Biological Activity of an Angiotensin II–Ferritin Conjugate on Rabbit Aortic Smooth Muscle Cells†

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ABSTRACT: Specific binding sites for [Asp[1],Ile[5]]angiotensin II (angiotensin) have been demonstrated in homogenates and subcellular fractions of aortic medial smooth muscle cells, but the localization of the angiotensin receptor responsible for contraction has not been determined [Devynck, M. A., & Meyer, P. (1976) *Am. J. Med. 61*, 758–767]. To establish the location of this receptor, we have prepared a membrane-impermeable analogue of angiotensin by acylating its N-terminal amino group with the *N*-hydroxysuccinimide ester of succinylated ferritin. This angiotensin–ferritin conjugate possessed the same intrinsic activity as angiotensin but was approximately 200 times less potent in inducing contraction in rabbit aortic strips. The stability of the conjugate was investigated, and approximately 5% of the contractile activity of the angiotensin–ferritin conjugate was attributable to low molecular weight components that were present before or after exposure to aortic strips. The time required for aortic strips to reach a plateau of contraction in response to angiotensin–ferritin was significantly longer than that required by free angiotensin to produce the same level of contraction. With enzymatically dispersed aortic smooth muscle cells, however, the time taken to produce contractions by both angiotensin and angiotensin–ferritin was indistinguishable. [Sar[1],-Ala[8]]angiotensin II, a competitive inhibitor of angiotensin, completely suppressed contractions induced by angiotensin or angiotensin–ferritin in aortic strips or dispersed aortic smooth muscle cells. These results suggest that angiotensin need not directly penetrate the plasma membrane to cause contraction and imply that the angiotensin receptor responsible for initiating contraction of aortic smooth muscle cells is located on the plasma membrane.

The octapeptide angiotensin II plays a role in the regulation of normal blood pressure and appears to be involved in establishment and/or maintenance of elevated blood pressure in some essential hypertensive patients (Gavras et al., 1978). Angiotensin has been shown to bind to a plasma membrane enriched fraction of homogenized rabbit aorta (Devynck et al., 1973) and to crude particulate fractions from homogenates of adrenal cortex and uterus (Capponi & Catt, 1979). Angiotensin receptors have also been reported in mitochondria-rich fractions from homogenates of cardiac muscle and uterus (Goodfriend et al., 1971). Autoradiographic studies using ^{125}I-labeled angiotensin have suggested that the hormone enters nuclei of cardiac endothelial cells and aortic smooth muscle cells when injected into the circulation (Robertson & Khairallah, 1971).

However, it has not been established whether the angiotensin receptor sites responsible for inducing contraction of aortic smooth muscle cells are present on the plasma membrane or are located within the cell. Therefore, we have investigated the ability of angiotensin covalently coupled to a membrane-impermeable ligand to induce contraction in cultures of rabbit aortic smooth muscle cells and in rabbit aortic strips.

In previous studies, intravenous infusions of macromolecular conjugates of angiotensin-elevated blood pressure in dogs (Deodhar, 1960) and rats (Arakawa et al., 1962) and a cytochrome *c*–angiotensin complex contracted rabbit aortic strips (Richardson & Beaulnes, 1971). On the basis of their histochemical reaction studies, Richardson & Beaulnes (1971) concluded that the vascular endothelium is the cellular site of action of angiotensin in vessels and not the smooth muscle cells.

We have prepared a membrane-impermeable conjugate of angiotensin by acylating angiotensin with the *N*-hydroxysuccinimide ester of succinylated ferritin. We have measured the biological activity of the angiotensin–ferritin conjugate by using rabbit aortic strips and enzymatically dispersed aortic smooth muscle cells. Also, we have determined the amount of biologically active material with low molecular weight which was present in the angiotensin–ferritin conjugate before and after contracting a rabbit aortic strip. Our results suggest that the angiotensin receptor responsible for initiating contraction of aortic smooth muscle cells is likely located on the plasma membrane.

Experimental Procedures

Materials

Angiotensin used in these experiments was synthesized by the solid-phase method as reported previously (Galardy et al., 1978) and was greater than 95% homogeneous as evaluated by countercurrent distribution and thin-layer chromatography. [Sar[1],Ala[8]]angiotensin was from Norwich Pharmaceuticals, Norwich, CT. ^{3}H-Labeled angiotensin (30 Ci/mmol) was from New England Nuclear, Boston, MA. Sephadex G-75 (40–120 μm) and Sepharose 6B were from Pharmacia Fine Chemicals, Piscataway, NJ. Ultrafiltration membranes were from Amicon Corp., Lexington, MA. Horse spleen ferritin was from Calbiochem, San Diego, CA, and soybean trypsin inhibitor was from Worthington Biochemical Corp., Freehold, NJ. Hepes,[1] Mes, and *N*-hydroxysuccinimide were from Sigma Chemical Co., St. Louis, MO, and were recrystallized 2 times from ethanol before use. *N*-Ethylmorpholine and 1-ethyl-[3-(dimethylamino)propyl]carbodiimide were from

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[1] Abbreviations used: angiotensin, [Asp[1],Ile[5]]angiotensin II; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; ED_{50}, effective dose which produces 50% of the maximum response.

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Aldrich Chemical Co., Milwaukee, WI, and *N*-ethylmorpholine was distilled over ninhydrin before use. Amino acid solutions, antibiotics, and fetal calf serum were from Grand Island Biological Co., Grand Island, NY. The modified Krebs–Ringer salt solution used for aortic strip contraction assays contained 105 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1 mM $MgSO_4$, and 2 mM $CaCl_2$ and was buffered at pH 7.4 with 25 mM Hepes. Culture dishes were from Falcon Plastics, Oxnard, CA. Medium 199 containing Hank's salts was from Flow Laboratories, Rockville, MD.

Methods

Coupling of Angiotensin with Ferritin. Angiotensin was covalently coupled to ferritin by using a modification of a procedure for preparing ferritin–antibody conjugates (Kishida et al., 1975). In the first step, succinic anhydride (2.9 g, 29 mmol) was added with stirring to 1 g of ferritin (theoretically containing 290 μmol of primary amino groups) in 75 mL of 2 M Hepes at pH 9 and reacted for 6 h at 23 °C. Succinylated ferritin was dialyzed overnight at 4 °C against 4 L of 1 mM Mes at pH 6 and then applied to a 1.5 × 100 cm column of Sephadex G-75 equilibrated and eluted in 5-mL fractions with 1 mM Mes, pH 6, at a flow rate of 0.3 mL/min. Succinylated ferritin fractions were located by absorbance at 440 nm and were pooled and concentrated at 4 °C over an Amicon PM-10 membrane to 10 mL. Five milliliters of succinylated ferritin (0.35 μmol of ferritin theoretically containing 394 μmol of carboxyl groups) was added with stirring at 4 °C to 12.5 mL of 1 M Mes at pH 6. A 2-fold molar excess of *N*-hydroxysuccinimide (90.75 mg, 788 μmol) and EDC (150 mg, 788 μmol) was added. After 5 h, the reaction mixture was applied to a 1.5 × 100 cm column of Sephadex G-75 equilibrated and eluted in 7.5-mL fractions of 4 °C with 1 mM Mes, pH 6, at a flow rate of 0.4 mL/min. The esterified ferritin peak was immediately pooled and concentrated as before and then added to 6.4 mL of 1 M Hepes at pH 7.6 containing 67 mg of angiotensin II (64 μmol) with 2.7 μCi of ^{3}H-labeled angiotensin added as a tracer.

After 16 h at 4 °C, the sample was applied to the first of three successive 1.5 × 100 cm columns of Sephadex G-75 which were equilibrated and eluted at 23 °C with 1 mM *N*-ethylmorpholinium acetate at pH 7.5 in 7.5-mL fractions at a flow rate of 0.4 mL/min. The ferritin peak from each Sephadex G-75 column was pooled, concentrated to 10 mL with an Amicon PM-10 membrane, and applied to the next Sephadex G-75 column. Following the third Sephadex G-75 column, the angiotensin–ferritin conjugate was applied to a 2 × 180 cm column of Sepharose 6B equilibrated and eluted at 23 °C with 10 mM *N*-ethylmorpholinium acetate in 10-mL fractions at a flow rate of 0.12 mL/min. The ferritin monomer peak was pooled and concentrated as before and passed over a fourth 1.5 × 100 cm column of Sephadex G-75 equilibrated and eluted at 23 °C with modified Krebs–Ringer salt solution containing 10 μg/mL soybean trypsin inhibitor, 100 U/mL penicillin, 0.25 μg/mL Fungizone, and 0.1 mg/mL streptomycin. The ferritin peak was pooled and concentrated as before and stored sterilely at 4 °C after passage through a 0.45-μm pore Millipore filter (Millipore Corp., Bedford, MA). The average number of angiotensin molecules coupled per ferritin molecule was determined from the amount of ^{3}H-labeled angiotensin coupled, assuming that the coupling of ^{3}H-labeled and unlabeled angiotensins to esterified ferritin proceeded identically. We presume that angiotensin is derivatized on its amino group on the basis of acylation of hemoglobin by *N*-hydroxysuccinimide esters (Lomant & Fairbanks, 1976).

Aortic Strip Contraction Assay. Rabbit aortic strip contraction assays were performed as described previously (Furchgott & Bhadrakom, 1953) with slight modifications. Male New Zealand white rabbits averaging 2 kg were sacrificed with a 4-mL intravenous injection of Nembutol, and the thoracic aorta was removed and placed in Krebs–Ringer solution. After excess adventitia were removed, a glass rod was inserted into the aorta, and a spiral strip 4 mm wide with a 45° pitch was cut by using a scalpel. A 2-cm length of aortic strip was tied by using loops of surgical silk between the bottom of a 10-mL water-jacketed vessel and a Grass FT 0.03 strain gauge which was connected to a Grass Model 70 polygraph. An initial stress of 5 gm was applied to the strip which was submerged in 10 mL of Krebs–Ringer solution supplemented with essential and nonessential amino acids as formulated in modified Eagle's medium (Eagle, 1959) and containing 100 U/mL penicillin, 0.25 μg/mL Fungizone, 0.1 mg/mL streptomycin, and phenol red as indicator. Air saturated with water was slowly bubbled from the bottom of the chamber. Dose–response curves to angiotensin were always run before and after a test sample. Molar concentrations reported for the angiotensin–ferritin conjugate always represent the amount of angiotensin in the conjugate. Following a contraction series, the strip was washed 6 times over a 20-min period and allowed to relax to a constant base line. Contraction forces are expressed as grams of tension generated. All assays were performed at 37 °C.

Isolated Rabbit Aortic Smooth Muscle Cells. Isolated smooth muscle cells were prepared by using a previously published procedure employing enzymatic digestion of the tunica media of rabbit aorta with purified collagenase and elastase in the presence of calcium (0.2 mM) followed by mild mechanical shear (Ives et al., 1978). The dispersed cells were cultured in Falcon 3001 tissue culture dishes for 16 h at 37 °C in medium 199 buffer with 25 mM Hepes at pH 7.4 containing 10% fetal calf serum and antibiotics, and saturated with air. Response of the isolated cells was recorded by photographing a field of cells before and after addition of an agonist with a Zeiss inverted phase contrast microscope. Experiments were performed at 37 °C.

Time Course of Strip Contraction. The time period from addition of a dose of agonist until the contraction reached a plateau was calculated from the strip chart of the Grass polygraph. A contraction was assumed to have reached a plateau when no additional contraction occurred during a subsequent 1-min period.

Hydrolysis of N-Hydroxysuccinimide Ester. The rate of hydrolysis of benzoyl-*N*-hydroxysuccinimide was monitored by following the increase in absorbance at 215 nm due to liberated *N*-hydroxysuccinimide and corrected for absorbance of benzoic acid and starting ester. A 0.1 M solution of benzoyl-*N*-hydroxysuccinimide in absolute ethanol was diluted 100-fold with 0.1 M Mes at pH 5.7. At various times, 200 μL of ester solution was acidified with 1 mL of 2 M HCl and then scanned with a Beckman Model 25 spectrophotometer equipped with a chart recorder. Extinction coefficients at 215 nm for benzoyl-*N*-hydroxysuccinimide, benzoic acid, and *N*-hydroxysuccinimide in 2 M HCl were determined to be 3.8 × 10^3, 4.6 × 10^3, and 7.6 × 10^3 M^{-1} cm^{-1}, respectively.

Results

Preparation and Chemical Properties of Angiotensin–Ferritin. The succinylation reaction reached a plateau after 6 h with 70% of the total primary amino groups succinylated as determined by ninhydrin reaction (Moore, 1968). The isoelectric point of the succinylated ferritin was 4.1–4.3 (Radola, 1973) compared to a p*I* of 4.2–4.6 for native ferritin

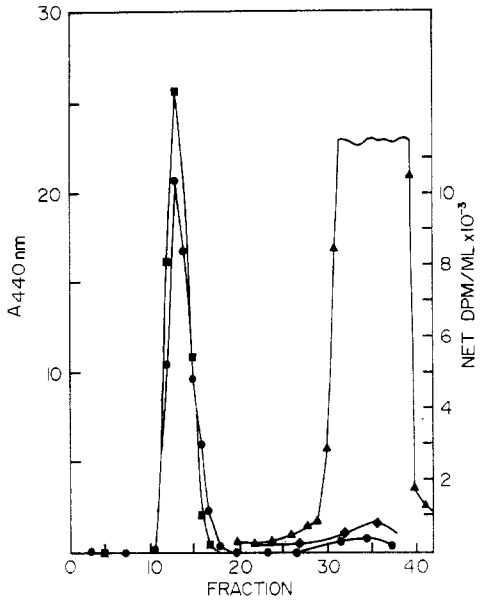

FIGURE 1: Three successive Sephadex G-75 column profiles of angiotensin–ferritin sample following the coupling reaction. Void volume fractions 10–17 containing angiotensin coupled to ferritin were pooled, concentrated, and reapplied to successive desalting columns as described under Materials and Methods. Symbols: absorbance of ferritin at 440 nm for third column (■); radioactivity from ^{3}H-labeled angiotensin for (▲) first column, (♦) second column, and (●) third column. The radioactivity in the ferritin peak is shown only for the third column.

(Urushizaki et al., 1971). Disodium succinate at pH 5.7 and triethylamine at pH 9.5 were tested as buffers for the reaction, but the extent of succinylation and stability of ferritin were superior in Hepes at pH 9.0.

Esterification of carboxyl groups of succinylated ferritin with 1-ethyl-[3-(dimethylamino)propyl]carbodiimide and *N*-hydroxysuccinimide was performed in Mes buffer at pH 5.7 rather than in phosphate buffer at pH 7 because of the greater stability of *N*-hydroxysuccinimide esters at lower pH and greater extent of esterification (Hoare & Koshland, 1967). The half-time for hydrolysis of benzoyl-*N*-hydroxysuccinimide in 0.1 M Mes, pH 5.7, was 13 h at 23 °C, and the rate of hydrolysis increased sharply in Hepes buffer at pH 7.4 and in *N*,*N*-bis(2-hydroxyethyl)glycine (Bicine) buffer at pH 8.5. Coupling of tritium-labeled leucine (specific activity 370 μCi/mmol) under the same conditions used to couple tritium-labeled angiotensin to activated ferritin indicated that the maximum esterification of succinylated ferritin with *N*-hydroxysuccinimide occurred after 5 h of reaction and that 43 mol of leucine was coupled per mol of ferritin. Following the coupling of angiotensin to ferritin, the reaction mixture was chromatographed 3 times on Sephadex G-75 columns (Figure 1). The first passage removed 99.5% of the free angiotensin. Two additional Sephadex G-75 columns were necessary to further remove free angiotensin in order to accurately measure the low biological activity of the angiotensin–ferritin conjugate in later experiments.

Commercial ferritin contains varying percentages of stable aggregates (Niitsu & Listowsky, 1973), and the ferritin sample used in this procedure before succinylation and esterification contained approximately 80% monomer on the basis of the elution position and area of peaks resolved by Sepharose 6B chromatography. Following the coupling procedure, the major peak of the angiotensin–ferritin conjugate (representing 60% of the total sample) was monomeric ferritin on the basis of its elution position with Sepharose 6B chromatography (Figure 2) and its appearance by electron microscopy.

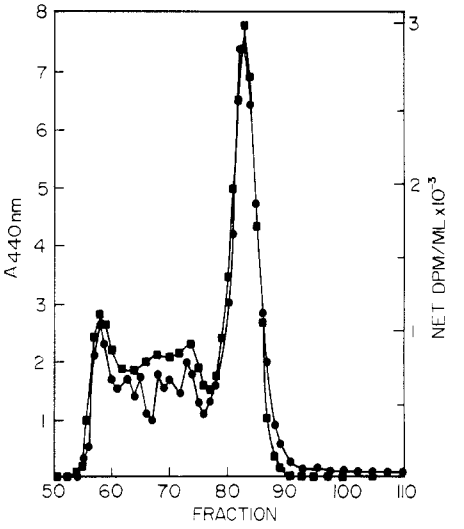

FIGURE 2: Sepharose 6B column profile of angiotensin–ferritin conjugate following third Sephadex G-75 column. Fractions 78–90 were pooled to obtain ferritin monomers. Symbols: (■) absorbance of ferritin at 440 nm; (●) radioactivity from ^{3}H-labeled angiotensin. Peak tube for ferritin monomers was tube 84, for ferritin dimers was tube 73, for ferritin trimers was tube 68, determined by electron microscopy of negatively stained samples. Column void volume was tube 56, and salt volume was tube 110.

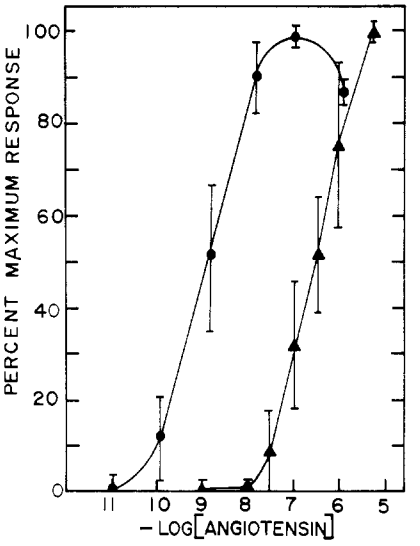

FIGURE 3: Dose–response curves of angiotensin and angiotensin–ferritin. Contractions of different aortic strips by angiotensin (●) or angiotensin–ferritin (▲) were normalized to the maximum tension produced by angiotensin for each strip. The mean ± 1 standard deviation are shown for 18 dose–response curves for angiotensin and 6 dose–response curves for angiotensin–ferritin, except the contraction produced by the highest dose of angiotensin–ferritin which is the average of two experiments.

Following the fourth Sephadex G-75 column, the sample of angiotensin–ferritin monomer was calculated to have an average of seven angiotensin molecules per ferritin molecule. The yield of coupling of angiotensin to ferritin was 0.33% on the basis of the starting amount of angiotensin, and the overall recovery of ferritin was 93%.

Biological Activity of Angiotensin–Ferritin. As shown in Figure 3, the average ED_{50} for angiotensin was 1.4 nM, and maximum strip contraction was produced by 0.1 μM angiotensin. On the basis of the contraction of angiotensin in the conjugate, the average ED_{50} for angiotensin–ferritin was 0.3 μM, indicating that the angiotensin–ferritin conjugate was approximately 200 times less potent than free angiotensin, although their intrinsic activities were similar.

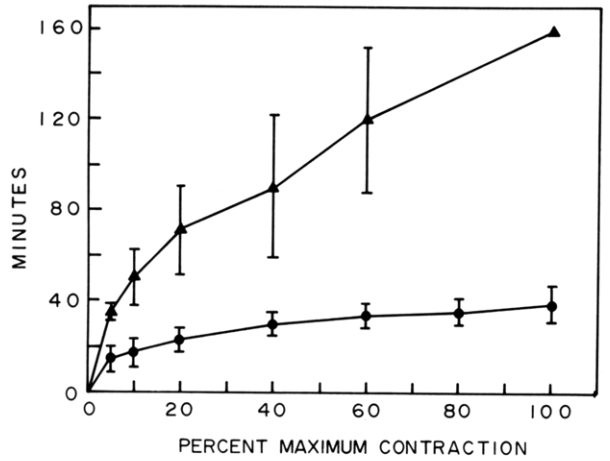

FIGURE 4: Cumulative time for aortic strip contraction. The time for each dose of agonist to reach a plateau of contraction was added to the amount of time that all preceding doses of agonist had taken to produce their plateaus of contraction. The accumulated time is plotted against the accumulated contraction produced by increasing doses of agonist. The mean ± 1 standard deviation are shown for seven angiotensin dose–response curves and for five angiotensin–ferritin dose–response curves. Symbols: (▲) angiotensin–ferritin; (●) angiotensin.

The specificity of contraction of aortic strips to angiotensin and angiotensin–ferritin was demonstrated by using [Sar^1,Ala^8]angiotensin which is a potent competitive inhibitor of angiotensin (Regoli et al., 1974). [Sar^1,Ala^8]angiotensin at 0.1 μM completely abolished the contraction elicited by 10 nM angiotensin and by 2 μM angiotensin–ferritin. Contraction induced by 1 μM norepinephrine was unaffected by [Sar^1,Ala^8]angiotensin (data not shown).

During the generation of dose–response curves for angiotensin–ferritin, the time required for a dose of angiotensin–ferritin to reach a plateau of contraction was always longer than that required for a dose of angiotensin to produce that same amount of contraction (Figure 4).

Since the aortic strip has an extensive extracellular matrix of collagen and elastin, penetration of angiotensin–ferritin may be hindered. Enzymatically dispersed aortic smooth muscle cells have freely accessible plasma membranes (Ives et al., 1978). When angiotensin–ferritin or angiotensin was added to dispersed aortic smooth muscle cells which had been cultured for 16 h, approximately 20% of the cells responded within 1 min by contracting and forming phase dense cytoplasmic blebs (Figure 5). Although evaluation of contraction by the dispersed cells was qualitative, all of the cells that underwent shape changes interpreted as contractions did so within 1 min and did not appear to develop more extensive contractions for the duration of exposure to angiotensin or to angiotensin–ferritin (15–20 min.). These responses were also totally inhibited by [Sar^1,Ala^8]angiotensin.

The competitor experiments described above established that angiotensin was the agonist present in the angiotensin–ferritin solution but did not indicate to what extent contraction was produced by angiotensin coupled to ferritin, by lower molecular weight conjugates containing angiotensin such as fragments of ferritin coupled to angiotensin, or by free angiotensin. For determination of how much contraction was produced by low molecular weight components, an aortic strip was exposed to 1 μM angiotensin–ferritin (8 nmol) that had been stored at 4 °C for 4 months following the last Sephadex G-75 column chromatography. After the strip had contracted fully (equal to 5 nM angiotensin), the medium surrounding the strip was removed and filtered through an Amicon PM-30 membrane which retains molecules with masses greater than 30 000 daltons. After the strip was washed and had relaxed to base line, the medium was replaced by the Amicon filtrate which produced a contraction equal to 0.25 nM angiotensin, suggesting that 5% of the biologically active angiotensin was not coupled to ferritin. This contraction was inhibited by [Sar^1,Ala^8]angiotensin. When the same experiment was performed with 1 nM angiotensin rather than with angiotensin–ferritin, the filtrate produced a contraction indistinguishable from that elicited by the initial angiotensin solution, indicating quantitative recovery of free angiotensin.

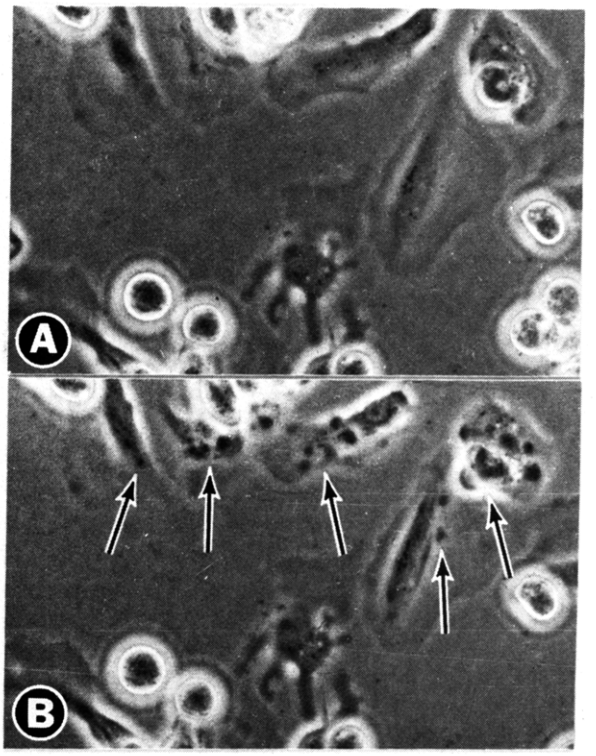

FIGURE 5: Cultured aortic smooth muscle cells exposed to angiotensin–ferritin. Smooth muscle cells were cultured for 1 day before experimentation. (Panel A) Before exposure to 1 μM angiotensin–ferritin. (Panel B) Same field 1 min after addition of 1 μM angiotensin–ferritin. Arrows indicate cells contracting and forming surface blebs. Magnification 387×.

By use of a different method, 8 nmol of angiotensin–ferritin (equal to 1 μM angiotensin–ferritin in aortic strip assay) was passed over a Sephadex G-75 column, and the included volume of the column was pooled, lyophilized, and tested for its ability to contract the aortic strip. The lyophilized sample produced a contraction equal to 0.20 nM angiotensin, while 8 nmol of angiotensin–ferritin which had not been chromatographed over the Sephadex G-75 column produced a contraction equal to 5 nM angiotensin, suggesting that 4% of the biologically active angiotensin was not coupled to ferritin.

Discussion

Angiotensin has been coupled previously through its N-terminal amino group to other molecules and proteins. As shown in Figure 6, the relative potency based on the amount of angiotensin in the conjugate decreased as the molecular weight of the N-terminal substituent of angiotensin increased. However, two of the conjugates (peptides 10 and 12, Figure 6) had relatively high potencies compared to the other macromolecular angiotensin conjugates. In these two cases, uncoupled angiotensin was separated from the conjugate by dialysis, and the degree of contamination by free angiotensin was not determined. It is possible that part of the biological activity of these two conjugates was due to angiotensin not

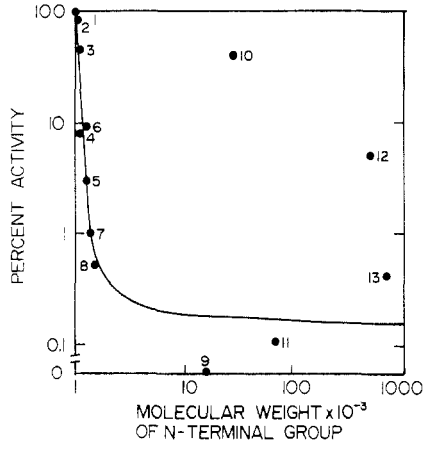

FIGURE 6: Biological activity of N-terminal linked angiotensin conjugates as a function of molecular weight. Percent activity is the ratio of the ED_{50} for angiotensin divided by the ED_{50} for N-terminal linked angiotensin conjugate. Peptide 1 is [Asp[1],Ile[5]]angiotensin; 2 is N^{α}-acetyl-1; 3 is N^{α}-hexanoyl-1; 4 is N^{α}-(formylbenzoyl)-[Asn[1],Val[5]]angiotensin II; 5 is N^{α}-(2-methoxy-2,4-diphenyl-3-(2*H*)-furanone)-1; 6 is N^{α}-(fluorescamine)-1; 7 is N^{α}-(2-nitro-5-azidobenzoylnorleucyl)-1; 8 is N^{α}-(*N*-fluoresceinthiocarbamoyl)-1; 9 is poly(L-lysine)-[Asn[1],Val[5]]angiotensin II; 10 is N^{α}-(poly(*O*-acetylserine))-1; 11 is avidin-N^{α}-(2-nitro-5-azidobenzoyl)-1; 12 is bovine γ-globulin-N^{α}-(azobenzoyl)-[Asn[1],Val[5]]angiotensin II; 13 is ferritin-1. Biological activities were determined by using rabbit aortic strip contraction assay except for peptides 2, 3, 9, 10, and 12 which were determined in rat colon contraction assay. Reference for peptides 4, 5, 7, 8, and 11 is Galardy et al. (1978); 12 is Deodhar (1960); 10 is Arakawa et al. (1962); 9 is Haber et al. (1965); 6 is Forget et al. (1975); 1–3 are Nouailhetas et al. (1977).

coupled to the macromolecules.

The angiotensin–ferritin conjugate (peptide 13, Figure 6) which we describe here has a low biological potency relative to free angiotensin and agrees with the trend seen with the carefully purified macromolecular angiotensin derivatives (peptides 9 and 11, Figure 6). If the angiotensin molecules (average of seven) are randomly coupled over the surface of the ferritin molecule, then some of the angiotensin molecules may not be able to interact with plasma membrane receptors for angiotensin due to the large diameter of ferritin (>100 Å; Ishitani et al., 1975). With the assumption that only one angiotensin on a ferritin molecule can interact at one time with a plasma membrane receptor for angiotensin, the maximum relative potency of the conjugate would be $^1/_7$ or 14%. However, the measured potency of the conjugate relative to free angiotensin is much less (0.4%), indicating that factors other than the accessibility of angiotensin in the conjugate to its receptor influence biological activity.

The biological activities of other small protein and peptide hormones coupled to membrane-impermeable ligands have been measured. Some have been reported to have high biological potency while others possessed low potency. For example, insulin was coupled to ferritin presumably through one or more of its three primary amino groups and was found to have equipotent biological activity relative to free insulin (Jarett & Smith, 1974, 1975, 1977). Insulin coupled to cyanogen bromide activated Sepharose was reported to be nearly as potent as free insulin (Cuatrecasas, 1969). In contrast to these results, acylation or succinylation of the A-chain amino terminus of insulin reduced its biological potency by 65%, and substitution by a slightly larger group (*tert*-butyloxycarbonyl) at this position reduced its potency by 83% (Pullen et al., 1976; Blundell et al., 1972). Modification of the other two primary amino groups of insulin showed a similar pattern of reduced biological potency with increasing size of the substituent.

Biotinylation of the amino terminus of the B chain of insulin or of its ε-amino group at lysine-β-29 did not significantly reduce its biological potency. However, when avidin (May et al., 1978) or avidin coupled to Sepharose (Hofmann et al., 1977) was added to these biotinylinsulins, their relative biological potencies were reduced by 95% and 85%, respectively.

Fluorescent derivatives of insulin and epidermal growth factor also have been prepared, and the biological potencies of the derivatized hormones decreased with increasing size of the substituent. $N^{\epsilon B\text{-}29}$-Rhodamine-insulin retained 75% of its biological potency and 89% of its relative binding affinity to isolated adipocytes, whereas the relative biological activity of insulin coupled through lysine B-29 with α-lactalbumin which itself had been labeled with seven rhodamines was 1%, and its binding affinity was 8% of that for native insulin (Schechter et al., 1978). Epidermal growth factor derivatized through its N-terminal amino group with fluorescein (Haigler et al., 1978) did not lose biological potency. However, when epidermal growth factor was coupled to ferritin in 1:1 molar ratio (Haigler et al., 1979) or to α-lactalbumin that was labeled with seven rhodamines (Schechter et al., 1978), it lost 25% and 60%, respectively, of its biological potency.

In all but two of these examples, the hormone conjugates had decreasing biological potency with increasing molecular size of the conjugate. One of the exceptions to this trend, insulin–Sepharose, may have contained insulin released from unstable bonds produced by coupling to cyanogen bromide activated Sepharose (Bolander & Fellows, 1975; Guilford, 1973). Indeed, superactive forms of insulin, prolactin, and placental lactogen were released following coupling of the native hormones to cyanogen bromide activated Sepharose (Wilchek et al., 1974; Topper et al., 1975). Such examples demonstrate the necessity of establishing the stability of the bonds between the hormone and the macromolecule since detection of the extracellular or intracellular location of hormone receptors by using a membrane-impermeable hormone conjugate requires that the hormone be stably coupled to the macromolecule.

The angiotensin in the conjugate reported here is nearly exclusively coupled through stable bonds to ferritin as evidenced by the low amount (4%) of biologically active material with low molecular weight which was present in the conjugate before exposure to the aortic strip. The biologically active low molecular weight components present in the conjugate at the 4% level may have been due to side reactions which produced less stable linkages such as esters formed by succinylation of tyrosine on ferritin (Riordan & Vallee, 1964).

Similarly, a low amount (5%) of biologically active material with low molecular weight was present in the medium surrounding an aortic strip contracted by the angiotensin–ferritin conjugate. It is possible that small fragments of ferritin containing biologically active angiotensin might have been absorbed by the Amicon filter or have become entrapped in the aortic strip so that manipulation of the medium would not have detected their presence. However, comparable amounts of biological activity were found with both the membrane ultrafiltration and gel filtration techniques, suggesting that there was not substantial release of biologically active low molecular weight components produced by the action of cellular proteases.

One might expect activity measurements of large hormone conjugates with intact tissue to be complicated by penetration barriers presented by the extracellular matrix. In our study, the time required for angiotensin–ferritin to produce a given level of contraction was longer than that required by free

angiotensin to produce the same amount of contraction. However, isolated aortic smooth muscle cells exposed to angiotensin–ferritin appeared to contract as rapidly as cells exposed to angiotensin, suggesting that a penetration barrier exists in the aortic strip which retards the interaction of angiotensin–ferritin with its receptor. In contrast, Richardson & Beaulnes (1971) did not observe a difference in the length of time required by angiotensin or angiotensin coupled to cytochrome *c* to produce an equal amount of contraction of rabbit aortic strips although angiotensin coupled to the larger tracer, horseradish peroxidase, did require a slightly longer time period to produce the same amount of contraction. In their parallel histochemical studies using rabbit aortic strips contracted by angiotensin–cytochrome *c*, a large amount of diaminobenzidine reaction product was found at the luminal surface of the endothelial cells, and relatively little was associated with underlying smooth muscle cells. Richardson & Beaulnes (1971) therefore suggested that the angiotensin receptor sites responsible for initiating contraction of the aorta were localized on the surface of endothelial cells. However, competition studies with free angiotensin to show displacement of angiotensin–cytochrome *c* were not performed, cytochrome *c* which was not coupled to angiotensin was not removed from the conjugate, and cytochemical reaction product was also found adhering to elastic tissue in an area of endothelial cell destruction, suggesting some nonspecific binding of the conjugate. Quantitative biological potencies of the angiotensin–cytocrome *c* or angiotensin–horseradish peroxidase conjugates used in their experiments were not reported.

Specific binding sites for angiotensin have been found in particular fractions of aortic smooth muscle (Devynck & Meyer, 1976), and angiotensin is capable of inducing contraction of pure cultures of isolated aortic smooth muscle cells (Ives et al., 1978), strongly indicating that they are a target cell for angiotensin. However, recent evidence indicates that cultures of endothelial cells respond to angiotensin by producing prostaglandin-E-like material (Gimbrone & Alexander, 1975) or by increasing cGMP levels (Buonassisi & Venter, 1976). These results, together with the morphological association of precapillary sphincter smooth muscle cells and endothelial cells described by Rhodin (1967), indicate the need for more information concerning angiotensin receptors and the interactions of these two cell types.

The results presented here for angiotensin–ferritin indicate that angiotensin likely does not directly penetrate the plasma membrane to induce contraction of aortic smooth muscle cells. This suggests that the angiotensin receptor responsible for inducing contraction in these cells is located on the plasma membrane. These results, however, do not exclude the possibility that angiotensin, its receptor, or both are internalized following hormone binding as has been observed with other peptide hormone receptors (Haigler et al., 1979) and that the biological response is secondary to internalization (Willingham et al., 1979).

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Inhibition of Pyruvate Dehydrogenase Multienzyme Complex from *Escherichia coli* with a Bifunctional Arsenoxide: Selective Inactivation of Lipoamide Dehydrogenase†

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ABSTRACT: The bifunctional reagent *p*-[(bromoacetyl)-amino]phenyl arsenoxide ($BrCH_2CONHPhAsO$) in the presence of excess reduced nicotinamide adenine dinucleotide has been shown to cause the irreversible active site directed inactivation of the lipoamide dehydrogenase (E3) component of the pyruvate dehydrogenase multienzyme (PD) complex from *Escherichia coli*. The ability of the lipoate acetyltransferase (E2) component to bind coenzyme A was decreased by about 50% in this system. In the presence of thiamine pyrophosphate, pyruvate, coenzyme A, and Mg^{2+}, E3 inactivation by $BrCH_2CONHPhAsO$ was selective (coenzyme A binding was unaffected) and stoichiometrically related to PD complex inactivation, indicating that a complement of E3 is necessary for full complex activity. The activity of the pyruvate dehydrogenase (E1) component was unaltered by $BrCH_2$-$CONHPhAsO$ in both systems. On inhibition of the PD complex with $BrCH_2CONHPhAsO$, the reagent mediated interchain cross-linking between E2 and about half of the E3 subunits. A marked change occurred in the quaternary structure of the PD complex, with some E1 and E3 subunits being dissociated from the E2 core. The mechanism outlined by Stevenson et al. [Stevenson, K. J., Hale, G., & Perham, R. N. (1978) *Biochemistry 17*, 2189] for the inhibition of the PD complex by $BrCH_2CONHPhAsO$ must be revised on the basis of these findings. E3 is only partially modified by delivery of the bromoacetyl moiety of the bifunctional reagent (covalently attached to lipoyl residues of E2 through dithioarsinite bonds) into the active site of bound E3. The inhibition of E3, dissociated from the PD complex during cross-linking, likely occurs via direct interaction of the free enzyme with $BrCH_2CONHPhAsO$ by initial dithioarsinite modification of the reduced active-site disulfide followed by alkylation of a nearby residue.

The pyruvate dehydrogenase multienzyme complex (PD complex)[1] from *Escherichia coli* catalyzes the overall reaction:

$$\text{pyruvate} + NAD^+ + CoA \rightarrow \text{acetyl-CoA} + NADH + H^+ + CO_2$$

The complex is composed of multiple copies of three different enzymes, which in order of participation are pyruvate dehydrogenase (lipoate) (E1) (EC 1.2.4.1), lipoate acetyltransferase (E2) (EC 2.3.1.12), and lipoamide dehydrogenase (E3 (NADH) (EC 1.6.4.3) (Reed, 1974; Hucho, 1975; Perham, 1975). A total of 24 apparently identical E2 chains form the structural core of the complex (Reed & Oliver, 1968; Reed, 1974). Recent evidence suggests that two (Danson & Perham, 1976; Brown & Perham, 1976; Speckhard et al., 1977; Collins & Reed, 1977; Bates et al., 1977) or possibly three (Hale & Perham, 1979) lipoyl residues are present per polypeptide chain of E2. The lipoyl residues seem to function in a series of transacetylation reactions within the E2 core of the complex (Bates et al., 1977; Collins & Reed, 1977), and these reactions are kinetically competent in the overall PD complex reaction (Danson et al., 1978).

For many years, trivalent arsenicals such as alkyldihaloarsines ($RAsX_2$) and alkyl arsenoxides (RAsO) have been known to react with dithiols such as reduced lipoic acid to form stable cyclic dithioarsinites (Whittaker, 1947; Stocken & Thompson, 1946).

We have recently begun to investigate the structure and function of the pyruvate dehydrogenase multienzyme complex from *E. coli* by using a new approach involving mono- and bifunctional arsenoxides (Stevenson et al., 1978). It is the purpose of the experiments reported here to clarify the mechanism of the inhibitory action of the bifunctional reagent

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[1] Abbreviations used: PD complex, pyruvate dehydrogenase multienzyme complex; $H_2NPhAsO$, *p*-aminophenyl arsenoxide; $BrCH_2CONHPhAsO$, *p*-[(bromoacetyl)amino]phenyl arsenoxide; $BrCH_2CONHPh$, *N*-phenyl-α-bromoacetamide; $NaDodSO_4$, sodium dodecyl sulfate; TPP, thiamine pyrophosphate; DHAT, dihydrolipoamide acetyltransferase; NADH, reduced nicotinamide adenine dinucleotide.

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