Combined neuroprotective action of JWH-015 and AM251 in the CA1 hippocampal area of transient global cerebral ischemia

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Running title: JWH-015 & AM251 in global cerebral ischemia

Abstract: Transient global cerebral ischemia (TGCI) induces by bilateral common carotid artery occlusion (BCCAO), and it mediates neuronal cell death of the CA1 hippocampal area. AM251 is a cannabinoid receptors type 1 (CB1) blocker that has been known to be protective against transient focal cerebral ischemia. JWH-015 is a selective agonist of CB2 and activator of CB1 that is involved in promotion of neuronal recovery and survival. The role of combined application of JWH-015 with AM251 in the rat model of GCI has been surveyed in this study. Male Wistar rats underwent 20 min of ischemia followed by reperfusion. Then, 1 mg/kg JWH-015 and 2 mg/kg AM251 were administered through caudal vein. The groups were control, sham, ischemia, vehicle, AM251, JWH-015 and AM251 + JWH-015. Animals were sacrificed at 14 days after reperfusion. The AM251 + JWH-015 group showed a significant increase in the protein expressions of AKT1, Bad 14-3-3, Bcl-2 and Bcl-XL, but it showed a considerable decrease in the protein expressions of Bad and JNK1/2 (p ≤ 0.05 vs. AM251, and JWH-015 groups). The AM251 + JWH-015 group had a significant higher number of alive cells and lower number of TUNEL-positive cells in the CA1 hippocampal area, and it also had a considerable improvement of spatial memory (p ≤ 0.05 vs. AM251, and JWH-015 groups). The results of this study showed that combined application of AM251 and JWH-015 could be neuroprotective against detrimental effects of ischemia probably via suppression of neuronal apoptosis and maintenance of their survival.

Keywords: CA1; survival; apoptosis; global cerebral ischemia

1. Introduction

Ischemic stroke is known as a brain damage results from reduced levels of glucose and oxygen supply caused by blockage of primary arteries [1, 2]. The transient global cerebral ischemia (TGCI) induces by bilateral common carotid artery occlusion (BCCAO) [3], and it
reduces brain blood flow [4]. TGCI mediates neuronal cell death resulting in severe neurological and neurobehavioral dysfunction [5]. Among various areas in brain, neurons of CA1 hippocampal area are highly vulnerable to ischemia [6] so that apoptosis of these neurons is an important phenomenon of brain ischemia/reperfusion injury [7].

A main function of endocannabinoids is to support tissue from pathological injury [8]. Cannabinoids effects are attributed to the activation of cannabinoid receptors type 1 (CB1) and type 2 (CB2) [9]. There is a rise in the levels of both CB1 and CB2 in the brain tissue after BCCAO and reperfusion (BCCAO/R) [4]. Both receptors are expressed in the brain [10]. CB2 has been linked to neuroprotection [11] through reduction of ischemic-induced microcirculatory dysfunction [4]. CB2 is involved in modulation of hippocampal CA1 synaptic plasticity [10]. Blockade of CB1 receptor by AM251 has been known to be protective against transient focal cerebral ischemia; however, using agonist of CB1 receptor could also capable of reducing neuronal loss of hippocampus following TGCI and decreasing the infarct volume following focal ischemia induced by middle cerebral artery occlusion [12]. JWH-015 is an indole that serves as a selective agonist of CB2 and activator of CB1 [13]. Viscomi et al. reported that application of JWH-015 against remote axotomy-induced apoptosis could decrease neuronal apoptosis through phosphatidylinositol 3-kinase (PI3-K)/AKT pathway and, consequently, promote neuronal recovery and survival [14]. The aim of the present study was to evaluate a possible role of combined exogenous application of JWH-015 with AM251 in the CA1 hippocampal area of TGCI in rats.

2. Results

2.1. Western blot analysis
Protein expressions of AKT1, Bad 14-3-3, Bad, JNK1/2, Bcl-2 and Bcl-XL were assessed by western blot. The AM251 + JWH-015 group showed about 2-fold increase in the protein expressions of AKT1 and Bad 14-3-3 with 1.84 ± 0.07 and 1.45 ± 0.11, respectively (for both p < 0.001 vs. ischemia). The AM251 + JWH-015 group also showed over 2-fold rise in the expression of Bcl-2 with 1.51 ± 0.1, and it had over 5.5-fold rise in the mRNA expression of Bcl-XL with 1.84 ± 0.19 (p < 0.001 vs. ischemia). This group showed over 6- and 2-fold fall in the mRNA expressions of Bad and JNK1/2 with 0.45 ± 0.06 and 0.58 ± 0.07, respectively (p < 0.001 vs. ischemia). The levels of protein expressions were significant in the AM251 + JWH-015 group compared with the AM251 and JWH-015 groups (for both p < 0.001) (Fig. 1).

2.2. Cresyl violet (Nissl) staining

Nissl staining was performed to evaluate survival rate of CA1 hippocampal neurons. The number of alive cells showed a significant fall in the ischemic group with 7.38 ± 2.63 (p < 0.001 vs. control). The AM251 + JWH-015 group had a high rate of neuronal viability by about 12-fold with 91.7 ± 4.62 (p < 0.001 vs. ischemia). The rate of survival in the AM251 + JWH-015 group was significant compared with either AM251 group (p < 0.001) or JWH-015 group (p < 0.02) (Fig. 2).

2.3. TUNEL assay

The number of TUNEL-positive cells rose significantly in the ischemic rats with 70.56 ± 0.62 (p < 0.001 vs. control). Conversely, the AM251 + JWH-015 group had a notable fall of the number of TUNEL-positive cells with 12 ± 1.06 (p < 0.001 vs. ischemia). Result of the AM251 + JWH-015 group was significant compared with the AM251 group (p < 0.001) and JWH-015 group (p < 0.05) (Fig. 3).

2.4. MWM
Spatial memory was investigated in rats by evaluation of MWM in the probe stage. Rats in the AM251 + JWH-015 group took lower speed with $13.12 \pm 3.20$ (p < 0.05 vs. ischemia) and spent longer time with $27.11 \pm 5.9$ in the target quadrant (p < 0.001 vs. ischemia). The number for speed was significant in the AM251 + JWH-015 group compared with the AM251 group and JWH-015 group (for both p < 0.05). Similarly, the number for time spent in the target quadrant was notable in the AM251 + JWH-015 group compared with the AM251 and JWH-015 groups (for both p < 0.001) (Table 1).

2.5. Figures, Table

Fig. 1. Western blot analysis of the role of combined application of AM251 and JWH-015 in protein expression of survival and apoptosis factors in the CA1 hippocampal area of rats underwent transient global cerebral ischemia (GCI) induction by bilateral common carotid artery occlusion and reperfusion (BCCAO/R). a, AKT1; b, Bad 14-3-3; c, Bad; d, JNK1/2; e, Bcl-2; and f, Bcl-XL. a, p < 0.001 vs all; b, p < 0.001 vs control and sham; c, p < 0.02 vs sham; d, p < 0.008 vs control; e, p < 0.01 vs sham; and f, p < 0.004 vs control.
Fig. 2. Cresyl violet (Nissl) staining to investigate a possible effect of combined application of AM251 and JWH-015 on survival rate of the CA1 hippocampal area in the model of transient global cerebral ischemia (GCI) induced by bilateral common carotid artery occlusion and reperfusion (BCCAO/R). a, control; b, sham; c, ischemia; d, vehicle; e, AM251; f, JWH-015; and g, AM251 + JWH-015. Viable neurons of CA1 hippocampal area are purple-stained cells with light cytoplasm and intact morphology, while dead neurons of CA1 hippocampal area are blue-stained cells with triangular shape (light microscope. Scale bar = 150 µm). h and i, Comparison of the percentage of alive and dead CA1 hippocampal neurons. a, p < 0.02 vs. JWH-015; b, p < 0.001 vs. AM251, Ischemia and Vehicle; c, p < 0.01 vs. JWH-015; d, p < 0.001 vs. Ischemia, Vehicle, control and Sham; and e, p < 0.001 vs. Control and Sham.
Fig. 3. TUNEL assay to evaluate a possible role of combined administration of AM251 and JWH0-15 in apoptosis of neurons of CA1 hippocampal area in the rat model of transient global cerebral ischemia (GCI) induced by bilateral common carotid artery occlusion and reperfusion (BCCAO/R). a, control; b, sham; c, ischemia; d, vehicle; e, AM251; f, JWH-015; and g, AM251 + JWH-015. Dark brown-stained cells are TUNEL-positive neurons of the CA1 hippocampal area (fluorescence microscope. Scale bar = 150 µm). h, Percentage of TUNEL-positive cells. a, p < 0.05 vs. JWH-015; b, p < 0.001 vs. AM251, Ischemia and Vehicle; c, p < 0.02 vs. Control and Sham; d, p < 0.01 vs. AM251; e, p < 0.001 vs. Vehicle, Ischemia, Control and Sham; f, p < 0.001 vs. Ischemia and Vehicle; g, p < 0.001 vs. Control and Sham.
3. Discussion

The combined additive neuroprotective action of AM251 and JWH-015 on the TGCI model induced by 20 min BCCAO has been shown in this study. Expression rates of factors related to the apoptosis or survival in the CA1 hippocampal area was assessed by western blot, and our results revealed high protein expressions of AKT1, Bcl-2, Bad 14-3-3 and Bcl-XL, but low protein expressions of JNK and Bad in the AM251 + JWH-015 group compared with each of the AM251 and JWH-015 groups. Bad is a pro-apoptotic protein with a key role in determination of cell death/survival. Bad inhibits Bcl-2 and Bcl-XL through which it promotes apoptotic cell death. Phosphatidylinositol 3-kinase (PI3-K)/AKT and JNK pathways are thought to be responsible for Bad regulation. AKT is an essential mediator of neuronal survival [15], but JNK is a mediator of cell death. PI3-K/AKT pathway is responsible for Bad inactivation, but JNK pathway is responsible for Bad activation. Phosphorylation of Bad is mediated by AKT; this phosphorylated form has an affinity to bind with 14-3-3 to form Bad.

Table 1. Spatial memory assessment by Morris water maze (MWM) to evaluate the role of AM251 combined with JWH-015 in rat model of cerebral ischemia induced by transient bilateral common carotid artery occlusion and reperfusion (BCCAO/R).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean ± SD</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Speed Q4 (cm/sec)</td>
<td>Time Q4 (sec)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>19.78 ± 4.75</td>
<td>26.43 ± 5.04</td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>19.12 ± 4.91</td>
<td>28.12 ± 5.12</td>
<td></td>
</tr>
<tr>
<td>Ischemia</td>
<td>36.91 ± 6.3 e</td>
<td>5.54 ± 4.22 e</td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>35.17 ± 6.71 e</td>
<td>5.23 ± 3.13 e</td>
<td></td>
</tr>
<tr>
<td>AM251</td>
<td>29.76 ± 4.68 d</td>
<td>13.11 ± 2.41 d</td>
<td></td>
</tr>
<tr>
<td>JWH-015</td>
<td>25.14 ± 3.50 c,d</td>
<td>18 ± 2.21 d</td>
<td></td>
</tr>
<tr>
<td>AM251+JWH-015</td>
<td>13.12 ± 3.20 a</td>
<td>27.11 ± 5.9 b</td>
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</table>

a, p < 0.05 vs. all groups; b, p < 0.0001 vs. JWH-015, AM251, Vehicle, and Ischemia; c, p < 0.02 vs. AM251; d, p < 0.0001 vs. Ischemia, Vehicle, Control and Sham; e, p < 0.0001 vs. Control and Sham
14-3-3, which by in turn decreases further attachment of Bad to Bcl-XL [16]. JNK activation may indirectly control the transcription of Bcl-2 family members, including Bcl-XL and Bcl-2 downregulation and Bax upregulation [8].

Activation of the PI3-K/AKT pathway has also been reported to be stimulated by CB2 receptor [17]. Murataeva et al. reported that JWH-015 is not only a CB2 selective agonist but also a potent activator of CB1 receptors in neurons [13]. Viscomi et al. used JWH-015 in targeting remote axotomy-induced apoptosis, and they found that it is capable of decreasing neuronal apoptosis and promoting neuronal recovery and survival through PI3-K/AKT pathway [14].

CB1 receptors are greatly expressed in neurons of various areas of CNS including hippocampus [18]. CB1 receptor blocker AM251 has been known to be protective against transient focal cerebral ischemia [12]. Dunbar et al. recently reported attenuation of CA1 injury by AM251 [19]. Interestingly, using CB1 receptor agonist could also capable of decreasing hippocampal neuronal loss following transient GCI and reducing the infarct volume after focal ischemia induced by middle cerebral artery occlusion [12].

Since neuronal DNA fragmentation is mostly used as a measure of neuronal loss, the TUNEL assay was further used to detect apoptotic cells based on labeling DNA strand breaks in the hippocampus [8]. In addition, Nissl staining was performed to evaluate cell survival. The results showed that ischemic rats had significant higher number of TUNEL-positive cells and lower number of alive cells compared to the control. To explain, the same results were obtained with 20 min induction of ischemia by BCCAO in the works performed by Sharifi et al. [20], Movassaghi et al. [21] in rats and Mori et al. [22] in mice. The results of TUNEL assay and Nissl staining in the ischemic rats were significantly counteracted by combined application of AM251 and JWH-015, as compared with each of the ischemia, AM251, and JWH-015 groups. The above findings of the present study indicate that combined administration of AM251 and
JWH-015 has a potential to suppress apoptosis and maintain further survival of CA1 hippocampal neurons, which may involve the inhibition of the JNK pathway and stimulation of the AKT pathway.

To see if the combined application of AM251 + JWH-015 could affect spatial memory, we evaluate MWM in the studying groups, and we found impairment of spatial memory in the ischemic group. To explain, pyramidal neurons of the CA1 hippocampal area are particularly sensitive to ischemia, and their apoptosis is an outcome of transient GCI [20]. Therefore, memory deficit is one of the outcomes of transient GCI [5], as in the study carried out by Movassaghi et al. who found that exposure of rats to BCCAO could impair spatial memory [21]. To evaluate learning and memory, MWM was used. There are several advantages of this test over other models including absence of motivational stimuli such as food and water deprivation, electrical stimulations and buzzer sounds {Morris, 1984 #37}. Memory deficit after BCCAO could be contributed to neurodegeneration in the hippocampus [22]. Our result showed that BCCAO impaired reference memory. We noticed that rats in the AM251 + JWH-015 group took lower speed and spent longer time in target quadrant which suggests low emotional state levels in these animals. To the best of our knowledge, these are the first findings denoting a protective role of combined application of AM251 and JWH-015 against spatial memory injury following GCI. CB1 and CB2 receptors modulate a vast variety of physiological functions including memory [10]. CB1 receptor expresses at high levels in the hippocampus [23]. Kim et al. found that CB2 receptor is capable of modulating hippocampal CA1 synaptic plasticity [10]. Data from MWM shed another light on beneficial role of combined administration of AM251 and JWH-015 in protecting neurons of CA1 hippocampal area.
4. Materials and Methods

4.1. Animals and surgical procedure

Male Wistar rats weighing 200 to 250 g were obtained from Pharmacy Faculty of Tehran University of Medical Sciences (TUMS). The standard conditions of constant temperature (22°C), illumination (12 hours light-dark cycle) and humidity (55-65%) were applied for all animals. Food and water ad libitum are easily provided for rats, and they were also allowed to be acclimatize to the new environment for one week without doing any procedures. Experiments were carried out on rats by adhering to the guides from Ethical Committee of Tehran University of Medical Sciences and the national and institutional guidelines for animal care.

Animals were kept fast overnight but allowed to have free access to water prior to surgery. Using 50 mg/kg ketamine (Sigma, USA) and 10 mg/kg xylazine (Serva Feinbiochemica, New York) rats were anesthetized. Anterior midline incision of cervical area was performed to isolate CCAs sheets. Both CCAs were detached from carotid sheet, then the vagus nerves were carefully separated. CCAs were occluded for 20 min using Yashargil Aneurism microclips [20], and then the clasps were removed. CCAs were visually inspected for blood flow recovery and no formation of blood clots within the vessels. After reperfusion, all animals were allowed to survive for 14 days. A rectal thermistor was used to monitor body temperature which maintained at 37 ± 5°C during ischemia. One rat died during ischemia, and two rats died post-ischemia.

Administration of 1mg/kg JWH-015 (Sigma, USA) and 2 mg/kg AM251 (Sigma, USA) both dissolved in dimethyl sulfoxide (DMSO, Sigma, USA) was carried out via tail vein at 1 h after reperfusion. There were seven groups (n = 8): control, sham, ischemia, vehicle, AM251, JWH-015 and AM251 + JWH-015. Control group had no specific treatment or surgery. Rats
in the sham group were underwent surgery with no ischemia induction. Vehicle group had ischemia induction and intracaudal injection of DMSO. Experimental groups had ischemia induction and intracaudal injection of 1mg/kg JWH-015, 2 mg/kg AM251 and 1mg/kg JWH-015 + 2 mg/kg AM251 at 1 h after reperfusion.

**4.2. Western blot analysis**

Extraction of total protein from hippocampus was performed using RIPA buffer containing phosphatase inhibitor and protease cocktails (Sigma, Louis, MO, USA). Protein concentration was evaluated using Bradford method (Bio-Rad, MI, USA), and separation of proteins was done using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Transferring of proteins onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad) incubated for 1h with 5% bovine serum albumin (BSA, Sigma, Louis, MO, USA) in 100 ml TBST was then performed. PVDF membranes were then probed with primary antibodies against Bad 14-3-3 (1:500 dilution, Santa Cruz Biotechnology, CA, USA), AKT1 (1:2000 dilution; Abcam, Cambridge, MA, USA), Bad (1:1000 dilution, Cell Signaling, USA), Bcl-XL (1:3000 dilution; Abcam, Cambridge, MA, USA), Bcl-2 (1:1000 dilution; Abcam, Cambridge, MA, USA), c-Jun N-terminal kinase (JNK) 1/2 (1:1000 dilution, Santa Cruz Biotechnology, CA, USA) and β-actin (1:500, Abcam, Cambridge, MA, USA). Membranes were washed three times in TBST buffer and then incubated for one hour at room temperature with horse radish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, CA, USA). Immunoreactive visualization of proteins were investigated using enhanced chemiluminescence system (ECL-plus, Lumigen, Inc. Southfield, MI, USA), and the band densities were normalized to β-actin and quantified using ImageJ analyzer (NIH, Bethesda, MD, USA). For each experiment three trials was performed.
4.3. Cresyl violet (Nissl) staining

Rats were perfused using 4% paraformaldehyde (Sigma, USA) in 0.1 M phosphate buffer (pH 7.3), and paraffin-embedded hippocampal tissue samples were sectioned (5 µm thicknesses with an interval of 120 µm). Then, slides were deparaffinized and stained with 0.5% cresyl violet. An Olympus microscope (CX31, Tokyo, Japan) equipped with a digital camera (Leica, München, Germany) was used for observation of sections at 400 X magnification. A total number of 100 cells were counted per three high power field. Purple-stained cells with intact morphology and light cytoplasm were chosen as viable cells, and blue-stained cells with triangular shapes were chosen as dead cells.

4.4. TUNEL assay

According to the manufacturer’s protocol, assessment of DNA fragmentation was performed using an in situ cell death detection kit (Roche). Deparaffinized sections were treated with 10 µg/ml proteinase K for 30 min and incubated with 20 µl terminal deoxynucleotidyl transferase enzyme. Samples were then incubated with 450 µl labeling solution for 1 h, horseradish peroxidase conjugated anti-fluorescein antibody Fab fragments for 30 min, and 50-100 µl DAB substrate for 10 min. After counterstaining with methyl green, the sections were assessed under fluorescence microscope (IX2 ILL100; Olympus, Tokyo, Japan) at 560 nm. Using ImageJ analyzer (NIH, Bethesda, MD, USA), A total number of 100 brown-stained apoptotic cells of CA1 hippocampal area were counted blindly in six randomly chosen areas per three high power field. Positive control samples were incubated with 2000 U DNase I recombinant in Tris-HCl (pH 7.5) for 10 min at 25ºC prior to labeling. Negative controls were coated only with the labeling solution.
4.5. MWM

Spatial memory was assessed by MWM. A large circular black pool with a diameter of 180 cc and a height of 60 cm filled with an opaque water (24 ± 1 °C) to a depth of 35 cm was used in this study. The pool was surrounded by non-motile external cues as a guide for rats in finding their roots. The tank was divided into four equal quadrants by using two threads fixed at right angles to each other on the rim of the pool. In the center of the fourth quadrant, a hidden platform was placed 1 cm below the water surface. The platform had a fixed position throughout the test. The test lasts for five consecutive days between 10:00 AM and 12:00 PM. Animal were allowed to have training four trials (training test). A video camera (Nikon, Melville, NY, USA) was installed above the pool for tracking the animals. Visual test was evaluated at day one by placing a platform coated by a transparent covering on the water surface (visible test). During trial days two, three and four the platform had no transparent covering at 0.5 cm below the water surface. During the training test, the rats were gently placed in the water from different sides (north, south, east and west) facing the wall of the pool randomly, and were allowed to find the platform in 60 sec. There was a 20 sec interval between the two consecutive trials. During each trial, animals were manually placed on the platform where they could not find the platform by themselves within 60 sec.

On the last day (day 5) or probe day the platform was removed, and long-term or reference memory was checked by allowing animals to swim for 60 sec in the pool that was not equipped with the platform. All rats in this day were placed in the pool from one side (west). At the end of day five, the period of time spent in the target quadrant (fourth quadrant where the platform was placed) and the speed of animals in recognizing the exact location of the place of platform were recorded {Tanwar, 2014 #36}. 
4.6. Statistical analysis

Homogeneity of variances (Levene’s test) was evaluated, and all variables had a normal distribution (Shapiro-Wilk’s test). Data were assayed with one-way analysis of variance (ANOVA) running by SPSS 22 (SPSS Inc., Chicago, IL). Tukey was used as a post-hoc for all equal variances. Data were presented as mean ± SD, and the differences between the groups were considered statistically significant at a \( p \leq 0.05 \).

5. Conclusion

The results obtained in the present study indicate an additive combined neuroprotective action of AM251 and JWH-015 on neurons of CA1 hippocampal area against destructive effects of GCI via probably suppression of apoptosis and maintenance of cell survival.

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Author Contributions: Shiasi Maryam, Abolhassani Farid and Hedayatpour Azim conceived and designed the experiments; Shiasi Maryam and Mortezaee Keywan performed the experiments; Shiasi Maryam, Mortezaee Keywan and Nadiasharifi Zahra analyzed the data; Shiasi Maryam and Mortezaee Keywan wrote the paper.

Conflict of interest: The declare no potential conflicts of interest

References


