Biochemical and Biophysical Research Communications 448 (2014) 414–417

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Metformin protects against seizures, learning and memory impairments and oxidative damage induced by pentylenetetrazole-induced kindling in mice

Ran-ran Zhao [a,b], Xiao-chen Xu [c], Fei Xu [c], Wei-li Zhang [d], Wen-lin Zhang [a], Liang-min Liu [a], Wei-ping Wang [a,*]

[a] Key Laboratory of Neurology of Hebei Province, The Second Hospital of Hebei Medical University, Shijiazhuang, Hebei 050071, PR China
[b] Department of Emergency, The First Hospital of Handan, Handan, Hebei 056002, PR China
[c] Department of Neurology, The First Hospital of Handan, Handan, Hebei 056002, PR China
[d] Department of Occupational Medicine and Enviromental Heath, Hebei Medical University School of Public Health, Shijiazhuang, Hebei 050071, PR China

ARTICLE INFO

Article history:
Received 11 April 2014
Available online 4 May 2014

Keywords:
Epilepsy
Oxidative stress
Cognition
Metformin
Morris water maze

ABSTRACT

Cognitive impairment, the most common and severe comorbidity of epilepsy, greatly diminishes the quality of life. However, current therapeutic interventions for epilepsy can also cause untoward cognitive effects. Thus, there is an urgent need for new kinds of agents targeting both seizures and cognition deficits. Oxidative stress is considered to play an important role in epileptogenesis and cognitive deficits, and antioxidants have a putative antiepileptic potential. Metformin, the most commonly prescribed anti-diabetic oral drug, has antioxidant properties. This study was designed to evaluate the ameliorative effects of metformin on seizures, cognitive impairment and brain oxidative stress markers observed in pentylenetetrazole-induced kindling animals. Male C57BL/6 mice were administered with subconvulsive dose of pentylenetetrazole (37 mg/kg, i.p.) every other day for 14 injections. Metformin was injected intraperitoneally in dose of 200 mg/kg along with alternate-day PTZ. We found that metformin suppressed the progression of kindling, ameliorated the cognitive impairment and decreased brain oxidative stress. Thus the present study concluded that metformin may be a potential agent for the treatment of epilepsy as well as a protective medicine against cognitive impairment induced by seizures.

© 2014 Elsevier Inc. All rights reserved.

1. Introduction

Epilepsy is a common neurological disorder that affects approximately 50 million people worldwide. Meanwhile, cognition impairment, which is the most common and severe comorbidity of epilepsy, greatly diminishes the quality of life [1,2]. "Indeed, many people with epilepsy, and their families, consider cognitive and behavioral consequences of seizures to be at least as troubling as the seizures themselves." Jonathan K reported [1]. Although antiepileptic drug therapy is useful for controlling seizures in many patients, current treatments have untoward impact on cognition rather than restoring it. As the goal of treating epilepsy goes beyond seizure control, there is an urgent need for new compounds with both anticonvulsant and cognition protective properties.

Oxidative stress, which has been reported as an underlying mechanism in the development and progression of epilepsy [3], may be responsible for the cognitive deficits [4]. Therefore antioxidants have been suggested as therapeutic design strategies for the treatment of epilepsy.

Metformin, a widely used medicine for the treatment of type 2 diabetes mellitus, has antioxidant properties which are not fully understood. Metformin has also been demonstrated to confer health and lifespan benefits in laboratory mice, partly by reducing oxidative stress and inflammation [5]. Previous study demonstrated that metformin could reduce reactive oxygen species (ROS) and associated DNA damage by inhibiting mitochondrial respiration [6]. Besides that, metformin also has a protective effect on the antioxidant defense system. It can upregulate uncoupled proteins 2 (UCP2) [7], glutathione and Nrf2 target gene activation [5]. What's more, metformin could enhance spatial learning and memory in C57BL/6 mice [8] and high-fat diet rats [9]. Previous studies demonstrated that metformin can rapidly cross the blood brain barrier (BBB) [10] and has neuroprotective effects [11]. Despite its potential benefits, the effect of metformin on epilepsy has not been investigated. The present study was aimed to

* Corresponding author. Fax: + 86 311 6600 2915.
E-mail address: wangweip2014@126.com (W.-p. Wang).

http://dx.doi.org/10.1016/j.bbrc.2014.04.130
0006-291X/© 2014 Elsevier Inc. All rights reserved.

evaluate if metformin can suppress the progression of pentylenetetrazole-induced kindling, ameliorate the cognitive deficits and oxidative stress induced by epileptic seizure.

2. Materials and methods

2.1. Animals

Adult male C57BL/6 mice weighing 20 ± 2 g (4–6 weeks old) were obtained from the Hebei Medical University and housed in groups of five per cage under standard laboratory conditions. They were kept at constant room temperature (25 ± 1 °C) and humidity (40–60%).The mice were kept on a 12 h light/dark cycle, with lights on at 08:00 AM and with free access to food and water. Animal experiments were performed according to the regulations of laboratory animal management promulgated by the Ministry of Science and Technology of the People's Republic of China [1988] No. 134, which coincides with internationally recognized NIH guidance.

2.2. Drugs and chemicals

All standard chemicals used in this study were of analytical grade. Metformin (MET) and pentylenetetrazole (PTZ) were purchased from Sigma (St. Louis, MO, USA). GSH detection kit and MDA detection kit were obtained from Nanjing Jiancheng Bioengineering Institute (China). Metformin and PTZ were dissolved in physiological saline freshly prior to the injections.

2.3. Induction of kindling and design of the experiment

A subconvulsive dose of PTZ (37 mg/kg, i.p.) was injected on alternating days for a total of 14 times [11]. The animals were observed for 30 min after each PTZ administration. Seizure stage was evaluated using the following scale [12]: stage 0, no response; stage 1, mouth and facial jerks; stage 2, convulsive waves axially through the body; stage 3, myoclonic jerks and rearing; stage4, clonic convulsions with the animal falling on its side; and stage 5, repeated severe tonic-clonic convulsions or lethal convulsions. The seizure severity during induction of kindling was recorded.

Animals were randomly divided into four groups with eight in each group. The control group received 0.9% saline i.p. every other day (10 ml/kg, 14 injections total). The PTZ group received saline pretreatment along with PTZ (37 mg/kg) every other day. The PTZ + MET group received MET pretreatment in dose of 200 mg/kg in addition to alternate day treatment of PTZ for 14 injections. Metformin was given 30 min before PTZ. The MET group received 200 mg/kg of metformin alone to study any effect of metformin on the cognitive functions and the biochemical parameters.

2.4. Learning and memory assessment

The Morris water maze (MWM) test was used for learning and memory behavior assessment [13]. The MWM test was done 24 h after the last administration of PTZ. Learning and memory behavior evaluations were performed in a 120-cm diameter water pool and virtually divided into four quadrants. The pool was filled with water (22 ± 1 °C) and was made opaque with wheat. A colorless escape platform (10 cm in diameter) was submerged 1 cm beneath the water surface, located in a designated target quadrant. The maze was located in a quiet test room, surrounded by many visual cues outsides of the maze which were visible from within the pool and could be used by the mice for spatial orientation.

Each test consisted two parts, learning trials (existed platform) and probe trials (non-existed platform). The acquisition test was performed for five consecutive days of training with four trials per day. Animals were given 60 s to locate the hidden platform, and any animals that did not find the platform within 60-second period were placed on the platform for 15 s. The acquisition time was recorded at the time the animal got into the water and ending at the time the animal reached the submerged platform. On the sixth day, probe trials without platform were assessed with only one starting point, and the time spent in the target quadrant where the platform had been located was recorded.

2.5. Tissue dissection

Following the behavioral tests, the animals were sacrificed and the brains were quickly harvested and frozen in liquid nitrogen and stored at −80 °C until further utilization.

The brain tissue samples were thawed and 10% (w/v) homogenates were made with ice-cold 0.1 M phosphate buffer (pH 7.4). The homogenates were used to determine the content of lipid peroxidation product and reduced glutathione.

2.6. Malondialdehyde (MDA) determination

MDA, an index of lipid peroxidation, was measured based on the reaction with thiobarbituric-acid (TBA) reaction described by Okhawa et al. [14]. MDA reacts with TBA as a thiobarbituric acid reactive substance to produce a pink complex with a peak absorbance at 532 nm. The quantification of MDA was determined by comparing the absorption to the standard curve of MDA equivalents.

2.7. Glutathione (GSH) estimations

Assay of GSH was performed in tissue homogenates by the method of Ellman [15]. The brain homogenates were precipitated in cooled trichloroacetic acid 10% and centrifuged at 2000×*g* for 15 min. Supernatants were incubated with DTNB in a 1 M phosphate buffer, PH 7.4. The colored complex formed by DTNB and GSH was measured spectrophotometrically at 412 nm. A standard curve of GSH was used to calculate GSH levels.

2.8. Statistic analysis

Data were expressed as mean ± SD. Significance of seizure stage was analyzed using Kruskal–Wallis one-way analysis of variance on ranks. Analysis of variance (ANOVA) for repeated measures was used to analyze the escape latencies in Morris water maze test among the groups over a period of 5 days. Other data were analyzed by one-way ANOVA. All statistical analysis was performed with SPSS 13.0 software and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Protective effect of metformin against pentylenetetrazole-induced kindling

In the PTZ group, repeated administration of subconvulsive PTZ (37 mg/kg) on every second day (for 28 days, 14 injections) resulted in a gradual increase in seizure score culminating in generalized clonic-tonic seizures. As shown in Fig. 1, pretreatment with metformin (200 mg/kg, i.p.) significantly suppressed the progression of kindling as evidenced by the decrease in seizure score as compared to the PTZ group. There were significant differences in seizure score between the PTZ and PTZ + MET groups from the 8th to the 14th PTZ administration ($p < 0.05$ from the 8th injection

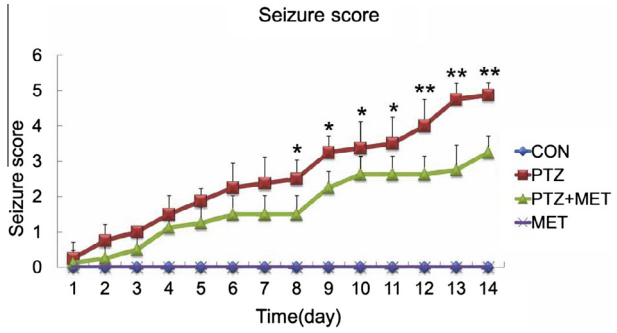

Fig. 1. Effect of metformin treatment on seizure scores. The 'Con', 'PTZ', 'PTZ + MET' and 'MET' represent control, kindling, kindling + metformin and metformin groups, respectively. Values are expressed as mean ± SD, and are shown for each injection ($^{*}p < 0.05$, $^{**}p < 0.01$).

to the11th injection and $p < 0.01$ from the 12th injection to the 14th injection).

3.2. Metformin improved cognitive impairment induced by epileptic seizures

As shown in Fig. 2, in the Morris water maze, all animals showed a progressive decline in the escape latency with training. Mice in the kindling group exhibited significantly prolonged escape latency as compared to the control group ($p < 0.01$). However, the poor performance was mitigated by pretreatment with metformin ($p < 0.01$). In the probe trial, the kindling group spent significantly less time in the target quadrant than the control group ($p < 0.01$), while pretreatment with metformin significantly improved the performance ($p < 0.01$). The number of crossing the platform in the kindling group obviously decreased as compared to the control group ($p < 0.01$), while pretreatment with metformin markedly increased the number of crossing ($p < 0.01$). In addition, metformin per se had no significant effects on cognition.

3.3. Metformin ameliorated the brain oxidative stress

As shown in Fig. 3, in the PTZ group, the level of malondialdehyde (MDA), an index of lipid peroxidation, was much higher as compared to the control group ($p < 0.05$), and the level of reduced glutathione (GSH), an endogenous antioxidant which plays a vital role as a free radical scavenger to protect cells against oxidative damage, in the PTZ group was significantly lower as compared to the control group ($p < 0.05$). However, pretreatment with metformin led to a noticeable decrease in the concentration of MDA ($p < 0.05$) and a significant increase in GSH level ($p < 0.05$) as compared to the PTZ group. Although metformin treatment ameliorated the oxidative damage, the oxidative stress level was still higher than the control group ($p < 0.05$). Metformin per se caused a decrease in the oxidative stress as indicated by the significant decrease in MDA levels and the significant increase in GSH levels as compared to the control group ($p < 0.05$).

4. Discussion

PTZ kindling is the most widely-accepted animal model used to study seizure mechanisms, epileptogenesis, learning and memory deficits induced by seizures and to discover novel treatments for seizures [11] and cognitive deficits [16,17].

Metformin is a first-line therapy for patients with type 2 diabetes. Previous studies demonstrated that metformin can rapidly cross the blood brain barrier [18] and has antioxidant [5–7], anti-inflammatory [5,19,20], and neuroprotective [21] properties.

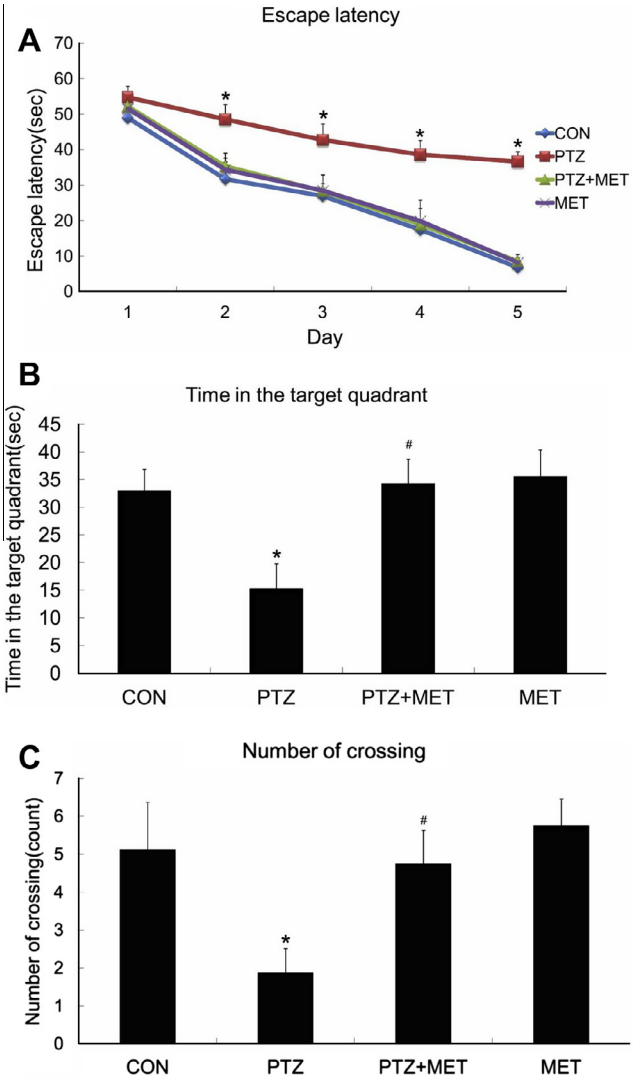

Fig. 2. Effect of metformin treatment on the performance of Morris water maze in PTZ kindling mice. (A) Latencies to reach escape platform. (B) Time spent in the target quadrant. (C) The number of crossing the platform. Values are expressed as mean ± SD, and are shown for each day. $^{*}p < 0.01$ vs control group; $^{\#}p < 0.01$ vs PTZ group.

Metformin has beneficial effects in animal models of multiple sclerosis [18], stroke [22] and Alzheimer's disease [23].

In the present study, we confirmed that repetitive administration of a subconvulsive dose of PTZ elicits behavioral seizures, oxidative stress and cognition deficits. However, metformin (200 mg/kg) significantly reduced the mean seizure score as compared to PTZ group, showing anticonvulsant activity. Furthermore, treatment with metformin restored the impaired learning and memory behavior and attenuated brain oxidative stress, showing cognition protective and antioxidant properties.

There is increasing evidence suggesting that oxidative stress plays an important role in development and progression of the seizures [3]. Epileptic seizure can increase the levels of free radicals and decreases the antioxidant defense mechanism. The brain is characterized by an elevated oxidative metabolism and low antioxidants enzymes, which increases the brain's vulnerability to reactive oxygen species. Since free radicals is supposed to mediate the convulsion development, it is of interest to search for antiepileptic compounds with antioxidant and neuroprotective effects. In the present study, metformin at a dose of 200 mg/kg significantly

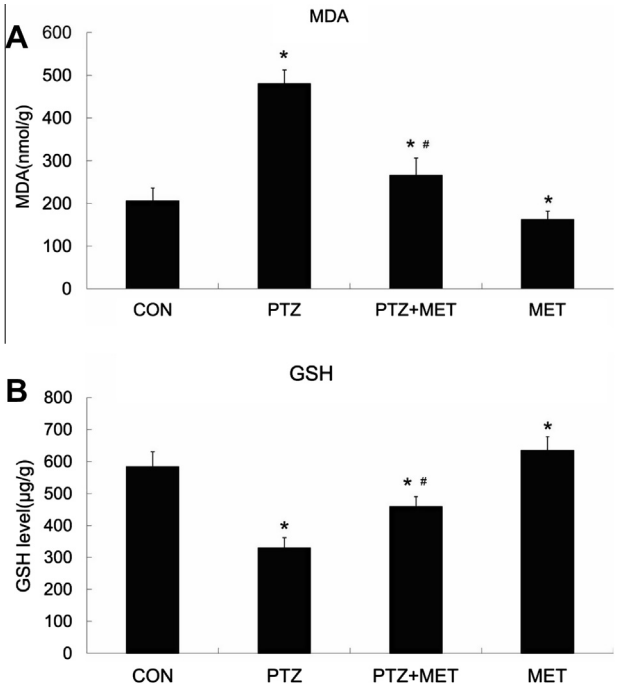

Fig. 3. The brain MDA (A) and GSH (B) levels in control, kindling, kindling + metformin and metformin groups. Values are expressed as mean ± SD. $^{*}p < 0.01$ vs control group; $^{\#}p < 0.01$ vs PTZ group.

decreased MDA level and increased GSH level relative to PTZ group. Therefore, it may be concluded that antiepileptic effect of metformin is at least partly mediated through its antioxidant property.

Previous studies documented that BBB disruption occurs during epileptogenesis and contribute to the progression of epilepsy [24], and increase of the BBB permeability was found in mice with generalized convulsive seizures induced by acute pentylenetetrazole [25], meanwhile, another study found that metformin may have considerable therapeutic benefits to prevent the formation and development of BBB breakdown [10]. Thus, the protection of metformin on seizures may be due to benefits of metformin on BBB, this is worth further investigation.

In line with previous studies [16], we found that PTZ kindling caused learning and memory deficits with a significant increase in escape latency in the training sessions and a significant decrease in time spending in the probe test in the MWM test. However, these changes were ameliorated by metformin. It has been well documented that learning and memory deficits are associated with the impairment of hippocampal neurogenesis, the enhancement of oxidative stress levels in the brain, and the reduction of synaptic plasticity in the hippocampus. In our study, metformin exerted its effect on improving the cognitive impairment induced by seizures. This improved cognitive function could be due to the beneficial effects of metformin on decreasing seizure score and brain oxidative stress. Furthermore, since metformin has been shown to rapidly cross the blood brain barrier, it is possible that metformin could have acted directly as a neuroprotective agent. It is well documented that metformin could active AMP-activated protein kinase (AMPK), an important sensor of energy balance [26]. Metformin has been shown to activate AMPK-atypical PKC-CBP pathway to promote neurogenesis and enhance spatial memory function [8]. Therefore, the beneficial effect of metformin on cognition in our study could be due to the metformin action through AMPK. Our study limitation is that we did not determine the AMPK in the present study.

Acknowledgment

This project was financially supported by the Natural Science Foundation of Hebei Province, PR China (C2010000548).

References

[1] J.L. Noebels, M. Avoli, M.A. Rogawski, R.W. Olsen, A.V. Delgado-Escueta (Eds.), Cognitive and behavioral co-morbidities of epilepsy, in: Jasper's Basic Mechanisms of the Epilepsies [Internet], fourth ed., National Center for Biotechnology Information, Bethesda, MD, 2012.
[2] H. Jokeit, A. Ebner, Effects of chronic epilepsy on intellectual functions, Prog. Brain Res. (2002) 455–463.
[3] K. Sudha, A.V. Rao, A. Rao, Oxidative stress and antioxidants in epilepsy, Clin. Chim. Acta 303 (2001) 19–24.
[4] J.N. Keller, F.A. Schmitt, S.W. Scheff, Q. Ding, Q. Chen, D.A. Butterfield, W.R. Markesbry, Evidence of increased oxidative damage in subjects with mild cognitive impairment, Neurology 64 (2005) 1152–1156.
[5] A. Martin-Montalvo, E.M. Mercken, S.J. Mitchell, et al., Metformin improves healthspan and lifespan in mice, Nat. Commun. (2013), http://dx.doi.org/10.1038/ncomms3192.
[6] C. Alqire, O. Moiseeva, X. Deschênes-Simard, et al., Metformin reduces endogenous reactive oxygen species and associated DNA damage, Cancer Prev. Res. 5 (2012) 536–543.
[7] A. Anedda, E. Rial, M.M. González-Barroso, Metformin induces oxidative stress in white adipocytes and raise uncoupling protein 2 levels, J. Endocrinol. (2008) 33–40.
[8] J. Wang, D. Gallagher, L.M. DeVito, et al., Metformin activates an atypical PKC-CBP pathway to promote neurogenesis and enhance spatial memory formation, Cell Stem Cell 11 (2012) 23–35.
[9] H. Pinana, N. Apaijai, W. Pratchayasakul, et al., Effects of metformin on learning and memory behaviors and brain mitochondrial functions in high fat diet induced insulin resistant rat, Life Sci. 91 (2012) 409–414.
[10] F. Takata, S. Dohgu, J. Matsumoto, et al., Metformin induces up-regulation of blood–brain barrier functions by activating AMP-activated protein kinase in rat brain microvascular endothelial cells, Biochem. Biophys. Res. Commun. (2013) 586–590.
[11] A. Dhir, Pentylenetetrazole (PTZ) kindling model of epilepsy, Curr. Protoc. Neurosci. (2012). 9.37.1–9.37.12.
[12] R.J. Racine, Modification of seizure activity by electrical stimulation. II. Motorseizure, Electroencephalogr. Clin. Neurophysiol. 2 (1972) 281–294.
[13] C.V. Vorhees, M.T. Williams, Morris water maze: procedures for assessing spatial and related forms of learning and memory, Nat. Protoc. 1 (2006) 848–858.
[14] H. Okhawa, N. Ohishi, K. Yagi, Assay of lipid peroxides in animal tissue by thiobarbituric acid reaction, Anal. Biochem. 95 (1979) 351–358.
[15] G.L. Ellman, Tissue sulphydryl groups, Arch. Biochem. Biophys. 82 (1959) 70–77.
[16] M. Pahuja, J. Mehla, K.H. Reeta, et al., Effect of *Anacyclus pyrethrum* on pentylenetetrazole-induced kindling, spatial memory, oxidative stress and Rho-kinase II expression in mice, Neurochem. Res. 38 (2013) 547–556.
[17] K.M. Choudhary, A. Mishra, V.V. Poroikov, et al., Ameliorative effect of curcumin on seizure severity, depression like behavior, learning and memory deficit in post-pentylenetetrazole-kindled mice, Eur. J. Pharmacol. 704 (2013) 33–40.
[18] K. Labuzek, D. Suchy, B. Gabryel, A. Bielecka, et al., Quantification of metformin by the HPLC method in brain regions, cerebrospinal fluid and plasma of rats treated with lipopolysaccharide, Pharmacol. Rep. 62 (2010) 956–961.
[19] N. Nath, M. Khan, M.K. Paintlia, et al., Metformin attenuated the autoimmune disease of the central nervous system in animal models of multiple sclerosis, J. Immunol. 182 (2009) 8005–8014.
[20] Y. Hattori, K. Suzuki, S. Hattori, K. Kasai, Metformin inhibits cytokine-induced nuclear factor κB activation via AMP-activated protein kinase activation in vascular endothelial cells, Hypertension 47 (2006) 1183–1188.
[21] M.Y. El-Mir, D. Detaille, G. Villanueva, et al., Neuroprotective role of antidiabetic drug metformin against apoptotic cell death in primary cortical neurons, J. Mol. Neurosci. 34 (2008) 77–87.
[22] J. Li, S.E. Benashski, V.R. Venna, L.D. McCullough, Effect of metformin in experimental stroke, Stroke 41 (2010) 2645–2652.
[23] J. Li, J. Deng, W. Sheng, Z. Zuo, Metformin attenuates Alzheimer's disease-like neuropathology in obese, leptin-resistant mice, Pharmacol. Biochem. Behav. 101 (2012) 564–574.
[24] E.A. Vanvliet, S. da Costa Araújo, S. Redeker, et al., Blood–brain barrier leakage may lead to progression of temporal lobe epilepsy, Brain 130 (2007) 521–534.
[25] S. Danjo, Y. Ishihara, M. Watanabe, et al., Pentylenetrazole-induced loss of blood-brain barrier integrity involves excess nitric oxide generation by neuronal nitric oxide synthase, Brain Res. 1530 (2013) 44–53.
[26] G. Zhou, R. Myers, Y. Li, et al., Role of AMP-activated protein kinase in mechanism of metformin action, J. Clin. Invest. 108 (2001) 1167–1174.