Systemic Hyperthermia Masks the Neuroprotective Effects of MK-801, but not Rosiglitazone in Brain Ischaemia

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(Received 18 August 2009; Accepted 26 January 2010)

Abstract: The use of neuroprotective agents has been under investigation for the treatment of ischaemic brain stroke. In this study, we examined the effects of rosiglitazone and MK-801, two potential neuroprotectants, on thromboembolic focal stroke in hyperthermic rats. The animals were assigned into groups of rosiglitazone, MK-801 and control, all under both normothermic and hyperthermic conditions. A focal ischaemia was induced by injection of preformed clot into the origin of the middle cerebral artery. The animals were assessed by measuring infarct size and brain oedema and also evaluating neurological deficit and seizure activity. Rosiglitazone improved infarct volume and neurological deficit in both normothermic (36%) and hyperthermic (63%) animals; but MK-801 only improved normothermic animals. Our results do not support the use of MK-801 in hyperthermic conditions of brain stroke but suggest that rosiglitazone may preserve its efficiency even in hyperthermia.

Brain stroke is still a leading cause of death and disability in the USA and other countries. Among various strategies for treatment of stroke, the use of neuroprotective drugs is being investigated at laboratory level to identify the most potent agents for clinical application [1]. By definition, any drug that is able to interfere with the cascade of events initiated by ischaemia is considered neuroprotectant [2]. Intravenous infusion of tissue plasminogen activator (tPA) is the only therapy for acute stroke proven to be effective in clinical trials [3].

Clinical studies have indicated that as large as 50% of stroke patients develop an elevated systemic temperature acutely after stroke insult [4]. Hyperthermia itself has damaging effects especially on neural tissue [5], and when it is accompanied by stroke the degree of insult is significantly intensified compared with normothermic conditions studied in a rat focal ischaemia model [6]. A hyperthermic condition even nullifies effects of some neuroprotective drugs and also masks the effects of tPA – the most relied drug in thrombotic stroke therapy [6–8]. The case for simvastatin, a drug of the statins family, is different as results suggest from our recent study (3-hydroxy-3 methylglutaryl coenzyme-A reductase inhibitors) neuroprotective effects especially on neural tissue [5], and when it is accompanied by stroke the degree of insult is significantly intensified compared with normothermic conditions studied in a rat focal ischaemia model [6]. A hyperthermic condition even nullifies effects of some neuroprotective drugs and also masks the effects of tPA – the most relied drug in thrombotic stroke therapy [6–8].

Thiazolidinediones are agonists of peroxisome proliferators-activated receptor-γ (PPAR-γ), which is a nuclear receptor and transcription factor. Activation of this receptor eventually leads to enhanced insulin sensitivity and reduced serum glucose in diabetic patients [10,11]. Rosiglitazone and pioglitazone are two kinds of thiazolidinediones which have been used extensively in clinics for diabetic patients without signs of significant toxicity [12]. Interestingly, evidence from animal models supports the efficacy of PPAR-γ of myocardial infarction [13], expanding its range of application for which anti-inflammation has been suggested to be the underlying mechanism [14,15]. Our experiments revealed its potential for reduction of infarct volume and neurological deficit in brain focal ischaemia in rats [16,17]. Following ischaemia, activation of glutamate receptors upon excessive release of the neurotransmitter pilots a major cascade of deleterious events collectively leading to neuronal death [18]. Targeting of glutamate receptors have thus been the focus of many studies [1,18]. The non-competitive antagonist of NMDA, MK-801 (dizocilpine) has shown promising results in focal ischaemic models in rats [1,16]. MK-801 in high doses has better effects, but the main drawback is sedative effects and increased mortality because of respiratory suppression. The least toxic dose of MK-801 (0.1 mg/kg) still preserves the neuroprotective effects of the drug [16].

We have previously shown that rosiglitazone and MK-801, alone or in combination, have revealed promising results in a model of thromboembolic focal ischaemia [16,17]. It is, however, unclear whether these drugs retain their efficacy in a hyperthermic brain. Using a model of focal ischaemia and by externally inducing hyperthermia, we investigated the effects of rosiglitazone and MK-801 in normo- and hyperthermic conditions in rat by means of morphometric and behavioural assessments.

Materials and Methods

A total number of 48 male Wistar rats weighing 250–300 g were used in this study (n = 8 per group). Experimental groups were: (1) normo-
mothermic-control, (2) hyperthermic-control, (3) normothermic-rosiglitazone, (4) hyperthermic rosiglitazone, (5) normothermic MK-801 and (6) hyperthermic MK-801. Care and surgical operations were under approval of the ethics committee of Tehran University of Medical Sciences.

Induction of cerebral focal ischaemia. We used a modified model which induces brain stroke by injection of a preformed clot into the origin of the middle cerebral artery [19]. Animals were anaesthetized by injection of a mixture of ketamine (40 mg/kg, i.p.) and chlorpromazine (5 mg/kg, i.p.). During surgery, a constant flow of oxygen was supplied through a face mask. Right common carotid artery and external carotid artery were exposed through first a midline incision on neck, followed by retraction of fat and muscle layers, and finally detachment of fascia and the vagus nerve. Injectable lidocaine was administered locally to minimize vagus nerve stimulation. The distal portion of the external carotid artery was ligated and cut, and then a perforation was made on it. For clot formation, blood was withdrawn into PE-50 tubing and left still in room temperature for 1–2 hr. Five-microlitre clot segments were then taken into PE-50 connected to a PE-10 tube, which was advanced 15–17 mm through the external carotid artery puncture into the internal carotid artery. In this position, the PE-10 approaches the origin of the middle cerebral artery. Clot was gently injected and the catheter was removed after 5 min. After ligation of the external carotid artery below the perforation, the wound was sutured and the animal was brought back to its cage.

Induction of hyperthermia. Hyperthermia was induced by means of a DIY-heating pad (Harvard Apparatus, Saint-Laurent, Canada) placed beneath the animal. For animals in hyperthermic groups, a rectal probe was inserted to monitor and regulate the systemic temperature which was maintained at 39.5 ± 0.2°C under deep anaesthesia. Brains were carefully extracted from the skull and loaded on a brain-sectioning chamber with 2 mm aperture coronal slits which was then cooled in ice-cold saline solution (1:10 v/v, 1 ml/kg), which is the vehicle of rosiglitazone and MK-801 (Alexis Biochemicals, Lausanne, Switzerland). Rosiglitazone (1 mg/kg, i.p.) and MK-801 (0.1 mg/kg, i.v.) were dissolved in vehicle and injected immediately after embolization (1 ml/kg).

Quantification of brain infarct volume and oedema. Quantification of infarct volume has been previously described [20]. Twenty-four hours after middle cerebral artery occlusion, animals were decapitated under deep anaesthesia. Brains were carefully extracted from the skull and loaded on a brain-sectioning chamber with 2 mm apart coronal slits which was then cooled in –70°C for 15 min. Using razor blade, seven ~2-mm-thick slices were obtained from each brain. The slices were then drenched with 2% triphenyl-tetrazolium chloride solution for 30 min. at 37°C. The infarct volume and brain oedema were determined manually. Per cent of infarct volume and brain oedema was calculated by the following formulas: infarct volume = ([volume of left hemisphere – (volume of right hemisphere – measured infarct volume)/volume of left hemisphere] × volume of left hemisphere)/volume of left hemisphere). Neurological deficit and seizure activity were assessed as median and interquartiles. While in both normo- and hyperthermic rosiglitazone-treated rats, neurological deficit was improved after 24 hr, MK-801 did not improve at all. For seizure activity, only normothermic-rosiglitazone-treated rats showed improvement and MK-801 again showed no improvement.

Behavioural assessments. Neurological deficit and seizure activity were assessed at 4, 8 and 24 hr. Neurological deficits were determined with the modification of the scoring system of Bederson et al. [21] as follows: 0, no observable deficit; 1, forelimb flexion; 2, forelimb flexion plus decreased resistance to lateral push; 3, unidirectional circling and 4, unidirectional circling plus decreased level of consciousness. Seizure activity was scored using Racine’s scale: 0, no seizure was observed; 1, rhythmic mouth and facial movement; 2, rhythmic head nodding; 3, forelimb clonus; 4, rearing and bilateral forelimb clonus; 5, rearing and falling [22].

Statistical analyses. Infarct volume and brain oedema are presented as mean ± S.E.M. and analysed with one-way ANOVA followed by Tukey’s test. Neurological deficit and seizure activity were analysed with the Kruskal–Wallis test and are reported as medians and interquartile ranges (25th and 75th percentiles). Differences were considered significant when p < 0.05.

Results
In total, six animals died either during or after surgery, and in five other rats embolization was defective as indicated by having no infarct area in the triphenyl-tetrazolium chloride stain. These animals were replaced in flawed groups. For normothermic animals, rectal temperature was generally below 37°C which is expected because of use of anaesthetics. In the hyperthermic rats, there was a variation of 0.2°C about the set point.

Infarct volume and brain oedema.
Embolization of clot into the middle cerebral artery resulted in the occlusion of this artery, which is evident as white areas in triphenyl-tetrazolium chloride staining. Animals in which no infarct area was observed were considered as having improper embolization and therefore removed from the study. Hyperthermia resulted in an almost double increase of infarct volume compared with the normothermic-control group (fig. 1). In animals treated with rosiglitazone, there was a decrease of 36% and 63%, respectively, in infarct volume in both normo- and hyperthermic conditions compared with their control counterparts. The swelling of right hemisphere was reduced by 46% only in the normothermic-rosiglitazone-treated group compared with the normothermic-control group (fig. 2). MK-801 reduced infarct volume in normothermic but not in the hyperthermic animals. This drug did not decrease brain oedema in either the normo- or hyperthermic groups (fig. 3).

Neurological deficit and seizure activity.
Tables 1 and 2 show neurological deficit and seizure activity presented as median and interquartiles. While in both normo- and hyperthermic rosiglitazone-treated rats, neurological deficit was improved after 24 hr, MK-801 did not improve at all. For seizure activity, only normothermic-rosiglitazone-treated rats showed improvement and MK-801 again showed no improvement.

Discussion
This study investigated the potency of rosiglitazone and MK-801 on an experimental thromboembolic stroke in conditions where systemic temperature was elevated. Our results showed that under hyperthermic conditions, rosiglitazone is still able to reduce brain infarction and improve motor performance, but signified no improvement for either brain...
oedema or seizure activity. MK-801 on the other hand, had no beneficial effects on hyperthermic animals.

Brain is one of the most sensitive organs to hyperthermia [23]. While neurons are the most vulnerable cells in brain tissue, glia and microvessels are affected as well [5]. Hyperthermia, when associated with brain ischaemia, exacerbates conditions and, as literature has suggested, increases infarction volume both in global [24,25] and focal brain ischaemia [6,26]. To date, hyperthermia is believed to exert its deleterious effects through various mechanisms such as release of excitatory neurotransmitters [6], destruction of brain endothelial cells [5], increased levels of free oxygen radicals [27], ischaemia-induced blood brain barrier opening [9] and up-regulation of nitric oxide synthase and haem oxygenase [28].

Fig. 1. Brain slices showing infarct area in a sample from each experimental group. From left to right: Normo/Rosi, Hyper/Rosi, Normo/MK-801, Normo/Control, Hyper/Control and Hyper/MK-801. Triphenyl-tetrazolium chloride staining, original magnification.

Table 1.

Scores of neurological deficits assessed 4, 8 and 24 hr after stroke.

<table>
<thead>
<tr>
<th>Duration (hr)</th>
<th>Normo/Control</th>
<th>Hyper/Control</th>
<th>Normo/Rosi</th>
<th>Hyper/Rosi</th>
<th>Normo/MK-801</th>
<th>Hyper/MK-801</th>
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<tr>
<td>4</td>
<td>4 (3–4)</td>
<td>4 (3–4)</td>
<td>4 (3–4)</td>
<td>4 (3–4)</td>
<td>2 (2–3.75)</td>
<td>4 (3–4)</td>
</tr>
<tr>
<td>8</td>
<td>3 (3–3)</td>
<td>4 (3–4)</td>
<td>2 (2–3)¹</td>
<td>3.5 (3–4)</td>
<td>2 (0–3)</td>
<td>3 (1.75–3.25)</td>
</tr>
<tr>
<td>24</td>
<td>3 (2–3)</td>
<td>3.5 (3–4)¹</td>
<td>1 (0–1.75)²</td>
<td>2 (1–2)²</td>
<td>1 (1–2)¹</td>
<td>2 (1–3)</td>
</tr>
</tbody>
</table>

Median and interquartiles are presented in parentheses. Difference compared with ¹normothermic-control group and ²hyperthermic-control group.

Fig. 2. Infarct volume 24 hr after embolization presented as per cent ± S.E.M. *Significant difference with the hyper/control group; **significant difference with the normo/control group.
Whatever the mechanism is, hyperthermia even masks the effects of a number of neuroprotectants and also tPA, which is the leading drug in thrombotic stroke therapy [29].

There is an ongoing debate about the mechanism of action of thiazolidinediones in neuroprotection. Studies suggest the anti-inflammatory effects of this family but the exact underlying mechanism is not documented [30]. Anti-oxidant effects [31] and inhibitory activities on pro-inflammatory enzymes such as inducible nitric oxide synthase and cyclooxygenase-2 have been proposed as mechanisms of neuroprotection of these drugs [14,32]. Up-regulation of PPAR-γ mRNA in ischaemic brains of rats is seen as another source of support for the role of thiazolidinediones in neuroprotection [33]. Pioglitazone and rosiglitazone are used for type-2 diabetes in the clinic [33]. These drugs act through enhancing 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, and thus do not affect serum glucose levels of non-diabetic humans or animals [14].

A similar controversy questions the mechanism by which MK-801 exerts its effect in the reduction of neurological damage following stroke. While being an NMDA antagonist, some have argued that MK-801 brings about neuroprotection by inducing hypothermia [34,35]; but others provided data supporting that the actions of MK-801 is independent from hypothermia [36–39]. If the neuroprotection of MK-801 is either dependent or independent from inducing hypothermia, our study does not support at least a hyperthemic-independent effect of MK-801. This implies that hyperthermia masks the effects of MK-801. Indeed, it has been reported that MK-801 maintains its efficacy when the core temperature is fixed at 37°C [40]. While release of excitatory neurotransmitters is heightened in hyperthermia, our study does not support a sustained neuroprotection of MK-801 in hyperthermic rats.

A previous study showed that hyperthermia exacerbates infarct volume, brain oedema, neurological deficit and mortality rate [6]. Except mortality rate, we also attained the same results for other assessments. The reduction of infarct volume observed in the normothermic-rosiglitazone group is a further support of our previous study showing the efficacy of rosiglitazone [16]. In the present study, the same effect was observed in hyperthermic conditions suggesting the potency of the drug even in elevated brain temperature. In this case, the anti-inflammatory effects of the drug may well have fought the inflammation intensified by hyperthermia. The reverse is true for brain oedema. While in a previous study, we reported no reduction of brain oedema in rosiglitazone-treated rats [16], this time we observed an almost 50% reduction of oedema in the normothermic group but not in the hyperthermic group. The dosage of rosiglitazone (1 mg/kg) was 10 times greater than our previous study [19]; maybe this is responsible for the observed effect in the

### Table 2.

<table>
<thead>
<tr>
<th>Duration (hr)</th>
<th>Normo/Control</th>
<th>Hyper/Control</th>
<th>Normo/Rosi</th>
<th>Hyper/Rosi</th>
<th>Normo/MK-801</th>
<th>Hyper/MK-801</th>
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<tbody>
<tr>
<td>4</td>
<td>1 (1–4)</td>
<td>1 (0.5–1)</td>
<td>1 (0–1)</td>
<td>1 (0.25–1.75)</td>
<td>0.5 (0–3.25)</td>
<td>3 (2.25–3.75)</td>
</tr>
<tr>
<td>8</td>
<td>1 (1–2)</td>
<td>1 (0.5–1)</td>
<td>0 (0–1)</td>
<td>1 (0–1)</td>
<td>0.5 (0–1.75)</td>
<td>2 (1.25–3.5)</td>
</tr>
<tr>
<td>24</td>
<td>0 (0–0)</td>
<td>0 (0–0.5)</td>
<td>0 (0–1)</td>
<td>0 (0–0)</td>
<td>0.5 (0–1)</td>
<td>1.5 (0.25–2)</td>
</tr>
</tbody>
</table>

Median and interquartiles are presented in parentheses.

*Difference compared with the normothermic-control group.

Fig. 3. Brain oedema 24 hr after embolization presented as per cent ± S.E.M. *Significant difference with the Normo/Control group.
present study. Brain oedema is one of the most dangerous acute complications of brain hyperthermia [23]. Considering the exaggeration of brain oedema by hyperthermia – as we also demonstrated – and improvement in infarct volume and neurological deficit, our results imply that rosiglitazone does not exert its beneficial effects through reduction of oedema.

Behavioural assessments, on the other hand, revealed different results. Improvement of neurological deficit was not accompanied by amelioration of seizure activity in the rosiglitazone-treated hyperthermic rats. In the normothermic group, however, rosiglitazone improved both behavioural parameters.

In summary, our study demonstrated the potential of rosiglitazone for reduction of stroke complications not only in normothermic but also under hyperthermic conditions. MK-801, on the other hand, remained useful only in normothermic but also under hyperthermic conditions. Regarding clinical use of rosiglitazone in diabetic patients, and the complication of diabetes on stroke thermic conditions. Regarding clinical use of rosiglitazone in diabetic patients, and the complication of diabetes on stroke

Source of funding

Tehran University of Medical Sciences, and Organization of Forensic Medicine, Tehran, Iran.

References


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