Decrease in Plasma Adiponectin Level and Increase in Adiponectin Immunoreactivity in Cortex and Hippocampus After Traumatic Brain Injury in Rats

Sıçanlarda Travmatik Beyin Hasarı Sonrasında Korteks ve Hipokampusta Adiponektin İmmünoreaktivitesinde Artış ve Plazma Adinopektin Seviyesinde Azalma

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ABSTRACT

AIM: Adiponectin plays an important role in the regulation of tissue inflammation. Recently, it has been reported that the plasma adiponectin levels in several acute illnesses decrease periodically, thus indicating that adiponectin may play a role in the inflammatory response in patients with acute illness. However, little is known about the effects of adiponectin following TBI. The aim of the present study was to examine the changes in the plasma adiponectin levels and the immunoreactivity of adiponectin in the brain after TBI.

MATERIAL and METHODS: Adult male Sprague-Dawley rats were subjected to lateral fluid percussion injury using the Dragonfly device. Plasma adiponectin levels were determined by ELISA kit. Immunohistochemistry and Western blot analysis were performed to assess the immunoreactivity of adiponectin.

RESULTS: The plasma adiponectin levels gradually decreased and were significantly lower at 48 h and 72h after injury than before injury. Immunohistochemistry and Western blot analysis showed that the adiponectin immunoreactivity was increased in the cerebral cortex at 24 hours after injury and in the hippocampus at 72 hours after injury.

CONCLUSION: Our findings suggest that adiponectin might participate in the pathophysiological process occurring after TBI.

KEYWORDS: Adiponectin, Traumatic brain injury, Lateral fluid percussion, Rat

ÖZ

AMAÇ: Adiponektin doku enflamasyonunun düzenlenmesinde önemli bir rol oynar. Son zamanlarda plazma adiponektin düzeylerinin çeşitli akut hastalıklarda düzenli olarak azalması nedeniyle adiponektinin akut hastalığı olan hastalarda enflamatuvar cevapta bir rol oynayabileceği bildirilmiştir. Ancak travmatik beyin hasarı sonrasında adiponektin etkileri hakkında çok az şey bilinmektedir. Mevcut çalışmanın amacı travmatik beyin hasarı sonrasında plazma adiponektin düzeylerinin değişiklikleri ve beyinde adiponektin immünoreaktivitesini incelemekti.

YÖNTEM ve GEREÇLER: Yetişkin erkek Sprague-Dawley sıçanlarında Dragonfly cihazları kullanılarak lateral sıvı perküsyon hasarı oluşturuldu. Plazma adiponektin düzeyleri ELISA kitiyle belirlendi. Adiponektin immünoreaktivitesini değerlendirmek üzere immünohistokimya ve Western blot analizi yapıldı.

BULGULAR: Plazma adiponektin düzeyleri giderek azaldı ve yaralanmadan 48 saat ve 72 saat sonra yaralanma öncesine göre önemli ölçüde daha düşüktü. İmmünohistokimya ve Western blot analizi adiponektin immünoreaktivitesinin serebral kortekste yaralanmadan 24 saat sonra ve hippokampuste yaralanmadan 72 saat sonra arttığını gösterdi.

SONUÇ: Bulgularımız adiponektinin travmatik beyin hasarı sonrasındaki patofizyolojik sürece katkıda bulunabileceğini düşündürmektedir.

ANAHTAR SÖZCÜKLER: Adiponektin, Travmatik beyin hasarı, Lateral sıvı perküsyonu, Sıçan

INTRODUCTION

Traumatic brain injury (TBI) remains one of the leading causes of death and disability. Research efforts in recent years have provided increasing evidence that the inflammatory response is largely responsible for the poor outcome of TBI (18). Among the various pathways underlying the inflammatory responses after TBI, recent attention has been paid to the AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor-α (PPARα) pathways because they exert strong anti-inflammatory effects (1, 15, 16, 18).

Adiponectin is a hormone secreted exclusively by adipose tissue, and plays an important role in the regulation of tissue

inflammation and insulin sensitivity (22, 23). Pertubations in the circulating adiponectin concentrations have been reported to be associated with metabolic syndrome, altered inflammation, and insulin resistance (7). Due to its effects, adiponectin is described as an anti-diabetic and anti-atherogenic adipokine (5). It has recently been reported that the plasma adiponectin levels in patients with several acute illness decreased periodically, thus indicating that adiponectin may play a role in the inflammatory response in patients with acute illness (21). Furthermore, recent experimental studies using a cerebral ischemia-reperfusion model have shown that high levels of adiponectin were detected in the ischemic hemisphere (24), and that adiponectin may exert a cerebroprotective action (13). The precise mechanism(s) underlying its effects remains elusive, but it has been reported that adiponectin exerts its effect mainly by activating the AMPK and PPARα pathways (9, 13, 22, 23). However, little is known about the effects of adiponectin on TBI. The aim of the present study was to examine the changes in the plasma adiponectin levels and the immunoreactivity of adiponectin in the brain after TBI.

MATERIAL and METHODS

Animals and Experimental Procedures

All experimental procedures were approved by the Animal Care and Use Committee of the National Defense Medical College. Thirty Sprague-Dawley rats (male, 300–400 g; 9–10 weeks of age) were used for the study. The rats were housed in individual cages under controlled environmental conditions (12/12 h light/dark cycle, 20–22°C; room temperature) with food and water freely available, for 1 week before the experimental surgery. The rats were anesthetized with isofluorane (1.5%) in a 30% oxygen to 70% nitrous oxide gas mixture via a nose cone and were fixed in a stereotaxic frame for the procedure. A 4.8 mm craniotomy was made over the right parietal cortex (3.8 mm posterior and 2.5 mm lateral to bregma), keeping the underlying dura intact. A plastic Luer-Loc was placed over the opening and secured with dental acrylic cement. The rats were returned to their cages and allowed free access to water overnight. The following day, the rats were anesthetized, intubated, and maintained on a mechanical ventilator after infusion of pancronium bromide (0.1 mg/kg; tidal volume: 2.5–3.0 ml/kg; respiratory rate: 60/min). The femoral artery was cannulated with a polyethylene catheter. The rats' blood pressure was monitored throughout the procedure, and arterial blood samples were intermittently analyzed ($PaCO_2$ was controlled at 30–40 mmHg). The rectal temperature was measured with a rectal probe and maintained at a constant level of approximately 37.0°C with a heating pad.

The rats were subjected to fluid percussion injury (FPI) at a moderate severity (2.5–3.0 atm, 16 msec in duration) using a Dragonfly fluid percussion device (model HPD-1700; Dragonfly R&D, Silver Spring, MD, USA), as previously described (11). Following injury, the connection cap was removed, the scalp was sutured, and the rats were returned

to their home cages with food and water available *ad libitum*. Sham control animals were subjected to the same procedures except for the actual insult.

Measurement of Plasma Adiponectin Levels

The plasma adiponectin levels in the six rats were determined via an adiponectin enzyme-linked immunosorbent assay (ELISA) kit (Otsuka Pharmaceutical Co., Tokyo, Japan). For this measurement, blood was collected from a femoral artery or tail artery before injury and at 2, 24, 48, and 72 h after injury (n=6 at each time point).

Tissue Preparation

For the Western blot analysis, the 12 rats were euthanized by decapitation under intraperitoneal anesthesia 24, 48, or 72 h after injury (n=4 per each time point). Tissue samples of the ipsilateral cortex and hippocampus (7-12 mm from the frontal polar) were immediately excised. These samples were then immediately submerged in ice-cold artificial cerebral spinal fluid (containing 0.6 mmol/L $NaH_2PO_4/2H_2O$, 3.35 mmol/L KCl, 138.6 mmol/L NaCl, 9.9 mmol/L dextrose, 21 mmol/L $NaHCO_3$, 2.5 mmol/L $CaCl_2$, and 1 mmol/L $MgCl_2$). The tissue samples were aliquoted, frozen, and maintained at -80°C until the analyses were performed. For the immunohistochemical studies, the other 12 rats were perfused transcardially with normal saline, followed by 4% buffered paraformaldehyde under intraperitoneal anesthesia 24 or 72 h after injury (n=4 per each time point). The brain was removed and embedded in paraffin after fixation in 4% buffered paraformaldehyde, followed by 0.1 mmol/L PBS (pH 7.4) for 24 h at 4°C. Serial 5 µm-thick coronal sections were prepared for the analyses.

Western Blot Analysis

The frozen tissue specimens were homogenized with ice-cold homogenization buffer (containing 50 mmol/L HEPES-KOH (pH 7.5), 250 mmol/L NaCl, 1 mmol/L EDTA, 1% Naridet P-40, 2 mmol/L Na_3VO_4, Protease Inhibitor Cocktail Set III (Calbiochem, Darmstadt, Germany)). The protein concentrations were quantified with a detergent-compatible protein assay reagent kit (Bio-Rad, Hercules, CA, USA). Equal amounts of protein (50 µg in all assays) were loaded in each lane on a 12% Tris polyacrylamide gel and were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with loading buffer (100 mmol/L Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate, 20% glycerol, 0.2% bromophenol blue, and 200 mmol/L dithithreitol). The samples were heated at 95°C for 5 min before loading. The protein was electrotransferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Buckinghamshire, UK) using an electrophoretic transfer system (TANK TRANSFER SYSTEM). The membranes were washed with Tris-buffered saline containing 0.05% Tween 20 (TTBS; pH 7.4), blocked in TTBS with 5% skim milk for 1h, and incubated for 18 h at 4°C with a primary antibody against adiponectin (Abcam, Cambridge, UK, 1:1000). The membranes were incubated for 1 h at room temperature with goat anti-rabbit IgG secondary antibody (Santa Cruz, CA, USA, 1:5000), followed by chemiluminescent detection using the

ECL-plus Western Blotting Detection System (GE Healthcare). To quantify the Western blot analysis data, a densitometric analysis was performed using the NIH ImageJ software program (http://rsb.info.nih.gov/ij/).

Immunohistochemistry

The immunohistochemical analyses were performed using the Universal Immuno- peroxidase Polymer method. After deparaffinization and hydration, the endogenous peroxidase activity was blocked with 3% hydrogen peroxidase. The sections were incubated overnight at 4°C with a polyclonal antibody aginst adiponectin (1:1000). The sections were washed with PBS and incubated with a Histofine Simple Stain Rat MAX-PO (Nichirei Co, Tokyo, Japan) for 30 min at room temperature. The sections were rinsed with PBS. Peroxidase activity was demonstrated with diamino benzidine. To evaluate the morphological changes, adjacent sections were counterstained with hematoxylin.

Statistical Analysis

The data are presented as the means ± SD. The statistical significance for the Western blot analyses was determined using a one-way ANOVA followed by the post-hoc Bonferroni/ Dunn test. The data about the plasma adiponectin levels were analyzed with a paired t-test. A value of $p < 0.05$ was considered to be significant. The Graphpad Prism 4.0 software program (San Diego, CA, USA) was used for all statistical tests.

RESULTS

Physiological Data

There were no significant differences in the physiological data between each group with regard to the mean arterial blood pressure, pH, pCO_2, pO_2, or body temperature (data not shown).

Changes in the Plasma Adiponectin Levels after TBI

The plasma adiponectin levels decreased gradually and were significantly lower at 48 h and 72h after TBI than before TBI (reduction rate; 38% at 48 h, 47% at 72 h, P < 0.05, respectively) (Figure 1).

The Immunoreactivity of Adiponectin in the Cortex after TBI

A Western blot analysis was performed to investigate the changes in the adiponectin immunoreactivity in the cortex. In the cortex, the immunoreactivity of adiponectin increased significantly until 24 h after TBI compared with before TBI (P < 0.05), and decreased thereafter until at least 72 h after TBI (Figure 2A, B). These findings were consistent with the results of the immunohistochemical analysis (Figure 2C).

The Immunoreactivity of Adiponectin in the Hippocampus after TBI

A Western blot analysis was performed to investigate the changes in adiponectin immunoreactivity in the hippocampus. In the hippocampus, the immunoreactivity
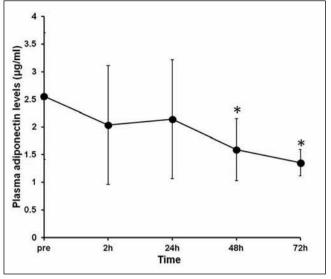

Figure 1: The serial changes in the plasma adiponectin levels following traumatic brain injury (TBI). The plasma adiponectin levels were significantly decreased at 48 h and 72h after TBI compared with before TBI. * $p < 0.05$. n = 6 per each time point. The data are the means ± SD.
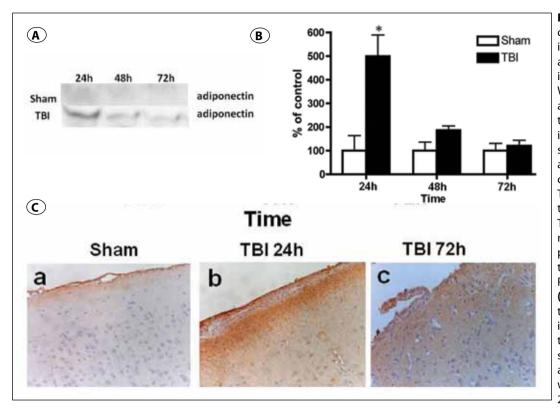
of adiponectin was not significantly different compared with the initial level until 48 h after TBI, but increased significantly thereafter until at least 72 h after TBI (P < 0.05, Figure 3A, B). These findings were consistent with the results of the immunohistochemical analysis (Figure 3C).

DISCUSSION

The current study showed, for the first time, that the immunoreactivity of adiponectin increased significantly in the cortex at 24 h after TBI and in the hippocampus (CA3-4) at 72 h after TBI.

It has been reported that the injured cortex and hippocampus, especially CA3 and CA4, are vulnerable to TBI in rats (4). Numerous experimental studies have identified a host of pathophysiological cascades caused by TBI. Several studies have suggested that pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), contribute to apoptotic programmed cell death and the glial reaction following TBI (3, 14). Increased pro-inflammatory cytokines stimulate inflammatory cells, leading to the release of damaging reactive oxygen and nitrogen species, elevating the glutamate levels to excitotoxic levels, impairing the ability of glia to buffer the extracellular potassium concentration, thereby compromising the blood-brain barrier and attracting more inflammatory cells to the brain (12, 17). Among the various pathways involved in the inflammatory responses after TBI, recent attention has been paid to the AMPK and PPARα pathways, which have strong anti-inflammatory properties (1, 15, 16, 18).

Adiponectin plays a role not only in chronic diseases, such as metabolic syndrome and atherosclerosis, but also in several
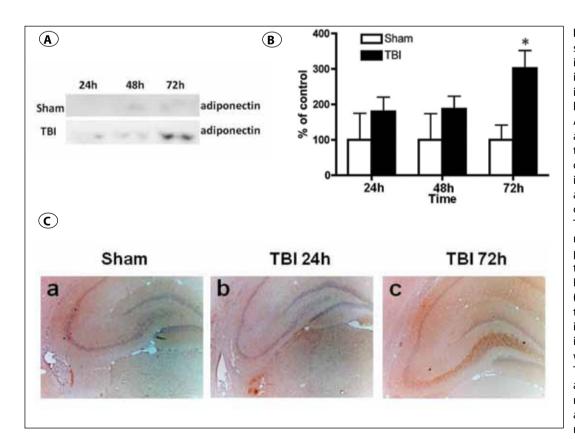
Figure 2: The serial changes in the immunoreactivity of adiponectin in the injured cortex. A Western blot analysis showed that the adiponectin immunoreactivity was significantly increased at 24 h after TBI compared with before TBI, and was decreased thereafter by 72 h after TBI **(A, B).** * $p < 0.05$. n = 4 per each time point. The data are the means ± SD. Photomicrographs **(C)** showed that the adiponectin immunoreactivity in the cortex was only slight before TBI (a) and at 72 h after TBI (c), but was remarkable at 24 h after TBI (b). Original magnifications: 400×.

Figure 3: The serial changes in adiponectin immunoreactivity in the ipsilateral hippocampus. A Western blot analysis showed that the immunoreactivity of adiponectin was increased significantly at 72 h after TBI compared with before TBI **(A, B).** * $p < 0.05$. n = 4 per each time point. The data are the means ± SD. Photomicrographs **(C)** showed that the adiponectin immunoreactivity in the hippocampus was only slight before TBI (a) and at 24 h after TBI (b), but was remarkable at 72 h after TBI (c). Original magnification: 50×.

acute illnesses. Yatomi et al. reported that, in a mouse model of ischemia-reperfusion, the plasma adiponectin level decreased significantly until 48 h after reperfusion (24). Marousi et al. reported that the serum adiponectin level was already significantly suppressed during the early phases of stroke, and that the levels remained unchanged 6 months later in humans (10). Furthermore, Venkatesh et al. recently reported that the plasma adiponectin levels in critically ill patients, including those with sepsis, burns, and trauma, decreased significantly on days 3 and 7 after injury (21), although they did not describe whether their studies included any patients with TBI. The precise mechanism by which adiponectin mediates these effects remains unclear, but it has been shown that adiponectin can activate the AMPK and PPARα pathways (1, 15, 16, 18), and it has been shown that adiponectin can exert a potent cerebroprotective function though its anti-inflammatory and/or anti-apoptotic actions in both the rat and mouse brain ischemia-reperfusion models (2, 13). We speculate that a high level of adiponectin immunoreactivity in the brain may act to reduce neuronal apoptosis after TBI via an anti-inflammatory and/or anti-apoptotic action as a result of AMPK and PPARα pathway activation. In addition, Conti et al. reported that apoptotic cells were increased mostly in the injured cortex at 24 h after TBI in rats, and that the apoptosis peak tended to be delayed in the hippocampus compared with that in the cortex (3). Therefore, we consider that the differences in the peak times of apoptosis between the cortex and hippocampus might contribute to the present results, which showed that the peak adiponectin immunoreactivity was delayed in the hippocampus.

The present results also showed that the plasma adiponectin levels were gradually decreased. The mechanism responsible for the decrease in the plasma adiponectin concentration at 48 hours after TBI in our study remains unclear. The serum TNF-α level has been reported to increase after severe TBI in a human study (19). Similarly, Kamm et al. reported that TNF-a showed a statistically significant increase in the plasma even at 24 hours after TBI in rats (8). Furthermore, it has been shown that there is a negative correlation between TNF-α and adiponectin (9). Therefore, we hypothesize that increased TNF-α expression might be one of the mechanisms responsible for the decrease in the plasma adiponectin level after TBI that was observed in the present study. Several authors have reported a high level of adiponectin immunoreactivity in the ischemic hemisphere in mice (20, 24), whereas adiponectin mRNA was not detected in the brain (24). Taken together with these past results, our data suggest that the immunoreactivity of adiponectin in the brain reflects not the production, but the accumulation, in the injured brain (24).

Based on these findings, we hypothesize that adiponectin may participate in cerebroprotective mechanisms through its anti-inflammatory and/or anti-apoptotic actions after TBI. In addition, we consider that the accumulation (recruitment) of adiponectin in the brain might contribute to the decrease in the plasma adiponectin levels.

CONCLUSIONS

Our findings suggest that adiponectin might participate in the pathophysiological process occurring after TBI. The relationship between adiponectin and its signal after TBI, and the effects of adiponectin administration after TBI should be the subject of future investigations.

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