Vasopressin mediates neuroprotection in mice by stimulation of V₁ vasopressin receptors: Influence of PI-3 kinase and gap junction inhibitors

Manoj G Tyagi* & K V Parthiban
Department of Pharmacology, Christian Medical College, Vellore 632 002, India

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Neuroprotective effect of vasopressin analogues, arginine Vasopressin (AVP) and lysine Vasopressin (LVP) was evaluated against MgCl₂ induced cerebral ischemia model. AVP significantly prevented (P<0.01) MgCl₂ (1M) induced cerebral ischemia as compared to lysine Vasopressin (LVP) which was less effective (P<0.05). Pretreatment with PI-3 kinase inhibitors, Wortmannin and LY-294002 (50 μg/kg, ip) significantly attenuated the protective effects of vasopressin. AVP was also effective in reducing the maximal electroshock (MES) induced convulsive time and this protective effect was blocked by PI-3 kinase inhibitors. On the other hand, pretreatment with gap junction intracellular communication (GJIC) blocker, mefenamic acid (30 mg/kg, ip) significantly potentiated the MgCl₂ induced cerebral ischemia. This enhancement of cerebral ischemia was not reversed by vasopressin analogue, LVP. The role of V₁ vasopressin receptor was evaluated by pretreating the animals with non-selective V₁ receptor antagonists, des Gly-NH₂ d (CH₂)₅ [D-Tyr², Thr⁴]² [OVT which reversed the effects of AVP suggesting a role for vasopressin V₁ receptors. This study suggests that neurohypophyseal hormone, AVP is neuroprotective against MgCl₂ induced cerebral ischemia and this effect is modulated by PI-3 kinase enzyme inhibitors and protein kinase C inhibitors through possible influence on the cerebral vascular tone. This study suggests that gap junctions have potential role in the induction of MgCl₂ induced cerebral ischemia.

Keywords: Arginine vasopressin, Cerebral ischemia, Gap junction, Lysine vasopressin, Neuroprotection, PI-3 kinase, Protein kinase C, Vasopressin receptors

Cerebral ischemia and haemorrhagic strokes resulting in ischemic cell death represent major cause of cerebrovascular disorders. Various potential treatment approaches have been developed to reduce the extent of tissue injury, approaches that have been derived from experimental models of cerebral ischemia¹.²

Neuronal damage mechanisms depend on complex interactions between neurotransmitters, neuropeptides and inflammatory molecules that modify survival and repair. A pathological consequence of cerebral ischemia is the hyperactivity of specific neuronal synapses and mechanisms targeting attenuation of excitotoxicity at the molecular receptor, and/or ion channel, protein and enzyme changes continue to be explored for therapeutic implications⁵. Drugs intended for treatment of cerebral ischemia might also be effective against some forms of epilepsy. Some novel anticonvulsant drugs emphasize this view as they also prevent cerebral ischemia due to constriction or occlusion of major cerebral arteries⁶.⁷.⁸

Arginine vasopressin (AVP) is an important hormone secreted by the posterior pituitary gland. AVP has also been shown to be a key regulator of physiological homeostatic balance and acts on two types of vasopressin receptors V₁ and V₂ in the brain. Vasopressin has been shown to have multifaceted action in the central nervous system affecting cognition, behaviour and neuronal excitability⁹. AVP has been found to induce depolarization of hippocampal neurons. AVP decreases the responsiveness of vasopressin neurons to acute changes in plasma osmolality. Data available on AVP plasma levels in ischemic stroke are few and discordant⁹. Several studies suggest that AVP may have either pro- or anticonvulsant effect depending on the specific animal models of epilepsy. Vasoconstrictor normally induces vasodilation peripherally and also induces vasodilation in some vascular beds by releasing nitric oxide and increasing cGMP levels⁹.¹⁰

On the other hand, PI-3 kinases induce the phosphorylation of inositol containing lipids, PI, PI-4P and PI (4,5) P₂ at the hydroxyl position on the inositol ring. AVP has been shown to activate the PI-3 kinase enzyme in some cell types¹¹.¹². The enzyme have been found to be expressed in the sympathetic and sensory

*Correspondent author:
Tel: (0416) – 262603
Fax: (0416) – 262788
E-mail: tyagi243@yahoo.co.in
This study was conducted in the dentate gyrus of the rats. Wortmannin, a potent PI-3 kinase inhibitor has been shown to inhibit long term potentiation in the dentate gyrus of the rats. Recent studies suggest that PI-3 kinase might also contribute in the cytokine induced neuroendocrine responses in some cell types.

The importance of Mg in homeostatic balance is well known. Mg ion can cause inhibition of NMDA responses in cultured rat neurons and has been shown to play an important role in neuronal depolarization. Mg is an important ion for regulation of myogenic tone of cerebral blood vessels and it is believed to play an important role in the autoregulation of cerebral blood flow. Dietary deficiency of magnesium (Mg) as well as abnormalities in Mg metabolism has been suggested to play important roles in hypertension, stroke, atherosclerosis and diabetic vascular disease.

We utilised MgCl2 for inducing cerebral ischemia in this study. MgCl2 induced reversible global cerebral ischemia in mice is an excellent in vivo model. Recent studies suggest an interaction of Mg ion and PI-3 kinase enzyme in maintaining the cerebral vascular tone.

The purpose of this study was to determine the role of signaling molecules in the neuroprotective action of vasopressin and to characterize the vasopressin receptor subtype responsible for neuroprotection. This study was hitherto carried out to elucidate the possible neuroprotective role of vasopressin against MgCl2 induced cerebral ischemia and maximal electroshock induced convulsions in mice. The influence of PI-3 kinase enzyme activity, gap junctions and nerve growth factor was also evaluated.

Materials and Methods

Drugs used for this study — Normal Saline (CMCH Pharmacy, Vellore), MgCl2 (Glaxo India Ltd, India), Arginine Vasopressin, Lysine Vasopressin (Shree Ganesh Pharmaceuticals, India, Sigma India Ltd), L-Y 294002, Wortmannin, Staurosporine and Nerve Growth Factor (Alomone Labs, Israel), Mephénylam Acid (Parke-Davis Ltd, India), DesGly-NH₂-d (CH₂) 5 [D-Tyr², Thr⁴] OVT (Dr. Maurice Manning, Ohio, USA).

Animal Care — This study was conducted on albino Swiss mice of either sex weighing between 25 and 35 g. The animals were kept under standard laboratory conditions and given food and water ad libitum. A 12 hr dark : light cycle was also maintained.

Induction of cerebral ischemia — Swiss albino mice were utilised for induction of cerebral ischemia. Global cerebral ischemia was induced by the intravenous injection (0.05 ml) of 1M MgCl2 into the tail vein. The indicative symptoms of cerebral ischemia were gasping, sedation and loss of righting reflex. The total time duration of ischemic episode beginning with onset and recovery of ischemic episode was noted.

Maximal electro shock method — Maximal Electro Shock seizures were induced in the animals using a technique described earlier. The animals were pre-treated with the test drugs prior to the start of experiments. The animals were subjected to electro shock (60 mA/0.2 sec) via transauricular electrodes attached to a electroconclusive meter. After induction of seizures, duration of tonic convulsion, clonic convulsion and total time to regain righting reflex or mortality of the animals were noted and tabulated.

Biostatistical evaluation — The data are represented as mean ± SE. Statistically significant difference was ascertained by 'P' value which is considered significant of P<0.05 and highly significant of P<0.01 as comparison of different groups were done using ANOVA and individual groups of Student's t test.

Results

The results of our study conducted on cerebral ischemia in the mice are shown in Table 1 and Figs 1 to 4. The experiments were conducted on different groups of mice (n=6). Our experiments suggest that

<table>
<thead>
<tr>
<th>Pretreatment (Dose, ip)</th>
<th>Treatment (Dose, ip)</th>
<th>MgCl2 induced cerebral ischemia time (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Saline (0.1 ml)</td>
<td>Saline</td>
<td>100 (124.2 ± 3.43)</td>
</tr>
<tr>
<td>2) Saline (0.1 ml)</td>
<td>NGF</td>
<td>81.21 ± 2.73*</td>
</tr>
<tr>
<td>3) AVP</td>
<td>NGF</td>
<td>59.14 ± 3.11*</td>
</tr>
<tr>
<td>4) LVP</td>
<td>NGF</td>
<td>64.16 ± 4.07*</td>
</tr>
<tr>
<td>5) Saline</td>
<td>Staurosporine</td>
<td>76.28 ± 3.63*</td>
</tr>
<tr>
<td>6) AVP</td>
<td>Staurosporine</td>
<td>47.02 ± 1.97**</td>
</tr>
</tbody>
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*P<0.05, **P<0.01, ***P<0.001.
pre-treatment of AVP and LVP (10 μg/kg, ip) produced significant neuroprotection against 1M MgCl₂ (0.05 ml, iv) induced ischemia i.e. a reduction in ischemic time by 78% and 37% respectively (P<0.01, <0.05).

In order to characterize the subtype of vasopressin receptor involved in the action of vasopressin, we treated the animals with a non-selective V₁ vasopressin receptor antagonist, DesGly-NH₂ d (CH₂) 5 [D-Tyr², Thr⁴] OVT (100 μg/100 g ip) and this antagonist reversed the protective actions of LVP. Thus signifying the importance of V₁ vasopressin receptors. These results are displayed in Fig. 1.

To evaluate the role of PI-3 kinase enzymes we used two potent inhibitors of PI-3 kinase, Wortmannin and LY 294 002. Pretreatment of mice with PI-3 kinase inhibitors, Wortmannin and LY294002 attenuated the neuroprotective actions of analogues AVP and LVP by 45% and 54% respectively (P<0.01, <0.05). The results of these experiments are shown in Fig. 2.

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Fig. 1 — Effect of pre-treatment with vasopressin analogues AVP, LVP (10 μg/kg, ip) and non-selective vasopressin antagonist des Gly-NH₂ d (CH₂) 5 D-[Tyr², Thr⁴] OVT (100 μg/100 g, ip) on MgCl₂ (1M, 0.05 ml, iv) induced cerebral ischemia in mice (n=6). The data are represented as mean ± SE. **P<0.01 and *P<0.05.

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Fig. 2 — Influence of Wortmannin and LY 294 002 (50 μg/kg, ip) on AVP (10 μg/kg, ip) induced neuroprotection against MgCl₂ (1M, 0.05 ml, iv) elicited cerebral ischemia in mice (n=6). The results are expressed as mean±SE.

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Fig. 3 — Effect of GJIC blocker, mephenamic acid (30 mg/kg, ip) on MgCl₂ (1M, 0.05 ml, iv) induced cerebral ischemia in mice and in the presence of LVP (10 μg/kg, ip). The results are expressed as mean±SE. **P<0.01.

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Fig. 4 — Bar graphs illustrate effects of AVP (10 μg/kg, ip) alone and in combination with LY 294 002 and wortmannin (50 μg/kg, ip) against maximal electroshock induced convulsions in mice (n=6). The data are depicted as mean±SE. *P<0.05.
We utilised mephenamic acid, an anthranilic acid analogue and GJIC inhibitor for evaluating our studies on gap junction intra cellular communication. Pretreatment of mice with gap junction inhibitor, mephenamic acid (30 mg/kg, ip) potentiated the actions of MgCl₂ on cerebral ischemia by increasing the ischemic time by almost 70% and this enhancement was significantly unaffected by LVP. The results are depicted in Fig. 3.

Table 1 illustrates the actions of NGF and Staurosporine against MgCl₂ induced cerebral ischemia. AVP potentiated the actions of NGF and the protein kinase C inhibitor, staurosporine against MgCl₂ induced cerebral ischemia (P<0.05, <0.01).

A separate group of mice were pretreated with vasopressin and PI-3 kinase inhibitor to evaluate the effect of MES induced convulsions. AVP produced a 30.9% decrease (P<0.05) in the convulsive time, while the PI-3 kinase inhibitors, Wortmannin and LY 294 002 potentiated the MES induced convulsive time (P<0.05) by 32% and 35% respectively. The results of these experiments are depicted in Fig. 4.

Discussion

The results of our study demonstrate that vasopressin analogues, AVP and LVP exhibit neuroprotective action against MgCl₂ induced cerebral ischemia and maximal electroshock induced convulsions in the mice. These results are shown in Fig.1. Vasopressin acts through the stimulation of specific V₁ and V₂ vasopressin receptors expressed in the discrete sites of the brain and the peripheral tissues and blood vessels. Vasopressin contributes to blood pressure regulation in haemorrhagic conditions and might also contribute significantly in cerebral ischemic condition. Intravenous injection of MgCl₂ causes a state of reversible transient global ischemia characterized by gasping, sedation and loss of righting reflex. The animals recover from this state after few minutes. MgCl₂ can induce cerebral ischemia by acting on NMDA receptors, affecting Ca²⁺ entry or affecting activity of enzymes e.g. PLA₂ / PI-3 Kinase/PKC. There is also possibly an increase in lipid peroxidation, which again is a consequence of stimulation by phospholipase enzymes like the PLA₂ (s). Thus it is a safe and effective method of inducing ischemia.

On the other hand, pretreatment with vasopressin can prevent this ischemic episode by inducing vasoconstriction and increasing the blood pressure via the stimulation of V₁ receptors in the peripheral vascular beds and increasing blood supply to the CNS. These actions are dependent on the Ca²⁺ entry into the vascular cells. Vasopressin also induces endothelium dependent vasodilatation in cerebral blood vessels by increasing the release of nitric oxide and cGMP levels. Vasopressin receptors are expressed in the brain stem region and particularly regulate the responses of the Rostral ventro lateral medulla (RVLM). Stimulation of RVLM also protects the brain against ischemic insult by excitation of sympathetic system and elevating arterial pressure and inducing expiratory responses. Another important area in the brain stem termed the medullary cerebral vasodilator area or MCVA might also interact with RVLM for inducing a neuroprotective response by vasopressin. Both the vasopressin analogues AVP and LVP exhibit this neuroprotective action against the MgCl₂ induced cerebral ischemia. The results are depicted in Fig. 3.

Phospholipid levels of phosphatidylinositol biphosphate and monophosphate (PIP₂) and (PIP) are reduced during 2-3 min of cerebral ischemia. Therefore our next objective was to elucidate the role of PI-3 kinase enzymes because some recent studies suggest that these enzymes might contribute to seizure activity and also affect the tone of cerebral blood vessels. Vasopressin has been shown to stimulate the PI-3 kinase enzyme activity in several cell types. We utilised two potent inhibitors of the PI-3 kinase enzyme, Wortmannin & LY 294002 and pretreated the mice with these inhibitors along with the vasopressin analogues. Both these inhibitors are effective in studying the physiological role of the PI-3 kinase in vivo models. The PI-3 kinase enzyme inhibitors blocked the protective action of arginine vasopressin. PI-3 kinase enzymes are key signalling molecules in the neuronal cell survival pathway and are also involved in sympathetic neuronal activation. PI-3 kinases are also involved in the regulation of retrograde axonal transport of nerve growth factor (NGF). Neurotrophic factors like Brain derived neurotrophic factor (BDNF) also utilise PI-3 kinase enzyme pathway. Thus it is suggested that these enzymes contribute effectively in maintaining the cerebral blood vessel tone during a state of cerebral ischemia. The results are shown in Fig. 4.

In order to characterize the vasopressin receptor subtype involved in the neuroprotective action of vasopressin, we utilised a nonselective V₁ receptor antagonist, des Gly NH₂ d (CH₂)₉[D- Tyr², Thr⁵] OVT. Pretreatment of mice with this V₁ receptor antagonist blocked the protective action of vasopressin
and caused an increase in ischemic time. There was significant enhancement in the time of the loss of righting reflex. This was indicative of the role for the $V_1$ vasopressin receptors. The results of this experiment are shown in Fig. 1.

Gap junctional communication can influence organism development, neuronal growth and homeostatic control by sharing ions, second messengers and other signalling molecules. Heterotrimeric gap junction proteins are expressed in many types of vascular beds, we intended to investigate the role of intracellular gap junctional communication in modulating MgCl$_2$ induced cerebral ischemia and for these experiments, we utilised mephenamic acid (30mg/kg, i.p) belonging to the fenamate class of non steroidal anti-inflammatory (NSAID) drugs.

Experimental evidence suggests that fenamates are important class of drugs which are effective blockers of the Gap junction intracellular communication and they may block anionic as well as cationic channels. They also interact with connexins and indirectly perturb the bulk membrane fluidity or the membrane protein interface that would affect the conformation of the connexins. The animals were pretreated with mephenamic acid 15 minutes before the injection of MgCl$_2$. GJIC blocker mephenamic acid caused an increase in the duration of ischemic time and potentiated MgCl$_2$ induced cerebral ischemia. This study illustrates that Gap junctions are critical for the ischemia inducing action of MgCl$_2$ and blockade of GJIC by mephenamic acid causes potentiation and enhancement of the cerebral ischemia. This is a novel finding suggesting the interaction of magnesium ion and gap junctional activity. This enhancement by mephenamic acid of the MgCl$_2$ induced cerebral ischemia was not significantly affected by pretreatment with LVP.

NGF was the first discovered and is the best known member of the neurotrophic factor family. NGF is produced in target tissues of the peripheral sympathetic and sensory nervous systems and binds to the tyrosine kinase receptor to elicit a cascade of intracellular events involved in neuronal differentiation, maturation and survival. Expression of NGF correlates with the density of sympathetic innervation in effector organs and the amount of NGF can affect the sympathetic nerve survival and synaptic transmission between neurons and cardiac myocytes. In this study NGF per se was unable to cause any significant change in the ischemia time, but pretreatment with AVP and LVP potentiated the effect of NGF, suggesting an interaction of the vasopressin receptors with nerve growth factors. The results are depicted in Table 1.

The concept that protein kinase C (PKC) plays a pivotal role in tonic contraction of smooth muscle is relatively recent. PKC is thought to phosphorylate smooth muscle myosin and myosin light chain kinase in vitro and increase the Ca$^{2+}$ sensitivity of contractile apparatus and influence sustained phase of agonist induced contraction. Earlier studies have shown the importance of PKC activation in cerebral arterial contractions. PKC activity is decreased after global ischemia and have importance in cell necrosis. Stauroporine has been shown to inhibit PKC activity and block the long opening of L-type Ca$^{2+}$ channels. Treatment with a PKC inhibitor stauroporine before an ischemic insult has protective effect. In this study, the non selective PKC inhibitor, stauroporine when injected (20 µg/kg, ip) in the mice potentiated the neuroprotective effect of AVP ($P<0.05$). These results are depicted in Table 1.

To examine the effect of vasopressin on the maximal electroshock induced convulsions we utilised the swiss albino mice. The animals were treated with AVP and subjected to MES induced convulsions. AVP decreased the convulsive time significantly. Pretreatment with PI-3 kinase inhibitors produced an enhancement of the MES induced convulsive time (Fig. 4). These results demonstrate that AVP is protective against MES induced cerebral convulsions. AVP has been shown to stimulate PI-3 kinase activity in various cell types. An important role for PI-3 kinase in neuronal excitability is well established. A maximal electroshock stimuli results in massive depolarization and PI-3 kinase inhibition possibly enhances the post tetanic potential. Our experiments using these inhibitors of the PI-3 kinase suggest the possible role of PI-3 kinase in modifying the AVP response to MES convulsions. AVP reduces the intensity of MES convulsive disorder probably by modulating the central opioidergic and alpha 2 adrenergic system.

In conclusion, this study demonstrates that vasopressin analogues stimulate the vasopressin $V_1$ receptors and mediate neuroprotection against global cerebral ischemia. In this process the signalling cascades involving the PI-3 kinase (s), and protein kinase C may significantly alter the effects of vasopressin. This study suggests that Gap junctions have a potential role in the induction of cerebral ischemia.
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