Br. J. Pharmac. (1972), **45**, 60–63.

Degradation of angiotensin II by plasmin

G. DUDEK, Z. S. LATALLO, J. PROKOPOWICZ AND K. WOROWSKI

From the Institute of Physiology and Biochemistry, Department of Clinical Biochemistry and Department of Physiological Chemistry, Medical Academy, Białystok (Poland) and Department of Radiobiology and Health Protection, Institute of Nuclear Research, Warsaw (Poland)

Summary

1. Synthetic angiotensin II (Hypertensin, Ciba, Basel) was incubated with a water-insoluble preparation of plasmin (E.C. 3.4.4.14) and the resulting products analysed by paper chromatography and N-terminal amino-acid analysis.

2. Only the N-terminal asparagine was split off from the peptide. This indicates that plasmin attacks the Asn-Arg but not the Arg-Val in angiotensin II molecule.

Introduction

Angiotensin, an octapeptide endowed with a high pressor activity, may easily be destroyed by a number of endo- and exopeptidases including such well defined enzymes as trypsin (E.C.3.4.4.4.) and chymotrypsin (E.C.3.4.4.5) and less well characterized angiotensinases isolated from plasma, red cells and kidneys (Riniker & Schwyzer, 1961; Glenner, McMillan & Folk, 1962; Khairallah, Bumpus, Page & Smeby, 1963; Yang, Erdös & Chiang, 1968; Kokubu, Akutsu, Fujimoto, Ueda, Hiwada & Yamamura, 1969).

In a series of papers (Prokopowicz, Worowski, Popławski, Gabryelewicz & Niewiarowski, 1967; Prokopowicz, Worowski, Popławski, Myśliwiec & Niewiarowski, 1967; Prokopowicz, Worowski & Niewiarowski, 1969) have confirmed previous observations concerning the relationship between the state of activation of the fibrinolytic system and various pathological conditions and have shown that the treatment of angiotensin II with plasmin resulted in destruction of the pressor activity to about 50%. The present experiments were designed to determine the peptide bond(s) in angiotensin II cleaved by plasmin.

Methods

Angiotensin II (Asn-Arg-Val-Tyr-Val-His-Pro-Phe, Hypertensin, CIBA, Basel). Plasmin, highly purified human plasminogen prepared according to the method of Wallen (1962), was activated with streptokinase (high purity preparation produced for intravenous injection by Biomed, Warsaw). Fifty units of streptokinase were added to 1 mg of plasminogen and incubated at 20° C until activation was complete. Dansyl (DNS; 1-Dimethylaminoaphthalene—5-sulphonyl chloride, Koch-Light, Laboratories Ltd.).

Ascending paper chromatography was carried out on Whatman paper No. 1 at 20° C for 8 h using a solvent mixture of n-butanol-acetic acid-water (4:1:5). Chromatograms were developed according to the method of Macek (1958).

Identification of the N-terminal amino acids was made according to the method of Gray & Hartley (1963). DNS-amino acids prepared by the method of Boulton & Busch (1964) were used as reference standards. Ascending chromatography on 20 × 20 cm glass plates covered with a 0·25 mm thick layer of activated silica gel G was performed at 20° C for 90 min using two of the solvent systems proposed by Morse & Horecker (1966):
(a) chloroform: tertiary amyl alcohol: acetic acid (70:30:0,5) and
(b) chloroform: tertiary amyl alcohol: formic acid (70:30:1).
The position of the spots was established using a UV-lamp.

Plasmin was transformed into a water insoluble form in order to avoid interference of the products of plasmin autocatalytic digestion or added inhibitors on the analysis of the products of angiotensin cleavage. The soluble enzyme was

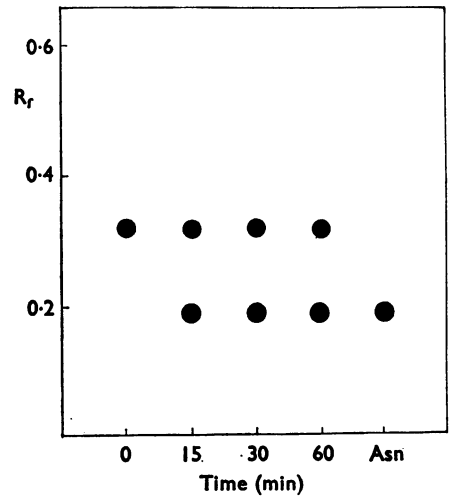

FIG. 1. Diagram indicating the chromatographic pattern of the products obtained at various times of incubation of angiotensin II with plasmin. At 0 min angiotensin had not been exposed to plasmin. Asn indicates asparagine alone.

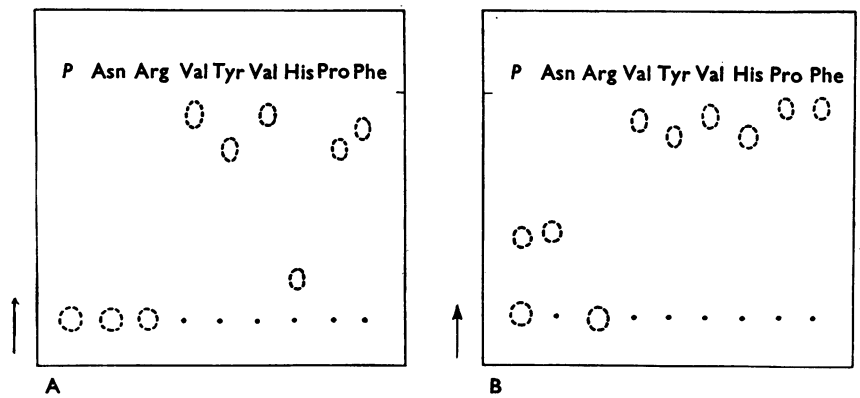

FIG. 2. Tracings of the thin-layer chromatography (silica gel) of dansylated derivatives of the products (*P*) of angiotensin degradation by plasmin, and of dansylated amino acids used as a reference. A and B represent different solvent systems (conditions described in text).

trapped in a lattice of a highly crosslinked polyacrylamide gel according to the method of Bernfeld & Wan (1963). The gel containing plasmin was homogenized, washed and used as a suspension. Incubation of angiotensin with plasmin was carried out at 37° C. One ml of the mixture contained 1 mg of angiotensin and such amount of the insoluble enzyme which corresponded to an equivalent of 0·55 units of soluble plasmin when tested by the method of Remmert & Cohen (1949) using casein as a substrate. Previous observations (Latallo & Bang, unpublished results) indicated, however, that the smaller the substrate molecule the easier was its access to the insoluble enzyme. Hence, the activity given here should, in fact, be much higher.

Phosphate buffer 0·15 M pH 7·65 was used as a medium, and constant stirring applied throughout the whole period of incubation. The proteolytic reaction was stopped by removing the enzyme by centrifugation for 10 min at 5,000 rpm and the products present in the supernatant were subjected to further study.

Results

Figure 1 illustrates the results of paper chromatography of undigested angiotensin (at 0 min) and products obtained after 15, 30, 60 minutes. After 15 min the products were resolved into two spots as indicated diagrammatically in Fig. 1. The position of one corresponded to the undigested angiotensin (Rf=0·32) although it probably was the peptide less asparagine. The position of the other, slower moving, spot (Rf=0·19) was identical to that of asparagine, thus indicating that the Asn-Arg bond was cleaved by plasmin. Prolongation of the incubation time changed neither the intensity of the spots, nor their number. Staining of the chromatograms with Sakaguchi reagent revealed the presence of one spot only (Rf=0·32), whereas ninhydrin stained both. These results were confirmed by the N-terminal amino acid analysis of the products obtained after 60 min incubation. DNS-derivatives of the products (P) and of free amino acids present in the angiotensin molecule were compared by thin-layer chromatography using two different solvent systems (Fig. 2) as described in the previous section on methods.

Discussion

Like trypsin, plasmin is considered to attack specifically arginyl and lysyl bonds (Troll & Sherry, 1955). The results presented here indicate that the enzyme does not always conform to this rule. In the case of angiotensin, it splits off the N-terminal asparagine instead of cleaving the next Arg-Val bond. Although a high purity plasmin preparation was used in this study it is impossible to exclude its contamination with other enzymes. However, angiotensinase A (Khairallah *et al.*, 1963) which is known to attack the Asn-Arg bond, is inactivated by the removal of Ca ions. Our experiments were performed in 0·15 M phosphate which has been shown to completely inactivate this enzyme (Khairallah, *et al.*, 1963).

The finding that incubation of angiotensin with plasmin resulted in the loss of about 50% of its pressor activity is consistent with our results, since Schwyzer (1961) showed that the removal from, or substitution of, asparagine in the angiotensin molecule resulted in a loss of about half its activity.

In conclusion, it can be said that activation of plasmin in blood may possibly decrease the blood pressure by various means. Besides the activation of the

kallikreinkinin system and the potentiating effect of the products of fibrinolysis on kinins (Buluk, Małofiejew & Czokało, 1966) plasmin, as confirmed here, may directly destroy the pressor activity of angiotensin.

However, it must be pointed out that naturally occurring angiotensin contains an aspartyl residue and not asparagine, the corresponding amino acid contained in the angiotensin used in the present investigation. It is possible that plasmin has a different specificity when acting upon the naturally occurring peptide.

We are grateful to Ciba Basel for a supply of angiotensin II.

REFERENCES

BERNFELD, P. & WAN, J. (1963). Antigens and enzymes made insoluble by entrapping them into lattice of synthetic polymers. *Science*, **142**, 678–679.

BOULTON, A. A. & BUSCH, J. E. (1964). Quantitative and qualitative analysis of amino acids as their "Dansyl" derivatives. *Biochem. J.*, **92**, 11*P*.

BULUK, K., MALOFIEJEW, M. & CZOKALO, M. (1966). Unknown properties of the products of plasmin degradation of fibrinogen and fibrin. *Bull. Pol. Acad. Sci.*, CI VI, **14**, 193–197.

GLENNER, G. G., MCMILLAN, P. J. & FOLK, J. E. (1962). A mammalian peptidase specific for the hydrolysis of N-terminal α-L-glutamyl and aspartyl residues. *Nature*, **194**, 867.

GRAY, W. R. & HARTLEY, B. S. (1963). A fluorescent end-group reagent for proteins and peptides. *Biochem. J.*, **89**, 59*P*.

KHAIRALLAH, P. A., BUMPUS, F. M., PAGE, I. H. & SMEBY, R. R. (1963). Angiotensinase with a high degree of specificity in plasma and red cells. *Science*, **140**, 672–674.

KOKUBU, T., AKUTSU, H., FUJIMOTO, S., UEDA, E., HIWADA, K. & YAMAMURA, Y. (1969). Purification and properties of endopeptidase from rabbit red cells and its process of degradation of angiotensin. *Biochem. Biophys. Acta*, **191**, 668–676.

MACEK, K. (1958). *Handbuch der Papierchromatographie*, Vol. 1, pp.905–908. Jena: Gustav Fischer Verlag.

MORSE, D. & HORECKER, B. L. (1966). Thin-layer chromatographic separation of DNS-amino acids. *Anal. Biochem.*, **14**, 429–433.

PROKOPOWICZ, J., WOROWSKI, K., POPLAWSKI, A., GABRYELEWICZ, A. & NIEWIAROWSKI, S. (1967). Fibrinolytic system in patients with hypertensive cardiovascular disease. *Thrombos. Diathes. haemorrh.*, **17**, 1–7.

PROKOPOWICZ, J., WOROWSKI, K., POPLAWSKI, A., MYŚLIWIEC, M. & NIEWIAROWSKI, S. (1967). Fibrinolytic system in the dog with experimental renal (Goldblatt) hypertension. *Experientia*, **23**, 224–225.

PROKOPOWICZ, J., WOROWSKI, K. & NIEWIAROWSKI, S. (1969). Partial inactivation of angiotensin II by plasmin. *Thrombos. Diathes. haemorrh., Stuttg.*, **21**, 166–167.

REMMERT, L. F. & COHEN, P. (1949). Partial purification and properties of a proteolytic enzyme of human serum. *J. biol. Chem.*, **181**, 431.

RINIKER, B. & SCHWYZER, R. (1961). Die sterische Einheitlichkeit des synthetischen Val^5-Hypertensin II Asp^1-β-amids. *Helv. Chim. Acta*, **44**, 658–667.

SCHWYZER, R. (1961). Synthetische Analoge des Hypertensins. I. *Einleitung. Helv. Chim. Acta*, **44**, 667–674.

TROLL, W. & SHERRY, S. (1955). The activation of human plasminogen by streptokinase. *J. biol. Chem.*, **213**, 881–891.

WALLEN, P. (1962). Effect of lysine and the purification of human plasminogen on cellulose ion exchangers. *Arkiv. Kemi*, **19**, 451–457.

YANG, H. Y. T., ERDÖS, E. G. & CHIANG, T. S. (1968). New enzymatic route for the inactivation of angiotensin. *Nature*, **218**, 1224–1226.

(*Received October* 13, 1971)