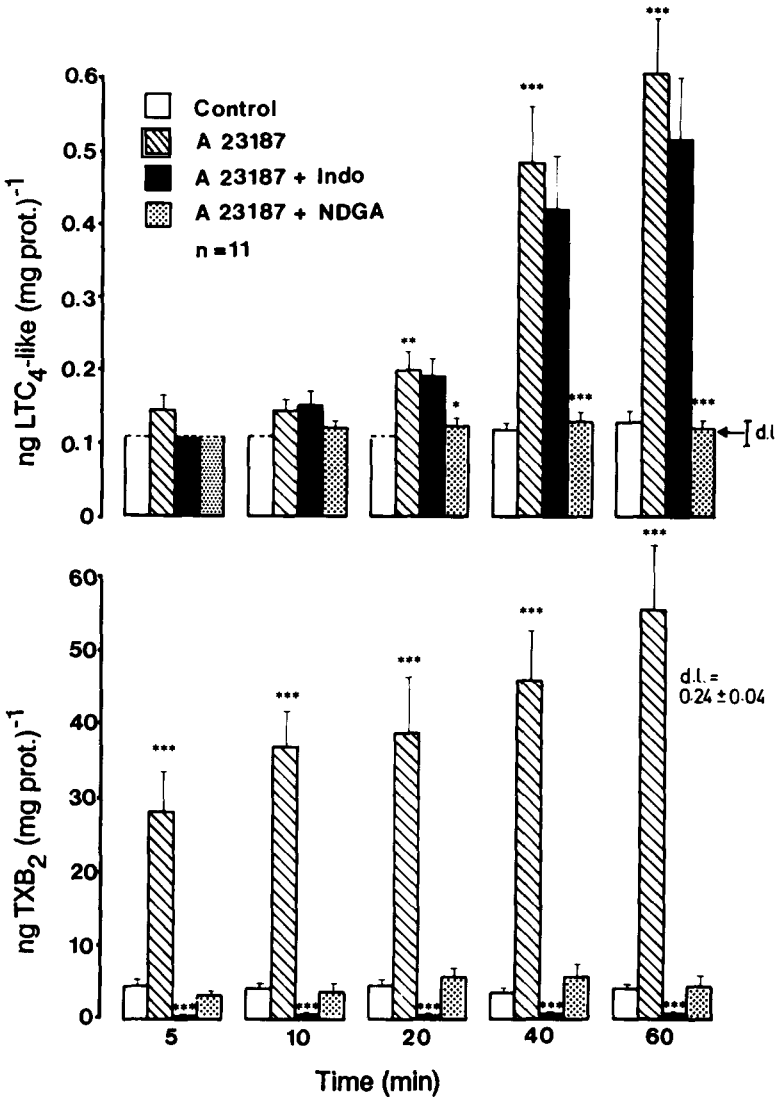

FIG. 1. Time course of the formation of LTC_4-like material (upper panel) and that of TXB_2 (lower panel) in the absence and presence of ionophore A23187 (10^{-5} M), and the effect of 5×10^{-5} M nordihydroguaiaretic acid (NDGA) and 10^{-6} M indomethacin (Indo) on A23187-stimulated eicosanoid synthesis in rat primary astroglial cultures. The results are the means of five experiments using independent cultures, performed in duplicate or triplicate ± s.e.m. d.l. = detection limit of the assay. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; Significantly different from the corresponding control as calculated by Student's t-test (two-tailed).

the A23187-induced appearance of LTC_4-like material in the supernatant of the cultures, showing that de novo synthesis of cysteinyl-LT by the cells, rather than their release from the cells is responsible for the effect of ionophore A23187. At the concentration used, NDGA also inhibited TXB_2 formation, which is in agreement with previous data showing NDGA to be a dual inhibitor (Brune et al 1984).

Like Hartung & Toyka (1987), we found that no increased formation of LTC_4-like material occurred in the presence of indomethacin, which blocked prostanoid synthesis. Thus, prostanoid formation via the cyclo-oxygenase pathway does not seem to compete for substrate arachidonic acid with cysteinyl-LT formation via the 5-lipoxygenase pathway in astrocytes. This might be due to a moderate synthetic capacity of astroglial 5-lipoxygenase, which can be supplied sufficiently with arachidonic acid, even if the astroglial cyclo-oxygenase of high specific activity (Keller et al 1985; Seregi et al 1987) is fully active. Moderate activity of some enzymes of the cysteinyl-LT forming enzyme system can also be rate-limiting. Compartmentalization of the two arachidonic acid metabolizing pathways within astroglial cells can also be taken into account.

It has to be mentioned that, unlike in astrocytes, inhibition of prostanoid synthesis enhanced cysteinyl-LT formation in brain tissue both in-vitro (Dembinska-Kiec et al 1984) and in-vivo (Simmet et al 1988a). This discrepancy suggests that cellular elements other than astrocytes in nervous tissue must exist which are capable of increasing cysteinyl-LT synthesis following cyclo-oxygenase inhibition, and that astrocytes are not the only source of cysteinyl-LT in the brain.

Fig. 2 shows that the LTC_4-like immunoreactive material generated by the rat primary astroglial cultures upon 60 min stimulation with ionophore A23187 can be resolved by reversed phase HPLC into two substances coeluting with authentic LTC_4 and LTD_4, respectively. Considering that the anti-cysteinyl-LT antiplasma used for the detection of cysteinyl-LT in the HPLC eluates crossreacts by 40% with LTD_4, it can be estimated that the ratio between LTC_4 and LTD_4 in the supernatants of the cultures was about 1:1. Although the 40% crossreaction of the

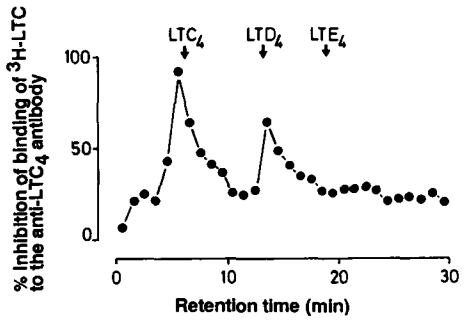

FIG. 2. Reversed phase HPLC profile of LTC_4-like material produced by astroglial cell cultures upon 60 min incubation in the presence of 10^{-5} M ionophore A23187. Supernatants from 10 culture dishes were pooled. Eluates were collected at 1 min intervals and cysteinyl-LT were detected by radioimmunoassay using the anti-cysteinyl-LT antiplasma described in Methods. Arrows indicate the retention times of appropriate standards.

anti-cysteinyl-LT antiplasma with LTE_4 also permits the identification of LTE_4, no significant amounts of immunoreactive material coeluting with authentic LTE_4 could be detected in cell incubates when subjected to HPLC analysis. These results show that primary astroglial cultures are capable of synthesizing LTC_4 and of transforming it into LTD_4, but they seem to have only very little, if any, dipeptidase activity to produce LTE_4. This is in contrast with the results obtained with rat (Lindgren et al 1984), or human (Simmet et al 1988b) brain slices in-vitro, as well as gerbil brain tissue after convulsions (Simmet et al 1988a) in-vivo, where in addition to LTC_4 and LTD_4, the formation of LTE_4 has also been reported. Furthermore, the distribution pattern among the cysteinyl-LT produced by rat astroglial cultures differs from the recently reported molecular composition of cysteinyl-LT synthesized by human malignant astrocytoma tissue slices, which produce mostly LTD_4 and LTE_4 (Simmet et al 1990). This suggests that LTE_4 in the rat brain does not originate from astrocytes. Thus, astroglia-derived LTD_4, is likely to be further metabolized into LTE_4 by other compartments of rat brain tissue.

Cysteinyl-leukotrienes represent biological effects on brain vasculature (Feuerstein & Hallenbeck 1987), neurons (Palmer et al 1980, 1981) and hormone producing cells (Hulting et al 1985; Gerozissis et al 1986) and are possibly involved in cerebral immunoinflammatory processes (Wolfe 1982; Hartung & Toyka 1987). Astrocytes, as LTC_4 and LTD_4 producing cells, seem to contribute to leukotriene-mediated physiological and pathophysiological processes in the brain.

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