Biochemical Pharmacology, Vol. 25, pp. 1733–1736. Pergamon Press, 1976. Printed in Great Britain.

IN VIVO AND *IN VITRO* EFFECTS OF STAPHYLOCOCCAL ALPHA-TOXIN ON AMINO ACID ACTIVATION IN THE AORTA*

J. PATELSKI, B. ZGORZALEWICZ, S. SZULC, Z. WALIGÓRA and J. JELJASZEWICZ

Department of Biochemistry, Department of Neurology, Academy of Medicine, Poznań, and National Institute of Hygiene, Warszawa, Poland

(*Received* 27 *October* 1975; *accepted* 19 *December* 1975)

Abstract—Increased amino acid activation in the aorta was found in rabbits injected with 25 μg of staphylococcal alpha-toxin (three times, every second day), compared with controls. The enzyme activity was increased also in the reaction mixtures containing aortic preparations from control and experimental rabbits (injected with 3 × 25 μg of the toxin) and low concentrations of the toxin (0.1 and 0.1–1.0 μg/ml respectively, and in those which contained the preparations from non-injected pigs and higher concentrations of alpha-toxin (50 μg/ml). No change in amino acid activation in the aorta was seen in rabbits injected with triple 150 μg and 250 μg dose of the toxin. Decreased enzyme activities were found in the reaction mixtures containing enzyme preparations from control and experimental rabbits (injected with 3 × 25 μg, 3 × 150 and 3 × 250 μg of the toxin), and different concentrations of the toxin (1 μg/ml, 50 μg/ml, 0.5 μg/ml and 0.1 μg/ml, respectively).

Staphylococcal alpha-toxin is a protein possessing a wide variety of biological activities [1]. In the search for sites of toxic action at the molecular level enhanced amino acid activation by enzyme preparations from pig aortas in the presence of the toxin [2] and a modified pattern of lipolytic enzyme activities of the arterial wall in rabbits injected with the toxin [3] were observed.

The present experiments were carried out to test whether the amino acid activation in the arterial wall might be influenced by staphylococcal alpha-toxin both injected in rabbits and added to the reaction mixtures containing enzyme preparations from the injected and control animals.

MATERIALS AND METHODS

Reagents. Hydroxylamine, inorganic pyrophosphatase (EC 3.6.1.1), protein amino acids and hydroxamates of the amino acids and other commercially available reagents of reagent grade were prepared and used as described previously [4]. Staphylococcal alpha-toxin was purified according to Bernheimer and Schwartz [5]. One μg of the freeze-dried toxin contained 2 minimum haemolytic doses when tested against rabbit erythrocytes. For experiments, saline solutions of the toxin were freshly prepared.

Experimental design. Belgian Giant rabbits of approximately 5 kg body wt and 12 months of age were used. Experimental animals were injected in the marginal ear vein with staphylococcal alpha-toxin: 25 μg (group II), 150 μg (III) and 250 μg (IV) in 5 ml saline, three times, every second day. Control animals (group I) were injected with saline. Twenty-four hours after last injection the animals were killed by decapitation and the aortas removed.

Enzyme preparation. Thoracic aortas from pigs were collected from the slaughter house and processed to obtain pooled acetone powders and enzyme preparations as described previously [4]. The rabbit aortas were prepared like pig aortas and cut into pieces in acetone at −20°. Acetone powders and enzyme preparations from each rabbit aorta were obtained in a similar way, except for different proportions of the powder and extractant (1:5, w/v) and the precipitate and solvent (1/1 of the initial volume).

Protein was determined by the biuret method according to Kingsley [6].

Assay of amino acid activation. Activation of amino acids was measured by determining amino acid hydroxamates [7] formed in 1-ml reaction mixtures containing 100 μmoles of an equimolar mixture of 20 amino acids and other components as described previously [4] and indicated in Table 1, in duplicate. Complete systems in the presence of heat-denatured enzyme preparations were used as controls. No hydroxamate formation is detected without the addition of amino acids to the reaction mixtures [4, 8], as neither the highly purified toxin [5] nor the enzyme preparation contain free amino acids [9].

Results are expressed in milliunits of enzyme sp. act., i.e. in nmoles of amino acid hydroxamates formed/min per mg of protein, or in nmoles/min per 0.05 ml of the enzyme preparation used.

RESULTS

There was no direct relationship between the protein content of the enzyme preparations and amino acid hydroxamates formed in the reaction mixtures. This is illustrated by lower enzyme sp. act. obtained at higher protein concentrations and *vice versa* (Fig. 1).

Protein concentration in enzyme preparations from 13 acetone powders from pooled pig aortas and the

* This investigation was supported by research grant 05-327-2 from the United State Public Health Service.

Table 1. *In vivo* and *in vitro* effect of staphylococcal alpha-toxin on amino acid activation in rabbit aorta

				Concentration of toxin in the reaction mixture† (μg ml)									
				0.1	0.2	0.5	1.0	2.0	5.0	10.0	25.0	50.0	100.0
		Protein	Activity	Difference in activity									
Group*	No. of Animals	mg/0.05 ml		nmole·min^{-1}·mg^{-1}									
I	13	0.99 ± 0.09‡	9.9 ± 2.0	0.7 (0.3) § P ≤ 0.05	0.9 (0.5)	−1.7 (1.1)	−2.5 (0.5) P ≤ 0.001	−3.0 (0.6) P ≤ 0.001	−3.6 (0.8) P ≤ 0.001	−4.0 (0.9) P ≤ 0.001	−4.2 (1.3) P ≤ 0.01	−4.5 (1.2) P ≤ 0.01	−4.8 (1.0) P < 0.001
II	6	0.97 ± 0.09	12.7 ± 2.1 P ≤ 0.01	3.6 (0.6) P ≤ 0.01	4.2 (0.7) P ≤ 0.01	4.7 (0.7) P ≤ 0.01	2.3 (0.8) P ≤ 0.05	2.0 (1.9)	0.5 (1.7)	−1.1 (1.0)	−2.0 (0.9)	−3.9 (1.0) P ≤ 0.02	−5.3 (1.0) P ≤ 0.01
III	6	0.95 ± 0.05	13.4 ± 6.8 P ≤ 0.01	−0.2 (0.5)	−0.6 (2.3)	−3.0 (0.8) P ≤ 0.02	−3.2 (0.7) P ≤ 0.01	−3.4 (0.6) P ≤ 0.01	−5.3 (0.6) P ≤ 0.001	−7.7 (0.8) P ≤ 0.001	−8.0 (1.1) P ≤ 0.001	−8.4 (1.7) P ≤ 0.01	−9.6 (2.0) P ≤ 0.01
IV	9	0.95 ± 0.05	9.1 ± 2.4	−2.4 (0.8) P ≤ 0.02	−2.6 (0.9) P ≤ 0.05	−3.6 (1.1) P ≤ 0.02	−3.9 (0.6) P ≤ 0.001	−4.0 (1.0) P ≤ 0.01	−4.5 (1.3) P ≤ 0.01	−5.4 (0.8) P ≤ 0.001	−5.7 (0.8) P ≤ 0.001	−5.9 (1.1) P ≤ 0.001	−6.8 (1.2) P ≤ 0.001

* Animals injected i.v. with (I) saline or staphylococcal α-toxin: (II) 25 μg, (III) 150 μg and (IV) 250 μg, in 5 ml saline, every second day, three times.

† Reaction mixture: $MgCl_2$, 10 μmole; Tris–HCl, 175 μmole, pH 7.8; staphylococcal alpha-toxin as indicated, equimolar mixture of 20 protein amino acids, 100 μmole; ATP, 10 μmole; pyrophosphatase, 25 μg; and enzyme preparation 0.05 ml, in 1 ml of total volume. Incubation at 37° for 30 min.

‡ Mean ± standard deviation. The means and standard deviations were compared by Student's *t*-test and comparison of variances, respectively. Where the means and standard deviations differ from control (I), the statistical significance is indicated.

§ Mean difference (standard error of the mean). The mean differences were estimated by Student's paired *t*-test. Statistically significant differences are indicated.

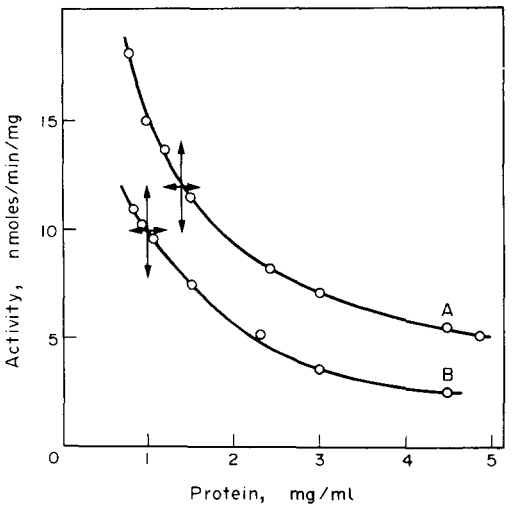

Fig. 1. Relation between the protein content of aortic enzyme preparations in the reaction mixtures and the enzyme specific activity. Activities obtained with different volumes of the enzyme preparations from (A) pig and (B) rabbit aortas are shown as circles (means from 3 series of determinations). Mean activities and protein concentrations ± standard deviations for 13 enzyme preparations from both pig and rabbit aortas are indicated by block dots with arrows. For reaction mixtures see Table 1.

corresponding activation rate of the equimolar mixture of amino acids was 1.41 ± 0.11 mg/0.05 ml and 11.7 ± 1.6 nmoles·min^{-1}·mg^{-1}, respectively. Significantly lower means were obtained for 13 rabbit aortas: 0.99 ± 0.09 mg protein/0.05 ml enzyme preparation ($P \leqslant 0.001$) and 9.9 ± 2.0 nmoles amino acids activated·min^{-1}·mg^{-1} ($P \leqslant 0.05$), as indicated by Student's *t*-test. The standard deviations for both the protein concentrations and enzyme activities are insignificantly different, as indicated by comparison of appropriate variances. The efficiency of protein extraction from acetone–butanol powders was 2.8% and 10% for pig and rabbit aorta, respectively, calculated by comparison of the mean protein concentrations and amounts of the powders used for the enzyme preparations.

Similar protein concentrations in the aortic enzyme preparations were obtained in all the rabbits studied. The enzyme activities were significantly higher in animals injected with the triple 25 μg dose of staphylococcal alpha-toxin (group II), compared with the control (I). In rabbits injected with 150 μg (group III) the activities were enhanced in 2 and unchanged in 4, compared with the range of values given by the mean ± double standard deviation in control animals, which resulted in markedly elevated standard deviation at insignificantly changed mean. Three out of 12 rabbits given the highest dose of the toxin (250 μg group IV) demonstrated a muscular tremor and excitation, and died after the first or second injection. In 7 of the remaining animals, the enzyme activities were within the range of mean ± double standard deviation in controls and in 2 the activity values were below this range (Table 1).

The amino acid activation of the aorta in control animals (group I) was increased by the toxin added to the reaction mixtures at a concentration of 0.1 μg/ml and 0.2 μg/ml. A much higher increase in the enzyme activity, which reached the maximum at 0.5 μg/ml concentration of the toxin in the reaction mixtures was seen in the aorta of animals previously injected with the 25 μg dose of the toxin (group II). In reaction mixtures containing the aortic enzyme preparations from rabbits injected with the 150 μg dose (group III), the initially high (previously enhanced) enzyme activities were increased and the relatively low (previously unchanged) were decreased by toxin added at 0.1–0.2 μg/ml; the means were insignificantly affected. The relatively low enzyme activities in animals injected with the highest 250 μg dose of the toxin (group IV) were considerably decreased by toxin added to the reaction mixtures (Table 1).

Highly significant positive correlation between the initial enzyme activities and the maximum activities at different concentrations of the toxin added to the reaction mixtures was found within groups, I, $r = 0.91$, $P \leqslant 0.001$ (for 0 and 0.1 μg toxin/ml reaction mixture); II, $r = 0.95$, $P \leqslant 0.01$ (0.5 μg/ml) and III, $r = 0.99$, $P \leqslant 0.001$ (0.1 μg/ml) but not within group IV, $r = 0.50$, $P > 0.1$ (0.1 μg/ml). The correlation between the appropriate mean activities in rabbits (groups I, II and IV; group III was omitted because of the elevated standard deviation) and pigs (group V) has also been calculated: $r = 0.97$, $P \leqslant 0.05$ (Fig. 2).

DISCUSSION

Species difference may account for the significantly higher enzyme specific activity at considerably higher protein concentration of the aortic preparations in

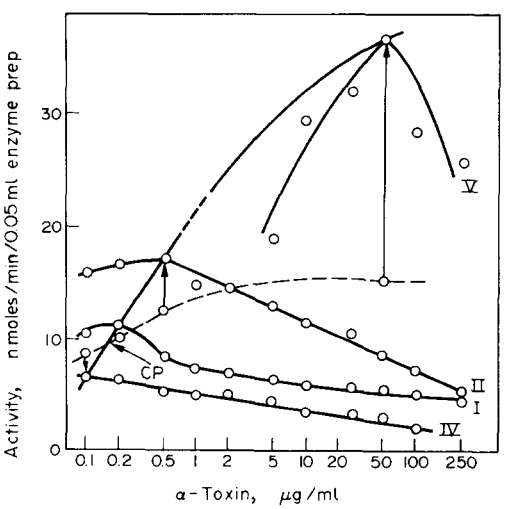

Fig. 2. Effects of staphylococcal alpha-toxin on amino acid activation in the aorta. I, II, IV—rabbits injected with saline, 25 μg and 250 μg of the toxin (three times, every second day), respectively, V—non-injected pigs; for details see text. Initial mean activities (dotted line) and maximum mean activities obtained at different concentrations of the toxin in the reaction mixtures (full line) are connected by arrows showing the differences; the decrease and increases are separated at mean 0.15 μg/ml concentration of the toxin, as indicated by the cross-point (CP) of the two curves. The initial and maximum mean activities are positively correlated ($r = 0.96$; $P \leqslant 0.05$).

pigs than in rabbits. However, differences in the extraction procedure (see Material and Methods) due to much smaller amounts of material available in rabbits may have influenced the results.

The lack of direct proportion between the amounts of reaction products and protein of the enzyme preparation present in the reaction mixture with 20 amino acids also requires comment. Different rates of formation of the individual amino acid hydroxamates [4], among other factors may be of importance. This cannot be overlooked when enzyme activities measured by this method are compared.

There is little doubt that the changes in the enzyme activity in rabbits injected with the toxin are due to differences in extractability of the enzyme(s) because the mean values of protein were not different between control and experimental animals.

The further *in vitro* increase in the enzyme activity which has already been elevated *in vivo* by the toxin indicates the importance of the enzyme/toxin concentration ratio. The higher the initial enzyme activity, the greater increase in the activity occurs at relatively higher concentration of the toxin in the reaction mixture. It is true for the enzyme activities both elevated by previous injection of the toxin and the originally high ones in control rabbits and pigs. This is shown by the high and significant coefficients of positive correlation between the initial activities and maximum activities obtained at different *in vitro* concentrations of the toxin.

Decreased amino acid activation in the aorta by staphylococcal alpha-toxin was definitely seen only in the *in vitro* experiment in rabbits. Thus, either the enzyme activity could not be diminished by the toxin given intravenously or the triple 250 μg dose was not high enough to obtain an effect. However, some inhibition of the enzyme system seems to have followed the injections as the lowest *in vitro* toxin concentration was sufficient to decrease the enzyme activity. The animals would not survive injections of higher dose because some of them were killed by 250 μg, which was above the known lethal dose of the toxin [10].

Thus, staphylococcal alpha-toxin has been shown to affect the amino acid activation in the aortic wall both *in vivo* and *in vitro*, depending on the dose applied. The mechanism of the dose/enzyme response relationship is not clear. Conformational changes of the enzyme(s) and/or other components of the complex system of amino acid activation may be involved. The effect of staphylococcal alpha-toxin on the initial step of protein synthesis can result in impaired metabolic processes in the arterial wall.

REFERENCES

1. J. Jeljaszewicz, in *The Staphylococci* (Ed. J. O. Cohen) p. 249. Wiley–Interscience, New York (1972).
2. J. Patelski, Z. Waligóra, B. Zgorzalewicz and S. Szulc, *Enzyme* **12**, 704 (1971).
3. J. Patelski, S. Szulc, B. Pniewska, Z. Przetakiewicz and J. Jeljaszewicz, in *Contributions to Microbiology and Immunology: Staphylococci and Staphylococcal Infections* (Ed. J. Jeljaszewicz) p. 352. Karger, Basel (1973).
4. J. Patelski, Z. Waligóra, B. Zgorzalewicz and S. Szulc, *Enzyme* **14**, 297 (1972).
5. A. W. Bernheimer and L. L. Schwartz, *J. gen. Microbiol.* **30**, 455 (1963).
6. M. Kingsley, *J. Lab. clin. Med.* **27**, 840 (1942).
7. M. B. Hoagland, E. B. Keller and P. C. Zamecnik, *J. biol. Chem.* **218**, 345 (1956).
8. M. B. Hoagland, *Biochim. biophys. Acta* **16**, 288 (1955).
9. P. Szafrański and E. Sułkowski, *Acta biochim. pol.* **6**, 133 (1959).
10. J. Jeljaszewicz, S. Szmigielski and C. Zak, *Zbl. Bakteriol. I. Orig.* **209**, 310 (1969).