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Abstract

Recent data suggested that platelet-activating factor (PAF) could play a pathophysiologically important role in the progression of hypoxic-ischemic brain injury. We investigated brain tissue PAF concentration in the hypoxic-ischemic brain of immature rats. Endogenous PAF concentration in brain tissue showed a marked increase in hypoxic-ischemic pups (Group 1, 85.6 +/- 15.5 pg/mg protein) when compared to that of the control (9.1 +/- 3.1 pg/mg protein). In addition, we studied the effects of pretreatment with L-carnitine (5 days and 2 h before the hypoxia) on endogenous PAF concentration in the hypoxic-ischemic brain. Endogenous PAF concentration in the short-term pretreatment group (Group 2, 81.6 +/- 9.7 pg/mg protein) was not different than in Group 1 rat pups. However, a significantly decreased PAF concentration was found in the group of pups that received carnitine pretreatment for 5 days (Group 3, 30.5 +/- 11.0 pg/mg protein). These results indicate that PAF is an important mediator in the immature rat model of cerebral hypoxic-ischemic injury. The suppressor effect of L-carnitine on PAF production may give new insight into the treatment of hypoxic-ischemic brain injury.

KEYWORDS: perinatal asphyxia, rat, carnitine, ischmia, brain, platelet-activating factor

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The Effect of L-Carnitine on Platelet Activating Factor Concentration in the Immature Rat Model of Hypoxic-Ischemic Brain Injury

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Recent data suggested that platelet-activating factor (PAF) could play a pathophysiologically important role in the progression of hypoxic-ischemic brain injury. We investigated brain tissue PAF concentration in the hypoxic-ischemic brain of immature rats. Endogenous PAF concentration in brain tissue showed a marked increase in hypoxic-ischemic pups (Group 1, 85.6 ± 15.5 pg/mg protein) when compared to that of the control (9.1 ± 3.1 pg/mg protein). In addition, we studied the effects of pretreatment with L-carnitine (5 days and 2h before the hypoxia) on endogenous PAF concentration in the hypoxic-ischemic brain. Endogenous PAF concentration in the short-term pretreatment group (Group 2, 81.6 ± 9.7 pg/mg protein) was not different than in Group 1 rat pups. However, a significantly decreased PAF concentration was found in the group of pups that received carnitine pretreatment for 5 days (Group 3, 30.5 ± 11.0 pg/mg protein). These results indicate that PAF is an important mediator in the immature rat model of cerebral hypoxic-ischemic injury. The suppressor effect of L-carnitine on PAF production may give new insight into the treatment of hypoxic-ischemic brain injury.

Key words: perinatal asphyxia, rat, carnitine, ischemia, brain, platelet-activating factor

Perinatal asphyxia remains one of the most important neurologic complications in the newborn (1, 2). Many interrelated mechanisms, including excitatory amino acids receptor overactivation, intracellular calcium accumulation, lipid peroxidation, reactive oxygen species and inflammatuar lipid mediators (prostaglandins, leukotrienes and platelet-activating factor (PAF)), contribute to the evolution of hypoxic-ischemic brain injury (3, 4).

Recent data suggest that PAF (1-0-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine (1-0-alkyl-2-acyl-GPC)) could play a pathophysiologically important role in the progression of hypoxic-ischemic brain injury (3-6). PAF is synthesized by vascular endothelium neutrophils, monocytes, platelets, glia cells and neurons through activation of membrane phospholipase A_2 (PLA_2) and acetyl transferase after calcium increase in these cells (4-7). The brain tissue is rich in phospholipids, such as alkyl-acyl-glyceryl-phosphoryl-choline, which are required for biosynthesis of PAF (8). In several experimental studies, it is demonstrated that PAF is produced by brain cells, and excessive production occurs in hypoxic-ischemic brain injury (9, 10). PAF exerts cytotoxic effects on neuronal cells, causes vasoconstriction, and increases the blood-brain barrier permeability (5, 11, 12). We have previously shown that plasma PAF levels are significantly higher in newborns with perinatal asphyxia (unpublished data).

Ischemia of the nervous tissue immediately activates calcium-dependent PLA_2 which catalyzes the hydrolysis of the fatty acid ester linkage at position 2 of the phospholipid molecule, generating arachidonic acid (AA) and lyso-PAF (the precursor for active PAF). During cerebral ischemia, there is a massive release of free AA from excitable membrane phospholipids. As a result, a significant amount of free AA accumulates as ATP decreases in the hypoxic-ischemic brain (3, 4, 13).

Carnitine (3-hydroxy-4-N-trimethylammounium-butyrate) is necessary for the transformation of acyl-compound (free long-chain fatty acids) to acyl-carnitines and for their transport from the cytosol into the mitochondrial matrix (14). We have recently shown that hypoxia leads to myocardial free carnitine depletion in young mice (15). In the absence of L-carnitine, the accumulation of free fatty

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acids in the cytoplasm produces a toxic effect on the cell, and an energy deficit arises from the unavailability of fatty acids within the mitochondria (14, 16). Recently, several reports have shown that L-carnitine and its esters have a significant neuroprotective effect in cerebral ischemia (17, 18).

As a result of many studies, PAF is accepted as a key mediator in the pathophysiology of brain injury. Therefore, it may be an important therapeutic approach to inhibit PAF biosynthesis in the hypoxic-ischemic brain. The aim of this experimental study was to investigate the effect of L-carnitine on endogenous PAF biosynthesis in the hypoxic ischemic brain of newborn rats.

Materials and Methods

Animals and experimental design. Seven-day-old Wistar-Albino rat pups of either sex, weighing between 14-18 g were used in the present study. Twenty-three rat pups were obtained from Ege University, Veterinary Research Institute and were randomly divided into four groups. Group 1 (n = 6; untreated group) pups were given physiologic saline (0.2 ml) by intraperitoneal injection immediately after the surgery. Group 2 (n = 5) pups were treated with 16 mmol/kg of L-carnitine as a 20 % (wt/vol) solution in water (Carneten, Sigma, Tau, Italy) by intraperitoneal injection immediately after the surgery. Group 3 (n = 6) pups were given the same dose of L-carnitine once a day for 5 days. The last administration took place immediately after the surgery. Group 4 (n = 6) pups served as the control.

Groups 1, 2 and 3 rat pups were anesthetized with ether. The right common carotid artery of each rat pup was ligated with 4-0 surgical silk. After surgery, the rat pups were returned to their dams for 2 h. Then, rat pups were subjected to a hypoxic environment in a Plexiglas chamber, consisting of 8 % oxygen (Oxygen Monitor, Minolta Company, Japan) and nitrogen for 60 min. The pups were then returned to their cages. All pups were sacrificed following a 60 min reoxygenation period, by cervical dislocation. Group 4 pups were not exposed to this procedure.

The cranium was opened and the cerebrum and cerebellum were removed. For the determination of PAF, the whole brain was pooled, immediately homogenized in ice-cold 2 ml PBS (pH 7.4, for 3 min in au ultra thorax homogenizer), and supernatants obtained by centrifugation (1,800 rpm for 10 min) were frozen at − 70°C in polypropylene tubes until assayed. Aliquots of the homogenate were assayed for total proteins by a modified Lowry method (19).

Measurement of PAF in the brain tissue. PAF was extracted from samples of tissue homogenates by the solid-phase extraction method using Amberlit XAD2 columns (Althech, CA, USA) (20). Extracted samples were dried with vacuum speed evaporator (Heto-1 Speed Vacuum Concentration, Denmark) under liquid nitrogen. Dried samples were dissolved in 20 ml high performance liquid chromatography (HPLC) solvent and kept at − 70°C. PAF was purified from dried samples by using the reversed-phase (RP)-HPLC method. PAF was collected from HPLC UV detector to its retention times and was dried again in a vacuum speed evaporator concentration. The amount of PAF was determined by a specific [^{3}H] PAF Scintillation Proximity Assay kit (Amersham Life Science, UK). Procedures were performed according to the instruction in the kits. PAF concentrations were measured by a Beta-liquid scintillation counter (TRI-CARB-1600 TR, LSA-Packard, Canberra Company, NY, USA). The minimum detectable concentration of PAF by this method was 20 picogram.

Results were expressed as picogram per mg tissue protein (pg/mg protein) in the homogenate supernatants of the brain.

All values are expressed as mean ± SEM. The means of the four groups were compared by one way analysis of variance. Stutdent-Newman-Keuls post hoc test was used in statistical analysis of the data. P values less than 0.05 were considered significant.

Results

PAF was in detectable concentrations in all of the samples. Brain tissue PAF concentration was found to be significantly higher in the saline group of rat pups (Group 1) (85.6 ± 15.5 pg/mg protein) when compared to that of the control group (Group 4) (9.1 ± 3.1 pg/mg protein) ($P < 0.01$). It was determined that there was an approximate 10-fold increase in PAF concentration in the hypoxic-ischemic brain of the immature rats (Table 1, Fig. 1).

Endogenous PAF concentration in Group 2 (81.6 ± 9.7 pg/mg protein) pups was not different when compared to Group 1 pups ($P > 0.05$). However, significantly decreased PAF concentration was found in the long-term carnitine-treated pups (Group 3) (30.5 ± 11.0 pg/mg protein) ($P < 0.01$) (Table 1, Fig. 1).

Table I Brain tissue platelet-activating factor (PAF) concentrations in rat pups with hypoxic-ischemic brain injury

	Group I	Group 2	Group 3	Group 4
Number of rat pups	6	5	6	6
Brain tissue PAF levels[a]	85.6 ± 15.5	81.6 ± 9.7	30.5 ± 11.0	9.1 ± 3.1
Range (pg/mg protein)	57.4 − 160.2	57.3 − 104.5	7.6 − 80.3	0.3 − 20.0

Group 1: Pups were given physiologic saline by intraperitoneal injection immediately after the surgery.
Group 2: Pups were treated with 16 mmol/kg of L-carnitine by intraperitoneal injection immediately after the surgery.
Group 3: Pups were given the same dose of L-carnitine once a day for 5 days.
Group 4: Pups served as the control.
a : Mean ± SEM (Group I *vs* Group 3 and Group I *vs* Group 4, $P < 0.01$; Group I *vs* Group 2, $P > 0.05$.)

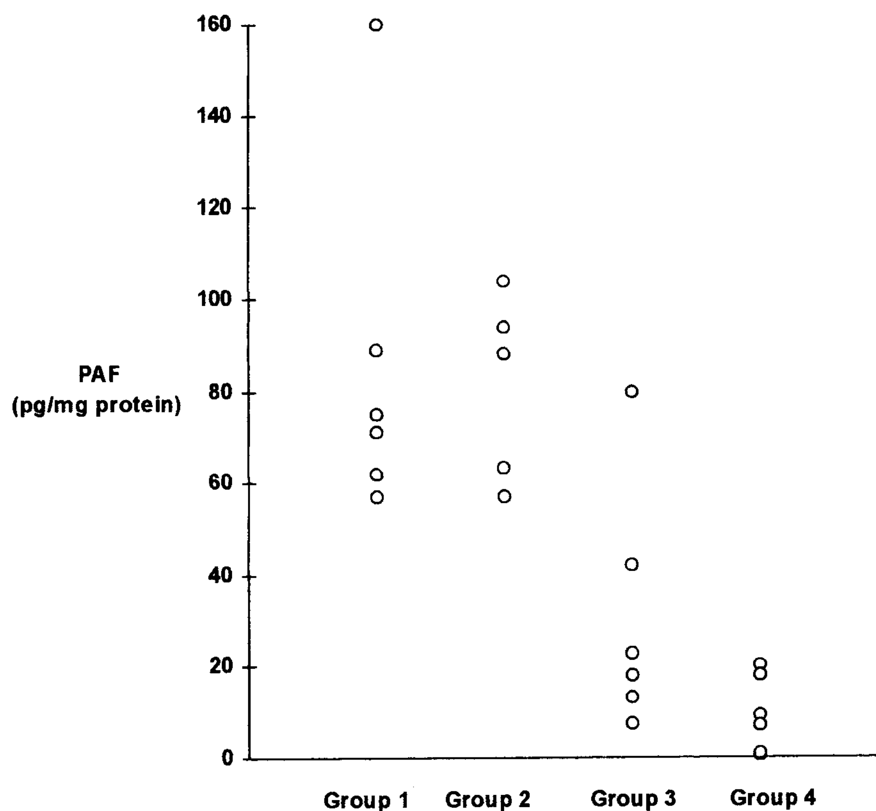

Fig. 1 Brain tissue platelet-activating factor (PAF) concentration in hypoxic-ischemic brain injury in each rat pup. See legend to Table 1.

Discussion

We have shown that the immature rat brain is able to produce PAF from an endogenous precursor, and our results support the hypothesis that PAF is an important mediator in the immature rat model of cerebral hypoxic-ischemic injury. Both cerebral (neurons, endothelium) and circulating cells (leucocytes, platelets) could act as sources of cerebral PAF production (3-5). Therefore, the main source of PAF is unclear. Lindsberg *et al.* (21) showed that concentration of PAF was increased by

20-fold after a stroke in the spinal cord tissue in rabbits. Furthermore, Kumar *et al.* (12) revealed that electroconvulsion caused a six fold increase in the rat brain PAF content. Our experiment suggested that PAF level was increased by approximately 10-fold in the hypoxic-ischemic brain tissue in an immature rat.

Low concentrations of PAF play a role in neuronal development and differentiation, whereas higher PAF concentrations cause neurodegeneration (4-6). The results of these studies show that a high concentration of PAF is an important toxic mediator in the pathophysiology of brain injury. Therefore, to inhibit PAF production may be a very important therapeutic target. Ischemia of the nervous tissue immediately activates the calcium-dependent PLA_2 and phospholipase C (PLC), which can hydrolyze phospholipids of cell membranes. Excitable membrane phospholipids contain relatively large proportions of polyunsaturated fatty acids, such as AA and display phospholipid-hydrolyzing enzymes, *i.e.*, PLA_2 and PLC (3, 4, 8). Ischemia-reperfusion induced cerebral injury results in the accumulation of AA (3, 13). Several studies support the idea that PLA_2-mediated release of AA plays a central role in the development of neuronal injury (3, 6, 8). In addition, Lyso-PAF is selectively acylated with AA in stimulated human neutrophils, and the resultant 1-0-alkyl-2-arachidonolly-GPC plays an important role in the PAF biosynthesis (3, 22, 23).

Carnitine is an essential co-factor in the transportation of fatty acyl groups into the mitochondrial matrix where they undergo beta-oxidation and result in the production of adenosine triphosphate (ATP). When carnitine stores are depleted, long-chain acyl-CoA and long-chain acylcarnitine esters accumulate in the cells. Elevated levels of these produces impair energy metabolism by inhibiting adenine nucleotide translocase activity and decreasing ATP production (14, 16). It was revealed that 15 min of global cerebral ischemia in dogs causes a 50 % decline in the concentration of free carnitine and a corresponding rise in the levels of acylcarnitine (24). Several studies have suggested that carnitine administration potentiates normalization of brain energy metabolites and substantially improves neurological outcome (17, 18, 25).

The effect of carnitine on PAF synthesis in ischemic brain injury has not previously been studied. Several studies have shown that carnitine treatment acts to lower plasma AA levels (26, 27). We therefore, hypothesized that L-carnitine pretreatment may decrease the availability of alkyl-arachidonoyl-GPC, the preferred substrate for PAF generation by PLA_2 enzyme. According to an earlier report, the passage of L-carnitine to the brain from blood *in vivo* is slow, indicating that L-carnitine may cross that blood brain barrier at a slow rate (28, 29). Yurdakok *et al.* (30) suggested that carnitine pretreatment 30 min before hypoxia did not prevent brain edema in newborn mice. In the current study, we have shown that carnitine pretreatment 2 h before hypoxia does not effect endogenous PAF synthesis in the immature rat model of the hypoxic-ischemic brain. However, long-term pretreatment with carnitine markedly inhibits endogenous PAF production in the hypoxic-ischemic brain. Although AA content of the brain has not been measured in this study, it may be speculated that L-carnitine plays a role in the decrease of AA concentration in the brain. However, the exact mechanism of its inhibitory effect on PAF production cannot be determined with certainty from this study.

In conclusion, the present study has shown that PAF is an important mediator in the immature rat model of cerebral hypoxic-ischemic injury and revealed that long-term pretreatment with L-carnitine reduces PAF concentration in hypoxic-ischemic brain tissue. The inhibitory effect of L-carnitine on PAF production may give new insight into the treatment of hypoxic ischemic brain injury.

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