Influence of Caffeine on Lysozyme Activity in the Blood Serum of Mice

A. Ramanavičienė[1],
V. Žukienė[1],
J. Ačaitė[1],
A. Ramanavičius[1,2]

[1] *Laboratory of Ecological Immunology, Institute of Immunology, Vilnius, Lithuania*
[2] *Department of Analytical and Environmental Chemistry, Vilnius University, Vilnius, Lithuania*

Observation show that the central nervous system plays a certain part in the regulation of lysozyme activity in different fluids of the organism. The aim of this study was to examine the effect of caffeine as a factor stimulating the central nervous system on lysozyme activity.

Four groups of mice were given a single oral dose of 2, 20, 40, 200 mg/kg caffeine solution in water by oral intubations. The control group of four mice was given water. Caffeine solutions were given at 3-day intervals during 30 days. Measurements of lysozyme in mouse blood serum were performed using a colorimetric rate-of-lysis *Micrococcus lysodeikticus* cell assay.

The lysozyme activity in the serum was found to depend on caffeine concentration in water. This dependence was investigated and is presented in this paper.

Key words: caffeine, lysozyme, mice

INTRODUCTION

Caffeine is a widely used nutrition factor. The use of caffeine has several advantages and disadvantages. It has a wide range of effects on cardiovascular activity including vasoconstriction, total peripheral resistance, blood flow and so forth (1). Caffeine produces acute elevations in both systolic and diastolic blood pressure in most individuals (2), has analgesic properties (3), but as a complex-forming agent decreases the effective concentration of the antitumor antibiotic actinomycin D (4). Caffeine is a potent central nervous system (CNS) stimulant. Some observations show that CNS plays a certain part in the regulation of lysozyme activity in different fluids of the organism (5).

Lysozyme (1,4-β-*N*-acetylmuramidase), a small (14.3 kDa) cationic protein, is generally assumed to be a component of the first-line host defense against bacterial invaders. This protein is a ubiquitous host factor found in secretions and inflamed tissue at infection sites. Lysozyme belongs to the class of enzymes that lyses the cell walls of bacteria, as the bond between the C-1 of N-acetylmuramic acid and the C-4 of *N*-acetylglucosamine of the peptidoglycan is cleaved. The antimicrobial activity of lysozyme is known to be directed against certain Gram-positive bacteria and to a lesser degree *in vitro* against Gram-negative bacteria (6–8). Besides its antimicrobial activity, lysozyme has many other functions including inactivation of certain viruses (9), surveillance of membranes of mammalian cells (10), enhancing of phagocytic activity of polymorphonuclear leukocytes and macrophages (11, 12), and stimulates proliferation and antitumor activity of monocytes (13). Neutrophilic granulocytes, monocytes and tissue macrophages produce lysozyme (14). Azurophilic and specific granules of neutrophilic granulocytes are the richest source of lysozyme (15). Lysozyme is found intracellularly in various blood cells and their bone marrow precursors. Lysozyme is also present in plasma/serum, tears, milk, spit, intestine mucous. In liver, spleen, kidney and lung particularly high levels of lysozyme are determined. Lysozyme is present in many animals, but its concentrations vary in a wide range depending on species (16, 17).

In most studies, lysozyme has been quantified by a functional assay using *Micrococcus lysodeikticus* and egg white lysozyme as standard. Measurement of the degradation of the high molecular weight substrate is a rather simple and quite sensitive method of

Correspondence to: A. Ramanavičius, Department of Analytical and Environmental Chemistry, Vilnius University, Naugarduko 24, LT-2009 Vilnius, Lithuania. E-mail: arman@bchi.lt

following the enzyme reaction. Commonly, three different assay procedures are employed: a turbimetric assay, a viscosimetric assay and a so-called lysoplate assay (18–20). In addition to these functional assays, which determine the enzymatic activity of lysozyme, immunological test systems (ELISA, RIA, LIA) have been established (21, 22).

The major goal of the present report was to investigate activity of lysozyme in mouse blood serum by the use of caffeine as a factor stimulating central nervous system. A second goal was to examine the nonspecific effects of caffeine on weight and activity of movement.

MATERIALS AND METHODS

Reagents

Lysozyme from chicken egg white, *Micrococcus lysodeikticus,* EDTA and caffeine were purchased from Sigma (St. Louis, USA). Other chemicals were of analytical grade and used as received. All solutions were prepared by using deionized water purified by Millipore S. A. water purification system (Molsheim, France).

Equipment

All colorimetrical measurements were performed with a KFK-2 colorimeter (Russia). The samples were mixed with a MM 2A magnetic stirrer (Czech Republic) and incubated in a water bath (MK-70, Germany). Serum was prepared by centrifugation with an Eppendorf centrifuge 5415 D (Germany). A Specord spectrophotometer (Germany) was used for detection of lysozyme activity.

Animals and housing

BALB/c mice obtained from the vivarium of Immunology Institute (Vilnius, Lithuania), weighing 27–28 g were used in this study. Twenty female mice were randomly allocated to treatment groups. The mice were distributed in groups of four, housed in groups in solid-bottomed cages containing bedding of wood shavings and were allowed food and water *ad libitum*. Room temperature was maintained at 21–24°C, and a 12 h light/dark cycle was employed. Approval of the Lithuanian Ethic Committee for Laboratory Animal Use was obtained prior to commencement of the experiments.

Procedure

The mice were given a single oral dose of 2, 20, 40, 200 mg/kg caffeine solution in water by oral intubations. The control group of four mice was given water. The caffeine solutions were given at 3-day intervals during 30 days.

On day 30 mice were given a single oral dose, and after 1.5 h the experiment was completed. The mice were killed by cervical dislocation. All groups of mice were killed at the same time. Blood samples were collected in tubes from four control and 16 caffeine-treated mice. Serum was prepared by centrifugation at 3000 rpm for 5 min.

Detection of lysozyme activity

The lysozyme activity in the purchased reagent was controlled following the instructions of supplier: one unit of lysozyme will produce a ΔA450 of 0.001 per min at pH 6.24 at 25 °C, using a suspension of *Micrococcus lysodeikticus* as substrate, in a 2.6 ml reaction mixture (1 cm light path).

Colorimetric lysozyme assay

The lytic activity of lysozyme (muramidase) toward a stock suspension of lyophilized *Micrococcus lysodeikticus* cells was studied in 0.01 M phosphate buffer, pH 6.2 with EDTA (0.15%) at 37 °C. This suspension was freshly prepared by dispersing 0.7 mg/ml of *M. lysodeikticus* cells in phosphate buffer and mixed with a magnetic stirrer for 0.5 h. For construction of th calibration curve, solutions with different lysozyme activity were used (0 U/ml, 40 U/ml, 80 U/ml, 120 U/ml, 160 U/ml, 200 U/ml, 240 U/ml). Each assay was initiated by adding and mixing 0.1 ml of egg-white lysozyme or investigated serum to 1 ml of bacterial cell suspension and 1 ml of phosphate buffer. In control, we used 0.1 ml deionized water instead of lysozyme. The solutions were mixed and incubated at 37 °C for 0.5 h. After that test-tubes were placed in ice. Changes in turbidity were monitored at a wavelength of 540 nm. No change of turbidity occurred under these conditions in the absence of enzyme.

A standard curve was plotted as a change of optical density *versus* the concentration of lysozyme. The concentration of lysozyme in mouse serum sample was obtained from a standard curve and expressed in U/ml.

RESULTS AND DISCUSSION

Body weight was assessed before mice were differently treated with caffeine and on day 30 of experiment. Mice preexposed to 2 mg/kg and 20 mg/kg caffeine weighed significantly less than those exposed to water, in agreement with previous observations that caffeine can have an anorexic effect in the rat (23). However, mice preexposed to high caffeine doses (40 mg/kg and 200 mg/kg) weighed more than those preexposed to

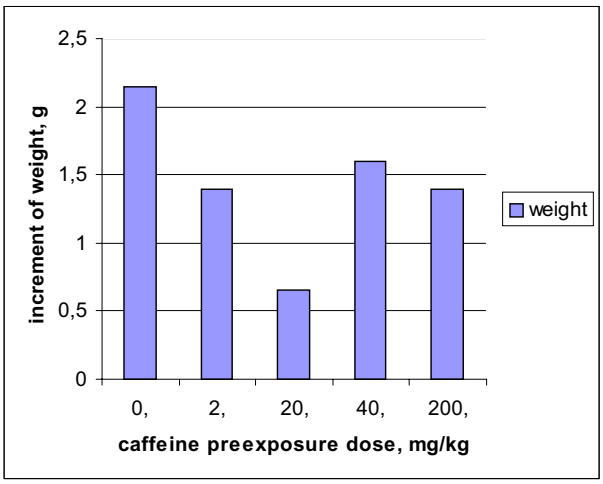

Fig. 1. Mean increase of weight of mice groups. Control group was given doses of water, Group 1 – 2 mg caffeine/kg of mouse weight, group 2 – 20 mg/kg, group 3 – 40 mg/kg, group 4 – 200 mg/kg

20 mg/kg caffeine, but less than those exposed to water (Fig. 1). The difference in weight was the result of caffeine exposure, because the weight was similar in the groups at the start of the experiment.

We have observed the behavior of mice during exposure to moderate doses of caffeine. The activity of movement and play fighting of each group was compared with those in the control group. Mice preexposed to 20 mg/kg caffeine solution exhibited a higher play fighting and movement activity. However, chronic exposure to a high dose (40 mg/kg and 200 mg/kg) had no effect on movement and play fighting. We have observed a correlation of movement activity and increased play fighting with mass change of mice. Mice preexposed to 20 mg/kg caffeine weighed significantly less than those exposed to water.

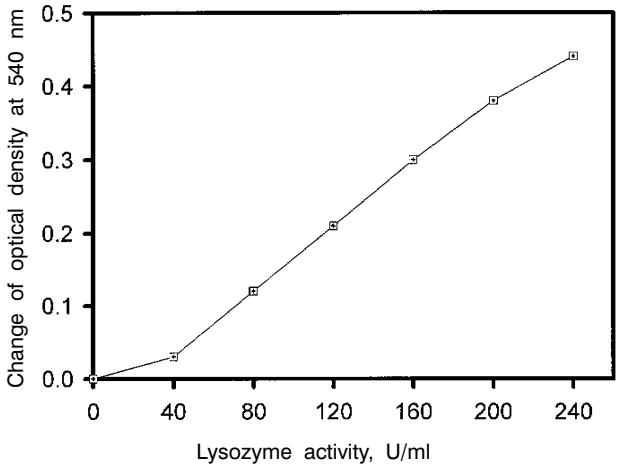

Fig. 2. Calibration graph, changes in optical density *vs.* lysozyme activity

We have found that lysozyme activity in the purchased reagent used for plotting the calibration was 7900 U/mg of solid lysozyme reagent instead of 45000 U/mg declared by purchaser. The standard curve (Fig. 2) obtained by using solutions of known lysozyme concentrations and activities was used to estimate the lysozyme concentration in the blood serum of each mouse from all five groups. Changes in the optical density of solution used for calibration were selected in the same range as those obtained during investigations of mouse serum.

The present experiment shows that different concentrations of caffeine increased lysozyme concentration in mouse serum. The obtained results (Fig. 3) show that even low concentrations of caffeine in the diet significantly increased lysozyme activity in the serum of mice, and confirmed the previous observations that CNS plays a certain part in the regulation of lysozyme activity and that caffeine is a potent CNS stimulant (5).

Our investigations allow to suggest that caffeine reinforces the first-line host defense against bacterial invaders.

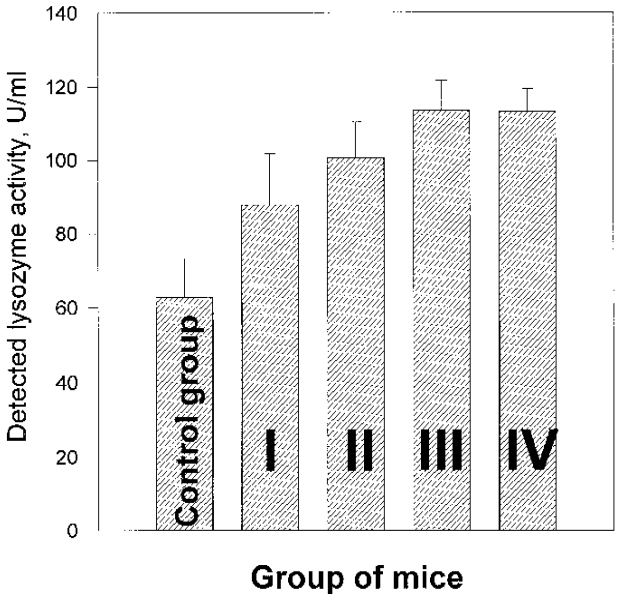

Fig. 3. Mean lysozyme activity found in each group of mice. Control group was given doses of water. Group 1 – 2 mg caffeine/kg, group 2 – 20 mg/kg, group 3 – 40 mg/kg, group 4 – 200 mg/kg

CONCLUSIONS

The results obtained in this experiment show that the state of CNS plays a particular role in the regulation of lysozyme activity in the serum of mice. Caffeine has an effect on mouse weight and movement activity.

ACKNOWLEDGEMENTS

This work was supported by Lithuanian State Science and Studies Foundation. Authors thank Dr. A. Dringelienė and Dr. A. Markevičius for advice and assistance in the research.

Received 29 April 2002
Accepted 10 September 2002

References

1. Fredholm BB, Bättig K, Holmén J, Nehlig A, Zvartau EE. Action of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacological Reviews 1999; 51: 83–133.
2. Nurminen ML, Niittynen L, Korpela R, Vapaatalo H. Coffee, caffeine and blood pressure: A critical review. Eur J Clin Nutr 1999; 53: 831–9.
3. Sawynok J, Yakahi TL. Caffeine as an analgesic adjuvant: A review of pharmacology and mechanisms of action. Pharmacol Rev 1993; 45: 43–85.
4. Veselkov DA, Kodintsev VV, Pakhomov VI, Djimant LN, Davies DB, Veselkov AN. NMR analysis of hetero-associations of caffeine with antibiotic actinomycin D in aqueous solutions. Biophysics 2002; 45: 193–202.
5. Novokrescenov MV. K voprosu o nervnoi reguliacii lizocimnoi aktivnosti syvopotki krovi. Medicina 1964; 8: 80–1.
6. Nakae T, Nikaido N. Outer membrane as a diffusion barrier in *Salmonella typhimurium*. J Biol Chem 1975; 250: 7359–65.
7. Pellegrini A, Thomas U, von Fellenberg R, Wild P. Bactericidal activities of lysozyme and aprotinin against Gram-negative and Gram-positive bacteria related to their basis character. J Applied Bacteriol 1992; 72: 180–7.
8. Cottagnoud P, Tomasz A. Triggering of pneumococcal autolysis by lysozyme. J Infec Diseases 1993; 167: 684–90.
9. Hasselberger FX. Uses of enzymes and immobilized enzymes. Chicago: Nelson- Hall Inc, 1978.
10. Osserman EF, Klockars M, Halper J, Fischer RE. Lysozyme. New York: Academic Press, 1974.
11. Kokoshis PL, Williams DL, Cook JA, Di-Luzio NR. Increased resistance to *Staphylococcus aureus* infection and enhancement in serum lysozyme activity by glucan. Science 1978; 199: 1340–2.
12. Anderson DC. The role of phagocytic cells in host defense and inflammatory disease. Nutrition 1990; 6: 5–15.
13. Dudley Youman J, Saarni MI, Linman JW. Diagnostic value of muramidase in acute myelomonocytic leukaemia and preleukemia. Clin Res 1969; 17: 348.
14. Zeman K, Kantorski J. Oxygen-independent microbiocidal systems of neutrophils. Postepy Biol Kom 1990; 17: 379–87.
15. Prokopowicz J, Kemona H, Wysocka J, Kilus S. Serum lysozyme activity in patients with lung carcinoma subjected to radiotherapy. Neoplasma 1985; 32: 605–11.
16. Gionfriddo JR, Davidson H, Asem EK, Krohne SG. Detection of lysozyme in llama, sheep, and cattle tears. Am J Net Res 2000; 61: 1294–7.
17. Roger P, Gascard JP, de Montpreville VT, Brink C. Basal secretion of lysozyme from human airways *in vitro*. Mediators of Inflammation 1999; 8: 319–23.
18. Ito Y, Yamada H, Imoto T. Colorimetric assay for lysozyme using *Micrococcus leuteus* labeled with a blue dye, Remazol brilliant blue R, as a substrate. Chem Pharm Bull 1992; 40: 1523–6.
19. Meyer K, Hahnel E. The estimation of lysozyme by a viscosimetric method. J Biol Chem 1946; 163: 723–32.
20. Becktel WJ, Baase WA. A lysoplate assay for *Escherichia coli* cell wall-active enzymes. Annal Biochem 1985; 150: 258–63.
21. Gao P, John MR, Schmidt-Gayk H, Arndt B, Scheida M, Theuer D. Solid-phase competitive luminescence immunoassay for lysozyme in faeces. Clin Chim Acta 1995; 239: 167–77.
22. Lollike K, Kjeldsen L, Sengelov H, Borregaard N. Purification of lysozyme from human neutrophils, and development of an ELISA for quantification in cell and plasma. Leukemia 1995; 9: 206–9.
23. Gans JH. Comparative toxicities of dietary caffeine and theobromine in the rat. Food Chem Toxicol 1984; 22: 365–9.

A. Ramanavičienė, V. Žukienė, J. Ačaitė, A. Ramanavičius

KOFEINO POVEIKIS PELIŲ KRAUJO SERUME NUSTATYTAM LIZOCIMO AKTYVUMUI

S a n t r a u k a

Tyrimai rodo, kad centrinė nervų sistema atlieka svarbų vaidmenį reguliuojant lizocimo aktyvumą organizmo skysčiuose. Šio tyrimo tikslas buvo nustatyti kofeino, kaip centrinę nervų sistemą stimuliuojančio agento, poveikį. Nustatyta, kad lizocimo aktyvumas laboratorinių pelių kraujo serume priklauso nuo kofeino tirpalo, kuriuo buvo girdomos pelės, koncentracijos.