## A Human Neutrophil-dependent Pathway for Generation of Angiotensin II

# PURIFICATION OF THE PRODUCT AND IDENTIFICATION AS ANGIOTENSIN II

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ABSTRACT A human neutrophil lysosomal protease interacts at physiologic pH with a 62,000-67,000mol wt plasma protein substrate to generate a vasoactive, smooth muscle-contracting "neutral" peptide. The peptide product of this system, previously designated the "neutral" peptide-generating pathway, was generated from purified components and purified by Bio-Gel P2 gel filtration and reverse-phase high performance liquid chromatography with a 50-60% yield of starting activity. The purified peptide had an amino acid composition of Asx, Pro, Val, Ile, Tyr, Phe, His, Arg, a composition identical to that of angiotensin II. The peptide and synthetic angiotensin II each filtered at 48-52% bed volume on Bio-Gel P2, had an isoelectric point of pH 7.8-8.1 at 4°C, migrated 3 cm toward the cathode during pH 6.4 low-voltage paper electrophoresis, and had a retention time of 44.8 min during reverse-phase high-performance liquid chromatography. In addition, the functional activity of the peptide at each purification step correlated with angiotensin II content determined by specific radioimmunoassay. The amino acid sequence of 25 nmol of the peptide was Asp-Arg-Val-Tyr-Ile-His-Pro-Phe, the same covalent structure as that of angiotensin II. Therefore, under physiologic conditions, in the absence of renin or angiotensin converting enzyme, a human neutrophil neutral protease cleaves a plasma protein to yield angiotensin II. This pathway provides a mechanism through which the neutrophil may alter local blood flow during inflammation by generation of a potent vasoactive peptide.

#### INTRODUCTION

Human neutrophils contain a neutral protease that cleaves a smooth muscle contracting and vasoactive peptide from a plasma protein substrate (1). This pathway is controlled by two plasma proteins:  $\alpha_1$ -antitrypsin inhibits the neutrophil protease and a plasma inactivator destroys the peptide product (1). The enzyme and substrate of this system, previously designated the "neutral" peptide-generating pathway, have been purified to homogeneity and characterized. The neutrophil enzyme is a neutral protease whose physicochemical characteristics and synthetic substrate specificity are identical to leukocyte cathepsin G (2, 3). The plasma protein substrate is a glycoprotein with physicochemical characteristics similar to angiotensinogen, the substrate of renin (4). The "neutral" peptide product of this pathway has now been purified to homogeneity and identified as angiotensin II on the basis of physicochemical, antigenic, functional, and structural characteristics. This human neutrophildependent pathway provides a mechanism for the generation of angiotensin II without requirement for renin or angiotensin converting enzyme.

### METHODS

*Materials.* Phenyl-Sepharose CL-4B and Sephacryl S-200sf were purchased from Pharmacia Fine Chemicals, Div. of

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Pharmacia, Inc., Piscataway, N. J.; Affi-Gel Blue, hydroxylapatite (Bio-Gel HTP), and Bio-Gel P2 from Bio-Rad Laboratories, Richmond, Calif.; angiotensin I and II from Sigma Chemical Co., St. Louis, Mo.; specific antisera to angiotensin I and II, Tyrosyl-<sup>125</sup>I monoiodinated 5-L-isoleucine angiotensin I and II from New England Nuclear, Boston, Mass.; dioxane, acetonitrile, and tetrahydrofuran (THF)<sup>1</sup> from Burdick and Jackson Laboratories., Inc., Muskegon, Mich.; pyridine, trifluoracetic acid, and 1,2-dichloroethane,1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (BOC-ON) from Aldrich Chemical Co., Milwaukee, Wis.; phenylisothiocyanate from Beckman Instruments, Inc., Palo Alto, Calif.; dimethyl formamide and aminoethylaminopropyl glass beads from Pierce Chemical Co., Rockford, Ill.; and porcine alpha-chymotrypsin from Millipore Corp., Bedford, Mass.

Assay of neutral peptide. Unless otherwise noted, neutral peptide, generated by interaction of the neutrophil protease and plasma protein substrate, was measured by its ability to contract the isolated, terminal guinea pig ileum that was previously standardized with partially purified neutral peptide. 1 U of neutral peptide activity was arbitrarily defined as the amplitude of the contraction caused by 25  $\mu$ l of the standard solution. Neutral peptide standard was made by incubation of 10  $\mu$ g neutral peptide-generating protease (2) with 1.5 ml partially purified, heat-denatured plasma protein substrate (10 mg protein/ml) for 60 min at 37°C in 0.01 M Tris, pH 8.0, 0.15 M NaCl. The reaction mixture was then filtered at 4°C at a flow rate of 3.0 ml/h through a  $1.5 \times 90$ -cm Bio-Gel P2 column equilibrated in 0.1 M NH4HCO3, pH 7.9, and the single peak of contractile activity was pooled and stored at -70°C in 200-µl portions. Partially purified plasma protein substrate, used for the generation of neutral peptide standard, was prepared as previously described (4).

Purification of components of the neutral peptide-generating pathway. The neutrophil neutral peptide-generating protease was purified from fresh human neutrophils as described (3) and stored at  $-70^{\circ}$ C in 50-µl portions (300-700 µg/ml) in 0.01 M Tris, pH 7.4, 1.0 M NaCl. Portions were thawed and each used only once.

The plasma protein substrate of the neutrophil neutral peptide-generating protease was purified from 300 ml of citrated human plasma by 45% ammonium sulfate precipitation of contaminating protein, Affi-Gel Blue affinity chromatography, hydroxylapatite chromatography, Phenyl-Sepharose hydrophobic chromatography, and Sephacryl S-200sf gel filtration (4). Final preparations contained 6.7 mg protein/ml in 0.01 M Tris, pH 7.4, 0.15 M NaCl, and gave a single stained band of 62,000-67,000 mol wt as assessed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis of reduced protein (5). At 1:50 enzyme:substrate molar ratios, neutral peptide activity could be generated from native plasma protein substrate or from material which had been heated for 2 h at 61°C. Because the yield of contractile activity was greater with heat-denatured substrate, it was used for production of the peptide for preparative purification.

Procedures for isolation and characterization of neutral peptide. Gel filtration was carried out at  $4^{\circ}$ C in a  $1.5 \times 90$ cm Bio-Gel P2 column equilibrated in 0.1 M NH<sub>4</sub>HCO<sub>3</sub>, pH 7.9, pumped at a flow rate of 3.0 ml/h. Fractions of 1.0% bed volume were collected.

Reverse-phase high performance liquid chromatography (HPLC) was carried out using an Altex Model 322 system

(Altex Scientific, Inc., Berkeley, Calif.) on a 4.6 × 250-mm Ultrasphere ODS column (Altex Scientific, Inc.). Peptides (1-50 nmol) were dissolved in 200  $\mu$ l 0.02 M H<sub>3</sub>PO<sub>4</sub>/0.1 M NaCl O<sub>4</sub>, pH 2.7, and the entire sample was injected into the column equilibrated in the same buffer. Peptide elution was accomplished with a linear gradient from 0-60% acetonitrile in 0.02 M H<sub>3</sub>PO<sub>4</sub>/0.1 M NaCl O<sub>4</sub>, pH 2.7. The gradient was initiated at the time of injection and pumped with a flow rate of 1.0 ml/min at room temperature. Alternatively, reversephase HPLC was carried out using a 0.02 M H<sub>3</sub>PO<sub>4</sub>/0.02 M KH<sub>2</sub>PO<sub>4</sub>, pH 2.8 buffer system. Elution of material was monitored by absorbance at 210 nm, and 1.0-ml fractions were collected. All buffers were filtered through a 0.22- $\mu$ m or 0.5- $\mu$ m millipore filter (Millipore Corp.) and degassed before use. When appropriate, peptides were desalted by gel filtration through a  $0.4 \times 15$ -cm Bio-Gel P2 column equilibrated in 0.1 M NH4HCO3, pH 7.9. The column was pumped at room temperature at a flow rate of 3.0 ml/h, and 1.0-ml fractions were collected. The conductivity of each column fraction was measured.

Isoelectric focusing was carried out at 4°C in a  $1.25 \times 15.3$ cm tube containing 2% pH 3.5-10 ampholytes in a linear 19ml 10-40% sucrose density gradient. The pH gradient was established by application of 9 mA for 3.5 h, after which a  $100\mu$  sample in 25% sucrose containing 2% pH 3.5-10ampholytes was inserted into the gradient via a 22-gauge spinal needle. A 500-V potential was applied for 3.5 h, at which time the current had remained stable for 1 h and the gradient was fractionated into 20 1.0-ml samples. The pH of each fraction was determined before direct bioassay.

Low voltage paper electrophoresis was conducted as described (6) in 4 M pyridine:acetic acid:H<sub>2</sub>O, 25:1:225 vol/vol/vol pH 6.4, and paper strips were stained for amino acids with ninhydrin (0.25% in acetone), or for arginine (7) or histidine (8). Peptide containing bands were cut from the remainder of each paper strip and eluted with 2.0 ml 0.01 M NH<sub>4</sub>OH and assayed directly for neutral peptide activity. The relative charge of neutral peptide was determined by comparing its migration to that of amino acid standards electrophoresed separately as described (9).

Competitive-binding radioimmunoassays for angiotensin I and angiotensin II were performed according to the methods of the manufacturer (New England Nuclear) using specific antisera for angiotensin I and II.

Procedures for structural analysis of neutral peptide. Chymotryptic digestion was performed with 10 nmol neutral peptide in 200  $\mu$ l, 0.1 M NH<sub>4</sub>HCO<sub>3</sub>, pH 7.9, containing 20  $\mu$ g alpha-chymotrypsin at 4°C for 4 h. The entire reaction mixture was subjected to reverse-phase HPLC as described above. Column fractions were bioassayed and the amino acid compositions determined.

Amino acid analyses were obtained by hydrolyzing peptide samples with 5.7 N HCl at 108°C for 24 h *in vacuo*. The amino acid compositions were determined using a Durrum D-500 amino acid analyzer (Dionex Inc., Sunnyvale, Calif.).

The amino acid sequence of purified neutral peptide was carried out by coupling the peptide to aminoethylaminopropyl glass beads via a water-soluble carbodiimide reagent (10). The peptide was then degraded over its entire length by manual phenylisothiocyanate degradation. At each step, one-half of the anilinothiazolinone derivative was back-hydrolyzed to the free amino acid (5.7 N HCl, 150°C for 18 h) and identified by amino acid analysis; the remainder was converted to the corresponding phenylthiohydantoin (PTH)-derivative with 25% trifluoroacetic acid at 55°C for 30 min and analyzed by HPLC on an Altex Ultrasphere ODS-PTH column (4.6 mm  $\times$  250 cm) at 45°C. The column was equilibrated in 5% THF in 0.009 M sodium acetate buffer,

<sup>&</sup>lt;sup>1</sup>Abbreviations used in this paper: HPLC, high performance liquid chromatography; PTH, phenylthiohydantoin; THF, tetrahydrofuran.

pH 5.11 (solvent A) and the PTH-amino acids were eluted with a linear gradient from 100% solvent A to 40% of 10% THF in acetonitrile (solvent B) for 20 min at a flow rate of 1.3 ml/min. The mobile phase composition was then changed to 30% solvent B and the remaining PTH-derivatives were separated isocratically at a flow rate of 2.0 ml/min.

#### RESULTS

Purification of neutral peptide. Neutral peptide was generated by incubating  $100 \,\mu g$  of neutral peptidegenerating protease with 10 mg purified plasma protein substrate for 1 h at 37°C in 1.4 ml of 0.01 M Tris, pH 7.4, 0.15 NaCl, and the reaction was stopped by cooling in ice. The reaction mixture was directly bioassayed for neutral peptide contractile activity; 30,000 U were detected. The entire reaction mixture was filtered through a previously standardized Bio-Gel P2 column and the column fractions were bioassayed for neutral peptide activity. A single peak of contractile activity was detected that corresponded to the angiotensin II marker at 48-52% bed volume (Fig. 1). Fractions with contractile activity were pooled and directly bioassayed; 19,000 U were detected. The pool was lyophilized and resuspended in 2.0 ml of HPLC equilibrating buffer, and 10% of this sample, which contained  $\sim$ 1,900 U of activity, was subjected to reverse-phase HPLC using the phosphate buffer system. Fractions were directly assayed for contractile activity, and a single region of activity with a retention time of 35.5-37.0 min was detected and coincided with a major peak of optical density at 210 nm (Fig. 2). Approximately 1.800 U of contractile activity were recovered. A 400- $\mu$ l portion of the fraction that corresponded to a retention time of 35.5-36.5 min and contained the highest amount of contractile activity was lyophilized, an aliquot hydrolyzed, and the amino acid composition determined (Table I). The amino acid composition of neutral peptide was Asx, Pro, Val, Ile, Tyr, Phe, His, Arg, all in equimolar ratio (Table I). This composition is identical to that of angiotensin II. The remaining 90% of column filtered contractile activity was purified by reverse-phase HPLC and used for physicochemical and structural studies. The overall recovery of starting activity was 58%. Additional material was purified by a similar protocol that used reversephase HPLC in the perchlorate buffer system, and under these conditions the retention time of neutral peptide was 44-45 min. Overall recovery of three such preparations was 50-60%.

Because neutral peptide was purified from material made using a heat-denatured protein substrate, 1,100 U of contractile activity were generated from 2.5 mg native plasma protein substrate and purified using a protocol similar to that described above. The neutral peptide product was detected at 48–52% bed volume



FIGURE 1 Bio-Gel P2 gel filtration of neutral peptide.

during Bio-Gel P2 gel filtration and the material eluted at 44-45 min during reverse-phase HPLC in perchlorate buffer. The amino acid composition was also Asx, Pro, Val, Ile, Tyr, Phe, His, Arg, all in equimolar ratio.

Characterization of neutral peptide and identification as angiotensin II. Because neutral peptide and angiotensin II were identical in terms of amino acid composition and behavior during Bio-Gel P2 gel filtration, the two peptides were compared during isoelectric focusing, low-voltage paper electrophoresis, and reverse-phase HPLC. 10 nmol of angiotensin II and 10 nmol of purified neutral peptide were subjected to isoelectric focusing on separate 10-40% sucrose density gradients using 2% vol/vol, pH 3.5-10.0 ampholytes. The contractile activity of each peptide was similar and was detected in fractions corresponding to pH 7.8-8.1 (Fig. 3).

To determine the electrophoretic mobility of the two peptides, 3 nmol neutral peptide, 50 nmol angiotensin II, and 20 nmol of an amino acid standard mixture were separately dissolved in 20  $\mu$ l of electrophoresis buffer and electrophoresed at pH 6.4 in parallel on separate 2.5 × 41.5-cm paper strips. Neutral peptide and angiotensin II each migrated 3 cm from the origin toward the cathode as assessed by bioactivity.

The elution pattern of neutral peptide and angiotensin II were compared on reverse-phase HPLC using the perchlorate buffer system and a linear



FIGURE 2 Reverse-phase high performance liquid chromatography of neutral peptide. HPLC was carried out using a 0.02-M  $H_3PO_4/0.02$  M  $KH_2PO_4$ , pH 2.8 buffer system in aqueous acetonitrile.

0-60% acetonitrile gradient. 2 nmol of neutral peptide and of angiotensin II were dissolved in 200  $\mu$ l of the equilibrating buffer and separately subjected to HPLC, and 1.0-ml fractions were collected and bioassayed. In three experiments the retention time (mean±SD) of neutral peptide and angiotensin II were 44.8±0.15 min and 44.8±0.21 min, respectively, as monitored by absorbance at 210 nm and bioactivity. In a similar protocol, 2 nmol each of angiotensin I and bradykinin had retention times of 48.9±0.12 min and 42.8±0.07 min, respectively. To confirm that the retention times of angiotensin II and neutral peptide were identical, 2 nmol neutral peptide and 2 nmol angiotensin II were combined in 200  $\mu$ l of equilibrating buffer, and the

 TABLE I

 Amino Acid Composition of Purified Neutral Peptide

Amino Acid	Residues/mol		
	Average mole %	Average values	
Asx*	14.69	1.17 (1)	
Pro	13.87	1.11(1)	
Val	13.62	1.09(1)	
Ile	10.63	0.85(1)	
Tvr	9.79	0.78 (1)	
Phe	14.39	1.15(1)	
His	10.60	0.85(1)	
Arg	12.40	0.99(1)	

\* The average number of residues of each amino acid was calculated on the basis of an octapeptide. The values are given as the averages of six runs of 24 h acid hydrolyses. In each run, only the eight calculated amino acids were present in the amino acid composition. mixture was subjected to reverse-phase HPLC as described. A single region of biologic activity corresponded to a peak of optical density with a retention time of 44.8 min.

To explore the functional and antigenic relationship between angiotensin II and neutral peptide, the angiotensin II content of material obtained from each step of neutral peptide purification was determined by guinea pig ileum bioassay standardized with angiotensin II and by radioimmunoassay. At each purification step, the total amount of angiotensin II as determined by radioimmunoassay was similar to that measured by bioassay (Table II). The angiotensin I content, as determined by radioimmunoassay, accounted for 0.5% of the bioactivity (Table II).

Because the octapeptide angiotensin II contains a chymotrypsin-susceptible Tyr4-Ile5 bond, 8 nmol neutral peptide and 10 nmol angiotensin II were subjected to chymotryptic digestion and the cleavage products were compared. To separate and identify the chymotryptic cleavage products, each reaction mixture was subjected to reverse-phase HPLC using the perchlorate buffer system and a linear 0-60% acetonitrile gradient. The neutral peptide and angiotensin II digestion mixtures each yielded two major peptidecontaining regions of optical density with retention times of 21.4 and 32.0 min, and 21.5 and 31.5, respectively. 30% of the material from each optical density peak detected in the neutral peptide mixture was separately lyophilized, acid hydrolyzed, and analyzed for amino acid composition. The amino acid compositions were Asx, Val, Tyr, Arg for the peptide eluting at 21.4 min (peptide A) and Pro, Ile, Phe, His for that at 32.0 min (peptide B). The amino acid composition of peptide A corresponded to that of the amino



FIGURE 3 Isoelectric focusing of neutral peptide ( $\bullet$ ) and angiotensin II ( $\bigcirc$ ).

terminal-tetrapeptide of angiotensin II, and the composition of peptide B corresponded to the carboxyterminal tetrapeptide of angiotensin II.

To confirm that neutral peptide was identical to angiotensin II, the amino acid sequence of neutral peptide was determined. Neutral peptide was generated, purified, and desalted as described, and 25 nmol were attached to glass beads via carbodiimide coupling. Manual Edman degradations were performed for nine cycles, and the PTH-amino acids were identified both by reverse-phase HPLC and amino acid analysis. The sequence of Asp-Arg-Val-Tyr-Ile-His-Pro-Phe obtained (Fig. 4) confirmed that neutral peptide was angiotensin II.

#### DISCUSSION

The neutral peptide contractile product generated by the interaction of a human neutrophil neutral protease and a plasma protein substrate (1) has been shown to be angiotensin II, an octapeptide whose structure is Asp-Arg-Val-Tyr-Ile-His-Pro-Phe. Using purified neutrophil protease (2) and plasma protein substrate (4), the smooth muscle contracting peptide was generated in vitro and isolated by a two-step procedure (Figs. 1 and 2). Physicochemical (Figs. 1 and 3), functional, antigenic (Table II), and structural analyses (Table I, Fig. 4) were used to show that the purified neutral peptide was identical to angiotensin II.

Angiotensin II, an octapeptide, is the principal biologically active peptide derived from the plasma protein angiotensinogen (11). Angiotensin II is generated from angiotensinogen by a pathway that requires two enzymes. Angiotensinogen is cleaved at a Leu-Leu bond by renin to release the decapeptide angiotensin I (12). Angiotensin I is then rapidly converted under physiologic conditions to angiotensin II by a dipeptidyl carboxypeptidase, which is designated

converting enzyme (13). In addition to this pathway, angiotensin II may be directly cleaved from a synthetic tetradecapeptide substrate or from angiotensin I by an enzyme designated tonin, which has been isolated from the rat salivery gland (14). A wide variety of biologic activities has been attributed to angiotensin II, including hemodynamic effects such as elevation of blood pressure (15), decreased mesenteric blood flow in cats, dogs, and humans (16-19), and reduced blood flow and vasoconstriction in perfused limbs of rabbits, cats and dogs (16, 20). Angiotensin II enhances vascular permeability (21, 22) and induces widening of interendothelial cell spaces in aortic, coronary, mesenteric, and peripheral arteries (16, 21, 23-25). In addition, this peptide is dipsogenic when administered into the central nervous system of rat (26) and stimulates aldosterone secretion from the adrenal cortex (27, 28).

The human neutrophil-dependent pathway which generates angiotensin II during interaction of a neutrophil protease and plasma protein substrate was previously designated the neutral peptide-generating pathway (1). The product of this pathway was appreciated because of its ability to contract the anti-

 TABLE II

 Angiotensin I and Angiotensin II Content of Neutral

 Peptide at Each Purification Step

	Angiotensin II content		Angiotensin I content	
Purification step	Bioassay	Radio- immunoassay	Radio- immunoassa	
		nmol		
Starting material	$82 \pm 10$	79±2	< 0.5	
Bio-Gel P2	$52 \pm 8$	$51 \pm 1.5$	< 0.5	
Reverse-phase HPLC	$51 \pm 1.5$	$48 \pm 2$	<0.5	



FIGURE 4 Amino acid sequence of neutral peptide. Panels represent HPLC chromatograms of PTH amino acids obtained following manual Edman degradation of 25 nmol neutral peptide coupled to glass beads. The yield of each residue is expressed as total nmol detected by HPLC. For each residue, the optical density peak corresponding to an amino acid as determined by amino acid analyzer is designated by the appropriate letter. For residue 7, the marked increase in background optical density is attributed to the longer hydrolysis at a higher temperature required for the proline residue. C, cysteic acid; D, aspartic acid; CMC, carboxymethyl cysteine; E, glutamic acid; N, asparagine; S, serine; Q, glutamine; T, threonine; G, glycine; H, histidine; Met SO<sub>2</sub>, methionine sulfone; A, alanine; I, isoleucine; K, lysine; L, leucine; NL, norleucine.

histamine-treated, atropinized terminal guinea pig ileum in vitro (1). Initial characterization showed that the 1,000-mol wt contractile principle was digested by trypsin and chymotrypsin, and had an isoelectric point of pH 7.2-7.4 (1). Although the contractile peptide was slightly basic, it was designated neutral peptide to distinguish it from the more basic kinin peptides (1). Each component of this pathway has now been purified to homogeneity and characterized (2-4).

The neutrophil enzyme is a neutral protease of 29,000–30,000 mol wt (2) that has been localized to the neutrophil granule by noncytotoxic release from cytocholasin-B treated human neutrophils and by subcellular fractionation of purified neutrophils (3). It is inhibited by  $\alpha_1$ -antitrypsin (1) and cleaves human fibrinogen and fibrin (29). The protease has been separated from leukocyte collagenase and elastase on the basis of physicochemical characteristics and synthetic substrate specificity (2). To generate angiotensin II from angiotensinogen, cleavage of a Phe-His bond is required. The ability of the neutrophil protease to catalyze this reaction, its ability to cleave N-benzoyl-L-tyrosine ethyl ester (3), a synthetic substrate of leukocyte cathepsin G, and the recognition of cathepsin G by antibody to the angiotensin II-generating neutral protease (3) show that the neutrophil protease is cathepsin G (30, 31).

The plasma protein substrate of the neutrophil enzyme has been purified to homogeneity (4). It has an isoelectric point of pH 4.6–5.0 and is a single polypeptide chain glycoprotein of 62,000–67,000 mol wt. The serum concentration of this protein is  $120\pm22 \ \mu g/ml$ (4). This protein is identical to angiotensinogen because it is similar in terms of physicochemical characteristics (3), cochromatographs with angiotensinogen during each purification step (32), is cleaved by renal renin to yield angiotensin I as measured by specific radioimmunoassay (32), and has an amino terminal amino acid sequence that is identical to the covalent structure of angiotensin I (32).

Although the physiologic and pathobiologic significance of this pathway are not clear, it is possible to speculate upon the biologic role of this neutrophildependent pathway in which angiotensin II may be generated under physiologic conditions without requiring renin or converting enzyme. In addition to generating angiotensin II from angiotensinogen, cathepsin G converts angiotensin I to angiotensin II (unpublished observations). Among the other substrates of cathepsin G are fibrin and fibrinogen (29, 30). The fibrinolytic potential in concert with generation of angiotensin II suggests that the modulated expression of this system may facilitate inflammatory cell movement into a local tissue site by generation of a potent permeability factor which regulates local blood flow, and by local control of fibrin deposition.

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