

The Neuroprotective Effect of Caffeic Acid Phenethyl Ester on Global Ischemia-Reperfusion Injury in Rat Brains ^[1]

Muhammed Enes ALTUĞ ¹  İsmet M. MELEK ² Suat ERDOĞAN ³
Vesile DÜZGÜNER ⁴ Atakan ÖZTÜRK ⁵ Altuğ KÜÇÜKGÜL ⁶

^[1] This study was presented as poster presentation at the XXVth International Symposium on Cerebral Blood Flow, Metabolism and Function and the Xth International Conference on Quantification of Brain Function with PET. May 24-28, 2011, Barcelona, Spain

¹ Department of Surgery, Faculty of Veterinary Medicine, Mustafa Kemal University, TR-31040 Hatay - TURKEY

² Department of Neurology, Tayfur Ata Sökmen Medical School, Mustafa Kemal University, TR-31034 Hatay - TURKEY

³ Department of Medical Biochemistry, School of Medicine, Zirve University, TR-27260 Gaziantep - TURKEY

⁴ School of Health Sciences, Ardahan University, TR-75000 Ardahan - TURKEY

⁵ Department of Physiology, Tayfur Ata Sökmen Medical School, Mustafa Kemal University, TR-31034 Hatay - TURKEY

⁶ Department of Biochemistry, Faculty of Veterinary Medicine, Mustafa Kemal University, TR-31040 Hatay - TURKEY

Article Code: KVFD-2014-11228 Received: 18.03.2014 Accepted: 14.04.2014 Published Online: 28.04.2014

Abstract

The aim of this study was to investigate the neuroprotective effects of caffeic acid phenethyl ester (CAPE) on phosphodiesterase 4 (PDE4) mRNA isoenzymes, oxidant and antioxidant defence in ischemia/reperfusion (I/R) injured rat brains. Twenty-one rats were randomly divided into three equal groups: sham-control, ischemia/reperfusion (I/R) and I/R+CAPE. Rats in sham-control group underwent only surgical intervention without bilateral common *carotid artery* occlusion. Ischemia/reperfusion was induced by bilateral common *carotid artery* occlusion with atraumatic clips for 30 min, followed by artery reopening. The I/R+CAPE group was subjected to the same surgical procedure as I/R group, but CAPE was administered intraperitoneally at the dose of 15 $\mu\text{mol kg}^{-1}$ twice, 1 h before occlusion and at 12th h of reperfusion. The rats were sacrificed 24 h after I/R. The cAMP concentration was analyzed by ELISA and PDE4 isozyme mRNA transcriptions were evaluated by qRT-PCR methodology in the brain cortex. Ischemia-induced NO production was significantly attenuated by CAPE in the cerebral cortex. CAPE significantly enhanced GSH-Px activity, while SOD, CAT and XO activities non-significantly changed, as compared to the I/R group. CAPE significantly decreased PDE4A and PDE4B transcripts, without changing cAMP levels compared to I/R group. Ischemia-induced neurologic deficit scores were reduced by CAPE. These results suggest that CAPE slightly modulates the antioxidant defense system and NO release in rat brain during global cerebral ischemia/reperfusion injury. In addition, CAPE treatments produce the neuroprotective effect by reducing the levels of some PDE4 transcriptions.

Keywords: CAPE, Brain, Ischemia/reperfusion, Antioxidant activity, cAMP-phosphodiesterase 4, Neuroprotective effect, Rat

Rat Beyinlerinde Global İskemi-Reperfüzyon Hasarı Üzerine Kafeik Asit Fenetil Esterin Nöroprotektif Etkisi

Özet

Bu çalışma iske-mi-reperfüzyon (I/R) hasarlı rat beyinlerinde fosfodiesteraz 4 (PDE4) mRNA izoenzimleri, oksidant ve antioksidant savunma sistemi üzerine kafeik asit fenetil ester (KAFE)'in nöroprotektif etkilerini araştırmak amacıyla yapıldı. Yirmi bir adet rat rastgele üç eşit gruba ayrıldı. Sham-kontrol, iske-mi/reperfüzyon (I/R) ve I/R+KAFE. Sham-kontrol grubundaki ratlara bilateral common *carotid arter* oklüzyonu yapılmaksızın sadece cerrahi müdahalede bulunuldu. İske-mi/reperfüzyon (I/R) bilateral common *carotid arter*lerin atravmatik klempler ile 30 dakika oklüzyonu ve takiben arter klempleri açılarak reperfüzyonu ile sağlandı. I/R+KAFE grubu I/R grubu ile aynı cerrahi üsüle tabi tutuldu fakat oklüzyondan 1 saat önce ve reperfüzyondan 12 saat sonra iki defa 15 $\mu\text{mol kg}^{-1}$ dozunda intraperitoneal KAFE verildi. Ratlar iske-mi/reperfüzyondan 24 saat sonra sakrifiye edildi. Beyin korteksindeki cAMP düzeyi ELISA ile, PDE4 mRNA izoenzim transkripsiyonları ise qRT-PCR ile değerlendirildi. KAFE iske-mi ile uyarılan beyin korteksindeki NO üretimini önemli oranda azalttı. I/R grubu ile karşılaştırıldığında SOD, CAT ve XO aktivitelerini KAFE anlamlı düzeyde değiştirmezken, GSH-Px aktivitesini önemli oranda arttırdı. KAFE cAMP düzeyini değiştirmeksizin PDE4A ve PDE4B düzeyini önemli oranda azalttı. İske-mi ile uyarılan nörolojik hasar skorları KAFE tarafından azaltıldı. Bu sonuçlar KAFE'nin global beyin iske-mi/reperfüzyon hasarı sırasında rat beyinlerinde antioksidant savunma sistemini ve NO salınımını hafifce dengelediğini önerir. Ayrıca KAFE bazı PDE4 izoenzim düzeylerini azaltarak nöroprotektif etki sağlar.

Anahtar sözcükler: KAFE, Beyin, İske-mi/reperfüzyon, Antioksidant aktivite, cAMP-fosfodiesteraz 4, Nöroprotektif etki, Rat



İletişim (Correspondence)



+90 326 2455845/1512



enesaltug@gmail.com

INTRODUCTION

Reactive oxygen radicals (ROS) are likely participants in the pathogenesis of cerebral ischemia-reperfusion (I/R) injury. Transient focal and global cerebral I/R triggers a plethora of cellular and molecular events that promotes neuronal cell death in several regions of the brain due to glutamate excitotoxicity, oxidative stress, inflammation and apoptosis [1-4]. Studies show that nitric oxide (NO) has beneficial properties to I/R injury including increase of blood flow produced by cerebral vasodilatation and inhibition of inflammation [1,5]. NO is also a free radical and initiates various pathophysiological events by reaction with superoxide anion to form peroxynitrite, on the contrary of its protective effects in various models of I/R injury [1,5,6].

Mammalian cyclic nucleotide phosphodiesterases (PDEs) are composed of 21 genes and are categorized into 11 families based on sequence homology, enzymatic properties, and sensitivity to inhibitors, the enzymes that hydrolyze and inactivate cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) [7]. As PDE4 is the major cAMP-hydrolysing family in many cells types, it represents a promising therapeutic target. The PDE4 family is composed of four subfamilies (PDE4A, PDE4B, PDE4C and PDE4D) encoded by different gene loci, and each of them has been shown to produce several mRNAs by alternative splicing [8]. Most recently, it has demonstrated that rolipram, selective PDE4 inhibitor, attenuates memory deficits produced by global brain ischemia [9].

Caffeic acid phenethyl ester (CAPE), an active component of propolis, has been shown to possess anti-inflammatory, immunomodulatory, anticarcinogenic, and antioxidant properties [1,2,10,11]. It has also been shown that CAPE treatment significantly reduces the infarction size and neuronal damage in ischemia-induced brain injury and suppresses cerebral lipid peroxidation [1,2,11]. To date, no studies have been reported that how CAPE treatment affects the levels of cAMP and PDE4s after transient global brain ischemia. For this purpose, the present study was designed to evaluate the neuroprotective effects of CAPE on PDE4 mRNA isoenzymes, oxidant and antioxidant defence in transient global cerebral ischemia rat model.

MATERIAL and METHODS

Transient Global Cerebral Ischemia in Rats

Experimental procedures were approved by Mustafa Kemal University, Veterinary Faculty Ethics Committee for the use and care of laboratory animals (25.04.2007, no:09). Experiments were performed on adult male wistar rats weighing 250-275 g. They were given free access to food and tap water. All animals were maintained under a controlled temperature ($21\pm 1^\circ\text{C}$) and humidity (55-60%) throughout the experiment. Twenty-one healthy rats were

randomly divided into three equal groups: Sham-control, ischemia/reperfusion (I/R), I/R+CAPE. The experimental and surgical procedures in groups were performed as in the following: All rats were anesthetized intraperitoneally with xylazine hydrochloride (4-5 mg/kg, Rompun, Bayer, Turkey) and ketamine HCl (40-50 mg/kg, Alfamine, Egevet, Turkey) and placed on heat blanket during surgical operation. Sham-control (sham-operated) group: Rats underwent only surgical intervention without bilateral common carotid artery (BCCA) occlusion. Briefly, the right and left common carotid arteries (CCAs) were isolated through a ventral midline cervical incision and separated carefully from vagosympathetic nerve. Ischemia-reperfusion (I/R) group: The common carotid arteries (CCAs) were isolated through a ventral midline cervical incision and separated carefully from vagosympathetic nerve by microsurgical procedures. Transient global cerebral ischemia was achieved by temporarily occluded (30 min) CCAs using atraumatic aneurysm clips and the opening in the skin was closed with wound clips. Reperfusion was achieved by declamping the arteries after 30 min. The surgery line was routinely closed. The rats in the sham-control and I/R groups were received intraperitoneally dimethyl sulfoxide (DMSO) at the dose of $15\ \mu\text{mol kg}^{-1}$ twice, 1 h before occlusion and at 12 h of reperfusion. Ischemia-reperfusion + CAPE (I/R + CAPE) group: The rats in this group were subjected to ischemia/reperfusion with the same procedure in I/R group as mentioned above and treated with CAPE (Sigma, Germany). CAPE was dissolved in sterile DMSO (Sigma, Germany) and administered intraperitoneally at the dose of $15\ \mu\text{mol kg}^{-1}$ twice, 1 h before occlusion and at 12 h of reperfusion. The CAPE doses used were chosen on the basis of previous experiments [2,11]. Body temperature was maintained at approximately $35.2\pm 0.4^\circ\text{C}$ with a heating pad until the animal had recovered from surgery. The rats were sacrificed 24 h after reperfusion with the anesthetic procedure reported above. Venous blood samples (5 ml) were taken by cardiac puncture into tubes with EDTA. Plasma was separated by centrifugation at 3,000 rpm for 15 min and was stored at -20°C until use. Immediately after taking blood specimen, brain was carefully removed, washed with ice-cold physiological saline and were rapidly stored at -20°C until analyses.

Assessments of Neurologic Deficit and Behavior

A neurologic evaluation was performed 24 h after the onset of the experiments by an investigator blinded to the study groups, using a neurologic deficit score (NDS) as described previously, with several modifications [3,12-14]. The four categories of reactions and the functions with associated tested were: the general behavioral deficit, cranial nerve reflexes, sensory-motor deficit, balance and coordination. The presence or absence of the appropriate reaction was scored. See [Table 1](#) for the exact procedure used. The NDS could range from 0 to 100, an NDS of 0 reflects normal brain function and an NDS of 100 reflects brain death.

Tissue Homogenization

Tissue samples were homogenized in a PBS buffer (pH 7.0) containing complete protease inhibitor mixture (Sigma, Germany). Homogenates were centrifuged at 4°C, 15.000 rpm for 10 min and the soluble fraction was retained. Protein concentrations of supernatants were measured by the method of Bradford [15] using bovine serum albumin as a standard.

The Analysis of Oxidant/Antioxidant Stress Markers

Lipid peroxidation levels were assessed by measuring malondialdehyde (MDA) concentration in tissues ($\mu\text{mol}/\text{mg}$ protein) [16]. The method was based on thiobarbituric acid (TBA) reactivity. 2.5 mL of 20% trichloroacetic acid was added to the 0.5 mL of plasma and then 1 mL of 0.675% TBA was added. The coupling of lipid peroxide with TBA was carried out by heating at 95°C water bath for 30 min. After cooling in cold water, the resulting chromogen was extracted with 4.0 mL of n-butyl alcohol by vigorous shaking. Separating of the organic phase was facilitated by centrifugation at 3.000 rpm for 10 min and its absorbance was determined at 535 nm by spectrophotometer.

Catalase (CAT) activity was measured according to the method of Luck [17]. One unit of CAT activity was defined as the amount of enzyme required to decompose 1 mol of H_2O_2 in 1 min in a tube containing 2.95 mL of a freshly prepared 30% H_2O_2 in phosphate buffer (pH 7.0), 50 μl of tissue supernatant or plasma were added.

The rate of decomposition of H_2O_2 was measured spectrophotometrically at 240 nm for 1 min. Using the reaction time (Δt) of the absorbance (A_1 and A_2), the following equation was generated to calculate the rate constant (k): $k = (2.3/\Delta t)(\log A_1/A_2)$. The enzyme activity was expressed as k/mg protein in tissues. Total superoxide dismutase (SOD) activity in the homogenates was determined according to the method of Sun and colleagues [18]. The method is based on the inhibition of nitroblue tetrazolium (NBT) reduction by the xanthine/xanthine oxidase system as a superoxide generation. The enzyme activity was measured in the ethanol phase of the lysate after addition of 1.0 ml ethanol/chloroform mixture (5/3, v/v) to the same amount of sample and the tubes were centrifuged. One unit of SOD was defined as the enzyme amount causing 50% inhibition in NBT reduction rate. SOD activity was expressed as U/mg protein in tissues. Nitric oxide (NO) concentration in plasma ($\mu\text{mol}/\text{L}$) and tissue ($\mu\text{mol}/\text{mg}$ protein) samples were determined indirectly by measuring the nitrite levels based on Griess reaction [19]. Samples were firstly deproteinized with 75 mmol zinc sulphate. Total nitrite was determined by spectrophotometer at 545 nm after conversion of nitrate to nitrite by copperized cadmium granules. Xanthine oxidase (XO) activity was measured as the rate of uric acid production when xanthine was incubated with tissue homogenates (U/g protein) [20]. Glutathione peroxidase (GSH-Px) activity was detected in the tissue homogenates by a kinetic method using a commercial kit (RANSEL by Randox Lab. UK). GSH-Px

Table 1. Neurologic deficit scores (NDS) for rats with global cerebral ischemia-reperfusion

Tablo 1. Global beyin iskemi-reperfüzyonlu ratlarda nörolojik hasar skorları

Neurologic Evaluations	Function Studied	Grades and Points
1. General behavioral deficits	Consciousness	Explore spontaneously (0)
		Sleepy (5)
		Epileptic seizures (10)
		No attempt (comatose) (20)
	Respirations	Normal (0) Abnormal (10)
2. Cranial nerve reflexes	Feeding	Normal (0) Absent (5)
	Water intake	Normal (0) Absent (5)
	Vision (follows hand)	Present (0) Absent (5)
	Corneal reflex	Present (0) Absent (5)
3. Sensory motor deficits	Whisker movement	Present (0) Absent (5)
	Hearing (turning to clapped hands)	Present (0) Absent (5)
4. Balance and coordination	Leg and tail movements	Normal (0) Paralysis (20)
	Ability to walk	Present (0) Absent (5)
	Righting reflex	Present (0) Absent (5)
Total score	Climbing the wall of a wire cage	Normal (0) Weakness of one limb (5) Weakness of more than one limb (10)
		100

activity was expressed as U/mg protein. The intracellular cAMP concentration was determined in the brain homogenates (Cayman Chemical, USA) using enzyme-linked immuno-sorbent assay (ELISA).

RNA Isolation and Real-Time QRT-PCR Analyses

Transcription levels of samples were performed by a qRT-PCR system (CFX96 Touch™-USA). Total RNA from tissues was extracted using TRIZOL reagent (Sigma, USA) according to the manufacturer's instructions. The rat primer sets (Thermo Electron Corporation, Germany) used for PCR reactions are given in [Table 2](#). B-Actin was used as endogenous control, and each sample was normalized on the basis of its β -actin content. Cycling conditions included reverse transcription at 42°C for 30 min, incubation at 94°C for 30 s and 40 cycles of 94°C for 10 s [a denature temperature of PCR profile at 95°C for 5 s, according to the manufacturer's instructions (SYBR Green Quantitative RT-PCR Kit, Sigma)] and 60°C for 10 s for annealing and 72°C for 30 s extension step. The cycle number required to achieve a definite fluorescence signal (crossing point, CP) was calculated by the second derivative maximum method (CFX Manager™ software, qbase^{PLUS}).

Statistics

Statistical analyses were accomplished with the use of the SPSS computer program (version 13.0). All data were expressed as mean±S.E. The differences between groups in biochemical, molecular and neurological deficit scores were evaluated using one-way analysis of variance (ANOVA) with Tukey's tests for post hoc comparisons. *P* values less than 0.05 were considered statistically significant.

RESULTS

CAPE Attenuates Ischemia-Induced Neurologic Deficit Scores

In the current study, three rats died as total, ischemia/reperfusion (I/R) group: 2, and I/R + CAPE group: 1. No rat died in the sham-control group. Ischemia-induced neurologic deficit scores were significantly higher than the sham-operated rats (67.4±12.3, 18.5±1.3, respectively, [Fig. 1](#), *P*<0.01). CAPE administration was able to attenuate ischemia-induced neurologic deficit scores 24 h after I/R (46.4±12.1, 67.4±12.3, respectively, [Fig. 1](#), *P*>0.05).

CAPE Slightly Modulates the Oxidant and Antioxidant Defense System

Transient global cerebral ischemia caused to significant increase in NO levels in brain homogenates, and this elevation was significantly inhibited by CAPE treatment (*P*<0.05, [Table 3](#)). On the other hand, NO production was decreased in plasma samples of occluded animal, but CAPE reserved plasma NO suppression (*P*<0.05, [Table 3](#)). CAPE non-significantly decreased ischemia-induced cerebral cortex MDA concentration ([Table 3](#)). The activity of cerebral cortex XO significantly increased in the ischemic group (*P*<0.05, [Table 3](#)), but CAPE non-significantly decreased this elevation. Significant increase in cerebral cortex SOD activity in the ischemia group (*P*<0.01, [Table 3](#)) was not effectively changed by CAPE. While there was no difference in the cerebral cortex CAT activity between the groups, CAPE significantly prevented the reduction in GSH-Px activity caused by ischemia (*P*<0.05, [Table 3](#)).

Table 2. List of primer sequences used for RT-PCR analyses

Tablo 2. RT-PCR analizi için kullanılan primer dizileri

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
PDE4A	GCG GGA CCT AGC TGA AGA AAT TCC	CAG GGT GAG TCC ACA TCG TGG
PDE4B	CAG CTC ATG ACC CAG ATA AGT GG	GTC TGC ACA AGT GTA CCA TGT TGC G
PDE4C	ACT GAG TCT GCG CAG GAT GG	CAC TCC TCT TCC TCT GCT CTC CTC
PDE4D	CCC TCT TGA CTG TTA TCA TGC ACA CC	GAT CCT ACA TCA TGT ATT GCA CTG GC
β -actin	CAT CGT CAC CAA CTG GGA CGA C	CGT GGC CAT CTC TTG CTC GAA G

Table 3. Effects of CAPE administration (15 μ mol kg⁻¹) on the antioxidant-oxidant enzyme activities and cAMP levels in cerebral cortex, and NO in plasma after 24 h ischemia-reperfusion injury

Tablo 3. İskemi-reperfüzyon hasarından 24 saat sonra plazma NO ile cerebral cortex antioksidant-oxidant enzim aktiviteleri ve cAMP düzeyleri üzerine KAFE (15 μ mol kg⁻¹)'in etkileri

Groups	MDA (μ mol/mg protein)	NO (μ mol/mg protein)	NO (μ mol/L plasma)	SOD (U/mg protein)	CAT (k/mg protein)	GSH-Px (U/mg protein)	XO (U/g protein)	cAMP (pmol/mg protein)
Sham-control	0.49±0.04	2.80±0.83	7.5±1.63	0.26±0.01	0.191±0.02	10.8±0.93	0.54±0.04	2.13±0.11
I/R	0.67±0.06	5.04±0.48*	2.1±0.86 [†]	0.53±0.04 [†]	0.186±0.02	6.9 ±0.29 [‡]	1.44±0.19 [#]	1.98±0.14
I/R+CAPE	0.57±0.14	3.02±0.77	9.6±1.27	0.55±0.06 [#]	0.219±0.04	10.6±1.22	1.32±0.19*	1.94±0.08

Data were presented as mean ± S.E. from six rats in each group. * *P*<0.05, [†] *P*<0.05, [‡] *P*<0.01 vs sham-control group, [#] *P*<0.05 vs sham-control and I/R+CAPE group, I/R: Ischemia-reperfusion

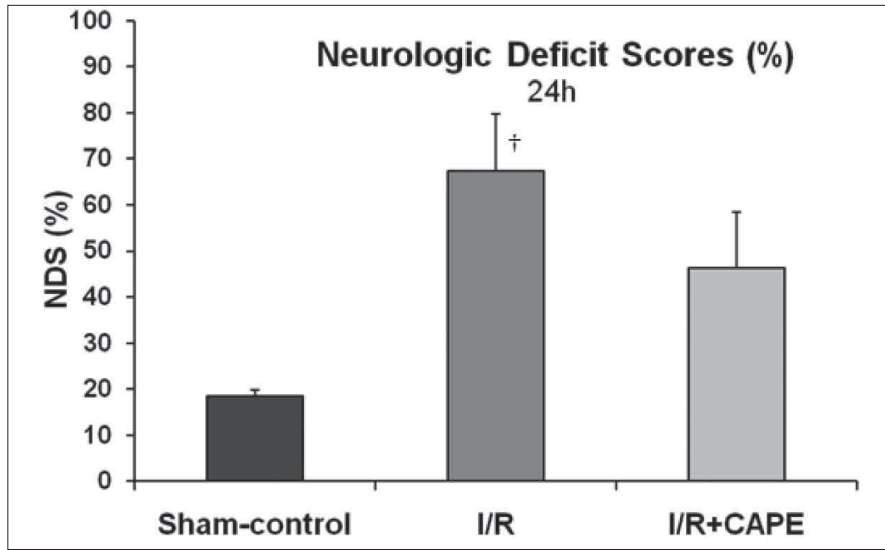


Fig 1. Effects of CAPE on neurologic deficit scores after 24 h ischemia-reperfusion injury. Ischemia-stimulated neurologic deficit scores were attenuated by CAPE. Data are presented as mean \pm S.E. from seven rats in each group. [†] P<0.01 vs sham-control group. I/R: ischemia/reperfusion

Şekil 1. İskemi-reperfüzyondan 24 saat sonra nörolojik hasar skorları üzerine KAFE'in etkisi. İskemi ile uyarılan nörolojik hasar skorları KAFE tarafından azaltıldı. Değerler her bir gruptaki 7 rat'ın ortalama \pm standart hatası olarak verildi. [†] P<0.01, sham-kontrol grubu ile karşılaştırıldığında. I/R: iskemi/reperfüzyon

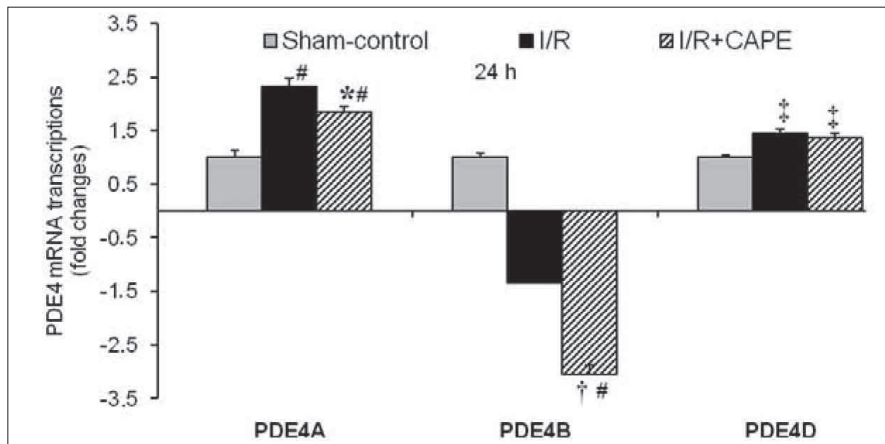


Fig 2. The fold change values of PDE4A, PDE4B and PDE4D mRNA transcriptions in qRT-PCR analyses after 24 h ischemia-reperfusion injury.

Global cerebral I/R compared to sham-control PDE4A and PDE4D transcriptions were elevated by 2.31 and 1.44-folds after 24 h, respectively. PDE4B transcription was decreased by 2.34-fold. CAPE compared to I/R significantly decreased PDE4A and PDE4B transcriptions by 1.25 and 2.26-folds, respectively. Non-significant decrease of PDE4D transcription was detected after 24 h. Neither global cerebral I/R nor CAPE unchanged PDE4C transcription in the rat cerebral cortex (data not shown)

Data were presented as mean \pm S.E. from six rats in each group. * P<0.05, [†] P<0.001 vs I/R group; [#] P<0.05, ^{*#} P<0.001, vs sham-control group; I/R: Ischemia/reperfusion

Şekil 2. İskemi-reperfüzyon hasarından 24 saat sonra qRT-PCR analizinde PDE4A, PDE4B ve PDE4D mRNA kat değişim değerleri.

Sham-kontrol ile karşılaştırıldığında global beyin I/R, PDE4A ve PDE4D transkripsiyonlarını 24 saat sonra söylendiği sıra ile 2.31 ve 1.44 kat arttırdı. PDE4B transkripsiyonu 2.34 kat azaltıldı. I/R ile karşılaştırıldığında KAFE, PDE4A ve PDE4B transkripsiyonlarını söylendiği sıra ile 1.25 ve 2.26 kat önemli oranda azalttı. PDE4D transkripsiyonu 24 saat sonra önemsiz oranda azaldı. Global I/R ve KAFE rat beyin korteksinde PDE4C transkripsiyonunu değiştirmede (veri gösterilmedi).

Değerler her bir gruptaki 6 rat'ın ortalama \pm standart hatası olarak verildi. * P<0.05, [†] P<0.001, I/R grubu ile karşılaştırıldığında; [#] P<0.05, ^{*#} P<0.001, sham-kontrol grubu ile karşılaştırıldığında; I/R: İskemi/reperfüzyon

CAPE Inhibits Global Cerebral Ischemia-Induced Increases in PDE4 mRNA Expression

Ischemia-induced cerebral cortex cAMP levels were not changed by CAPE treatment (Table 3). Global cerebral I/R compared to sham-control raised PDE4A ($P < 0.001$) and PDE4D ($P < 0.05$) mRNA transcripts by 2.31 and 1.44-folds in rat cerebral cortex 24 after I/R, respectively (Fig. 2), and also decreased PDE4B by 2.34-fold (Fig. 2, $P < 0.001$). Whereas, CAPE treatment significantly decreased PDE4A ($P < 0.05$) and PDE4B ($P < 0.001$) expressions compared to I/R by 1.25 and 2.26-folds 24 h after I/R, respectively (Fig. 2). CAPE has also non-significantly decreased PDE4D transcription by 1.05-fold. Neither ischemia-reperfusion nor CAPE unchanged the PDE4C mRNA transcription in the rat cerebral cortex (data not shown).

DISCUSSION

So far, CAPE treatment has not been reported how it affects the levels of cAMP and PDE4 transcripts. We found that the global cerebral ischemia/reperfusion (I/R) and CAPE treatment non-significantly decreased in cerebral cortex cAMP levels after 24 h reperfusion (Table 3). Our cAMP findings are partially consistent with those of Choi et al.^[21] 24 h after I/R. As similar to that reported in an earlier study^[22], CAPE treatment may influence the cAMP levels by increasing activity of cAMP-responsive element binding protein, and thus it may inhibit ischemia-induced oxidative stress and inflammation. A recent study^[9] has demonstrated that cerebral ischemia led to increases in activity of PDE, primarily PDE4. However, it is still unknown whether specific PDE4 subtypes are differentially expressed after global cerebral I/R injury in rat cerebral cortex. This study firstly explains that the cerebral I/R significantly raised PDE4A and PDE4D expressions in the rat cerebral cortex at 24 h reperfusion, and also PDE4B mRNA was decreased (Fig. 2). Accordingly, a more recent study reports increased PDE4D expression following global cerebral ischemia^[23]. In addition, despite different experimental models, our data are in agreement with PDE4A increases newly reported by traumatic brain injury^[24]. The phosphodiesterase (PDE4) is the predominant PDE isozyme in various leukocytes and plays an important role in the regulation of inflammatory cell activation^[7]. Consistent with our data previous studies reported that the brain PDE4A, PDE4B and PDE4D mRNA transcripts are highly expressed, whereas PDE4C is absent^[25,26]. Furthermore, previous studies observed that chronic antidepressant treatment increased PDE4A and PDE4B gene expression in rat cerebral cortex, but PDE4D gene expressions were unchanged^[25,27,28]. Additionally, our results firstly shown that CAPE treatment significantly decreased PDE4A and PDE4B transcripts 24 h after I/R compared to I/R (Fig. 2). However, PDE4D transcription was not significantly changed (Fig. 2). The current study clearly suggests that CAPE treatments produce the neuro-

protective effect by reducing the levels of some PDE4 isozyme transcriptions with a mechanism similar to phosphodiesterase inhibitors, and also may be useful for the treatment of cerebral ischemia.

The present study clearly showed that the cerebral cortex NO production was stimulated 24 h after global cerebral I/R (Table 3), and this was inhibited by CAPE treatment. CAPE, a structural derivative of flavonoids, possesses its antioxidant properties by inhibiting the gene expressions and/or catalytic activity of certain free radical producing enzymes such as NOS^[10,29]. In consistent with the NO sera data presented here (Table 3), Tsai and colleagues^[1] noted that pretreatment with CAPE increased NO bioavailability in plasma at 24 h of reperfusion in rats subjected to focal and global brain ischemia. CAPE is able to inhibit the reaction of NO with superoxide anion to prevent the formation of peroxynitrites which is more toxic oxidant than either NO or superoxide anion alone^[1]. It was previously reported that NO has protective effects during ischemic injury, although in a narrow concentration range, overproduction may facilitate or mediate neurotoxicity^[5,6,30]. Indeed, NO could act with a dual action either protective or pro-oxidant^[6]. This dual effect of NO in cerebral ischemic injury has been suggested that the effects depend on the stage of evolution of tissue damage and NOS isoforms. Immediately after induction of ischemia, NO is synthesized by endothelial NOS (eNOS) and neuronal NOS (nNOS), but later times after ischemia NO is synthesized by iNOS^[5]. As previously reported^[5,6], we suggest that an increase in NO production may be due to stimulation of all NOS subtypes expression in the brain. In addition, the increase in NO bioavailability induced by CAPE is attributed to its strong free radical scavenging ability in lipophilic environments.

Xanthine oxidase (XO) is an important oxidant enzyme which catalyzes the reduction of O_2 initiating to the formation of superoxide anion and H_2O_2 . In the present study, ischemia-stimulated increases in the XO activity could be reduced by CAPE (Table 3). A few studies have reported similar results in the XO activity in relation to I/R injury, which is time and dose dependent manner^[1,11]. Similar to our study, it is demonstrated that ischemic circumstances lead to the accumulation of hypoxanthine and stimulates XO activity^[29]. The activity of GSH-Px, which detoxifies H_2O_2 while oxidizing reduced GSH to oxidized GSSG, was depressed during I/R^[4]. In the present study, cerebral cortex GSH-Px activity significantly decreased after 24 h ischemia. However, the decrease in the GSH-Px activity was prevented by CAPE treatment (Table 3). The curative effect of CAPE on GSH-Px activity could be due to scavenging ROS produced during oxidative stress^[31]. Superoxide dismutase (SOD) is an antioxidant enzyme which plays key role to convert the superoxide anion to less toxic compound H_2O_2 and molecular oxygen. Toyoda and Lee^[32] showed that SOD activity enhanced in some

ischemic regions of brain parallel to this study (Table 3). Horakova and colleagues [33] have reported a reduction in GSH-Px activity, but an increase in the SOD activity in rat brain ischemia model induced by the ligation of the common carotid artery for 260 min and followed by a reperfusion of 10 min. Another study [34] reported that MnSOD increased in hippocampus 24, 48 and 72 h after ischemia, coincident with the marked reduction in the activity of glutathione-related enzymes. In the presented study, CAPE exhibited a slight antioxidant effect with regarding to cerebral cortex SOD and CAT enzyme activities (Table 3). Our CAT results are in agreement with the study of Mishra and colleagues [35]. It has also been explained the stimulation of antioxidant enzymes in brain I/R as the transient substrate induction [36]. Therefore, it may be explained that free radicals produced in moderate ischemia were not intense enough to affect the catalase activity which is kept in peroxisomes.

The neurological evaluations shown that CAPE treatment could attenuate ischemia-induced cerebral neurologic deficit scores (Fig. 1). This finding is in agreement with our previous study showing that CAPE reduces the infarction percentage and neurological damage against focal permanent middle cerebral artery occlusion [11]. In addition to its antioxidant properties, this neuroprotective effect is supported further by the decrease of PDE4 isoforms in this current study. Therefore, we propose that CAPE plays a protective role for therapy against neuronal death after transient BCCA occlusion with its preconditioning and therapeutic effects.

In conclusion, these results suggest that CAPE administrations slightly modulate the antioxidant defense system and NO release in rat brain during peracute global cerebral ischemia-reperfusion injury. In addition, CAPE treatment produces the neuroprotective effect by reducing the levels of some PDE4 isosyme transcriptions.

REFERENCES

1. Tsai SK, Lin MJ, Liao PH, Yang CY, Lin SM, Liu SM, Lin RH, Chih CL, Huang SS: Caffeic acid phenethyl ester ameliorates cerebral infarction in rats subjected to focal cerebral ischemia. *Life Sci*, 78, 2758-2762, 2006.
2. Irmak MK, Fadillioglu E, Sogut S, Erdogan H, Gulec M, Ozer M, Yagmurca M, Gozukara ME: Effects of caffeic acid phenethyl ester and alpha-tocopherol on reperfusion injury in rat brain. *Cell Biochem Funct*, 21, 283-289, 2003.
3. Zhou Q, Cao B, Niu L, Cui X, Yu H, Liu J, Li H, Li W: Effects of permissive hypercapnia on transient global cerebral ischemia-reperfusion injury in rats. *Anesthesiology*, 112, 288-297, 2010.
4. Islekel S, Islekel H, Guner G, Ozdamar N: Alterations in superoxide dismutase, glutathione peroxidase and catalase activities in experimental cerebral ischemia-reperfusion. *Res Exp Med*, 199, 167-176, 1999.
5. Atochin DN, Clark J, Demchenko IT, Moskowitz MA, Huang PL: Rapid cerebral ischemic preconditioning in mice deficient in endothelial and neuronal nitric oxide synthases. *Stroke*, 34, 1299-1303, 2003.
6. Gourine AV, Bulhak AA, Gonon AT, Pernow J, Sjoquist PO: Cardio protective effect induced by brief exposure to nitric oxide before myocardial ischemia reperfusion *in vivo*. *Nitric Oxide*, 7, 210-216, 2002.
7. Omori K, Kotera J: Overview of PDEs and Their Regulation. *Circ Res*, 100, 309-327, 2007.
8. Houslay MD, Adams DR: PDE4 cAMP phosphodiesterases: Modular enzymes that orchestrate signalling cross-talk, desensitization and compartmentalization. *Biochem J*, 370, 1-18, 2003.
9. Li LX, Cheng YF, Lin HB, Wang C, Xu JP, Zhang HT: Prevention of cerebral ischemia-induced memory deficits by inhibition of phosphodiesterase-4 in rats. *Metab Brain Dis*, 26, 37-47, 2011.
10. Song YS, Park EH, Hur GM, Ryu YS, Lee YS, Lee JY, Kim YM, Jin C: Caffeic acid phenethyl ester inhibits nitric oxide synthase gene expression and enzyme activity. *Cancer Lett*, 175, 53-61, 2002.
11. Altug ME, Seraslan Y, Bal R., Kontas T, Keci F, Melek IM, Aslan M, Duman T: Caffeic acid phenethyl ester protects rabbit brains against permanent focal ischemia by antioxidant action: A biochemical and planimetric study. *Brain Res*, 1201, 135-142, 2008.
12. Katz L, Ebmeyer U, Safar P, Radovsky A, Neumar R: Outcome model of asphyxial cardiac arrest in rats. Outcome model of asphyxial cardiac arrest in rats. *J Cerebr Blood F Met*, 15, 1032-1039, 1995.
13. Geocadin RG, Ghodadra R, Kimura T, Lei H, Sherman DL, Hanley DF, Thakor NV: A novel quantitative EEG injury measure of global cerebral ischemia. *Clin Neurophysiol*, 111, 1779-1787, 2000.
14. Brambrink AM, Koerner IP, Diehl K, Strobel G, Noppens R, Kempinski O: The antibiotic erythromycin induces tolerance against transient global cerebral ischemia in rats (pharmacologic preconditioning). *Anesthesiology*, 104, 1208-1215, 2006.
15. Bradford MM: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 72, 248-254, 1976.
16. Yoshiko T, Kawada K, Shimada T: Lipid peroxidation in maternal and cord blood and protective mechanism against activated-oxygen toxicity in the blood. *Am J Obstet Gynecol*, 135, 372-376, 1979.
17. Luck H: Catalase. In, Bergmeyer HU (Ed): *Methods in Enzyme Analysis*. Verlag-Chemic. Weinheim/Bergstrasse, Germany. 1965.
18. Sun Y, Oberley LW, Ying L: A simple method for clinical assay of superoxide dismutase. *Clin Chem*, 34, 497-500, 1988.
19. Cortas NK, Wakid NW: Determination of inorganic nitrate in serum and urine by a kinetic cadmium-reduction method. *Clin Chem*, 36, 440-443, 1990.
20. Prajda N, Weber G: Malignant transformation-linked imbalance: decreased xanthine oxidase activity in hepatomas. *FEBS Lett*, 59, 245-249, 1975.
21. Choi JM, Shin KS, Kim KY, Lee JH, Hong KW: Neuroprotective effect of cilastazol against focal cerebral ischemia via antiapoptotic action in rats. *J Pharm Exp Therap*, 300, 787-793, 2002.
22. Montpied P, Bock F, Rondouin G, Niel G, Briant L, Courseau AS, Lerner-Natoli M, Bockaert J: Caffeic acid phenethyl ester (CAPE) prevents inflammatory stress in organotypic hippocampal slice cultures. *Brain Res Mol Brain Res*, 23, 111-120, 2003.
23. He Z, He B, Behrle BL, Fejleh MPC, Cui L, Paule MG, John L: Greenfield ischemia-induced increase in microvascular phosphodiesterase 4 d expression in rat hippocampus associated with blood brain barrier permeability: Effect of age. *Neuroscience*, 3, 428-432, 2012.
24. Oliva AA, Kang Y, Furones C, Alonso OF, Bruno O, Dietrich WD, Atkins CM: Phosphodiesterase isoform-specific expression induced by traumatic brain injury. *J Neurochem*, 123, 1019-1029, 2012.
25. Takahashi M, Terwilliger R, Lane C, Mezes PS, Conti M, Duman RS: Chronic antidepressant administration increases the expression of cAMP phosphodiesterase 4A and 4B isoforms. *J Neurosci*, 19, 610-618, 1999.
26. D'Sa C, Eisch AJ, Bolger GB, Duman RS: Differential expression and regulation of the cAMP-selective phosphodiesterase type 4A splice variants in rat brain by chronic antidepressant administration. *Eur J Neurosci*, 22, 1463-1475, 2005.
27. Ye Y, Conti M, Houslay MD, Farouqi SM, Chen M, O'Donnell JM: Noradrenergic activity differentially regulates the expression of rolipram-sensitive, high-affinity cyclic AMP phosphodiesterase (PDE4) in rat

brain. *J Neurochem*, 69, 2397-2404, 1997.

28. Ye Y, Jackson K, O'Donnell JM: Effects of repeated antidepressant treatment of type 4A phosphodiesterase (PDE4A) in rat brain. *J Neurochem*, 74, 1257-1262, 2000.

29. Hosnuter M, Gurel A, Babuccu O, Armutcu F, Kargi E, Isikdemir A: The effect of caffeic acid phenethyl ester on lipid peroxidation and nitric oxide levels in the plasma of rats following thermal injury. *Burns*, 30, 121-125, 2004.

30. Dalkara T, Yoshida T, Irikura K, Moskowitz MA: Dual role of nitric oxide in focal cerebral ischemia. *Neuropharmacology*, 33, 1447-1452, 1994.

31. Griffith OW: Biologic and pharmacologic regulation of mammalian glutathione synthesis. *Free Radic Biol Med*, 27, 922-935, 1999.

32. Toyoda T, Lee KS: Differential induction of superoxide dismutase

in core and penumbra regions after transient focal ischemia in the rat neocortex. *Neurosci Lett*, 235, 29-32, 1997.

33. Horakova L, Uraz V, Ondrejickova O, Lukovic L, Juranek I: Effect of stobadine on brain lipid peroxidation induced by incomplete ischemia and subsequent reperfusion. *Biomed Biochim Acta*, 50, 1019-1025, 1991.

34. Candelario-Jalil E, Mhadu NH, Al-Dalain SM, Martínez G, Leó OS: Time course of oxidative damage in different brain regions following transient cerebral ischemia in gerbils. *Neurosci Res*, 41, 233-241, 2001.

35. Mishra OP, Papadopoulos MD, Wagerle LC: Anti-oxidant enzymes in the brain of newborn piglets during ischemia followed by reperfusion. *Neuroscience*, 35, 211-215, 1990.

36. Stanimirovic DB, Micic DV, Markovic M, Spatz M, Mrsulja BB: Therapeutic window for multiple drug treatment of experimental cerebral ischemia in gerbils. *Neurochem Res*, 19, 189-194, 1994.