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The effects of valproate on the arachidonic acid metabolism of rat brain microvessels and of platelets

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Abstract

Long-term administration of the antiepileptic drug valproate can induce hematologic, hepatic and endocrine abnormalities and morphologic alterations in the brain capillaries and glial cells. Valproate elicits bone marrow suppression, reducing the number of red blood cells and platelets, and causes platelet functional abnormalities. Various data suggest that more than one mechanism of valproate-associated toxicity may exist, but the pathomechanism of cell function alterations elicited by valproate has not yet been elucidated. The reported ex vivo experiments were designed to investigate the effects of valproate on the arachidonic acid cascade of rat brain capillaries and platelets. Valproate was administered (300 mg/kg body weight/day) in the drinking water to male Wistar rats for 2 weeks. Isolated platelets and brain microvessels were labelled with [^{14}C]arachidonic acid and the released [^{14}C]eicosanoids were separated by overpressure thin-layer chromatography and determined quantitatively by liquid scintillation counting. Valproate treatment reduced the synthesis of cyclooxygenase and lipoxygenase products in rat platelets. In brain microvessels valproate stimulated the synthesis of lipoxygenase metabolites and attenuated the cyclooxygenase pathway. Modifications of the arachidonate cascade in platelets and brain microvessels may contribute to the cell function alterations caused by valproate.

Keywords: Valproate; Arachidonic acid cascade; Platelet; Brain microvessel, rat

1. Introduction

Valproate is a well-established, commonly administered first-line antiepileptic drug. Long-term administration of this drug can induce hematologic, hepatic and endocrine abnormalities and morphologic alterations in the brain capillaries and glial cells.

Valproate elicits bone marrow suppression, reducing the red blood cell and platelet counts (May and Sunder, 1993; Ozkara et al., 1993; Gidal et al., 1994). It also inhibits platelet aggregation and prolongs the bleeding time (Tohen et al., 1985; Delgado et al., 1994; Gidal et al., 1994). In consequence of the valproate-induced prolonged bleeding time, some authors (May and Sunder, 1993; Delgado et al., 1994) support four main features: (1) valproate reduces the platelet count in a dose-related manner; (2) valproate alters platelet aggregation in a concentration-specific way; (3) the valproate-elicited prolonged bleeding time may be indicative of either thrombocytopenia or an altered platelet function; (4) in valproate-treated patients, a normal bleeding time does not allow the assumption of a normal platelet function. These data suggest that more than one mechanism of valproate-associated alterations may be operative. The suppression of bone marrow function is attributed to the direct toxic effect of valproate on hemopoetic precursor cells (Hauser et al., 1996). The dysfunction of platelets may be a consequence of the formation of platelet antibody (Sandler et al., 1978) or valproate may affect the phospholipid membrane structure (Davis et al., 1991). In spite of the fact that several mechanisms have been proposed to explain these hematologic disturbances, the exact explanation is not yet known.

Some metabolites of the arachidonic acid cascade are important second messengers in cells (Khan et al., 1995)

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and in platelets (Nozawa et al., 1991; Khan et al., 1992). The inhibition of thromboxane synthesis by the blocking of cyclooxygenase with acetylsalicylic acid leads to a decreased aggregability of platelets and results in hemorrhagic diathesis. The anti-inflammatory effects of aspirin are mediated via inhibition of the cyclooxygenase pathway in cells. Acetylsalicylic acid is a very effective inhibitor of platelet-mediated thrombosis at sites of vascular injury (Goodnight, 1996).

Long-term administration of valproate likewise elicits changes in endothelial cells and in the vascular basement membrane of the blood–brain barrier, and can cause the proliferation of astrocytes and oligodendrocytes (Sobaniec-Lotowska and Sobaniec, 1996). Damage to the capillaries is accompanied by marked alterations to neuroglial cells and neurons of the cerebellum. These morphologic and functional changes may play a role, directly or indirectly, in the pathogenesis of ''valproate encephalopathy''.

Endothelial cells of the cerebral microvasculature are known to metabolize arachidonic acid via the lipoxygenase and cyclooxygenase pathways (Abdel-Halim et al., 1980; Gecse et al., 1982). Many data support the view that arachidonic acid metabolites play a role in the autoregulation of the cerebral circulation, in either physiologic or pathologic processes (Siesjö, 1992; Aranda et al., 1993; Parfenowa et al., 1994; Chemtob et al., 1996). They are involved in the pathomechanism of the vasospasm after subarachnoid hemorrhage (Simmet and Peskar, 1990) and contribute to the increased local cerebral blood flow during epileptic seizures (Busija and Leffler, 1989).

The present ex vivo experiments were designed to investigate the effects of valproate on the arachidonic acid cascade of rat brain capillaries and platelets.

2. Materials and methods

2.1. Chemicals

Arachidonic acid (grade I), 12-L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) as unlabeled standards were from Sigma, St. Louis, MO (USA). [^{14}C]Arachidonic acid (specific activity: 2035 MBq/mmol) was from Amersham (England). Serum-free tissue culture mediums, Medium 199, was from Sigma, St. Louis, MO (USA). Silica gel thin-layer plates (0.25 mm) were from Merck, Darmstadt (Germany). Prostaglandin E_2, prostaglandin D_2, thromboxane A_2, thromboxane B_2 (the stable metabolite of thromboxane A_2), prostaglandin $F_{2\alpha}$ and 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-prostaglandin $F_{1\alpha}$, the stable metabolite of prostacyclin) were generously provided by Upjohn Kalamazoo (USA). Sodium valproate (Depakine®) was from Sanofi Winthrop/Chinoin.

2.2. Isolation of platelets

Male Wistar rats (body weight at the beginning of the treatment: 170–190 g and at the end of the study: 260–280 g) were used in this study. Sodium valproate was administered (300 mg/kg body weight/day) in the drinking water to rats for 2 weeks. The weight gain of valproate-drinking animals (6.43 ± 0.23 g/day) was not significantly different from that of water-drinking (6.6 ± 0.5 g/day) animals. We controlled the drinking habits of animals. Every day we measured the quantity of water drunk. There was no significant difference between the drinking habits of the control (15.73 ± 3.86 ml/day) and the valproate-treated animals (14.36 ± 1.54 ml/day). The plasma level of valproate was 4.84 ± 0.78 μg/ml. The eicosanoid profiles of the platelets and microvessels were determined 24 h after the last administration of valproate. Under light ether anesthesia, blood was drawn from the abdominal aorta and diluted with phosphate buffer (pH 7.4) containing EDTA (5.8 mM) and glucose (5.55 mM). Platelet-rich plasma was collected after the whole blood had been centrifuged at $200 \times g$ for 10 min at room temperature. The platelets were sedimented from the supernatant by centrifugation at $2000 \times g$ for 10 min. The pellet was contaminated with red blood cells, which can metabolize arachidonic acid by the lipoxygenase pathway and release 12-HETE and leukotrienes (Kobayashi and Levine, 1983). The erythrocytes were therefore lysed with hyposmic ammonium chloride (0.83%, nine parts) containing EDTA (0.02%, one part) at 4°C for 15 min. The platelets were then washed with phosphate buffer (pH 7.4, containing 5.8 mM EDTA and 5.55 mM glucose) and centrifuged at $2000 \times g$ for 10 min at room temperature. During the separation procedure, activation of the platelets was inhibited by applying Ca^{2+}-free medium and by using siliconized glassware. The washed platelet suspension was free from other cellular elements of blood (red blood cells, leukocytes, etc.) and from plasma proteins. After the last centrifugation, the platelets were resuspended (10^8 platelets/ml) in serum-free Medium 199. Animal experiments were performed with the permission of the Ethics Committee for the Protection of Animals in Research (Albert Szent-Györgyi Medical University, Szeged, Hungary).

2.3. Isolation of brain capillaries

We used transcardiac perfusion of male Wistar rats (body weight at the beginning of the treatment: 170–190 g and at the end of the study: 260–280 g) to remove blood from the brain vessels. Under light ether anesthesia, the thorax of rats was opened and the heart was exposed. A needle (20 ga) was introduced into the left ventricle, and an incision was made on the right atria. The aorta descendens was occluded, and 50 ml cold (4°C) normal saline containing EDTA (5.8 mmol/l) and 5.55 mM glucose was administered into the left ventricle.

The eicosanoid profile of the microvessels was determined in blood-free brain microvessels isolated by a micromethod (Hwang et al., 1980), with the following modifications. The cerebral cortex (300 mg wet weight) was freed of myelin and pial membranes. The chopped tissue was homogenized in 9 volumes of 3 mM HEPES buffer (containing 0.32 M sucrose, pH 7.3) with 10 strokes (100 revolutions/min), and then centrifuged at $1000 \times g$ for 10 min at 2°C. The pellet was resuspended in 5 volumes of 3 mM HEPES buffer, homogenized with two strokes (1000 rpm), and then centrifuged again at $1000 \times g$ for 10 min at 2°C. The pellet was resuspended in the above-mentioned buffer, the suspension was centrifuged at $100 \times g$ for 15 s and the pellet was diluted with 1 volume of 3 mM HEPES buffer, followed by centrifugation at $100 \times g$ for 15 s. The supernatants of the last two centrifugations were combined and centrifuged at $200 \times g$ for 1 min. The pellet was resuspended in 2.5 volumes of plasma-free TC Medium 199 (pH 7.4) and centrifuged at $200 \times g$ for 1 min. The pellet was then taken up in 1 ml of protein-free TC Medium 199 and centrifuged again at $200 \times g$ for 1 min. The pellet, which contained the microvessels, was resuspended in TC Medium 199. An aliquot was examined for purity by light and phase-contrast microscopy. The contamination was less than 6%, and the microvessels were free of blood cells.

2.4. Analysis of eicosanoids

Platelets (10^8 cells/ml in each sample) or brain capillaries (isolated from 300 mg wet weight of brain cortex/ml in each sample) were preincubated at 37°C for 5 (platelets) or 10 (microvessels) min. The enzyme reaction was started by the introduction of tracer substrate, [^{14}C]arachidonic acid (3.7 kBq, 0.172 pmol), into the incubation mixture. Ten (platelets) or thirty minutes (microvessels) later, the enzyme reaction was stopped by bringing the pH of the incubation mixture to 3 with formic acid. Samples were then extracted with ethyl acetate (2×3 ml) and the organic phases were pooled and evaporated to dryness under nitrogen. The residues were reconstituted in 150 μl ethyl acetate and quantitatively applied to silica gel G thin-layer plates. The plates were developed to a distance of 15 cm in an organic phase of ethyl acetate:acetic acid:2,2,4-trimethylpentane:water (110:20:30:100) by means of overpressure thin-layer chromatography (Labor MIM, Hungary) (Abdel-Halim et al., 1980). Each 3 mm band of the chromatograms was then scraped off and the radioactivity was determined in a liquid scintillation counter (TRI-CARB 2100TR, Canberra Packard, USA), in disintegration per min (dpm), using 5 ml toluene containing 0.44% w/v 2,5-diphenyloxazole, 0.02% w/v 1,4-di [2-(5-phenyl)oxazoyl]benzene and 10% v/v ethanol. The radiolabeled products of arachidonic acid were identified by means of unlabeled authentic standards, which were detected with anisaldehyde reagent (Kiefer et al., 1975). For the separation of lipoxygenase products of the arachidonic acid cascade, high-performance liquid chromatography (HPLC, ISCO 2350, USA) was also applied, using a reversed-phase column (4.6×250 mm) connected to a guard column (4.6×25 mm), both packed with Nucleosil ^{18}C (5 μm particles). The eluent consisted of acetonitrile:water (700:300), and phosphoric acid was added to adjust the pH to 4. In pre-experiments, we determined the absolute amounts of lipoxygenase products (9.65 ± 1.42 nmol/l) in the incubation mixtures with a UV detector (Hewlett Packard 1050, USA) at 235 nm, following HPLC separation. On the same sample, we also determined the amount of radiolabelled lipoxygenase (98.13 ± 13.24 fmol/l) with a liquid scintillation counter. The ratio between the platelet and labelled platelet lipoxygenase pools was 100 000:1.

Statistical analysis was carried out by using SPSS for Windows (version 6.1.2). Analysis of variance was performed, followed by the Student's *t*-test.

3. Results

The formation of arachidonic acid metabolites was significantly attenuated ex vivo in the platelets of rats after valproate administration. Valproate inhibited both the cyclooxygenase (32%) and lipoxygenase (24%) pathways. The synthesis of vasoconstrictor (prostaglandin $F_{2\alpha}$ and

Table 1
The effect of valproate treatment on the arachidonic metabolism of rat platelets. Results are reported as means ± S.E.M. ($n = 8$), in 10^3 dpm. Platelets of valproate-treated and untreated Wistar male rats were incubated with [^{14}C]arachidonic acid as a tracer substrate for 10 min at 37°C. The metabolites were extracted, separated by overpressure thin-layer chromatography and quantitatively determined with a liquid scintillation counter

Products	Control rats	Valproate-treated rats
Total amount of platelet eicosanoids	98.255 ± 5.255	72.795 ± 2.998[a]
Lipoxygenase metabolites	72.984 ± 4.505	55.566 ± 2.726[b]
Cyclooxygenase (COX) metabolites	25.270 ± 1.094	17.228 ± 1.270[a]
12-Hydroxyheptadecatrienoic acid	4.785 ± 0.400	3.223 ± 0.255[b]
Prostaglandin D_2	2.839 ± 0.111	2.376 ± 0.172[c]
Thromboxane B_2	11.996 ± 0.508	8.286 ± 0.804[b]
Prostaglandin E_2	2.011 ± 0.141	1.471 ± 0.171[c]
Prostaglandin $F_{2\alpha}$	2.445 ± 0.317	0.993 ± 0.075[a]
6-keto prostaglandin $F_{1\alpha}$	1.193 ± 0.117	0.878 ± 0.049[c]

[a] $P < 0.001$ statistically significant differences as compared to values for platelets from untreated Wistar animals.
[b] $P < 0.01$ statistically significant differences as compared to values for platelets from untreated Wistar animals.
[c] $P < 0.05$ statistically significant differences as compared to values for platelets from untreated Wistar animals.

thromboxane A_2) and platelet aggregator cyclooxygenase metabolites (thromboxane A_2) was significantly reduced in the platelets of valproate-treated animals as compared with the controls. The formation of vasodilator and platelet antiaggregator cyclooxygenase products (6-keto-prostaglandin $F_{1\alpha}$, prostaglandin E_2 and prostaglandin D_2) was significantly attenuated in the platelets of valproate-treated rats. The synthesis of HHT, which is an indirect vasodilator eicosanoid released by the cyclooxygenase pathway in platelets, was also inhibited by valproate treatment (Table 1). The synthetic ratio of vasoconstrictor and antiaggregator, vasodilator cyclooxygenase products was similarly significantly reduced in the platelets of valproate-treated rats as compared with the controls.

The synthesis of the metabolites of the arachidonic acid cascade was stimulated in the brain microvessels of valproate-treated rats, relative to the controls. This alteration in the arachidonic acid cascade was a result of the significantly increased ex vivo formation of lipoxygenase products after valproate administration. Each component (prostaglandin $F_{2\alpha}$, thromboxane A_2, prostaglandin E_2, prostaglandin D_2, 6-keto-prostaglandin $F_{1\alpha}$ and HHT) of the cyclooxygenase pathway of arachidonic acid in the microvessels was attenuated in the valproate-treated animals as compared with the controls (Table 2). The ratio of vasodilator and vasoconstrictor cyclooxygenase metabolites for the microvessels was not modified by valproate administration.

Table 2
The effect of valproate treatment on the arachidonic acid metabolism of rat brain capillaries. Results are reported as means ± S.E.M. ($n = 8$), in 10^3 dpm. Brain capillaries of valproate-treated and untreated Wistar rats were incubated with [^{14}C]arachidonic acid as a tracer substrate for 30 min at 37°C. The metabolites were extracted, separated by overpressure thin-layer chromatography and quantitatively determined with a liquid scintillation counter

Products	Control rats	Valproate-treated rats
Total amount of brain capillary eicosanoids	72.770 ± 1.234	79.825 ± 1.877[a]
Lipoxygenase metabolites	65.784 ± 1.204	75.603 ± 1.818[b]
Cyclooxygenase (COX) metabolites	6.985 ± 0.798	4.222 ± 0.083[a]
12-Hydroxyheptadecatrienoic acid	1.949 ± 0.278	0.958 ± 0.059[a]
Prostaglandin D_2	1.275 ± 0.182	0.679 ± 0.036[c]
Thromboxane B_2	1.768 ± 0.226	1.177 ± 0.036[c]
Prostaglandin E_2	0.671 ± 0.085	0.456 ± 0.018[c]
Prostaglandin $F_{2\alpha}$	0.650 ± 0.073	0.458 ± 0.020[c]
6-keto prostaglandin $F_{1\alpha}$	0.671 ± 0.073	0.494 ± 0.018[c]

[a] $P < 0.01$ statistically significant differences relative to values for platelets from untreated Wistar animals.
[b] $P < 0.001$ statistically significant differences relative to values for platelets from untreated Wistar animals.
[c] $P < 0.05$ statistically significant differences relative to values for platelets from untreated Wistar animals.

4. Discussion

Studies suggest that the etiology of valproate-related hematologic disturbances is multifactorial. The valproate-associated side-effects are either dose related (Gidal et al.,1994), metabolic (Davis et al.,1991) or idiosyncratic (Barr et al.,1982) in origin. The arachidonic acid cascade is one of the most important regulator systems in the platelets and the endothelial cells of the cerebral microvasculature (Moore et al., 1988b; Simmet and Peskar, 1990; Kálmán et al., 1992; Mezei et al., 1997).

In our study, the formation of arachidonic acid metabolites was significantly attenuated in the platelets of rats after valproate administration. The synthesis of both vasoconstrictor (prostaglandin $F_{2\alpha}$ and thromboxane A_2) and vasodilator antiaggregator cyclooxygenase metabolites (prostaglandin E_2 and prostaglandin D_2) was significantly reduced in the platelets of valproate-treated rats as compared with the controls. These results demonstrate that valproate inhibited both the cyclooxygenase and lipoxygenase pathways of the arachidonic acid cascade of rat platelets.

Thromboxane A_2 is functionally the most important product of the platelet arachidonic acid cascade, and its synthesis was inhibited by valproate. Thromboxane A_2 synthesis in platelets is accompanied by the parallel production of HHT and malondialdehyde (Chignard and Vargaftig, 1988). Our results are in agreement with the findings of Voss et al. (1978), who demonstrated a decreased malondialdehyde production in patients on valproate therapy.

Different types of platelet activator substances (collagen, ADP, thrombin, arachidonic acid and ristocetin) are used to test platelet function. Thrombin induces arachidonic acid cascade-independent, and arachidonic acid itself induces arachidonic acid cascade-dependent, platelet activation and aggregation (Vargaftig et al., 1981). Gidal et al. (1994) reported a decreased arachidonic acid-induced platelet activation (the platelet ATP release was measured) and aggregation in valproate-treated subjects, but did not observe differences in thrombin-induced platelet activation as compared to that of their control group. Their results demonstrated that the valproate-induced platelet dysfunction involve alterations in the arachidonic acid cascade. We found a lower activity of the arachidonic acid cascade and a decreased production of thromboxane A_2 in rat platelets following valproate treatment, which lends support to the findings of Gidal et al. (1994).

The mechanism of action of valproate on the platelet arachidonic acid cascade has not yet been elucidated. Valproate may cause alterations in the membrane phospholipids of the platelets, as in erythrocytes (Davis et al., 1991), or reduce the Ca^{2+} current in the platelets, as in neurons (Kelly et al., 1990), modifying the intracellular Ca^{2+} level, which plays an important role in the activation of the arachidonic acid cascade of platelets. However,

Kusumi et al. (1994) reported that valproate did not alter the Ca^{2+} concentration in platelets.

There is controversy as to whether valproate induces an increased bleeding tendency or not (Tetzlaff, 1991; Ranganathan and Verma, 1992; Ward et al., 1996). Our data revealed that a low dose of valproate inhibited the thromboxane A_2 production of platelets, which may lead to a decreased platelet activation and aggregation.

The formation of the arachidonic acid metabolites was significantly increased ex vivo in the brain capillaries but not in the platelets of valproate-treated rats. The synthesis of arachidonic acid metabolites was shifted more toward the lipoxygenase pathway after valproate treatment. The formation of each component (prostaglandin $F_{2\alpha}$, thromboxane A_2, 6-keto-prostaglandin $F_{1\alpha}$, prostaglandin E_2, prostaglandin D_2 and HHT) of the arachidonic acid cyclooxygenase pathway of microvessels was attenuated, and the activation of the arachidonic acid cascade was therefore a result of the significantly stimulated lipoxygenase pathway. How can these results be explained? It is known that the endothelial cells of the cerebral microvessels can metabolize arachidonic acid via the lipoxygenase and cyclooxygenase pathways (Moore et al., 1988b; Kálmán et al., 1992). The most abundant eicosanoid in the brain microvessels is the 12-HETE released by 12-lipoxygenase. In high concentrations, 12-HETE has been reported to inhibit vascular prostanoid production (Moore et al., 1988a). Our ex vivo experiments allow us to conclude that the valproate-stimulated synthesis of lipoxygenase eicosanoids and the inhibition of prostanoid production in the brain microvessels may be consequences of an increased 12-HETE concentration. 12-HETE might also be incorporated into membrane phospholipids, altering membrane function and subsequently the permeability of the blood–brain barrier (Moore et al.,1988a; Simmet and Peskar, 1990) and may contribute to the morphologic alterations of the capillaries (Sobaniec-Lotowska and Sobaniec, 1996) caused by valproate treatment.

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