PAF antagonism modifies neuroprotective action of histone deacetylase and calcineurin phosphatase inhibitors in mice

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Received 1 March 2006; revised 1 September 2006

To evaluate the hypothesis that platelet activating factor (PAF) antagonism may affect the functional recovery following the nerve injuries and also to evaluate the effect of PAF receptor antagonism on the neuroprotective effect of tacrolimus and sodium valproate, effect of PAF receptor antagonist, WEB2086 was evaluated in animal models of sciatic nerve crush and endothelin-1 induced focal cerebral ischemia. WEB2086, per se, while attenuating spontaneous sensory motor recovery after sciatic nerve crush, enhanced functional recovery after focal cerebral ischemia. WEB2086 also attenuated the neuroprotective effect of tacrolimus and sodium valproate subsequent to peripheral nerve injury, while it significantly improved the neuroprotective action of tacrolimus and sodium valproate following cerebral ischemia reperfusion injury. These results suggest that PAF receptor antagonists alone and in combination with tacrolimus/sodium valproate could be used in the treatment of cerebral ischemia reperfusion injuries however, their use following peripheral nerve injuries could be detrimental.

Keywords: Calcineurin phosphatase, Histone deacetylase Neurotrophins, Neuroprotection, Platelet activating factor

Platelet-activating factor (PAF) is a potent phospholipid released from many cell types, including stimulated basophils, platelets, macrophages, and polymorphonuclear neutrophils. Biological actions of PAF are mediated through activation of a specific, high-affinity receptor on the target cell surface as well an intracellular receptor. PAF is involved in variety of biological and immune responses in the peripheral and central nervous system (CNS). In the CNS, PAF is synthesized in neurons and microglia and PAF receptors are expressed predominantly in neurons, microglia, on astrocytes and CNS endothelium. PAF levels are elevated in the brain and cerebrospinal fluid following an ischemic attack and as a result of the breakdown of membrane phospholipids. PAF is a known inflammatory mediator in ischemia reperfusion injury. However, the role of PAF in peripheral nerve injuries is not clear even though studies indicate the beneficial role of Ginkgo biloba extract, which includes PAF antagonist, BN 52021 in peripheral neuropathies. The triazolobenzodiazepine analogue, WEB2086 is a specific PAF receptor antagonist and it inhibits both the intracellular and extracellular PAF receptors. WEB2086 inhibits the aggregation of platelets and thromboxane synthesis.

In the present study, two models causing neurological deficit i.e the ET-1 induced cerebral ischemia and sciatic nerve crush were utilised. We specifically intended to utilise the ET-1 induced cerebral ischemia because ET-1 is a potent vasoconstrictor peptide and can be used to produce small and highly localized ischemic lesions with a minimally invasive surgical procedure. On the other hand, nerve crush injuries cause axonal degeneration and motor end plate loss. The regeneration process involves axonal regeneration, restoration of the blood nerve barrier and limiting the extent of motor end plate loss.

The neuroprotective effects of immunosuppressant tacrolimus and anti-epileptic drug sodium valproate have been demonstrated in various peripheral nerve injury and stroke models. Tacrolimus acts by inhibiting calcineurin-mediated T-cell activation via complex formation with FK506 binding protein 12 (FKBP12). Combination therapy including tacrolimus and PAF antagonists significantly reduced ischemia-reperfusion injury in cardiac tissue and preserved energy metabolites following cerebral ischemia. These studies along with calcineurin mediated pathway playing a vital role in platelet activating factor-induced chemokine gene expression indicate a

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potential interaction between calcineurin phosphatase and PAF pathways. On the other hand, sodium valproate is a known histone deacetylase inhibitor and histone acetylation has been shown to be critical for neurogenesis and cell differentiation.

The present study has been designed to test the role of PAF antagonist WEB2086 in recovery following sciatic nerve crush injury and focal cerebral ischemia. Also, the effect of WEB2086 on the neuroprotective role of tacrolimus and HDAC inhibitor sodium valproate using the similar models has been evaluated.

Materials and Methods

The following drugs were used: Tacrolimus (Fujisawa, Japan), WEB 2086 (Boehringer Ingelham, Germany), Urethane and Endothelin-1 (Sigma, USA), Sodium valproate (Sun Pharma, India), Ether and Normal Saline (CMCH Pharmacy).

Animals—Swiss albino mice (25-30 g) of either sex were randomly allocated into 7 different treatment and control groups. Animals received food and water ad libitum and were kept on a 12-hr light/dark cycle. Animals were kept as per the protocols approved by the institutional Animal Care and Use Committee.

Sciatic nerve crush

Surgery—Mice were subjected to sciatic nerve crush according to Siconolfi and Seeds. Adult mice were anesthetized with 150 mg/100g intraperitoneal urethane. The area above the right lower thigh was shaved and sterilized with betadine and 70% surgical spirit. A 1 cm incision was made in the skin above the lower thigh between the gluteus maximus muscle and the biceps femoris muscle. The muscles were teased apart with scissors and the sciatic nerve exposed. For crush injury the nerve was placed in a 1 mm wide needle holder and crushed for 20 sec. The holder was rotated 90° and the crush was repeated at the same site. The nerve was replaced under the muscle and the incision was sutured. Completeness of the crush was established by examining the loss of sensory and motor function in the hind limb of the operated mice. Pinching the hind limb digits and pricking the footpad without eliciting a foot withdrawal and vocalization was noted as loss of sensory and motor function. For sham controls the sciatic nerve of the right hind limb was surgically exposed but no crush was made.

Functional evaluation of regeneration—Evaluation of sensory and motor functions in the hind limbs was done on day 0 i.e. before surgery and on days 1, 3, 7, 10, 14 following surgery as per Varejao et al. The animals in six groups (n=7 each) received normal saline (0.9% NaCl, 0.5 ml, ip), WEB 2086 (20 mg/kg/day, ip), tacrolimus (2 mg/kg/day, sc), sodium valproate (200 mg/kg/day, sc) and combination of tacrolimus (2 mg/kg/day, sc)/sodium valproate (200 mg/kg/day, sc) with WEB 2086 (20 mg/kg/day, ip) respectively for 13 days.

Sciatic function index (SFI) —The mice were held by the chest and their hind feet were pressed down onto a stamp pad soaked with water soluble black ink. The animals were immediately allowed to walk along a confined walkway 6 cm wide by 30 cm long with a dark shelter at the end the corridor leaving its foot prints on the paper that is cut to the appropriate dimensions and placed on the floor of the corridor. The tracks were evaluated for three different parameters: (1) distance from the heel to the third toe, the print length (PL); (2) distance from the first to the fifth toe, the toe spread (TS); and (3) distance from the second to the fourth toe, the intermediary toe spread (ITS). All three measurements were taken from the experimental (E) and normal (N) sides. Using the following formula derived by Bain et al, SFI was calculated as,

\[
SFI = -38.3 \left( \frac{(EPL - NPL)}{NPL} \right) + 109.5 \left( \frac{(ETS - NTS)}{NTS} \right) + 13.3 \left( \frac{(EIT - NIT)}{NIT} \right) - 8.8
\]

The SFI was analysed as: An SFI equal to 100 indicates significant impairment, whereas an SFI oscillating around 0 is considered to reflect normal function.

Nociceptive function—Nociceptive function was evaluated by observing the withdrawal reflex of the hind limb and vocalization in response to noxious stimulation like mechanical stimulation (pinch test) and pricking the plantar aspect of the lateral part of the foot with a needle.

Gait—Animals were allowed to walk on a platform as well as on an inclined plane for 2 min each. Subjective scores were assigned on the basis of hind limb movement and its posture while ambulating. Mice moving both the hind limbs uniformly given 3, if the operated limb was moving with deformity it received 2, scored as 1 if the operated limb was moving seldom and 0 – when no movement was seen in the operated hind limb.
Focal cerebral ischemia

Surgical procedure—In mice under continuous ether anaesthesia the skin overlying the left side of the skull was incised and skull was exposed under total aseptic precautions. A 26 gauge needle was inserted 2.5 mm posterior to bregma and 3.0–3.5 mm lateral to midline about 2 mm deep dura. ET-1 (60 pmol delivered in 5 μl sterile water) was then delivered via micro syringe. Sham controls received 5 μl sterile water perivascularly. After the injection the needle was removed and scalp was sutured.

The animals in six groups (n=7 each) received normal saline (0.9% NaCl, 0.5 ml, ip), WEB 2086 (20 mg/kg/day, ip), tacrolimus (2 mg/kg/day, sc), sodium valproate (200 mg/kg/day, sc) and combination of tacrolimus (2 mg/kg/day, sc)/sodium valproate (200 mg/kg/day, sc) with WEB 2086 (20 mg/kg/day, ip) respectively for 6 days.

Postural hang test—Postural hang test as described by Moyanova et al. found to be highly sensitive to sensorimotor function, was conducted before surgery (day 0) and on the days 1, 3, 5 and 7 after surgery. Mice were suspended by the tail 50 cm above a platform and then slowly lowered toward it. Mice which extended both forelimbs toward the platform were given a score 3. If a forelimb flexion toward the body occurred, it received score 2, and scored as 1, if a forelimb flexion together with a reduced resistance to lateral push was detected. Severe neurological deficits such as twisting of the thorax or circling when mice were allowed to move freely on the platform was given a score 0.

Statistical evaluation—Statistical evaluation was conducted using multiple comparisons and Mann Whitney U test. Data are depicted as mean ± SE of 7 experiments. P < 0.05 were considered significant.

Results

PAF antagonism reduces the spontaneous recovery following sciatic nerve crush in mice and enhances the functional recovery following transient focal cerebral ischemia in mice (Table 1 and Fig. 1)—In all animals SFI score prior to surgery was 100, gait score was three and nociceptive function was intact. Following sciatic nerve crush, in all animals except sham controls, SFI scores and gait score were reduced to 0 and there was loss of nociceptive function. In sham controls there was no reduction in SFI score and gait score, while nociceptive function remained intact. As on day 14 following injury there was a spontaneous recovery of sensorimotor function in saline treated mice as shown by improvement in SFI scores, in gait scores and recovery of nociceptive function (Table 1). In comparison with the saline treated control animals, WEB2086 treated animals significantly reduced improvement in SFI scores by 14.5%, in gait scores by 5.8% and recovery in nociceptive function by 3.3 days. (Table 1).

In all animals postural hang test score prior to surgery was three. Following ET-1 induced focal cerebral ischemia, in all animals except sham controls, postural hang test scores reduced to 0. In sham controls there was no reduction in postural hang test scores. On day 7 following ET-1 induced focal cerebral ischemia WEB2086 treated animals showed significantly high improvement in postural hang test scores.

<table>
<thead>
<tr>
<th>Treatment groups (n = 7 animals in each group)</th>
<th>SFI scores (as on day 14)</th>
<th>Improvement in gait (%) as on day 14</th>
<th>Time taken for sensory recovery (number of days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham control</td>
<td>43.2 ± 1.8</td>
<td>28.1 ± 2.2</td>
<td>17.8 ± 2.9</td>
</tr>
<tr>
<td>WEB2086</td>
<td>28.7 ± 2.4</td>
<td>22.3 ± 1.7a</td>
<td>21.1 ± 3.1a</td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>55.3 ± 1.7a</td>
<td>69.6 ± 3.5a</td>
<td>13.2 ± 2.6a</td>
</tr>
<tr>
<td>Tacrolimus + WEB2086</td>
<td>36.2 ± 2.9</td>
<td>50.3 ± 3.1b</td>
<td>16.9 ± 3.4b</td>
</tr>
<tr>
<td>Sodium valproate</td>
<td>47.1 ± 2.1a</td>
<td>42.6 ± 2.7a</td>
<td>15.1 ± 2.7a</td>
</tr>
<tr>
<td>Sodium valproate + WEB2086</td>
<td>34.5 ± 2.0</td>
<td>30.5 ± 3.3c</td>
<td>20.1 ± 3.0</td>
</tr>
</tbody>
</table>

P values: <0.05; a vs control; b vs tacrolimus; c vs sodium valproate (Mann-Whitney U test with multiple comparisons)
scores (28.7±1.8%,* P < 0.05) (Fig. 1) in comparison with saline treated (4.3 ± 2.4 %) control animals.

**PAF antagonism modifies the neuroprotective action of tacrolimus in mice. (Fig. 1 and Table 1)—**In mice receiving tacrolimus following sciatic nerve crush/transient cerebral ischemia, functional recovery was significantly higher (P<0.01) than the saline treated control animals in both the models of nerve injuries. Co-administration of PAF antagonist WEB2086 with tacrolimus following sciatic nerve crush significantly reduced the improvement in SFI scores by 19.1%, in gait scores by 19.3% and recovery in nociceptive function by 3.7 days in comparison with tacrolimus alone treated mice. However, in transient focal cerebral ischemia model PAF antagonism positively modified the neuroprotective action of tacrolimus. As on day 7 following ET-1 induced focal cerebral ischemia co-administration of PAF antagonist WEB2086 with tacrolimus significantly improved (54.3 ± 3.1%, P <0.05) the postural hang test scores in comparison with tacrolimus alone (39.2 ± 2.9%) treated animals.

**PAF antagonism modifies the neuroprotective action of sodium valproate in mice (Table 1 and Fig. 1)—**In mice receiving sodium valproate following sciatic nerve crush/transient cerebral ischemia, functional recovery was significantly higher (P<0.01) than the saline treated control animals in both the models of nerve injuries. Co-administration of PAF antagonist WEB2086 with sodium valproate following sciatic nerve crush significantly reduced the improvement in SFI scores by 12.6%, in gait scores by 12.1% and recovery in nociceptive function by 5 days in comparison with sodium valproate alone treated mice. However, in transient cerebral ischemia model PAF antagonism positively modified the neuroprotective action of sodium valproate. As on day 7 following ET-1 induced focal cerebral ischemia co-administration of PAF antagonist WEB2086 with sodium valproate (37.3 ±1.3 %, P<0.05) improved the postural hang test scores in comparison with sodium valproate alone (31.1 ± 2.2 %) treated animals.

**Discussion**

In the present study the antagonism of PAF using a potent antagonist like WEB 2086 *per se* resulted in worsening of the regeneration of the sciatic nerve and recovery of sensory and motor deficits following a crush injury. Following nerve injury neutrophils and other leukocytes release various inflammatory mediators, including PAF. PAF induces platelet activation and platelet aggregation. Specific PAF receptor antagonists present in *Gingko biloba* extracts and triazolobenzodiazepine WEB 2086 antagonize this neutrophil induced platelet aggregation. Platelet aggregation in turn releases inflammatory mediators which are involved in Wallerian degeneration and phagocytosis. PAF also releases neurotrophins like vascular endothelium derived growth factor (VEGF) and nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin - 3 (NT-3) and insulin like growth factor – 1 (IGF-1). Both these growth factors orchestrate axon regeneration and recovery of function after nerve injury. Therefore, through release of neurotrophins, which promote survival of existing Schwann cells and support neurite outgrowth, PAF may act as a neuro regenerative agent *in vivo*.

The behavioural effects of unilateral middle cerebral artery occlusion (MCAO) were induced by perivascular injection of endothelin-1 (ET-1). During ischemia–reperfusion, cerebral vascular endothelial cells have been shown to release/express various products of arachidonic acid metabolism with both vasoactive and pro-inflammatory properties, including prostaglandins, leukotrienes, and platelet-activating factor. Studies indicate that PAF promotes neuronal cell death following ischemia–reperfusion. Studies indicate that treatment with the *Gingko biloba* extracts containing PAF receptor antagonists decreases hypoxic-ischemic brain injury in different models of cerebral ischemia. The results of the present study (Fig. 1) show improved neurological recovery following WEB2086 treatment in ET-1 induced cerebral ischemia. PAF antagonist WEB2086 *per se* by reducing vasoconstriction, thrombus formation, lipid peroxidation and nitric oxide synthesis confers neuroprotective effect in focal cerebral ischemia.

The neuroprotective action of tacrolimus in peripheral nerve injuries involves nerve regeneration by increasing the rate of axonal regeneration, early restoration of the blood-nerve barrier and limiting the extent of motor end plate loss. It improves functional recovery of denervated targets by increasing both regenerative and collateral re-innervation in axotomy models thereby limiting permanent functional loss. Tacrolimus is known to potentiate nerve growth factor induced neurite outgrowth via the Ras/Raf/MAP kinase pathway.
Based on the results of the present study it is proposed that tacrolimus may depend on PAF for its neuroprotective action following peripheral nerve injuries. The neuroprotective effect of tacrolimus has been established in several models of CNS ischemia reperfusion injuries. It presumably involves the inhibition of calcineurin, preventing the dephosphorylation of nitric oxide synthase and its subsequent activation. Through its immunosuppressant action it reduces the inflammatory reaction in the infarct region and reduces the extension of infarct rim. It also ameliorates long-term neurologic deficits in a rodent model of stroke, strengthening the view that tacrolimus may prove beneficial in treating stroke patients. WEB 2086 by enhancing the immunosuppressant action of tacrolimus as well as with the abovementioned mechanisms improves the neuroprotective action of tacrolimus.

The histone deacetylase inhibitor valproate promotes neurite outgrowth stimulated by neurotrophins, activates the extracellular signal regulated kinase pathway, increases growth cone-associated protein 43 and anti-apoptotic bcl-2 genes. Recently, it has been shown that sodium valproate enhances axonal regeneration and recovery of motor function after sciatic nerve axotomy in adult rats. It also suppressed ischemia-induced neuronal caspase-3 activation in the cerebral cortex and time-dependent increase in acetylated histone H3 levels in the cortex and striatum of both ipsilateral and contralateral brain hemispheres in animal models of stroke. In the present study, sodium valproate enhanced the functional recovery following neuronal injuries in both models. WEB 2086, while enhancing the neuroprotective effect of sodium valproate following focal cerebral ischemia, attenuated its neuroprotective action in sciatic nerve crush model. The results suggest that PAF could play an important role in the neuroprotective pathways of sodium valproate as well.

In conclusion, the present study has demonstrated that PAF plays critical but paradoxical roles in peripheral and central nervous system injuries. In traumatic peripheral nerve injuries it appears to be neurotrophic while in ischemia – reperfusion injuries in CNS it is neurotoxic. PAF receptor antagonism with WEB 2086 also modifies the neuroprotective action of tacrolimus as well as sodium valproate. It is suggested that WEB 2086 alone as well as in combination with tacrolimus/sodium valproate could be used in the treatment of CNS ischemia reperfusion injuries while its use in peripheral nerve injuries could be detrimental.

Acknowledgement

The authors would like to thank Fujisawa Ltd (Japan), Alomone Labs (Israel) and Boehringer Ingelheim (Germany) for gift of drug samples.

References