ORIGINAL ARTICLE

Simvastatin reduces VEGF and NO levels in acute stages of experimental traumatic brain injury

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Abstract This study was undertaken to evaluate the effect of simvastatin, a cholesterol-lowering agent, on vascular endothelial growth factors (VEGFs), nitric oxide (NO) levels and neuroprotection, in rats with experimentally induced traumatic brain injury (TBI). Forty Wistar albino rats were categorized into four groups: sham operated (S), trauma (T), trauma + vehicle (T + V) and trauma + simvastatin (T + S). The T, T + V and T + S groups were subjected to TBI. The T + V group was administered vehicle [ethanol:saline (1/2)] and the T + S group was administered 1 mg/kg of simvastatin 3 h after the injury insult. Blood and brain tissue specimens were obtained 24 h after the trauma to measure VEGFs and NO levels and perform histopathological examinations. The histopathological injury scores of brain tissues were significantly higher in the T group, and simvastatin significantly prevented brain injury in the T + S group. In the T group, significant increases of VEGF levels in serum and brain tissues were noted, which were prevented with simvastatin treatment in the T + S group. The markedly high levels of NO in brain tissues of the T group were decreased by simvastatin treatment in the T + S group. It can be concluded that, as evidenced by histopathological findings, simvastatin treatment improves neuropathology in acute stages of TBI.

Keywords Simvastatin · Traumatic brain injury · Rat · VEGF · NO

Introduction

Traumatic brain injury (TBI) is a common cause of long-term neurological morbidity, with devastating personal and

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social consequences. TBI results from direct impact to the head or from acceleration–deceleration injury, and results in functional deficits due to both primary and secondary mechanisms. Primary injury results from the immediate mechanical damage that occurs at the time of injury. So that primary injury cannot be prevented. Secondary injury evolves over a period which can range from hours to, in some cases, months, induced by biochemical and physiological events after the primary insult [1].

Several biochemical abnormalities responsible for secondary injury have been demonstrated, including disruption of cellular calcium homeostasis, mitochondrial dysfunction [2], increased free radical generation and lipid peroxidation [3–5], inflammation, apoptosis, and diffuse axonal injury [1]. NO, a mediator of biological effects in the brain, is produced by three different isoenzymes: the endothelial form (eNOS), the neuronal form (nNOS) and the inducible form (iNOS). iNOS has been reported to play a potential role in inflammatory reactions and catalyze the synthesis of harmful NO in the injured brain [6, 7]. In addition, iNOS-derived NO is implicated in the pathophysiological mechanisms of secondary brain injury after trauma, via peroxynitrite-related effects [8]. Peroxynitrite ($ONOO^-$) is a powerful oxidant and neurotoxin which is synthesized from NO and superoxide anion (O_2^-) radicals and it may lead to neuronal necrosis and apoptosis [9]. Vascular endothelial growth factor (VEGF) has been described as a vascular permeability factor and as a major angiogenic factor, which promotes proliferation and survival of endothelial cells, and vascular formation [10]. The various effects of VEGF on vascular and endothelial cell permeability in the peripheral circulation have been reported, and appear to involve an increase in endothelial cell calcium influx [11] and synthesis of NO with subsequent activation of guanylyl cyclase [12]. Furthermore, VEGF has been proposed to enhance angiogenesis in the ischemic brain and reduce neurological deficits during stroke recovery; however, the inhibition of VEGF at the acute stage of stroke may reduce the blood–brain barrier (BBB) permeability after focal cerebral ischemia [13].

The therapeutic efficacy of several drugs such as calcium channel blockers, erythropoietin, free radical scavengers, growth factors and statins in animal TBI models by targeting secondary injury mechanisms has been reported [14]. Statins are potent inhibitors of cholesterol biosynthesis. Improvement of endothelial function, increased NO bioavailability, antioxidant properties and inhibition of inflammatory responses, immunomodulatory actions, upregulation of endothelial nitric oxide synthase (eNOS) and decreases in platelet activation are the cholesterol-independent pleiotropic effects of statins. [15]. Therefore, investigation of possible mechanisms of protective effects of statins can be a new clinical approach to treatment of brain injury.

In this study, we aimed to assess the effect of a single low-dose simvastatin treatment on VEGF and NO levels and neuroprotective roles in the acute stage of an experimental TBI model.

Materials and methods

Forty male Wistar albino rats, weighing 300–330 g, from the Animal Laboratory of Duzce University (Duzce, Turkey), were randomly categorized into four groups: Group 1 (S), control rats receiving sham operation only; group 2 (T), rats with TBI; group 3 (T + V), rats with TBI and treated with vehicle [ethanol:saline (1/2)] and group 4 (T + S), rats with TBI and treated with 1 mg/kg simvastatin (ZOCOR®, Merck & Co. Inc., Whitehouse Station, NJ, USA) [16]. This study was approved by the Animal Care and Ethics Committee of Duzce University School of Medicine.

Experimental procedure of TBI

A special weight-drop device developed by Marmarou et al. [17] was used to deliver a standard diffuse traumatic impact. Under ketamine hydrochlorure anesthesia (50 mg/kg intraperitoneally (i.p.), a midline incision was made in the scalp and a metal disk was placed on the skull. After rats were placed in a prone position on the bottom plate of the weight-drop device, a 450-g weight was allowed to fall freely from a height of 2 m onto the metal helmet to induce TBI. Animals which survived after 3 min were used in the study, and those which died during trauma and observation were excluded.

Both vehicle and simvastatin were injected i.p. 3 h after TBI. At 24 h after impact, physiological parameters of the rats were noted and the rats were anaesthetized with an i.p. injection of ketamine hydrochlorure. After intracardiac blood drawing for biochemical analysis, all rats were sacrificed and the brains were carefully removed. The anterior part was finally placed into 10 % formaldehyde for pathological examination and the posterior part was immediately frozen in a nitrogen tank for biochemical analysis. Both brain tissues and blood serums were kept at −80 °C until analysis.

Biochemical analyses

Homogenization of tissues

Tissues were weighed and homogenized in 10 volumes of ice-cold phosphate buffer solution (PBS) (50 mM/L, pH 7.4) using a homogenizer (Heidolph Instruments GmbH 8 Co.K6 Schwabach, Germany DIAX 900). Supernatants of

samples were used for determination of VEGFs and NO levels.

Measurement of lipid parameters

Total cholesterol (TC), low density lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C) levels of serum were measured by the enzymatic colorimetric method (Architect C8000, Abbott Diagnostics, Japan).

Measurement of VEGF

VEGF levels of serum and brain tissue homogenates were measured by the enzyme-linked immunoassay method using commercially available kits (Rat VEGF ELISA kit, RayBiotech, Inc., Norcross, GA, catalog no. ELR-VEGF-001).

Measurement of NO

NO levels of brain tissue homogenates were measured colorimetrically (Cayman Inc., Ann Arbor, MI, USA, kit catalog no. 780001) [18] using a Griess reaction in which nitrate reductase reduces NO, and the end products, nitrite (NO_2^-) and nitrate (NO_3^-), were then measured.

Measurement of protein levels in brain tissue

The protein levels in supernatants of brain tissue homogenates, which were prepared in 50 mM cold potassium phosphate tampon (1:9, m/v), were measured using a method that denatures proteins by benzethonium chloride and measuring the absorbance of turbidity at 404 nm (Shimadzu UV-1201V, Japan) [19].

Histopathological examination

All specimens were fixed in 10 % buffered paraformaldehyde for histopathological examination. Tissue samples were taken from hippocampus, pons and cerebellum. Axial sections were stained with hematoxylin and eosin (H&E). Stained specimens were examined blindly under an Olympus BX40 light microscope by a pathologist.

In the histopathological examination of brain tissues, hemorrhage, edema, neuronal damage (pink ischemic neurons—perineural vacuolization), retraction ball-diffuse axonal damage, vascular congestion and their extensity were estimated.

The semi-quantitative scores reflect the approximate percentage of axonal, neuronal and vascular changes observed in the section. Results were scored as 0 (no changes), 1 [mild changes (<10 %)], 2 [moderate changes

(11–50 %)] or 3 [severe changes (>50 %)] for hemorrhage and edema. Results were scored as 0 (no changes), 1 [mild changes (0–25 %)], 2 [moderate changes (26–50 %)] or 3 [severe changes (>50 %)] for neuronal damage and retraction ball-diffuse axonal damage.

Statistical analyses

Statistical analyses were performed using SPSS (SPSS for Windows, version 13.0, SPSS Inc., Chicago). Variance analysis was performed using a one-way analysis of variance test followed by a Scheffe test for normally distributed data and a Kruskal–Wallis test for data which were not distributed normally. For binary comparisons, a Mann–Whitney U test was used with Bonferonni correction. For statistical analyses of histopathological data, score values were grouped into low (score 0 + score 1) or high (score 2 + score 3) and data were analyzed using four-eyed tables and Chi-square tests. A p value of <0.05 was considered statistically significant.

Results

Physiological measurements

During the experimental procedures, eight rats died and six rats were excluded because of hemolyzed serums, which could not be measured. The remaining rats survived all experimental procedures. There was no difference between the physiological measurements of rats before trauma (0 h) and after trauma (24 h) as regards weight, rectal temperature, respiration rate and heart rate.

Histopathological results

Light microscope images of S, T and T + S groups are shown in Fig. 1. Neuronal damage ($p = 0.015$), retraction ball ($p = 0.015$) and hemorrhage ($p = 0.001$) were significantly higher in the T group than the S group, and these findings were lower in the T + S group than the T group ($p = 0.005$; $p = 0.005$ and $p = 0.001$, respectively) (Table 1).

Biochemical analyses

There were no differences between the T + S group and the other groups in serum levels of TC, LDL-C and HDL-C.

The levels of VEGF and NO are shown in Table 2. The VEGF levels were increased significantly in the T group compared with the S group in terms of brain tissue ($p = 0.004$) and serum ($p = 0.04$). In addition, VEGF levels were significantly decreased with simvastatin
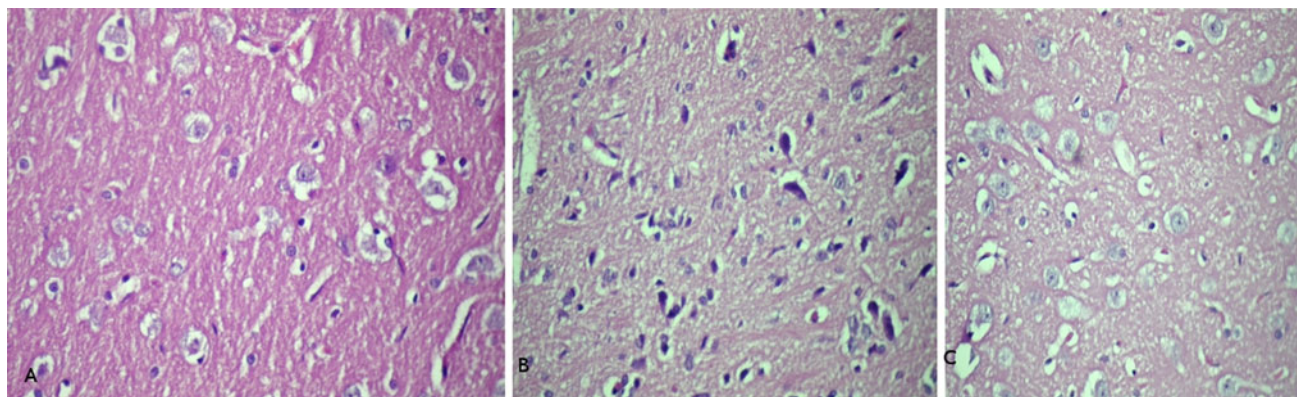
Fig. 1 Light microscopic images of histopathologic sections (H&E ×400). **a** Sham group: normal neuronal distribution. **b** Trauma group: severe neuronal damage and retraction. **c** Trauma + simvastatin group: normal neurons, occasional retraction ball formation

Table 1 The distribution of histopathologic results with high scores (score 2 + score 3) of brain tissues

Groups	Neuronal damage n (%)	Retraction ball-DAD n (%)	Vascular congestion n (%)	Edema n (%)	Hemorrhage n (%)
S ($n = 6$)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
T ($n = 6$)	5 (83.3)[a]	5 (83.3)[a]	2 (33.3)	3 (50.0)[a]	6 (100)[a]
T + V ($n = 7$)	6 (85.7)	6 (85.7)	1 (14.3)	1 (14.3)	6 (85.7)
T + S ($n = 7$)	0 (0)[b]	0 ((0)[b]	0 (0)	0 (0)[b]	0 (0)[b]

n number of the rats, *DAD* Diffuse axonal damage

[a] $p < 0.05$ (compared with S group)

[b] $p < 0.05$ (compared with T group)

treatment in brain tissue ($p = 0.003$) and serum ($p = 0.02$). The VEGF levels of the T and T + V groups did not differ. The NO levels of brain tissues were significantly higher in the T group ($p < 0.049$) than the S group and significantly lower in the T + S group ($p < 0.045$) than the T group. There was no difference between the T and T + V groups.

Discussion

The simvastatin treatment of rats markedly ameliorated the TBI findings as confirmed by microscopic examination and biochemical assays.

Central and lateral fluid impact, wounding with hard objects, acceleration (weight drop from a height), local stress, injection, cold injury and penetrating injury models have been developed for mimicking the brain injury in humans. Acceleration and fluid impact models have been reported to simulate cerebral contusion, however, the acceleration model has been suggested to be a useful model for diffuse brain injury [20], and therefore, the acceleration model developed by Marmarou et al. was chosen in this study.

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors have been developed as drugs which reduce the mortality and morbidity of coronary and cerebrovascular diseases [21]. In addition to the lipid-lowering effects, statins have been reported to reduce thrombosis, lesion volume and vascular damage after TBI [22]. Also, statins have been proposed to enhance neurogenesis, angiogenesis and synaptogenesis after stroke and to induce significant neurological improvement [23]. Urbich et al. [24] determined that low concentration of atorvastatin (0.01–0.1 µmol/L) stimulates angiogenesis in endothelial cells and high concentrations of atorvastatin (>0.1 µmol/L) have antiangiogenic effects. So it is suggested that the different effects of statins on angiogenesis can depend on dose, cell type and the degree of hypoxia.

Simvastatin was chosen from a large number of statins for this study because it is the most lipophilic statin and is able to pass the BBB [25]. In experimental TBI models, low-dose (1 mg/kg) and high-dose (37.5 mg/kg) simvastatin was given to rats but the treatment was continued for 14–35 days after injury [26, 27]. In this study, 1 mg/kg simvastatin given 3 h after trauma ameliorated the histopathological results of brain tissues and decreased the VEGFs and NO levels in acute stages of the trauma.

Table 2 The VEGF and NO levels of serum and brain tissue

	S ($n = 6$)	T ($n = 6$)	T + V ($n = 7$)	T + S ($n = 7$)	p
VEGF (pg/mg protein)**	2.3 ± 0.19 (1.7–2.8)	4.2 ± 1.32[a,b] (3.6–12.0)	2.4 ± 0.83 (1.8–8.1)	2.3 ± 0.12 (1.7–2.5)	0.006
VEGF serum (pg/mL)*	4.61 ± 1.7	8.42 ± 3.0[c,d]	3.86 ± 2.6	4.10 ± 1.3	0.004
NO (μmol/mg protein)*	6.3 ± 1.2	8.6 ± 1.5[a,b]	7.2 ± 1.0	6.4 ± 1.6	<0.019

* Data are given as mean ± SD. ** Data are given as median ± SEM (min–max)

[a] $p = 0.049$ when compared with S group

[b] $p = 0.045$ when compared with T + S group

[c] $p = 0.04$ when compared with S group

[d] $p = 0.02$ when compared with T + S group

Deterioration of the BBB under hypoxic conditions is multifactorial, and among the reasons are the increases in VEGF, NO and inflammatory cytokines [13]. Hypoxia stimulates hypoxia-induced factor-1α (HIF-1α) which regulates the gene transcription of VEGF [28]. Also, HIF-1α is a transcriptional activator of iNOS and increases NO formation [29]. Blood vessels which are developed in response to VEGF are leakier than normal blood vessels and increase cerebral edema and ischemic damage. Recombinant human VEGF (rhVEGF) treatment 48 h after stroke enhanced cerebral microperfusion and functional neurological recovery and rhVEGF treatment 1 h after stroke enhanced the leakage of the BBB, hemorrhage and ischemic damage [13]. It has been suggested that VEGF increases vascular permeability by stimulating NO synthesis and release. The inhibition of VEGF in acute stages after brain damage has been reported to reduce BBB permeability [30]. Zhu et al. [31] have shown that simvastatin prevented the angiogenesis by inhibiting the VEGF increase in a pro-inflammatory environment created by tumor necrosis factor-α. In addition, simvastatin treatment decreased the VEGF levels 3 h after TBI in an inflammatory environment in this study. Moreover, it has been shown that simvastatin treatment decreased the hemorrhage, edema, neuronal damage, diffuse axonal damage, vascular congestion and their extensity in histopathological examinations.

During normal metabolism, NO molecules are synthesized by nitric oxide synthase (NOS) from L-arginine and have many roles in many biological mechanisms, which involve arranging transport of ions and other molecules in the BBB. However, NO synthesized by iNOS has harmful effects after TBI, via increasing BBB permeability and contributing to vasogenic edema [32]. Terpolilli et al. [33] observed that administration of a new NOS inhibitor, 4-amino-tetrahydro-L-biopterine, to inhibit the formation of iNOS in brain edema, provides a significant reduction in long-term neurological dysfunction. In addition, studies have reported that inhibition of NO synthesis by minosycline, melatonin and simvastatin reduced the hypoxia-induced BBB damage [29]. In this study, simvastatin treatment significantly decreased the levels of VEGF in both serum and brain tissue and the NO levels in brain tissue after TBI.

In conclusion, it can be considered that simvastatin treatment in acute stages of experimental TBI improves neuropathological changes of diffuse axonal injury by reducing the levels of VEGF and NO in inflammation and preventing the development of vasogenic brain edema due to BBB leakage independent of cholesterol-lowering effects. In further investigations, histochemical and electron microscopy analysis of neurons may provide supportive information in respect of the simvastatin effects on TBI.

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