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Influence of farnesyltransferase inhibitor (*Manumycin A*) upon the changes of sulfur-containing amino acids level in brain ischemia

L. Shanshiashvili,* E. Sekoyan,** D. Mikeladze,* I. Sekoyan**

*Institute of Physiology, Georgian Academy of Sciences**
*SRI for Spa Treatment and Physical Medicine of the Armenian Ministry of Health***

14 Gotua St., 0160 Tbilisi, Georgia

41 Orbeli St., 375028 Yerevan, Armenia

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The recent studies have demonstrated that moderate elevation of homocysteine levels is a causal risk factor for cardiovascular diseases, including atherosclerosis and thrombosis [3,9]. Homocysteine is a sulfur-containing amino acid biosynthesized from methionine that occupies a key place between the folate and activated methyl cycles. The first step of the methyl cycle is the formation of S-adenosylmethionine (SAM) from methionine and ATP by methionine S-adenosyl-transferase; demethylation of SAM, coupled with methylation of an acceptor (DNA, RNA, proteins, lipids, neuromediators, etc.), accompanied by the synthesis of adenosylhomocysteine (SAH), is the next one. The hydrolysis of S-adenosylhomocysteine when acted on by S-adenosylhomocysteine hydrolase leads to the formation of homocysteine and adenosine. The resulting homocysteine is either catabolized into cystathionine (transsulfuration pathway) or remethylated into methionine (remethylation pathway). The homocysteine remethylation is catalysed by methionine synthase, which uses N5-methyltetrahydrofolate as a methyl donor and cobalamin as a cofactor. By the transsulfuration pathway, homocysteine is converted to cystathionine from the pyridoxal-5'-phosphate-dependent cystathionine b-synthase (CBS) and then into cysteine, a precursor of glutathione, which is the main antioxidant compound of the cells. The oxidative stress in result of impaired homocysteine metabolism might play a central role in the molecular mechanisms underlying moderate hyperhomocysteinaemia- mediated vascular disorders [3]. Hypoxia increases intracellular adenosine concentrations, which in the presence of homocysteine is efficiently converted to S-adenosylhomocysteine, a potent inhibitor of methyltransferase reactions [5]. It has been reported that physiologically relevant concentrations of homocysteine in the presence of adenosine inhibit the growth of vascular endothelial cells and cause apoptosis by a mechanism involving a decreased carboxymethylation of Ras [8,14]. The production of intracellular reactive oxygen species (ROS) has been implicated in the pathogenesis of several human disorders, including cerebral ischemia [7]. Recent evidence suggests that Ras is directly involved in the regulation of the intracellular redox state [4,6,12]. This GTP-binding protein (specifically Ha-Ras) stimulates intracellular ROS levels by activating NADPH oxidase, furtherly amplifying the cascade initiated by H2O2 formed during ischemia. Inhibition of Ras signalling by farnesylation inhibitors increases the resistance to apoptosis in Ha-Ras-expressing cells [2]. The present study has been conducted to examine the effects of Manumycin A, an inhibitor of farnesyltransferase, on ischemia-evoked homocysteine, on methionine and cysteine levels in the cerebral cortex.

Material and Methods

Cerebral ischemia was induced by bilateral carotid artery occlusion in male white rats for 20 minutes after anesthesia with sodium pentobarbital (4 mg/100 g body weight). After 20 min, the animals were decapitated and the quantity of sulfur-containing amino acids in the brain were determined. Intraperitoneal injection of Manumycin A (3 mg/kg body weight) was carried out 2 hours before anesthesia. The quantity of homocysteine was determined by the method of Pfeiffer et al. [11]. Briefly, the cerebral cortex samples were homogenized in a phosphate buffer saline and 10 μM cysteamine hydrochloride (internal standard) pH 7.4, then incubated with 10 μL of 100 g/L tris(2-carboxyethyl)phosphine (TCEP) (Pierce Chemical Co.) for 30 minutes at room temperature to reduce and release protein-bound thiols, after which 90 μL of 100 g/L trichloroacetic acid containing 1 mmol/L EDTA was added for deproteinization. The sample was centrifuged for 10

minutes at 13 000 g, and 50 μL of the supernatant was added to a vial containing 10 μL of 1.55 mol/L NaOH; 125 μL of 0.125 mol/L borate buffer (pH 9.5) containing 4 mmol/L EDTA; and 50 μL of 1 g/L 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F, Sigma Chemical Co.) in the borate buffer. The sample was then incubated for 60 minutes at 60 °C. HPLC was carried out on a solvent delivery system using a fluorescence detector (385 nm excitation, 515 nm emission), both from Waters Technologies Corp. Separation of the SBD-derivative thiols was performed on a NovaPak C18 analytical column (particle size 5 μm, 100 x 8 mm i.d., Waters Technologies Corp.) using a 40-μL injection volume and 0.1 mol/l acetic acidacetate buffer pH 5.5, containing 30 ml/L methanol as mobile phase at a flow rate of 0.7 ml/min and a column temperature of 29 °C. The determination of the methionine and cysteine contents of the cerebral cortex began with its homogenization in ice-cold HClO4 containing 40 μmol/L norleucine as an internal standard. The homogenate was then centrifuged at 3000 g for 10 min. After centrifugation, the supernatant was neutralized by KHCO3 and the supernatant obtained after the second centrifugation was used for HPLC analysis. Amino acid analysis was performed after phenylisothiocyanate (PITC) derivatization by a Pico Tag analyser (Waters Technologies Corp.). The equipment consisted of two model 6000A pumps, a model 660 solvent programmer, a U6K injector, a model 441 absorbance detector and PicoTag C18 column (particle size 5 μm, 100 x 8mm i.d.). The data were treated by one-way ANOVA analysis.

Results and Discussion

The effects of Manumycin A on ischemia-evoked changes of sulfur-containing amino acids were observed in the rat brain four-vessel occlusion model. It was found that after ischemia the contents of methionine and homocysteine were decreased, whereas the content of cysteine was increased. In the rats pretreated with Manumycin A, cerebral ischemia did not change the content of methionine, homocysteine or cysteine (Figure). These results confirm the suggestion that oxidative stress reduces remethylation and enhances transsulfuration to maintain the intracellular glutathione pool, which is essential for the redox-regulating capacity of the cells. Ischemia-induced oxidative stress through the Ras-dependent process changes the direction of homocysteine metabolism toward the direction of transsulfuration.

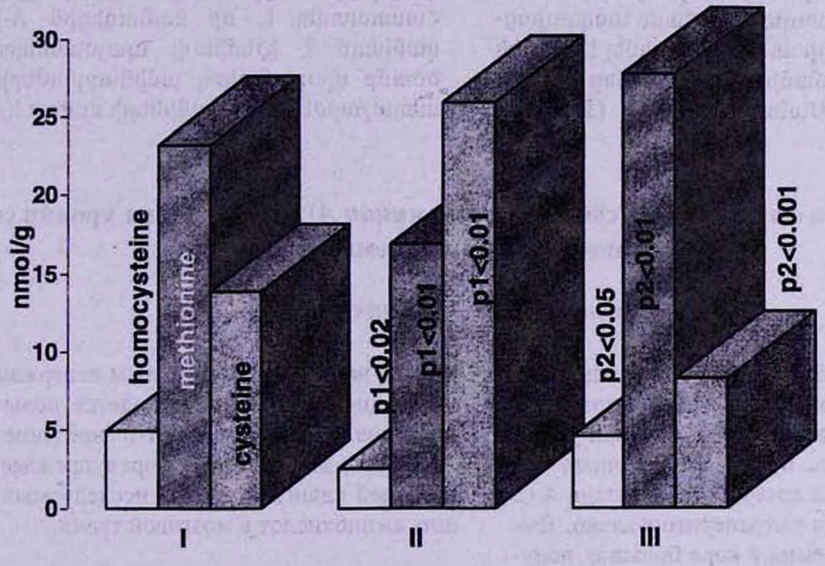

Figure. Alteration of sulfur-containing amino acids in the brain: control (I), ischemia (II), ischemia+Manumycin A (III). p_1 – control/ischemia, p_2 – ischemia/ischemia+Manumycin A

Reactive oxygen species (ROS) formed during ischemia can activate the Ras signalling system. Ha-Ras is an important activator of the NADPH oxidase complex because it participates in the assembly under the plasma membrane of the oxidase complex [6]. The activation of the NADPH oxidase complex results in a significant increase in cellular ROS. We have found out that after ischemia, the content of homocysteine and methionine in the brain is diminished, while the amount of cysteine is greatly increased. These results suggest that the remethylation pathway in homocysteine metabolism was diminished during ischemia. The observations confirm the assumption that oxidative stress reduces remethylation and enhances transsulfuration to maintain via an adaptive

process the intracellular glutathione pool, which is essential for the redox-regulating capacity of cells [3, 10]. It is interesting to note that methionine synthase is inactivated by oxidation and requires reductive methylation for reactivation [1], while cystathionine betasynthase is a hem protein and active in the oxidized form [13]. Thus, increasing the amount of free radicals in the brain of rats in which ischemia has been evoked can augment synthesis of cysteine and glutathione. In the Manumycin A-treated animals, neither the content of homocysteine, nor the amount of methionine and cysteine changes.

The obtained results show that the main source of ROS during ischemia action on homocysteine metabolism is the Ras-dependent enzyme cascade. In fact, there are reports showing that the inhibition of p21 Ras signalling by farnesylation inhibitors increase resistance to apoptosis in Ha-Ras-expressing cells [2]. All these results suggest that Ras and the Ras-dependent downstream effectors switch homocysteine/methionine metabolism from remethylation to transsulfuration pathways, and through the formation of cysteine accelerate synthesis of the main antioxidant component of neuronal cells – glutathione.

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Գլխուղեղի իշեմիայի պայմաններում ֆարնեզիլտրանսֆերազի ինհիբիտորի (մանումիցին A) ազդեցությունը ծծումբ պարունակող ամինաթթուների մակարդակի փոփոխությունների վրա

Լ.Շանշիաշվիլի, Է. Սեկոյան, Դ.Միկելադզե, Ի. Սեկոյան

Սպիտակ առնետների մոտ գլխուղեղի իշեմիայի պայմաններում ուսումնասիրված է ֆարնեզիլտրանսֆերազի ինհիբիտոր՝ մանումիցին A-ի ազդեցությունը ծծումբ պարունակող ամինաթթուների քանակական տեղաշարժերի վրա: Գլխուղեղի իշեմիան անզգայացված սպիտակ առնետների մոտ առաջացվել է քնային զարկերակների երկկողմանի 20-րոպեանոց օկլյուզիայի ճանապարհով: Մանումիցին A-ն (3 մգ/կգ) ներմուծվել է ներորովայնային: Հայտնաբերված է, որ իշեմիայի պայմաններում գլխուղեղի կեղևում մեթիոնինի և հոմոցիսթեինի պարունակության իջեցումը զուգորդվում է ցիսթեինի մակարդակի բարձրացմամբ: Հաստատվել է, որ մանումիցին A-ի ներմուծումը կանխում է իշեմիայի պայմաններում զարգացող ծծումբ պարունակող ամինաթթուների քանակական տեղաշարժերը կենդանիների ուղեղի հյուսվածքում:

Влияние ингибитора фарнезилтрансферазы (*манумицин А*) на изменения уровня серосодержащих аминокислот при ишемии мозга

Л.Шаншиашвили, Э. Секоян, Д.Микеладзе, И. Секоян

Изучено влияние ингибитора фарнезилтрансферазы – манумицина А на уровень серосодержащих аминокислот в коре при ишемии мозга, вызванной у анестезированных белых крыс путем двусторонней 20-минутной окклюзии сонных артерий. Манумицин А (3 мг/кг массы тела) вводился интраперитонеально. Выявлено, что в условиях ишемии в коре больших полушарий, наряду с уменьшением содержания метионина и гомоцистеина, обнаруживается повышение уровня цистеина. Установлено, что введение подопытным животным манумицина А предупреждает вызываемые ишемией сдвиги в уровне исследуемых серосодержащих аминокислот в мозговой ткани.

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