Combination Therapy of Rosiglitazone, a Peroxisome Proliferator-ACTivated Receptor-γ Ligand, and NMDA Receptor Antagonist (MK-801) on Experimental Embolic Stroke in Rats

Mohammad Allahtavakoli¹, Alireza Shabanzadeh², Ali Roohbakhsh¹ and Aliasghar Pourshanazari¹

¹Department of Physiology and Pharmacology, School of Medicine, Rafsanjan University of Medical Sciences, Rafsanjan, Iran, and
²Department of Physiology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

(Received April 5, 2007; Accepted May 29, 2007)

Abstract: Peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists have been found to have potent anti-inflammatory actions and suggested as potential therapies for brain ischaemia. Glutamate is the most common excitatory neurotransmitter in the central nervous system and is released excessively during ischaemia. Stroke therapy will require combinations of drug classes, because no single drug class has yet been proven efficacious in human beings. The present study was conducted to assess whether N-methyl-d-aspartate (NMDA) receptor antagonist (MK-801) treatment can improve recovery from ischaemic brain injury and whether rosiglitazone, a PPAR-γ ligand, can increase its neuroprotective effect in an embolic model of stroke. Stroke was induced in rats by embolizing a preformed clot into the middle cerebral artery. Rosiglitazone (0.1 mg/kg, intraperitoneally) and MK-801 (0.1 mg/kg, intravenously) were injected immediately after embolization. Forty-eight hours later, the brains were removed, sectioned and stained with triphenyltetrazolum chloride and analysed by a commercial image processing software programme. Rosiglitazone and MK-801 alone or in combination decreased infarct volume by 49.16%, 50.26% and 81.32%, respectively (P < 0.001). Moreover, the combination therapy significantly decreased the infarct volume when compared to any drug used alone (P < 0.05). MK-801 reduced the brain oedema by 68% compared to the control group (P < 0.05), but rosiglitazone or combination did not show any significant effect. The drugs alone or in combination also demonstrated improved neurological function, but combination therapy was more effective on neurological deficits improving. Our data show that the combination of MK-801 and rosiglitazone is more neuroprotective in thromboembolic stroke than given alone; this effect perhaps represents a possible additive effect in the brain infarction.

According to the report of the World Health Organization, stroke is the secondary leading cause of death and the first cause of major adult disability in the world [1,2]. Among the stroke patients, 85–90% of the cases are ischaemic stroke with a predominant (75–80%) cause of cerebral arterial thrombosis, and majorities of ischaemic episodes occur as a result of occlusion of the middle cerebral artery or its branches [3,4]. Elevated extracellular glutamate after cerebral ischaemia is thought to play a key role in the development of cerebral infarction via N-methyl-d-aspartate (NMDA) receptors. Under pathological conditions, overactivation of these receptors cause an excessive amount of Ca2+ influx into the nerve cells, which then triggers a variety of processes that can lead to necrosis or apoptosis [4]. In line with this, it has been shown that NMDA receptor antagonists reduce damage in animal models of stroke [5,6].

Thiazolidinediones are agonists of the nuclear receptor and transcription factor peroxisome proliferator-activated receptor-γ (PPAR-γ) that act to enhance insulin sensitivity and reduce serum glucose in diabetic patients without significant alterations in serum glucose of non-diabetic animals or human beings [7,8]. Rosiglitazone and pioglitazone, two thiazolidinedione drugs have been used in clinical practice extensively without signs of significant toxicity [9]. There is good evidence from animal models supporting the potential utility of PPAR-γ agonists in the treatment of myocardial infarction [10]. Thiazolidinediones drugs may have protective effect against ischaemic injury through inhibition of inflammation [11,12]. However, the exact mechanism of PPAR-γ ligands in stroke is less clear [11].

Unfortunately, those high doses of NMDA antagonists that have shown neuroprotective effects in animal models of stroke failed to enter clinical practice due to their severe side effects. It has been reported that the dose of 0.1 mg/kg of MK-801 (NMDA receptor antagonist) is the maximal level that can be applied in vivo without side effects. However, higher doses of MK-801 are more neuroprotective but they may cause excessive sedation and mortality due to respiratory suppression [6]. Because no single drug class has yet been proven efficacious in human beings, effective stroke therapy will require combinations of different drug classes [13]. Previous reports revealed that NMDA receptor antagonists and thiazolidinediones exert their neuroprotective effects by complementary and overlapping mechanisms [2,5,6,11].

The embolic model has been used previously in different experiments to induce experimental stroke in rodents [14,15]. This model mimics human stroke and is more relevant to
Materials and Methods

Animals. Animals were handled in accordance with criteria outlined in the Guide for Care and Use of Laboratory Animals (US NIH publication, revised 1996; http://books.nap.edu/read/readingroom/books/labrats/). Adult male Wistar rats (250–320 g) were maintained on a 12-hr light:dark cycle with food and water available ad libitum. Eight animals were used in each group.

Clot formation. One day before ischaemic onset, arterial blood was withdrawn from donor rats into PE-50 tubes, stored at room temperature for 2 hr, and then kept at 4°C for 22 hr [16].

Physiological parameters monitoring. Rats were anaesthetized with chloral hydrate (400 mg/kg, intraperitoneally). In separate experiments (n = 5 for each group), arterial blood was obtained via the catheter placed in the femoral artery, for continuous blood pressure monitoring. A blood gas, oximetry, electrolyte and metabolic system machine (Radiometer Medical A/S, Copenhagen, Denmark) was used to monitor arterial PaO₂, PaCO₂, pH and glucose. Blood was analysed only 5 min. before and after embolization. Body temperature was maintained at 37 ± 0.5°C during the surgery and the immediate post-operative period until the animal fully recovered from anaesthesia.

Focal embolic cerebral ischaemia. Once surgical level of anaesthesia was attained (assessed by absence of hind leg withdrawal to pinch), the focal cerebral ischaemia was induced by embolizing a preformed clot into the middle cerebral artery as reported previously [18]. Briefly, a longitudinal incision of 1.5 cm in length was made in the midline of the ventral cervical skin. The right common carotid artery, right internal carotid artery and right external carotid artery were exposed. The distal portion of the external carotid artery was ligated and cut. Coagulated blood was subsequently sectioned into 20 mm segments, washed with saline and transferred to a modified PE-50 catheter with a tip diameter of 0.3 mm for injection. The modified PE-50 tube with the 20 mm clot (5 μl) was connected to a 50-μl Hamilton lock syringe, and advanced 17 mm in the internal carotid artery until its tip was 1–2 mm away from the origin of the middle cerebral artery. The preformed clot was then injected, and the catheter was removed. Relative regional cerebral blood flow was monitored with laser Doppler monitor (Moor Instruments Ltd., Devon, UK) for a minimum of 10 min. prior to embolization, continuously throughout the surgery and for a minimum of 5 min. after clot injection. A minimum initial reduction of 70% in the laser Doppler reading was considered a successful occlusion of middle cerebral artery perfusion territory [17]. Animals that did not show 70% reduction in the laser Doppler reading, were deemed to have had failed successful clot placement and were excluded from any further study. For sham-operated animals, 5 μl saline was injected. The wound was closed and after the recovery the animal returned to its cage. The duration of surgery did not exceed 30 min. in any case.

Quantification of brain infarct volume and oedema. The quantification of infarct volume has been previously described in detail [18]. Briefly, 48 hr after middle cerebral artery occlusion, animals were killed under deep anaesthesia induced by sodium pentobarbital.
Statistical analysis. Infarct volume and brain oedema are presented as mean ± S.E.M. and analysed with one-way ANOVA and t-test followed by Tukey’s test. Neurological deficits and sedation assessments are reported as medians and interquartile ranges (25th and 75th percentiles). Neurological and sedation assessment scores were analysed with Kruskal–Wallis test. The rates of seizure occurrence and mortality after different treatments were compared using chi-squared test. Differences were considered significant when P < 0.05.

Results

We did not observe any infarction, brain oedema, neurological deficits and sedation scores in sham-operated animals.

Evaluation of physiological parameters during surgery.

Physiological parameters are shown in table 1. There were no differences between groups in blood gases, pH, heart rate, blood glucose and arterial blood pressure.

Effects of DMSO on infarct volume and brain oedema.

The results did not show any significant difference between two groups. These data are presented in fig. 2.

Evaluation of behaviour.

Changes of neurological deficits scores in different groups are shown in table 2. Compared to the control group, combination therapy improved neurological deficits at both 24 and 48 hr after embolic stroke (P < 0.05). Furthermore, compared to the MK-801 group, combination therapy at 48 hr after stroke improved neurological deficits (P < 0.05). Administration of rosiglitazone or MK-801 alone also decreased neurological deficits only at 48 hr after embolization (P < 0.05). Changes of sedation assessment scores in different groups are shown in table 3. Compared to the control group, rosiglitazone, MK-801 or combination of rosiglitazone and MK-801 improved sedation assessment at 24 and 48 hr after embolization (P < 0.05).

Twenty-four hours after embolization, seizure activity was seen in one animal of the control group. Seizure occurred in one rat in each group that treated with rosiglitazone, MK-801 or vehicle 48 hr after stroke. None of the combination therapy animals showed seizure activity at 24 or 48 hr after stroke. Compared to the control group, the rates of seizure occurrence were not significant at either 24 or 48 hr after embolization between the control group and any drug-treated group.

Table 1.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MK-801</th>
<th>Rosiglitazone</th>
<th>Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dl)</td>
<td>220 ± 14</td>
<td>206 ± 24</td>
<td>214 ± 19</td>
<td>217 ± 20</td>
</tr>
<tr>
<td>pH</td>
<td>7.377 ± 0.017</td>
<td>7.374 ± 0.003</td>
<td>7.412 ± 0.026</td>
<td>7.380 ± 0.009</td>
</tr>
<tr>
<td>P&lt;sub&gt;O2&lt;/sub&gt; (mmHg)</td>
<td>174 ± 8</td>
<td>173 ± 6</td>
<td>174 ± 7</td>
<td>168 ± 9</td>
</tr>
<tr>
<td>P&lt;sub&gt;CO2&lt;/sub&gt; (mmHg)</td>
<td>35.5 ± 1.4</td>
<td>33.5 ± 1.6</td>
<td>33.8 ± 1.5</td>
<td>34.1 ± 1.8</td>
</tr>
<tr>
<td>*MAP (mmHg)</td>
<td>91 ± 4</td>
<td>90 ± 4</td>
<td>93 ± 3</td>
<td>92 ± 3</td>
</tr>
<tr>
<td>Heart rate (beats/min.)</td>
<td>362 ± 34</td>
<td>382 ± 30</td>
<td>380 ± 34</td>
<td>354 ± 28</td>
</tr>
</tbody>
</table>

Data are presented as mean ± S.E.M. n = 5 in each group. Differences between the groups were not significant. *MAP, mean arterial pressure.

Effects of MK-801 and rosiglitazone alone or in combination on infarct volume and brain oedema.

Embolization of a preformed clot resulted in an infarction in the ipsilateral hemisphere, mainly located in the middle cerebral artery-irrigated region.

Effects of rosiglitazone and MK-801 alone or in combination on infarct volumes are shown in fig. 3. Compared to the control group, rosiglitazone and MK-801 alone or in combination decreased the infarct volume by 49.16%, 50.26% and 81.32%, respectively (P < 0.001). Moreover, combination therapy significantly decreased the infarct volume when compared to MK-801 or rosiglitazone-treated groups (P < 0.05).

Brain oedemas are shown in fig. 4. The brain oedema, 48 hr after the embolic injury for the control group, was 8.98 ± 2.15%. Administration of MK-801 significantly reduced the brain swelling by 68% compared to the control animals (P < 0.05), but rosiglitazone or combination did not show any significant effect.

Graphical representation:

Fig. 2. Infarct volumes and brain oedemas were determined at 48 hr after embolic stroke and expressed as mean ± S.E.M. No statistically significant differences between the two groups were observed. Dimethyl sulfoxide (DMSO) and saline-treated animals received 10% DMSO (0.1 ml DMSO in 0.9 ml saline/kg) or saline alone (1 ml/kg) after embolization, respectively (n = 8).
Mortality rates.

All rats survived up to 24 hr after stroke, except one rat of the control group that died at 18 hr. Within 24 to 48 hr after ischaemia, two rats died in each group that received vehicle, rosiglitazone or MK-801. All animals in combination therapy survived until the end of the experiments. Compared to the control group, the rates of occurrence of death were not significant at either 24 or 48 hr after stroke among groups.

Discussion

In the present study, we examined the neuroprotective effect of an NMDA receptor antagonist, MK-801, on ischaemic brain injury using an experimental embolic stroke model. Because a single drug that alone acts against the complex series of pathological events after stroke may not exist, the

---

**Table 2.** Neurological deficits scores in different groups.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Rosiglitazone</th>
<th>MK-801</th>
<th>Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 hr</td>
<td>3.5 (2.25–4) n = 8</td>
<td>3 (2.25–3.75) n = 8</td>
<td>3.5 (2.25–4) n = 8</td>
<td>3 (2–3.5) n = 8</td>
</tr>
<tr>
<td>24 hr</td>
<td>4 (4–4) n = 7</td>
<td>2 (1–4) n = 8</td>
<td>2.5 (2–3) n = 8</td>
<td>1.5 (1–2.5)* n = 8</td>
</tr>
<tr>
<td>48 hr</td>
<td>4 (3–4) n = 5</td>
<td>1.5 (1–2.25)* n = 6</td>
<td>2 (1.75–3)* n = 6</td>
<td>1 (1–1)*† n = 8</td>
</tr>
</tbody>
</table>

Data are expressed as medians and interquartile ranges; 25th and 75th percentiles are shown in parentheses. The drugs studied were dimethyl sulfoxide (0.1 ml/kg, control), rosiglitazone (0.1 mg/kg), MK-801 (0.1 mg/kg) and the combination of rosiglitazone (0.1 mg/kg, intraperitoneally) plus MK-801 (0.1 mg/kg, intravenously). *P < 0.05 compared to the control group and †P < 0.05 compared to the MK-801-treated group.

**Table 3.** Sedation assessment scores in different groups.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Rosiglitazone</th>
<th>MK-801</th>
<th>Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 hr</td>
<td>4 (3.25–4.75) n = 8</td>
<td>3 (2.25–4) n = 8</td>
<td>3.5 (3–5) n = 8</td>
<td>3 (3–3.5) n = 8</td>
</tr>
<tr>
<td>24 hr</td>
<td>4 (4–5) n = 7</td>
<td>2.5 (2–4)* n = 8</td>
<td>3 (2.25–3)* n = 8</td>
<td>2 (1.25–3)* n = 8</td>
</tr>
<tr>
<td>48 hr</td>
<td>4 (3–5) n = 5</td>
<td>2 (1–3.25)* n = 6</td>
<td>2 (2–3)* n = 6</td>
<td>1.5 (1.25–2.75)* n = 8</td>
</tr>
</tbody>
</table>

Data are expressed as medians and interquartile ranges; 25th and 75th percentiles are shown in parentheses. The drugs studied were dimethyl sulfoxide (0.1 ml/kg, control), rosiglitazone (0.1 mg/kg), MK-801 (0.1 mg/kg) and the combination of rosiglitazone (0.1 mg/kg, intraperitoneally) plus MK-801 (0.1 mg/kg, intravenously). *P < 0.05 compared to the control group.
combination of neuroprotectants with distinct mechanisms has been proposed for stroke therapy [13]. Therefore, we examined the possible effect of rosiglitazone (PPAR-γ agonist) alone or in combination with MK-801 on brain injury. These two drugs are logical to combine, because they both limit excitotoxic cell damage by complementary and overlapping mechanisms [6,11].

Our results showed that treatment with MK-801 or rosiglitazone significantly decreased infarction volume, but the combination of these two drug classes had more therapeutic efficacy compared to each of them alone. The drugs alone or combination-treated rats also improved their neurological function at 48 hr after stroke and this included both neurological deficits scores (motor function) and sedation assessment scores (level of arousal). Combination therapy was also more effective in neurological deficits compared to MK-801 alone at 48 hr.

These data show that MK-801 could significantly decrease infarct volume. In agreement with our results, Suzuki et al. [6] reported that intravenous administration of MK-801 at the dose of 0.1 mg/kg immediately after middle cerebral artery occlusion showed neuroprotective effects without side effects. Moreover, Gerriets et al. [5] also found that MK-801 was neuroprotective after macrosphere model of ischaemic brain injury in rats. Behavioural tests showed that MK-801 could improve both neurological deficits scores (motor function) and sedation assessment scores (level of arousal). This clinically relevant end-point is of critical importance. Functional outcome does not always correlate with infarct size, but is the measure used in clinical trials of neuroprotective agents [24]. It has been reported that MK-801 prevents binding of glutamate at the NMDA subtype ionophore and limits opening of voltage-gated calcium channels [25,26]. This drug reverses the cascade of steps leading to neuronal cell death include: activation of the p38 MAPK–MEF2C (transcription factor) pathway, toxic effects of free radicals such as nitric oxide and reactive oxygen species, and activation of apoptosis-inducing enzymes including caspases [4].

The present study shows that administration of rosiglitazone also decreased infarct volume and improved neurological functions. These results are in agreement with the recently reported effects of thiazolidinediones following transient [11], permanent [2] and embolic [23] stroke models. In these studies, thiazolidinediones reduced infarction volume and improved neurologic function when rats were treated prior to middle cerebral artery occlusion. Regarding the effects of thiazolidinediones on risk factors of stroke a clinical trial has been founded by the National Institute of Neurologic Disorders and Stroke to test the effectiveness of thiazolidinediones as preventative agents for stroke [11]. In a recent clinical study, it has been reported that use of rosiglitazone was associated with enhanced functional recovery for patients with stroke and type 2 diabetes [27].

Reduction in infarct size is associated with reduced expression of inflammatory mediators such as cyclooxygenase-2 and ICAM (intercellular cell adhesion molecule) suggesting that suppression of inflammatory gene expression may be one of the mechanisms of neuroprotection by thiazolidinediones [28]. Interestingly, PPAR-γ mRNA is up-regulated in ischaemic brains of rats showing the importance of this target in stroke treatment [29]. The neuroprotective effects of thiazolidinediones may be mediated through antioxidant effects [30] and the inhibitory activity of these drugs on pro-inflammatory enzymes such as inducible nitric oxide synthase and cyclooxygenase-2 [11,31]. Pioglitazone and rosiglitazone are in clinical use for type 2 diabetes [29]. These drugs enhance insulin-mediated glucose uptake into skeletal muscle, thereby reversing the primary deficit in insulin resistance and decreasing serum glucose levels in type 2 diabetic patients without alterations in serum glucose of non-diabetic humans or animals [11]. The dose of rosiglitazone used in this study is almost similar to that used clinically without evidence of significant toxicity [9].

The new finding of this study is that the combination of rosiglitazone and MK-801 has more therapeutic efficacy compared to each of them alone. The combination-treated rats also show better neurological function at 24 and 48 hr after stroke, and this included both neurological deficits scores and sedation assessment scores. Combination therapy was also more effective in neurological deficits compared to MK-801 alone at 48 hr. In agreement with these results, it has been reported that troglitazone could attenuate glutamate neurotoxicity by interfering with some event downstream of NMDA receptor overactivation [32]. Similar observations have been made using MK-801 and α-phenyl-tert-butyl nitrone, a free radical scavenging agent [33] or muscimol, a GABA₄ agonist [26]. Regarding these findings, one can conclude that thiazolidinediones may augment the therapeutic effects of MK-801. By finding the drugs that have additive or synergistic effects with NMDA receptor antagonists, it is probably possible to decrease the dose and hence the toxic effects of NMDA receptor antagonists while the beneficial effects remain or even increase.

The dose of MK-801 in this study is the maximal level that can be applied in vivo without side effects, although higher doses of MK-801 are more neuroprotective but may cause excessive sedation and mortality due to respiratory suppression [6]. At these dosages, MK-801 often induces stereotypic behaviours (head weaving and circling, jerky movements) and loss of balance and ataxia [24,33] that we did not observe in our experiments. One interesting feature of our data was inability of rosiglitazone and combination therapy to decrease brain oedema. While we initially found this surprising, further investigation revealed that thiazolidinediones have significant high oedema rates, and fluid retention remains a major safety obstacle to be overcome by discovering novel PPAR-γ activators [34].

In conclusion, our data show for the first time that the combination of MK-801 and rosiglitazone is more neuroprotective in embolic stroke than given alone at maximally tolerated dose of MK-801. These results perhaps represent a possible additive effect between thiazolidinediones and NMDA antagonists in brain infarction.
References


© 2007 The Authors