Manumycin restores the levels of cortical homocysteine, methionine and cysteine changed in ischaemia-evoked animals

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Reactive oxygen species (ROS) formed during ischaemia can activate the Ras signalling system, which amplifies the intracellular formation of ROS. Ras proteins induce senescence by altering the intracellular levels of ROS which leads to the impairment of homocysteine metabolism. The effects of the farnesyltransferase inhibitor, manumycin, were examined on ischaemia-evoked changed sulfur-containing amino acids. A rat four-vessel occlusion model was utilized to examine the effects of manumycin on cortical levels of homocysteine, methionine and cysteine. Cerebral ischaemia was induced by occlusion of the carotid arteries for 20 min in anaesthetized animals. Intraperitoneal injection of manumycin A (3 mg/kg body weight) was carried out 2 hours before anaesthesia. It was found that after ischaemia the quantities of methionine and homocysteine were decreased, whereas the amount of cysteine was increased. In the rats pretreated with manumycin, cerebral ischaemia does not change the content of methionine, homocysteine or cysteine. These results suggest that ischaemia-induced oxidative stress, acting through Ras-dependent processes, change homocysteine metabolism in the direction of trans-sulfuration.

Keywords: homocysteine, ischaemia, Ras

1. INTRODUCTION

Recent studies have suggested that moderately elevated homocysteine levels are a causal risk factor for cardiovascular disease, 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) and accompanied by the synthesis of adenosylhomocysteine (SAH) is the next. 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 (trans-sulfuration pathway) or remethylated into methionine (remethylation pathway). The homocysteine remethylation is catalysed by methionine synthase, which uses N^5-methyltetrahydrofolate as the methyl donor and cobalamin as the cofactor. By the trans-sulfuration pathway, homocysteine is converted to cystathionine from the pyridoxal-5'-phosphate-dependent cystathionine β-synthase (CBS) and then into cysteine, a precursor of glutathione, which is the main antioxidant compound of the cells.

The oxidant stress that results from impaired homocysteine metabolism might play a central rôle 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 ischaemia [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, further amplifying the cascade initiated by H_2O_2 formed during ischaemia. Inhibition of Ras signalling by farnesylation inhibitors increases the resistance to apoptosis in Ha-Ras-expressing cells [2]. The present study was conducted to examine the effects of manumycin, an inhibitor of farnesyl transferase on ischaemia-evoked homocysteine, on methionine and cysteine levels in the cerebral cortex.

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2. MATERIAL AND METHODS

Cerebral ischaemia was induced by bilateral carotid artery occlusion in male white rats for 20 minutes after anaesthesia with sodium pentobarbital (4 mg/100 g body weight). After 20 minutes, the animals were decapitated and the quantity of amino acids, ATP, ADP, AMD, adenosine and lactate in the brain were determined using a Kone (Kone Corporation, Helsinki, Finland) biochemical analyser. The local cerebral blood flow was monitored in the parietal cortex by Doppler flowrimetry BLF21 (Transonic System Inc., Ithaca, USA). Intraperitoneal injection of manumycin A (3 mg/kg body weight) was carried out 2 hours before anaesthesia. The Armenian National Cardiovascular Centre's guidelines for animal care and experiments were followed.

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 × 8 mm i.d, Waters Technologies Corp.) using a 40-μL injection volume and 0.1 mol/L acetic acid-acetate 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 $HClO_4$ 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 $KHCO_3$ and the supernatant obtained after a 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 × 8 mm i.d.).

The data were treated by one-way ANOVA analysis.

3. RESULTS

The effects of manumycin on ischaemia-evoked changes of sulfur-containing amino acids were observed in the rat brain four-vessel occlusion model. Confirmation of ischaemia in the control group for the animal's cerebral cortex ATP, ADP, AMP, adenosine and lactate levels were determined. ATP, ADP, AMP, adenosine and lactate levels in the control group prior to and following ischaemia are presented in Table 1. ADP, AMP, adenosine and lactate levels were significantly elevated during ischaemia, whereas the content of ATP was decreased. This data evidenced successful induction of ischaemia.

Table 1. Ischaemia-evoked changes in the cerebral cortex levels of ATP, ADP, AMP, adenosine, lactate and local blood flow. The data are presented as the means ± SEM of triplicate determination.

	Control	Ischaemia 20 min
ATP μmol/g	2.95 ± 0.14	2.02 ± 021
ADP μmol/g	1.85 ± 016	1.96 ± 0.19
AMP μmol/g	0.78 ± 0.06	0.98 ± 0.14
Adenosine μmol/g	1.42 ± 021	1.82 ± 0.08
Lactate μmol/g	2.87 ± 0.14	6.22 ± 0.23
Local blood flow mL/min/100g	56.2 ± 9.7	36.4 ± 8.9

In another group of animals, the levels of sulfur-containing amino acid in the cerebral cortex were determined. It was found that after ischaemia the contents of methionine and homocysteine were decreased, whereas the content of cysteine was increased. In the rats pretreated with manumycin, cerebral ischaemia does not change the content of methionine, homocysteine or cysteine (Fig. 1). These results confirm the suggestion that oxidant stress reduces remethylation and enhances trans-sulfuration to maintain the intracellular glutathione pool, which would be essential for the redox-regulating capacity of the cells. Ischaemia-induced oxidative stress through the Ras-dependent process changes the direction of homocysteine metabolism toward the direction of trans-sulfuration.

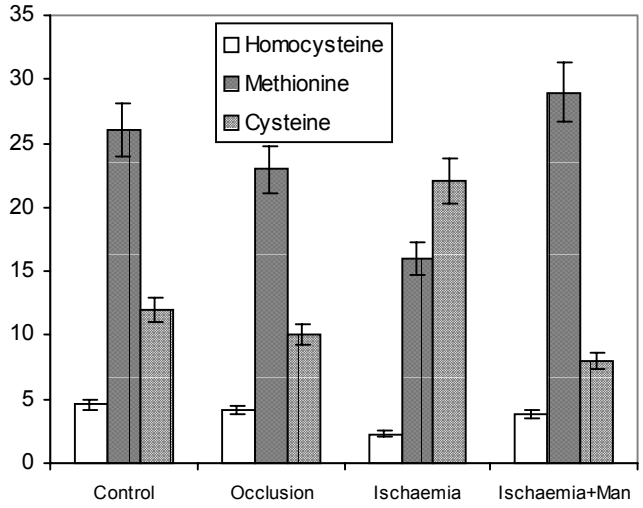

Figure 1. Alteration of sulfur-containing amino acids in the brain after occlusion and ischaemia (nmol/g wet weight). Man = manumycin. The data are presented as the means ± SEM for quintet determination.

4. DISCUSSION

Reactive oxygen species (ROS) formed during ischaemia 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 of cellular ROS. We have found that after ischaemia, the content of homocysteine and methionine in the brain is diminished, while the amount of cysteine is greatly increased (Fig.1). These results suggest that the remethylation pathway in homocysteine metabolism was diminished during ischaemia. The observations confirm the assumption that oxidant stress reduces remethylation and enhances trans-sulfuration to maintain, via an adaptive process, the intracellular glutathione pool, which would be 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 beta-synthase is a haem protein and active in the oxidized form [13]. Thus, increasing the amount of free radicals in the brain of rats in which ischaemia has been evoked can augment synthesis of cysteine and glutathione.

In the manumycin-treated animals, neither the content of homocysteine, nor the amount of methionine and cysteine changes (Fig.1). These results show that the main source of reactive oxygen species during ischaemia acting 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 increased 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 trans-sulfuration pathways and, through the formation of cysteine, accelerate synthesis of the main antioxidant component of neuronal cells—glutathione.

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