行政院國家科學委員會補助專題研究計畫成果報告

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※花生四烯酸引起大鼠小腦顆粒神經細胞內酸化※
※機制之研究　　　　　　　　　　　　　　　　※
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計畫主持人：陳惟浩　臺大醫院內科部

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花生四烯酸引起大鼠小腦顆粒神經細胞內酸化機制之研究

The mechanism of arachidonic acid-induced intracellular acidosis in rat cerebellar granule cells

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一、中文摘要

花生四烯酸(arachidonic acid, AA)是生物体內最重要的不飽和脂肪酸之一,對於許多細胞生理功能都有相當大的影響.例如可以造成細胞內鈣離子上升及細胞內酸鹼值下降(酸化)等等.在某些病理狀態下如中風(腦缺血),癲癇及神經退化性疾病床(如Alzheimer氏症),AA與其他一些不飽和脂肪酸的濃度可能大量增加,而對神經細胞產生嚴重損害.其機制至今尚未明瞭.因此, 本研究中,我們以初級培養的大鼠小腦顆粒細胞,利用螢光染劑,來研究AA引起細胞內酸鹼值改變機制,並且嘗試探討此種酸化機制在腦缺血初期,對神經細胞功能的重要性.

我們發現10 μM AA可以引起顯著而持續性的細胞內酸化,由7.11±0.01 (n=32)造成0.32單位之酸化,而相同程度的細胞內酸化則須40mM propionate.我們證明此種酸化是由AA被動地由細胞外擴散入的細胞內所致(此即Kamp & Hamilton所提出之Flip-Flop模式)而非經由細胞膜上傳輸器.主要證據如下:(1)含一COOH基之脂肪酸(如AA,linoleic acid, α-linolenic acid等)皆可引起相當之酸化,而帶一COOCH3基者則否,至於帶一氨基者反而引起鹼化; (2)所有AA/FAs引致的酸鹼變化均可被0.3%BSA所恢復.此外我們也證實氫離子進入胞內的速率與AA濃度成正比顯示賜此過程為單純擴散機制.另外,DIDS不能抑制此酸化過程表示此酸化非由脂肪酸傳輸器造成.當我們使用抑制脂肪酸代謝之第一個酵素時也無法阻止酸化發生表示此酸化非由脂肪酸之下游代謝產物所造成.除此之外我們也排除自由基,PKC,鈉氫交換器,與氫離子孔道之角色故而我們認為AA之酸化是由AA分子擴散入細胞引起不須藉由傳輸器或離子孔道.在引起細胞內酸化之同時我們也初步觀察到AA也引起細胞核內的同步酸化現象.因為細胞內酸化與細胞核內的同步酸化現象,可能與基因表現有關,因此這一現象值得我們更深入研究.

關鍵詞：花生四烯酸, 細胞內酸鹼值, flip-flop, 細胞核內酸鹼值

Abstract

Arachidonic acid (AA), one of the most important unsaturated fatty acids in nervous system, exerts many cellular effects, including intracellular acidosis and altered intracellular ionic homeostasis that are associated with many physiological functions and pathophysiological conditions. For example, during stroke, convulsion and neurodegenerative disorders, AA and other Non-esterified fatty acids might accumulate in the neurons with resultant injury. In the present study, by continuously measuring intracellular pH with BCECF-loaded cultured rat cerebellar granular cell, we investigated the mechanisms and potential physiological relevance of AA-induced intracellular acidosis.

We found that treatment with 10 μM AA (20 min) results in marked intracellular acidosis (~0.32 pH units). A much higher concentration of propionic acid (40 mM) is

needed to induce a similar pH_i change. AA-induced acidosis occurs by predominantly passive diffusion of the unionized form of AA, as proposed by Kamp and Hamilton for fatty acids (FAs) in artificial phospholipid bilayers (the flip-flop model), rather than a protein carrier. The results supporting this conclusion include: (i) FAs containing a –COOH group (AA, linoleic acid, α-linolenic acid, and docosahexaenoic acid) induce intracellular acidosis, and a FA with a $-COOCH_3$ group (AA methyl ester) has little effect on the pH_i, whereas tetradecylamine, a FA amine, induces intracellular alkalosis, and (ii) both the AA-/FAs- and FA amine-induced pH_i changes are reversed by 0.3 % bovine serum albumin. There is a linear relationship between the initial rate of acid flux and the concentration of AA, and the acidosis is not inhibited by 4,4'-diisothiocyanostilbebe-2,2'-disulfinic acid (DIDS). Moreover, metabolites of AA/FAs are not involved in the acidosis, as inhibition of the first enzyme involved in metabolism of the two parent FAs for the omega-3 (α-linolenic acid) and omega–6 (linoleic acid, i.e. AA precursor) families had no effect. Possible involvement of free radicals, PKC, the Na-H exchanger, or H^+ channels in AA-induced acidosis was also ruled out. It is therefore concluded that AA-induced acidosis is mediated by simple diffusion of AA, rather than by transporters or channels. We have also preliminarily found that AA might alter synchronously the nuclear pH and the cytosolic pH. Since AA can be released under physiological and pathophysiological conditions, the potential relevance of the AA-evoked increases in H^+ in both the cytoplasm and nucleoplasm deserve further investigations.

Keywords: arachidonic acid, intracellular pH, flip-flop, nuclear pH

二 緣由與目的

Arachidonic acid (AA, 20:4) is one of the major unsaturated fatty acids (FAs) in biological membranes.AA and its metabolites participate many physiological and pathophysiological events in a variety of cell types (for reviews, see Katsuki and Okuda, 1995). For example, during stroke, convulsion or neurodegenerative disorders, AA and other Non-esterified fatty acids (FAs) might be released and accumulate in the neurons and results in neuronal damage (Farooqui et al., 1997).. In addition, AA is associated with many other important physiological and pathophysiological events (Dumuis et al., 1988; Miller et al., 1992; Lynch et al., 1989). AA can induce intracellular acidosis, and several mechanisms have been raised for the AA-induce intracellular acidosis: (1) inhibition of NHE, (2) increase in intracellular calcium, (3) increased metabolic production of lactic acids, (4) generation of oxygen-derived free radicals, (5) activation of PKC, and (6) activate membrane NADPH oxidase to result in the membrane depolarization, and therefore the activation of an outward-rectifying proton conductance (a H^+_i efflux or a pH_i increase). Furthermore, oleic acid (18:1) and -linoleic acid (18:2), another two non-esterified FAs, have been found to evoke significant intracellular acidosis in artificial phospholipid vesicles (Kamp and Hamilton, 1992; Kamp and Hamilton, 1993; Kamp et al., 1995). A "flip-flop" transportation was proposed to account for free the transmembrane movement of FAs (*i.e.* a H^+_o influx or a pH_i decrease). The FAs-induced acidosis in living cells has been reported in only two studies, involving pancreatic β-cells (Hamilton et al., 1994) and adipocytes (Civelek et al., 1996).
In this study, we investigate the mechanism of AA-induced intracellular acidosis and discuss the potential relevance of AA-induced acidosis in physiological and pathophysiological states.

三 結果與討論

AA-induced acidosis

The resting pH_i in nominally HCO_3-free Hepes-buffered media was 7.11 ± 0.01 (n=32) in granule cells. Continuous perfusion with 10 μM AA induced a profound persistent intracellular acidosis (0.32 ± 0.01 pH units, n=60). Though the amplitude of the acidosis

plateaued at a concentration of 10 μM. The initial rate of acid flux, J_{H_i} (= β_i X ΔpH_i/min, where β_i =33.3 ± 0.3 mM [n=9] at pH_i 7.1, Wu et al., 1994), increased linearly with increasing concentration of AA over the range 2-100 μM. In subsequent experiments, we used 10 μM AA to investigate the mechanism(s) involved .

The basal pH_i decreased following total removal of Na_o (complete inhibition of the NHE), and a greater acidosis was induced by AA under Na-free conditions, showing that the NHE was not responsible for the acidosis and that the real magnitude of the acidosis was uncovered when acid-extruding mechanism was blocked. AA metabolism can generate free radicals to induce marked intracellular acidosis in glial cells. However, pretreatment with free radical scavengers (U 78517F, an O_2^{-}•/OH• scavenger, or a mixture of superoxide dismutase, an O_2^{-}• scavenger, and catalase [reduction of H_2O_2 into H_2O]), no inhibitory effect was seen. Unsaturated FAs, including AA, can activate protein kinase C (PKC, Katsuki and Okuda, 1995) and PKC regulates the pH_i in other cell types. However, pretreatment with 200 μM H7 (a PKC inhibitor) or 1 μM TPA (a PKC activator) had no effect on AA-induced acidosis. AA activation of NADPH oxidase can result in the production of large amounts of intracellular protons. However, a mixture of diphenyleneiodonium (DPI, an NADPH oxidase inhibitor) and BiCNU (a glutathione reductase inhibitor) had no effect on the AA-induced acidosis.

Evidence for a simple diffusion

Another possibility to be considered was simple FA diffusion (flip-flop) demonstrated in lipid bilayers. Albumin can extract FAs from the lipid bilayer and increase the rate of H_i efflux (Kamp and Hamilton, 1992, 1993; Hamilton, 1998). In the presence of 10 μM AA, addition of 0.3 % BSA resulted in pH_i recovery by 70 ± 1 % toward resting level (n=6). Moreover, the initial rate of pH_i recovery (R_1= 0.017 ± 0.002 pH unit/min, n=6) following removal of external AA was significantly lower than that seen when BSA was subsequently added (R_2= 0.191 ± 0.020 pH unit/min, n=6, $p<0.05$), suggesting that BSA extracts AA from the lipid membrane. This effect of BSA on pH_i recovery was specific to AA-induced acidosis, since no effect was seen on acidosis (0.30 ± 0.02 pH units, n=6) induced by another weak acid loading method using 40 mM propionate in the presence of 10 μM 5-(N-ethyl-N-isopropyl)-amiloride (EIPA).

We also tested other polyunsaturated essential FA members of the omega-6 and omega-3 families, namely linoleic acid and α-linolenic acid, the respective parent FAs of these two families, and docosahexaenoic acid (DHA), the end metabolite of α-linolenic acid which plays an important role in neuronal development. All three FAs caused a considerable intracellular acidosis which was reversed by 0.3% BSA. In contrast, addition of AA methyl ester had little effect on the pH_i (0.02 ± 0.01 pH units, n=6). Tetradecylamine, in its neutral form, attracts intracellular protons, thus producing an intracellular alkalosis in the vesicles, and is suggested to be transported by a simple diffusion mechanism, rather than a protein carrier. tetradecylamine (10 μM) induced pH_i increase of 0.19 ± 0.02 pH units, which was again reversed by addition of 0.3% BSA (n=7).

Since a transmembrane carrier protein can transport FAs, we tested whether DIDS, a potent inhibitor of the FA membrane transport protein (Amumrad NA, et al., 1991), inhibited AA-induced acidosis, and we found 0.2 mM DIDS had no significant effect on the AA-evoked acidosis.

The first enzyme involved in the metabolism of both linoleic acid (the precursor of AA) and α-linolenic acid is Δ^6 desaturase. We found that a potent Δ^6 desaturase inhibitor, salicylhydroxamic acid (SHAM) (Khozin-Goldberg et al., 1999) did not alter the acidosis evoked by either linoleic acid or α-linolenic acid, suggesting that AA/FAs metabolites do not play a role in the production of acidosis.

Effects of AA on nuclear H^+ levels

It has recently been shown that changes in the pH_i play an important role in gene expression. AA regulates the expression of immediate early genes (Rao GN et al., 1993, Danezch U et al., 1994) and it was therefore

of interest to know whether it had any effect on nuclear H^+ levels. In some preliminary experiments, we used confocal microscopy to measure nuclear pH, since optical sectioning allows independent nuclear and cytoplasmic fluorescent signals to be obtained. We found that pH_i decreased in both the cytosol and nucleus (pH lowered by 18 ± 5% and 20 ± 5%, respectively, compared to resting levels, n=8). We showed that AA causes a similar and simultaneous increase in H^+ levels in both the nucleus and cytosol. The simplest explanation is that H^+ ions diffuse freely through the nuclear pores, which may be important in neuronal gene expression. This AA-induced increased permeability of the nuclear envelope to cytosolic H^+ is of interest, since a lowered pH_i and increased Ca^{2+} levels in the nucleus may have a marked effect on immediate early gene expression or nuclear protein phosphorylation (for review, see Finkbeiner and Greenberg, 1998). The present study shows that AA produced increased nuclear concentrations of both H^+ that were sustained for at least 15-20 min. Since AA is suggested to be involved in the expression of many growth-associated immediate early genes (Rao et al., 1993; Danesch et al., 1994), it would be interesting whether AA-induced long-term increase in H^+ levels has a more direct effect on gene expression. Besides, During brain hypoxia and ischemia, the dramatic pH_i fall to 6.3, an effect that may result in neuronal death(Fujiwara et al., 1992). The present study suggests that AA and other unsaturated fatty acids, including linoleic acid released during ischemia may contribute to the marked intracellular acidosis seen under these condition (Van der Vusse et al., 1982). Further studies are necessary to clarify this important issue.

四 計畫結果自評

由本計畫之詳細設計與成果我們首度瞭解在神經細胞AA引起之酸化是由AA分子(而非氫離子)擴散入細胞所造成不須藉由傳輸器或離子孔道(即flip-flop model).在引起細胞內酸化之同時我們也初步觀察到AA也引起細胞核內的同步酸化現象.此種細胞內與細胞核的同步酸化現象,可能與基因表現甚至其他細胞生理活動有關,因此這一現象值得我們更深入研究.

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