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Neuroprotective Effect of ACE Inhibitors in Glutamate - Induced Neurotoxicity: Rat Neuron Culture Study

Glutamatın İndüklediği Nörotoksisitede ACE İnhibitörlerinin Nöroprotektif Etkisi: Sıçan Nöron Kültürü Çalışması

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ABSTRACT

AIM: Glutamate is known to be neurotoxic at concentrations of 10-6M and 10-7M. Angiotensin converting enzyme (ACE) inhibitors can be assumed to be neuroprotective as they open the mitochondrial adenosine triphosphate-sensitive potassium channels by inhibiting the degradation of bradykinin. In this study, we investigated whether the ACE inhibitors captopril, ramipril and perindopril have protective effects in glutamate-induced neurotoxicity in newborn rat cerebral cortex cell cultures.

MATERIAL and METHODS: Viability tests were performed among ACE inhibitors by constituting groups of control and 10-7M and 10-6M glutamate doses in newborn rat cortex cultures.

RESULTS: While the mean viable cell number was 0.47±0.06 in the control group, it was 0.37±0.03 in the group exposed to 10-7M glutamate ($p<0.05$) and 0.37±0.01 in the group exposed to 10-6M glutamate ($p<0.05$). Captopril was used at a dose of 10 µM, perindopril was used at a dose of 1 µM, and ramipril was used at a dose of 30 µM against 10-7M and 10-6M glutamate. Ramipril and perindopril reversed the toxicity against 10-6M glutamate ($p<0.05$). The neuroprotective properties of captopril, perindopril and ramipril were not found to be statistically significant against 10-7M glutamate at the doses mentioned above.

CONCLUSION: Data obtained from this study indicate that ramipril and perindopril can prevent 10-6M glutamate-induced neurotoxicity.

KEYWORDS: ACE inhibitors, Glutamate, Neuroprotection, Neurotoxicity

ÖZ

AMAÇ: Glutamatın 10-6M ve 10-7M konsantrasyonlarında nörotoksik olduğu bilinmektedir. ACE inhibitörleri, bradikinin yıkımını engelleyerek mitokondriyal adenozin trifosfat duyarlı potasyum kanallarını açtığından dolayı nöroprotektif olabileceği düşünülebilir. Bu çalışmada, Anjiotensin dönüştürücü enzim inhibitörleri olan kaptopril, ramipril ve perindoprilin yenidoğan sıçan beyin hücre kültürlerinde, glutamatla oluşturulan nörotoksik hasarda koruyucu etkilerinin olup olmadığını araştırdık.

YÖNTEM ve GEREÇ: Yenidoğan sıçan korteks kültürlerinde kontrol grupları ve 10-7M ve 10-6M glutamat dozlarında gruplar oluşturularak ACE inhibitörlerinin arasında hücre kültürlerinde canlılık testleri yapıldı.

BULGULAR: Canlı hücre ortalamaları kontrol grubunda 0,47±0,06 iken 10-7M glutamata maruz kalan grupta 0,37±0,03 ($p<0,05$), 10-6M glutamata maruz bırakılan grupta 0,37±0,01 idi ($p<0,05$). 10-7M ve 10-6M glutamata karşı kaptopril 10 µM, perindopril 1 µM ve ramipril 30 µM dozlarında kullanıldı. 10-6M glutamata karşı ramipril ve perindopril toksisiteyi geri çevirdi ($p<0,05$). 10-7M glutamata karşı yukarıdaki dozlardaki kaptopril, perindopril ve ramiprilin nöroprotektif özelliği istatistiksel olarak anlamlı değildi.

SONUÇ: Bu çalışmadan elde edilen veriler, 10-6M glutamatın oluşturduğu nörotoksisiteyi ramipril ve perindoprilin engelleyebileceğini ortaya koydu.

ANAHTAR SÖZCÜKLER: ACE inhibitörleri, Glutamat, Nöroproteksiyon, Nörotoksisite

INTRODUCTION

While glutamate is the most important neurotransmitter of the brain, it is also toxic. Alterations in glutamate concentration lead to neurotoxicity and thereby cell death. Occurance of the injury and its outcomes are frequently permanent and irreversible as cell death in brain is irreversible (10, 11, 17). ACE inhibitors are frequently used in the treatment of essential hypertension that lead to impairment, especially in the cerebral circulation. ACE inhibitors are assumed to prolong the neuronal lifetime due to their free radical collecting effect (16, 27). ACE inhibitors pass the blood-brain barrier very easily and can modulate the central neurotransmitter level. However, very little is known about the relationship between ACE inhibition and neuroprotection (18, 27). Excessive release of glutamate, which is an excitatory aminoacid, is held responsible for the neurotoxicity in the development of brain ischemic injury (6). Intracellular calcium increases with activation of excitatory aminoacid receptors and this leads to impairment of mitochondrial functions via activation of protein kinase, phospholipase, protease, nitric oxide synthase and release of free radicals (28).The role of free radicals induced with activation of excitatory aminoacid receptors in cell death is still a wide field of interest. ACE inhibitors can exhibit a neuroprotective effect due to the radical collecting effect (26). In this study, the neuroprotective effect of ACE inhibitors was investigated in glutamate neurotoxicity generated in primary cell cultures made from newborn rat cortex.

MATERIAL and METHODS

This study was conducted at the Medical Experimental Research Center, Ataturk University. The Ethical Committee of Ataturk University approved the study protocol. All procedures were performed in accordance with the National Institute of Health Principles of Laboratory Animal Care. A total of three newborn Sprague-Dawley rats were used in the study. The rats were decapitated by making a cervical fracture in the cervical midline and the cerebral cortex was dissected and removed. The cerebral cortex was placed into 5 ml of Hank's balanced salt solution (HBSS; Sigma Co., St Louis, MO, USA), which had already been placed in a sterile petri dish and macromerotomy was performed with two lancets. This composition was pulled into a syringe and treated at 37°C for 25-30 min as 5 ml HBSS + 2 ml Trypsin-EDTA (% 0.25 trypsin- % 0.02 EDTA; Biol. Ind. Haemek, Israel) and chemical decomposition was achieved. 8,5-9 microliters of DNAse type 1 (120u/ml, Sigma, St Louis, USA) was added to this solution and treated for 1-2 minutes, and centrifuged at 800rpm for 3 min. After having thrown away the supernatant, 31,5 ml of Neurobasal Medium (NBM) and 3,5 ml fetal calf serum (FCS, Biol, Ind.) were added to the residue. The single cell which was obtained after physical and chemical decomposition was divided into 3,5 ml samples in each of 10 flasks coated with poly-D-lysine formerly dissolved in phosphate buffer solution (PBS). The flasks were left in the incubator including 5% CO2 at 37°C in the ventile position. The flasks were then changed with a fresh medium of half of their volumes every 3 days until the cells were branched and had reached a certain maturity and in vitro neurotoxicity experiments were performed 8 days later (7). Each experimental group was tested in at least 4 culture medium (n=5). The drugs that were to be used for the test were administered into the flask 16 hours prior to the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) analysis and the flasks were placed into the incubator again. Poly D-lysine coating was not required as the 24-48 flasks had already been coated (10). Poly D-lysine (25 mg/total) was obtained as trypsin-EDTA (100 ml 0.25%), fetal calf serum 100 ml. 9,8 gr of HBSS was used for every 1 liter of water. The solution was stored in the dark at 2-8°C. This solution was controlled in terms of pH alteration, precipitations and participations, blurring, change of color and infections prior to every use. Attempts at saving the cultures were made by adding 100,000 U/ml of penicillin, 10 mg/ml of streptomycin and 0.025 mg/ml of amphotericin B, which are the recommended antibiotic prophylaxis, to the flasks on the first day (10, 12). The cells lived for 5-10 days in the cultures in which germination was observed in the single cell suspension. Captopril, ramipril and perindopril were administered in doses of 10, 30 and 1 micromolar, respectively, with the same doses used in cell cultures (20, 24, 31). In this study, glutamate was used in two distinct doses as concentrations of 10-6 and 10-7M .We waited for half an hour after administration of the ACE inhibitor and glutamate was added to the cultures at the aforementioned doses. The MTT kit was used in order to differentiate dead cells after having waited for 16 hours. Cell evaluation was made spectrally with the Microquant reader at 570 nm wavelength.

SPSS 13.0 (Statistical Package Program for Social Sciences version 13.0) for Windows was used for the statistical analysis. The mean viability values ± standard deviations were calculated by spectrally counting the viable cells in the Microquant reader utilizing the MTT kit and the obtained numerical values were assessed using the Oneway ANOVA test. A P value of <0.05 was accepted as statistically significant in the intergroup relationships.

RESULTS

It was observed that there was a difference between the control groups and the glutamate-administered groups in terms of viability when the newborn rat cerebral cortex cultures were exposed to glutamate. While the mean number of viable cells in the control group was 0.47±0.06, it was 0.37±0.03 in the group exposed to 10-7M glutamate and 0.37±0.01 in the group exposed to 10-6M glutamate ($p<0.05$). Captopril, perindopril and ramipril were used at doses of 10 µM, 1 µM and 30 µM, respectively, against 10-7M and 10-6M glutamate. 10-7M and 10-6M glutamate doses seemed to be neurotoxic. The neuroprotective effect was not found to be statistically significant in case of use of captopril, perindopril and ramipril alone compared to the control (Table I) ($p<0.05$). The mean viability value against 10-6M glutamate was 0.52±0.06 for ramipril and 0,48±0.07 for perindopril and reversed toxicity (Table II) ($p<0.05$). The neuroprotective properties of captopril,

Table I: Neurotoxic Effects of Administered Drugs in Neuron Cell Cultures

	n (total number of wells in 2 distinct studies)	Mean of viable cells	p value according to control
10-6 Glutamate	n=4	0,37±0.01	0.035*
10-7 Glutamate	n=5	0,37±0.03	0.018*
10 µM captopril	n=5	0,46±0.05	0.74
1 µM perindopril	n=5	0,44±0.06	0.48
30 µM ramipril	n=4	0,46±0.03	0.77
CONTROL	n=21	0,47±0.06	

**p<0.05 was considered significant according to the control group.*

Table II: Effects of Captopril, Perindopril and Ramipril on Neuronal Cell Death at 10^{-6}M Glutamate Concentration

	n (total number of wells in 2 distinct studies)	Mean of viable cells	p values according to 10^{-6}M Glutamate
10-6 Glutamate +10 µM captopril	n=5	0,47±0.11	0.10
10-6 Glutamate+1 µM perindopril	n=6	0,48±0.07	0.045*
10-6 Glutamate +30 µM ramipril	n=4	0,52±0.06	0.027*
10-6 Glutamate	n=4	0,37±0.01	

** p<0.05 was considered significant according to the 10^{-6}M glutamate group*

Table III: Effects of Captopril, Perindopril and Ramipril on Neuronal Cell Death at 10^{-7}M Glutamate Concentration

	n (total number of wells in 2 distinct studies)	Mean of viable cells	p values accordin to 10^{-7}M Glutamate
10-7 Glutamate +10 µM kaptopril	n=5	0,45±0.09	0.11
10-7 Glutamate +1 µM perindopril	n=5	0,40±0.03	0.62
10-7 Glutamate +30 µM Ramipril	n=4	0,43±0.11	0.52
10-7 Glutamate	n=5	0,37±0.03	

** p<0.05 was considered significant according to the 10^{-7}M glutamate group.*

perindopril and ramipril were not statistically significant at the aforementioned doses against 10-7M glutamate. 10-6M and 10-7M glutamate concentrations were found to produce equal toxic doses. Perindopril was found to be most effective at a dose of 1 µM in 10-7M glutamate concentration; however, this was not statistically significant (Table III).

DISCUSSION

In this study, the neurotoxic effect of glutamate generated in the newborn rat cerebral cortex cells at certain doses and the ability of captopril, perindopril and ramipril to reverse this toxic effect were investigated. This study was designed with the hypothesis that different drug subgroups of ACE inhibitors could exhibit different properties. These 3 ACE inhibitors were used in the study: captopril which contains a sulphydryl group and ramipril and perindopril which do not contain sulphydryl groups.

The mean of the viable cells exposed to glutamate at toxic dose (10-6M) was tested as 0.52±0.06 in the ramipril-administered group and 0,48±0.07 in the perindopril-administered group, which reversed toxicity, and a statistically significance was obtained. The lack of statistical significance in the captopril-administered group despite reversal of toxicity makes us think that the chemical structures of ACE inhibitors are not sufficient alone in producing a neuroprotective effect. While some ACE inhibitors exhibited a neuroprotective effect at the 10-6M glutamate dose, they were found not to exhibit a neuroprotective effect at the other neurotoxic dose (10-7M glutamate). Here, glutamate transporters that prevent toxicity at high glutamate doses are considered to work faster. This effect against the concentration in which glutamate caused neurotoxicity was investigated in order to assess the statistically significant results of ACE inhibitors. According to our data obtained from the test results, the differences had appeared in terms of viability between the control groups and the glutamate-administered groups when the newborn rat cortex cultures were exposed to glutamate. Similar to previous studies, glutamate concentrations of 10-7M and10-

6M were found to cause statistically significant toxicity in our study (10, 12). Glutamate levels in the central nervous system are balanced between glutamate release and re-uptake. The basal extracellular glutamate levels are maintained with release from the neurons and re-uptake by the astrocytes and neurons. Exogenous glutamate is removed by uptake and re-uptake mechanisms. This event plays an important role in glutamate toxicity (10, 11). Astrocyte stimulation was previously shown to reduce excitatory and inhibitory transmission by selective activation of synaptic transmission in mixed cultures including astrocyte and neuron cultures (1). The endoplasmic reticulum (ER) is the major site of intracellular storage of calcium and provides cellular calcium homeostasis. Oxidants, sulphydryl active agents and free radicals inhibit the calcium-ATPase (1, 10). In a study, captopril was reported to maintain the membrane integrity by inhibiting the calcium-ATPase in ischemic injury (15). Inhibition of calcium-ATPase releases calcium from the ER. This condition makes us think that the ER calcium plays a role in oxidant toxicity (1, 10).

ACE inhibitors are differentiated as those that include sulphydryl group, carboxyl group and phosphoryl group according to their chemical structures (19). In various studies, captopril has been put forth as having a free radical collecting property along with the sulphydryl group in its composition. This tissue-protective effect of captopril is achieved by binding the sulphydryl group to thiol groups generated after ischemia (2, 5, 13, 22, 25). In our study, the sulphhydryl-containing property of captopril is thought not to be the only effective factor for viability of neurons, as drugs not containing sulphydryl group like ramipril and perindopril were found to reverse toxicity significantly in glutamate toxicity-related ischemia. Free oxygen radicals are under the strict control of superoxide dismutase enzymes, catalase and gluthation peroxidase. In spite of this, an increase in free radical formation and a decrease in antioxidant defense cause oxidative stress. The main producers of free radicals are the mitochondria. Oxygen radicals contribute to cell, tissue and organ injury. Free oxygen radicals may inhibit the re-uptake of glutamate. Furthermore, NMDA can lead to superoxide release, intracellular calcium increase and NO formation via receptor activation (1, 10). NMDA and KA receptor activation have been shown to induce NO synthesis from arginine in cerebellar cells. NO plays a dual role in cerebral ischemia; neuroprotection and neurotoxicity. NO as a neurotoxin increases the ischemic excitotoxic brain injury with glutamate release and excessive activation of NMDA receptors. On the other hand, it increases the blood supply of the ischemic region in the early phases of cerebral ischemia. Nitric oxide accompanies with the effect of NMDA receptor in many brain functions (such as memory and learning). NO has been shown to play a protective role in glutamate neurotoxicity by closing the NMDA receptor-bound ion channels; however, increased concentrations of NO has been shown to be related to oxygen radical formation and to commence toxicity in retinal neuron cultures (29). It has been shown that oxidative stress plays a role in the pathophysiology of stroke, free radical-caused brain edema by breaking the blood-brain barrier, migration of inflammatory cells to ischemic region and alterations in blood flow. Hydroxyl radical and superoxide ions which are free oxygen radicals, are reactive and injure the nucleic acids, lipids, carbohydrates and proteins in neurons by binding to them (4). ACE inhibitors have been shown to exhibit a neuroprotective effect by reducing the major oxidative stress indicators (ie. lipid peroxidation and protein oxidation) (3, 21, 26, 32). Rats in which NADPH oxidase was deleted were found to be resistant to ischemic brain injury (4). Oxidative stress emerging in cerebral ischemia causes local ACE and angiotensin II production. Angiotensin II induces the formation of superoxide by increasing the vascular NADPH oxidase. As a result, the NO level decreases, and the oxidative stress increases (8, 14). ACE inhibitors may reduce the harmful effects of free radicals by exhibiting an antioxidant effect (by reducing the angiotensin II and NADPH oxidase). ACE inhibitors are kininase-II inhibitors and prevent the degradation of bradykinin, which is a vasodilator peptide (23). Bradykinin has been reported to exhibit a directly protective effect from glutamate neurotoxicity via the bradykinin-B receptor in retinal neuron cultures (9). ACE inhibitors prevent angiotension-1 from converting to angiotensin-2. Reduced angiotensin-2 levels show their protective effect by decreasing the superoxide anion production in vessels (9). The neuroprotective effect of bradykinin in glutamate excitotoxicty has been demonstrated in retinal neuron cells by opening the mitochonrial adenosine triphosphate (ATP)-sensitive potassium channels (Mit K (ATP)) . Glutamate is thought to be induced and the superoxides are thought to be inhibited by the opening of Mit K(ATP) channels (30). ACE inhibitors may have helped in opening the Mit K(ATP) channels by preventing bradykinin degradation via inhibition of kininase-II and in exhibiting a neuroprotective effect in glutamate neurotoxicity by reducing the glutamate-induced superoxide radicals.

In conclusion, we consider that ACE inhibitors can exhibit a neuroprotective effect in glutamate neurotoxicity by increasing the endogenous antioxidant defence mechanisms or by reducing the free radicals.

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