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Research Report

Platelet-activating factor antagonist (ABT-491) decreases neuronal apoptosis in neonatal rat model of hypoxic ischemic brain injury

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ABSTRACT

Hypoxic ischemic brain injury (HIBI) is a common cause of neonatal mortality and morbidity. To date, no study has investigated the role of platelet-activating factor (PAF) antagonists on neuronal apoptosis in neonatal rat model of HIBI. In the present study, we evaluated the effect of a highly potent and selective PAF antagonist (ABT-491) on neuronal apoptosis in neonatal rat model of HIBI. Seven-day-old Wistar rat pups were subjected to right common carotid artery ligation and hypoxia (92% nitrogen and 8% oxygen) for 2 h. They were treated with ABT-491 or saline either immediately before or after hypoxia. In sham group animals, neither ligation, nor hypoxia was performed. Neuronal apoptosis was evaluated by the terminal-transferase mediated dUTP biotin nick-end-labeling (TUNEL) and caspase-3 staining methods. Administration of ABT-491 either before or after hypoxia resulted in significant reduction of the numbers of apoptotic cells in both hemispheres, when compared to saline treatment group. The numbers of apoptotic cells in right hemispheres in all groups were significantly higher than that in the left hemispheres. These results suggested that ABT-491, a highly potent and selective PAF antagonist, administration either before or after hypoxia reduces apoptosis and we propose that ABT-491 may be a novel approach in the treatment of HIBI.

1. Introduction

Hypoxic ischemic brain injury (HIBI) is a common cause of neonatal mortality and morbidity including cognitive, sensory, and motor disabilities (Vannucci, 2000; Ferriero, 2004; Perlman, 2006). Although the mechanisms underlying neonatal HIBI remain inadequately understood, there is good evidence that hypoxic ischemia triggers a pathophysiological cascade including activation of neutrophils, inflammatory cytokines and adhesion molecules with increased thrombogenicity, release of massive intracellular calcium, and generation of oxygen-derived free radicals (Johnston et al., 2001;

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Hossain, 2005; Rodrigo et al., 2005; Oechmichen and Meissner, 2006). Reactive oxygen species including superoxide anions, hydrogen peroxide or hydroxyl radicals, and nitric oxide or peroxynitrite cause DNA damage, neuronal injury, neurodegeneration, and cell death (Rodrigo et al., 2005; Blomgren and Hagberg, 2006).

Platelet-activating factor (PAF) is a lipid mediator-released by many types of cells such as platelets, monocytes/macrophages, neutrophils and endothelial cells- and potently activates neutrophils, contributing to the pathogenesis of inflammation, endotoxic shock and lipopolysaccharide mediated tissue injury (Bazan, 2003). During ischemia and in other pathologic conditions involving oxidative stress, PAF concentration increases and it becomes a pro-inflammatory messenger and a mediator of neurotoxicity (Chen and Bazan, 2005). In recent years, an increased proportion of basic science research has been directed toward evaluating mechanisms involving HIBI, and PAF was thought to have a possible role in the pathogenesis of this condition (Bazan, 2003; Chen and Bazan, 2005). Excessive PAF promotes neuronal damage and this event has critical consequences for neuronal survival.

Over the past decade, a few research has been done to investigate the role of PAF and PAF antagonists in HIBI (Liu et al., 1996, 2001; Viswanath et al., 2000). However, no study has investigated the role of PAF antagonists on neuronal apoptosis in neonatal rat model of HIBI. The aim of this study was to evaluate the efficacy of ABT-491, a highly potent and selective PAF antagonist on neuronal apoptosis in neonatal rat model of HIBI.

2. Results

The numbers of TUNEL-positive apoptotic cells of each group are presented in Fig. 1 and some examples are shown in Fig. 2. The numbers of apoptotic cells in both hemispheres were significantly higher in saline treatment group than that of sham group ($p<0.0001$). The animals treated with ABT-491 either before or after hypoxia had a significant decrease in the numbers of TUNEL-positive apoptotic cells compared with the saline group ($p<0.0001$). The number of apoptotic cells in the animals treated with ABT-491 before hypoxia were not statistically significantly different than in the animals treated with ABT-491 after hypoxia ($p>0.05$).

The numbers and some examples of caspase-3 positive apoptotic cells of each groups are shown in Figs. 3 and 4. Compared with sham group, caspase-3 positive apoptotic cells obtained from saline group were significantly higher in both hemispheres ($p<0.0001$). The numbers of caspase-3 positive apoptotic cells decreased significantly in the ABT-491 treated group compared with the saline group ($p<0.0001$). There was no significant difference in the number of caspase-3 positive apoptotic cells between the animals treated with ABT-491 before or after hypoxia ($p>0.05$).

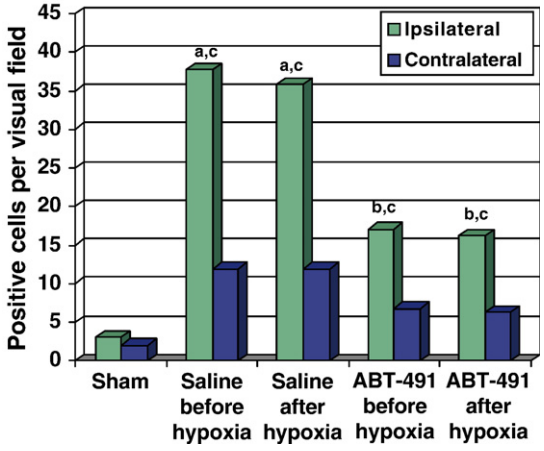

Fig. 1 – TUNEL-positive apoptotic cells in the ipsilateral and contralateral hemispheres of the sham, saline and ABT-491 treated animals. [a]$p<0.0001$ vs. sham animals, [b]$p<0.0001$ vs. saline treated animals, [c]$p<0.0001$ vs. contralateral hemisphere.

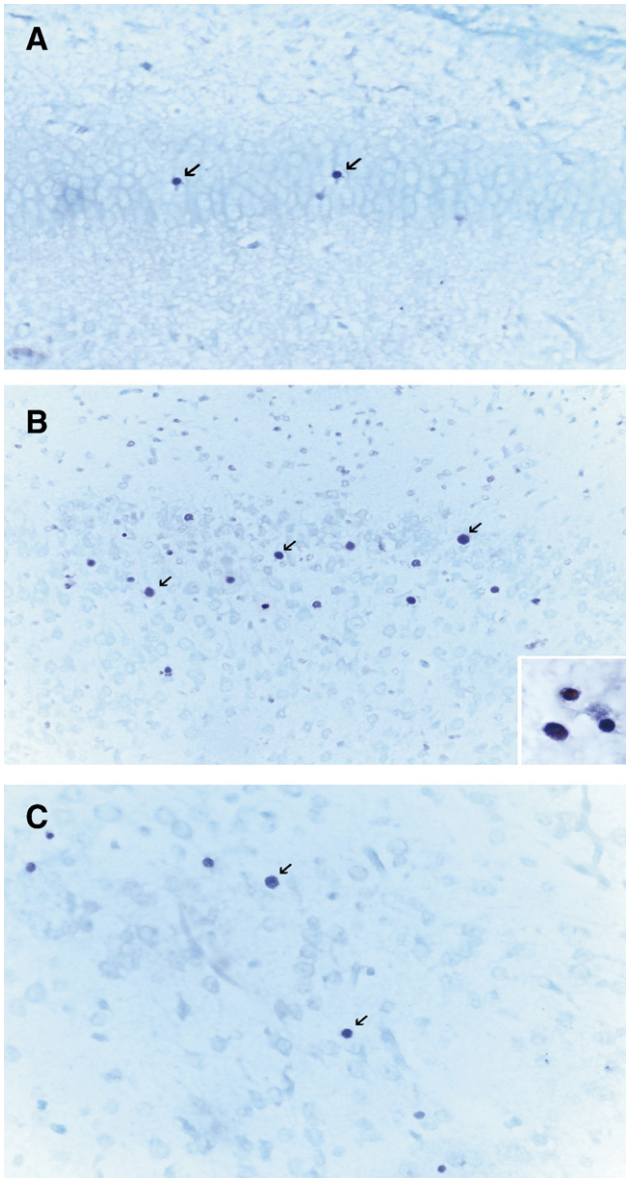

Fig. 2 – TUNEL-positive apoptotic cells in hippocampus in the (A) sham group, (B) saline and (C) ABT-491 treatment groups. Original magnification, ×400.

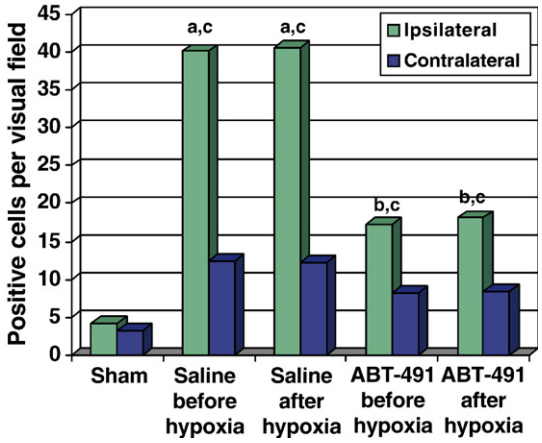

Fig. 3 – Caspase-3 positive apoptotic cells in the ipsilateral and contralateral hemispheres of the sham, saline and ABT-491 treated animals. [a]$p<0.0001$ vs. sham animals, [b]$p<0.0001$ vs. saline treated animals, [c]$p<0.0001$ vs. contralateral hemisphere.

Except the sham group, the numbers of apoptotic cells in right hemispheres were significantly higher than that in the left hemispheres in all of the groups (Figs. 1 and 3) ($p<0.0001$). Mortality rates did not differ among the groups ($p>0.05$).

3. Discussion

HIBI is an important contributor to perinatal mortality and long-term neurological impairments in term and preterm survivors (Vannucci, 2000; Ferriero, 2004; Perlman, 2006). More recently, extensive neuropathologic study of the animal models has been done for evaluating different neuroprotective therapies in perinatal HIBI (Ferriero, 2005; Hossain, 2005; Northington et al., 2005; Vannucci and Vannucci, 2005). In the present study, we used modified Levine–Rice procedure of unilateral carotid ligation plus hypoxia to the 7-day-old rat pups. This well-characterized model is the most often used model of neonatal HIBI. The rat's brain at this stage is histologically similar to that of a 32–34-week gestation human fetus or newborn infant and this model has proved useful in many studies (Vannucci and Vannucci, 2005; Northington, 2006).

In the present study, hypoxic ischemia resulted in a significant increase in the numbers of apoptotic cells in the brain. There is good evidence that hypoxic ischemia causes neuronal injury, which has been attributed to excitotoxicity, oxidative stress, and inflammation (Johnston et al., 2001; Hossain, 2005; Rodrigo et al., 2005; Blomgren and Hagberg, 2006; Oechmichen and Meissner, 2006). Neuronal cell death after hypoxic ischemia has generally been attributed to either rapid necrosis or delayed apoptosis. Although there is no doubt that necrosis plays major role in the neuronal cell death, accumulating data suggest that apoptosis plays an important role in the evolution of hypoxic ischemic injury in the neonatal brain (Sastry and Rao, 2000; Johnston et al., 2001; Northington et al., 2005). We demonstrated that the numbers of apoptotic cells in the animal groups of hypoxic ischemia were significantly higher than those in the sham group. Our current results are in agreement with those of previous studies.

PAF is a membrane-derived bioactive phospholipid and this bioactive lipid may be significant in the pathophysiology of brain trauma, seizures, and ischemia (Bazan, 2003). PAF is physiologically involved in excitatory neurotransmitter release, neuronal plasticity, and memory formation (Chen and Bazan, 2005). Although low concentrations of PAF modulate cell function, relatively higher concentrations may become neurotoxic. PAF concentrations of brain rise acutely after neonatal hypoxic ischemic conditions. Maki et al. (1988) reported that neonatal animals may have higher PAF concentrations than those in the adults as the neonate has low amounts of the acetylhydrolase. For this reason, the contribution of PAF to injury of the immature rat brain may be more significant than in the adult.

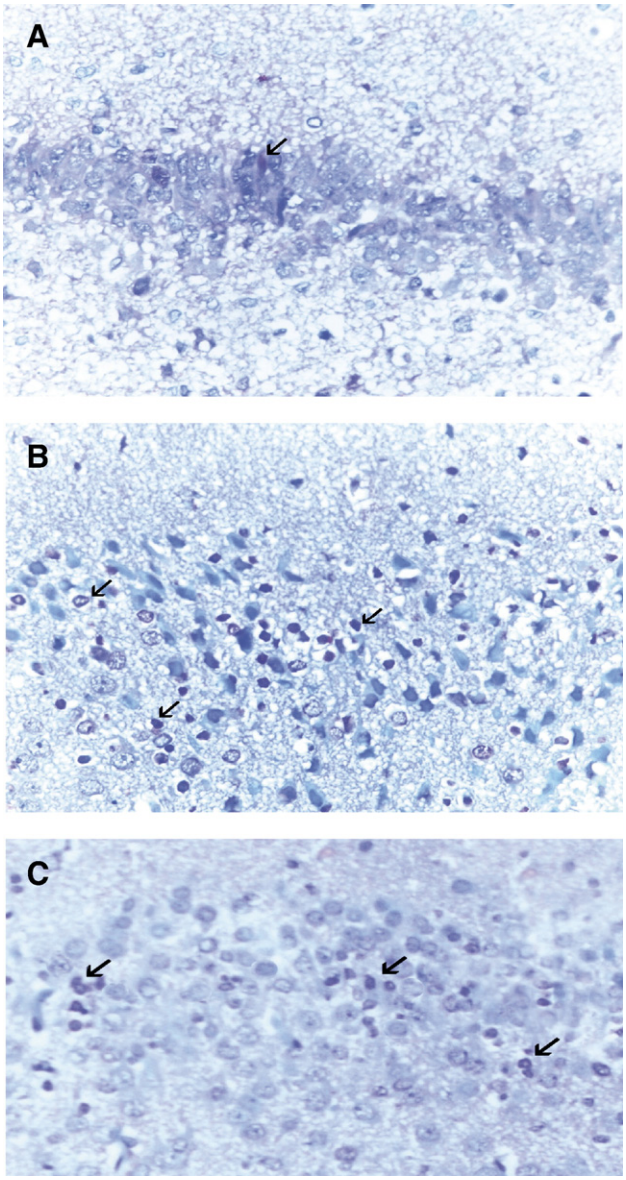

Fig. 4 – Caspase-3 positive apoptotic cells in hippocampus in the (A) sham group, (B) saline and (C) ABT-491 treatment groups. Original magnification, ×400.

In recent years, an increased proportion of basic science research has been directed toward evaluating mechanisms and treatment involving cell injury, and use of PAF antagonists after ischemia and reperfusion has been shown to improve the status of various organs following ischemia in animal models. Administration of PAF antagonists led to a reduction of the ischemia–reperfusion injury to the heart muscle (Katoh et al., 1993), a protection against ischemic injury to the kidney (Torras et al., 1999), a reduction of the ischemia–reperfusion damage to the lung (Stammberger et al., 1999), and an amelioration of the ischemia–reperfusion injury to the liver (Aldemir et al., 2004). This success has led us to attempt such treatment with models of HIBI.

There has been relatively little research about the role of PAF antagonists in neonatal HIBI. Liu et al. (1996) reported that administration of a PAF antagonist BN 52021 reduced cerebral infarction in neonatal rat model of HIBI. They also demonstrated that treatment with the PAF receptor antagonist BN 50730 resulted in approximately 60–80% reduction in brain tissue loss in 7-day-old Sprague–Dawley rats (Liu et al., 2001). In contrast, Viswanath et al. (2000) found that PAF antagonist treatment either with WEB 2170 or BN 52021 had no effect on brain damage in the neonatal Wistar rat. On the other hand, it has been demonstrated that PAF receptor antagonism with BN 52021 before the circulatory arrest period can significantly improve recovery of cerebral blood flow and oxygen metabolism (Liu et al., 1996). However, all those previous studies did not evaluate the effect of PAF antagonist treatment on the neuronal apoptosis. To our knowledge, the effect of ABT-491 on neonatal rat model of HIBI is unknown. In the present study, we found that treatment with ABT-491 either before or after hypoxia reduced neuronal apoptosis in the neonatal rats subjected to hypoxic ischemic insult.

ABT-491 is a recently described highly potent and selective PAF antagonist (Albert et al., 1997). It is a potent antagonist of responses linked to the PAF receptor at the cellular level, especially platelets and neutrophils. Albert et al. (1997) found that ABT-491 was also effective in blocking platelet activation in blood, indicating that the presence of high concentrations of protein and other serum factors slightly alters the ability of ABT-491 to interact with PAF receptor. In the present study, we used 0.4 mg/kg ABT-491 intraperitoneally. The dose of ABT-491 in the treatment of HIBI has not been reported previously. It has been demonstrated that ABT-491 was effective in inhibiting lipopolysaccharide-induced hypotension (ED_{50}=0.04 mg/kg) and gastrointestinal damage (0.05 mg/kg), it was found to be lethal at a dose of 1 mg/kg (Albert et al., 1997).

The exact mechanism of the apoptotic function of PAF is still unclear. However, it has been demonstrated that PAF elicits apoptosis in enterocytes via a mechanism that involves Bax translocation to mitochondria, collapse of mitochondrial membrane potential, and caspase activation (Lu et al., 2004). There is strong evidence that PAF was involved in cellular apoptosis with Fas–Fas ligand (FasL) and cytochrome-C release in several cell types. Wu et al. (2003) demonstrated that PAF promotes mucosal apoptosis via FasL-mediating caspase-9 active pathway in rat small intestine after ischemia–reperfusion. On the other hand, recent studies have emphasized the anti-apoptotic effect of PAF antagonists and the protective effect of PAF antagonists against apoptotic changes in several tissues and cells (Murohisa et al., 2002; Loucks et al., 2003; Grypioti et al., 2006). Our study is the first report in the literature to show that the neuronal apoptotic changes were reduced by ABT-491, a highly potent and selective PAF antagonist, in neonatal rat model of HIBI. In light of these data, we propose that inhibition of apoptosis in hypoxic ischemia represents an important protective effect provided by PAF antagonist.

Therapy using PAF antagonists may be a new approach in the treatment of HIBI. Although there has been relatively little research about the role of PAF antagonists in neonatal HIBI, no study has investigated the role of PAF antagonists on neuronal apoptosis in this model. The current study shows that ABT-491, a highly potent and selective PAF antagonist, administration either before or after hypoxia reduces apoptosis and we propose that ABT-491 may be a novel approach for the therapy of HIBI. Although the present study demonstrated the immediate neuroprotective effect of ABT-491 in HIBI, the long term effects of this drug should be investigated and further studies are required to elucidate the mechanism of HIBI that involves PAF activation and the possible role of platelets in HIBI.

4. Experimental procedures

4.1. *Animals*

Seven-day-old Wistar rat pups (n=100) of either sex, delivered spontaneously, were used in this experimental study. All animal experiments followed a protocol approved by the ethical committee on animal research at our institution.

4.2. *Animal preparation and surgical procedure*

Rat pups were anaesthetized by halothane inhalation and duration of anaesthesia was less than 5 min. Hypoxic ischemia was induced according to the Levine–Rice model (Rice et al., 1981). A median incision was made in the neck. Under the microscopic magnification, the right common carotid artery was dissected and ligated with a 6-zero silk suture. After the wound was sutured, the animals were allowed to have 3 h recovery and feeding period. Except for the sham group, rats were then placed in a plastic chamber and exposed to a continuous flow of 8% oxygen–92% nitrogen for 2 h. After hypoxic period, the rats allowed to have a 2 h recovery period in an open chamber without any supplemental oxygen. The animals in the sham group were placed in an open chamber for the same intervals. The chambers were partially submerged in a water bath at 37 °C to maintain a constant thermal environment. After these procedures, all the pups were euthanized by decapitation. The brains were removed and paraffin-embedded for pathological evaluation.

Saline treatment group: 0.5 mL saline was injected intraperitoneally either immediately before (n=20) or after (n=20) hypoxia. Fourteen rats (6 before hypoxia, 8 after hypoxia) in this group were died during the procedure.

ABT-491 treatment group: The rat pups were administered intraperitoneally 0.4 mg/kg ABT-491 which was dissolved in

saline, either immediately before ($n=20$) or after ($n=20$) hypoxia. Five rats (4 before hypoxia, 1 after hypoxia) in this group were died during the procedure.

Sham group ($n=20$): After median neck incision was made, neither ligation, nor hypoxia were performed. Four rats in this group were died during the procedure.

4.3. *Histopathological evaluation*

TUNEL (Terminal Deoxynucleotidyl Transferase-mediated-dUTP Nick End Labeling) Method: To investigate DNA fragmentation at neurons, TUNEL method (in Situ Apoptosis Detection Kit, Biogen, USA) was selected. After deparaffinized and rehydrated, sections were pretreated with proteinase K for 15 min at room temperature, then endogen peroxidase activity was quenched with 2% H_2O_2. Slices were then incubated at 37 °C for 60 min in moist chamber with 50 μl of TdT buffer. Finally, the reaction was visualized by streptavidin–biotin–peroxydase complex and diaminobenzidine. TUNEL labeled slides were counterstained with 1% methyl green.

Caspase-3 Method: Serial sections from paraffin-embedded coronal brain sections were deparaffinized in xylene and dehydrated through graded concentrations of ethanol. After the blocking of endogenous peroxidase activity with hydrogen peroxide, the sections were heated in 0.01 mol/L citrate buffer in a microwave cooker for 20 min. Sections were incubated using caspase-3 polyclonal antibody (dilution 1/100, Neomarkers, RB-1197-B0, USA) for 1 h at room temperature in a humidified chamber, and were then stained using the avidin–biotin complex (ABC; Labvision, Fremont, USA) immunoperoxidase technique with a commercially available reagent. The sections were counterstained with Mayer's haematoxylin and mounting media (Labvision, Fremont, USA).

4.4. *Apoptotic cell counting*

Apoptotic cell counting was performed in subtalamic nuclei, hippocampus and parietal cortex of both right and left hemispheres (Vannucci, 2000; Johnston et al., 2001; Rodrigo et al., 2005). In evaluating numeric density, total TUNEL and caspase-3 positive stained neurons were calculated in 5 high power fields (5×400) under the light microscope (Zhu et al., 2004). Microscopic examinations were made by a single pathologist who was unaware of the characteristics or treatment of the animals.

4.5. *Statistical analysis*

All data were expressed as mean±standard deviation (S.D.). Animal mortality rate was evaluated by chi-square testing using Yates correction whenever necessary. Unpaired *t* test and ANOVA was used for statistical analysis. Multiple comparisons were made using Tukey's procedure with $p<0.05$ considered statistically significant.

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