Phenylalaninylargininylarginine: a novel tripeptide exerting Zn^{2+} -dependent, insulin-mimetic inhibitory action on myocardial proteolysis

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A novel tripeptide, Phe-Arg-Arg, was found to exert a potent, insulin-mimetic inhibitory action on lysosomal proteolysis in the Langendorff-perfused rat heart. This tripeptide was synthesized based upon its partial structural analogy to the biguanide anti-hyperglycaemic agent, phenformin (phenylethylbiguanide), which has previously been found to exert a Zn^{2+} -dependent inhibitory action on lysosomal proteolysis. Hearts were biosynthetically labelled with [3H]leucine *in vitro*. The percentage change in subsequent release of [3H]leucine (2 mM non-radioactive leucine) was determined in non-recirculating perfusate. The background Zn^{2+} content of the perfusate was determined to be 20 nM. Major endogenous Zn^{2+} buffers were present in molar excess of Zn^{2+} : 0.1 mM citrate, 0.2 % BSA, and complete physiological amino acids. Infusion of maximally effective levels of chloroquine (30 μ M) or insulin (5 nM) caused a 38 % inhibition of total proteolysis, which corresponds to the lysosomal sub-

component. In the presence of background levels of perfusate Zn^{2+} the infusion of Phe-Arg-Arg (10 μ M), insulin (5 nM), or phenformin (2 μ M) maximally caused a 39 % inhibition of [3H]leucine release. Combined infusion of maximally effective levels of insulin and Phe-Arg-Arg, or maximal levels of chloroquine and Phe-Arg-Arg did not cause additive inhibition of [3H]leucine release greater than the 39 % inhibition caused by either agent alone, regardless of the order of infusion. Addition of physiological concentrations of Zn^{2+} (1 μ M) to the background perfusate Zn^{2+} accelerated the insulin-mimetic action of submaximally effective levels of Phe-Arg-Arg, and increased its potency. Prior chelation of background Zn^{2+} by a 3 h perfusion with $CaNa_2EDTA$ (2 μ M) reversibly delayed the time course of Phe-Arg-Arg action and decreased its potency at submaximal concentrations.

INTRODUCTION

Inhibition of lysosomal protein degradation is a fundamental action of insulin; and circulating insulin contains bound Zn^{2+} ions (Cunningham, et al., 1955; Emdin et al., 1980; Brill and Venable, 1968). The exact function of Zn^{2+} in the action of insulin remains uncertain, due to experimental difficulties in manipulation of Zn^{2+} activity in living tissues; however, some role seems likely (Huber and Gershoff, 1966, 1973; Hendricks and Mahoney, 1972; Quarterman and Florence, 1972; Coulston and Dandona, 1980; Roth and Hirschgessner, 1981; May and Contoreggi, 1982; Fields et al., 1983; Ueda et al., 1984; Herrington, 1985; Pang and Shafer, 1985; Reeves and O'Dell, 1985; Park et al., 1986; Eckel et al., 1987; Ezaki, 1989).

After reporting that the biguanide anti-hyperglycaemic drugs inhibit myocardial lysosomal proteolysis we accidentally discovered that a slightly supraphysiological extracellular Zn^{2+} concentration also mimics this proteolytic inhibitory action of insulin (Thorne and Lockwood, 1990, 1991). Among other cations investigated, only copper was weakly insulin mimetic at supraphysiological levels. Biguanides form complexes with various metal ions including Zn^{2+} (Ray, 1961; Schwarzenbach and Anderegg, 1962). The proteolytic inhibitory action of the biguanide drug phenformin (phenylethylbiguanide) at the maximally effective concentration (2 μ M) could be completely inactivated by prior perfusion with $CaNa_2EDTA$ at a concentration of 2 μ M. This inhibited action of phenformin in the presence of chelator could be promptly restored by concurrent infusion of a physiological excess of non-chelated Zn^{2+} . Pursuit of these findings revealed that the time course and potency of Zn^{2+} -free or previously Zn^{2+} -crystallized insulin could be

decreased at submaximally effective concentrations by prior chelation of background perfusate Zn^{2+} by $CaNa_2EDTA$ (2 μ M) (Thorne and Lockwood, 1991). This inhibited action of submaximally effective levels of insulin in the presence of chelator could be promptly restored by concurrent infusion of a physiological excess of non-chelated Zn^{2+} in the same preparations (3 μ M total infused Zn^{2+} , 2 μ M $CaNa_2EDTA$, 1 μ M non-chelated Zn^{2+}). The action of maximal Zn^{2+} -free insulin (5 nM) was not appreciably changed by Zn^{2+} chelation or supplementation, presumably due to acquisition of sufficient Zn^{2+} from tissues and/or the background perfusate.

The active moieties of various biguanide drugs are the vicinal two guanide groups (guanidylguanidine); however, the differing hydrophobic substituents can alter the potency greatly (Schafer, 1983). We considered possible structural similarities between the vicinal guanides of biguanide drugs and vicinal guanides of arginines in peptides. Accordingly we investigated the insulin-mimetic, proteolytic inhibitory action of the novel synthetic tripeptide Phe-Arg-Arg, and its Zn^{2+} -dependence. We presently report that Phe-Arg-Arg exerts a potent insulin-mimetic action that can be altered by experimental manipulation of the Zn^{2+} availability.

EXPERIMENTAL

Perfusion of hearts

Hearts were perfused as Langendorff preparations by methods described previously (Lockwood, 1985a,b, 1988; Thorne and Lockwood 1993). A constant flow rate of 7.0 ml/min sustained a low perfusion pressure of 40 mmHg (5.33 kPa) for 8 h. The non-recirculating perfusate (95 % O_2 :5 % CO_2) contained

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Figure 1 (a) Remaining myocardial $[^3\text{H}]$ leucine versus time and (b) effect of Phe-Arg-Arg on $[^3\text{H}]$ leucine release in the presence of background perfusate Zn^{2+} or physiological Zn^{2+} concentrations or CaNa_2EDTA

(a) The remaining incorporated myocardial $[^3\text{H}]$ leucine was plotted versus time after a 10 min incorporation period. The log-linear component from 275 to 380 min was extrapolated to the origin and subtracted from the upper trace. The progress of the difference between the upper trace and the extrapolated component (broken trace) yielded the rapidly declining log-linear component that was eliminated within 3 h. The indicated fitted equation from this single heart describes the relationship shown to within 2% at any time. Constants k_1 and k_2 are shown $\pm$ S.D. from three separate hearts (see text). (b) Percentage inhibition of $[^3\text{H}]$ leucine release at 330 min is shown for continuous 90 min prior Phe-Arg-Arg infusion in the presence of background perfusate Zn^{2+} (20 mM) ($\bullet$), or CaNa_2EDTA , (2 μM , 3 h prior exposure) (Δ), or added physiological levels of Zn^{2+} (1 μM) ($\circ$) (see Figures 2 and 3). Measurements are shown $\pm$ S.D. ($n = 3-5$) except for two which are the average of two experiments each. The maximal inhibition produced by chloroquine (30 μM) is shown $\pm$ S.D. ($\blacksquare$). The half-maximally effective values shown are for background Zn^{2+} .

Krebs-Henseleit salts, 11 mM glucose and the physiological concentrations of 0.1 mM citrate, 0.1 mM pyruvate, 0.3 mM lactate, and complete amino acids all adjusted to a pH value of 7.42 after addition. BSA was added at a concentration of 0.2%. Only rhythmically contracting preparations were used and these were monitored throughout.

Biosynthetic labelling of proteins

Proteins were biosynthetically labelled *in vitro* by an infusion of L-[4,5- ^3H]leucine (40–60 mCi/mmol, New England Nuclear, Boston, MA, U.S.A.) for 10 min at a perfusate tracer concentration of 4.5 $\mu\text{Ci}/\text{ml}$ without additional non-radioactive leucine. Recycling of the $[^3\text{H}]$ leucine degradation product back into protein synthesis was prevented by addition of supra-physiological levels (2 mM) of non-radioactive leucine immediately after radiolabelling (Lockwood, 1985b).

Determination of protein degradation

To determine the cumulative percentage release of $[^3\text{H}]$ leucine from labelled proteins, hearts were homogenized using a Brinkman Polytron homogenizer after collection of the total non-recirculating perfusate at 1.5 min intervals, from 20 min to 6 h after labelling (Figure 1a). Macromolecular $[^3\text{H}]$ leucine from tissue homogenates was precipitated from 6% (v/v) ice-cold trichloroacetic acid and washed three times. The macromolecular radioactivity of tissue homogenates was then determined after alkaline protein hydrolysis as described previously (Lockwood et al., 1982). Retained tissue radioactivity was more than 97% trichloroacetic acid-precipitable. Radioactivity in tissue hydrolysates was compared with total radioactivity released in the effluent perfusate after adjustment of both to pH 5 and after quench determination.

To determine the percentage change in the baseline rate of $[^3\text{H}]$ leucine release from the preparation a 3 h preliminary perfusion period was routinely employed to permit degradation of the rapid-turnover subclass of proteins (Figure 1a). Beginning 3 h after labelling, the control baseline rate of $[^3\text{H}]$ leucine release was determined at 1.5 min intervals using a fraction collector. A minimum of 40 measurements of $[^3\text{H}]$ leucine release/min were statistically fitted and extrapolated over the subsequent period of degradative inhibition as described previously (Thorne and Lockwood, 1990, 1993).

Pig crystalline insulin (25 international units/mg, 0.5% zinc) and drugs were obtained from Sigma; other methods and materials were as described (Falchuk and Vallee, 1988; Thorne and Lockwood, 1991). The tripeptide Phe-Arg-Arg was synthesized by the Bachem Corporation (Torrance, CA, U.S.A.) and assayed by h.p.l.c.

RESULTS

Release of $[^3\text{H}]$ leucine from multiple myocardial proteolytic processes

Immediately after a brief 10-min labelling period $[^3\text{H}]$ leucine is released, largely from the rapid-turnover class of proteins which are eliminated within 3 h (Figure 1a). Beginning at 20 min after labelling the amount of $[^3\text{H}]$ leucine remaining in the heart with time could be approximated by a two-component relationship of the form $Y = Ae^{-k_1t} + Be^{-k_2t}$. Coefficients A and B are the amounts of $[^3\text{H}]$ leucine released from rapid and slower turnover components, and are 30% and 70% respectively. The degradative rates of rapid and slower turnover components are $0.99 \pm 0.05\%/\text{h}$ and $0.029 \pm 0.002\%/\text{h}$ respectively ($\pm$ S.D., $n = 3$). These values are merely approximations because of the heterogeneity of proteins comprising both classes and the possibility of change in degradation as a function of hormone-free perfusion time. However, the error in determination of k_1 is small, due to the large difference in degradation rates; and the value of k_2 closely approximates to the degradative rate from 4 to 6 h. Changes in the rate of protein degradation are measured as changes in the control baseline rate of $[^3\text{H}]$ leucine release/min

Figure 2 Effects of sequentially combined infusions of maximally effective concentrations of chloroquine, phenformin and isoprenaline on the proteolytic inhibitory action of maximally effective levels of Phe-Arg-Arg

Non-additive proteolytic inhibitory actions of (a) maximally effective concentrations of Phe-Arg-Arg (10 μ M) or insulin (5 nM) followed by chloroquine (30 μ M) infusion ($\pm$ S.D.) (see also Thorne and Lockwood, 1990, 1991, 1993), and of (b) maximally effective Phe-Arg-Arg (15 μ M) followed by phenformin (2 μ M) infusion ($\pm$ S.D.). (c) Additive proteolytic inhibitory actions of maximally effective Phe-Arg-Arg (10 μ M) followed isoprenaline (0.5 μ M) infusion ($\pm$ S.D.). The 35% inhibitory effect of isoprenaline was similar to that shown when the β -agonist was infused without prior Phe-Arg-Arg exposure (Lockwood, 1985b). Abbreviations: Chloroq., chloroquine; Phenf., phenformin; ISO, isoprenaline.

Figure 3 Effect of increasing the background perfusate Zn^{2+} concentration on the inhibitory action of various concentrations of Phe-Arg-Arg, and the effect of prior perfusion with $CaNa_2EDTA$ on this inhibitory action

(a) Effect of increasing the background perfusate Zn^{2+} concentration on the proteolytic inhibitory action of a minimally effective Phe-Arg-Arg concentration. After infusion of Phe-Arg-Arg (5 nM) under background Zn^{2+} (20 nM) conditions infusion of physiological Zn^{2+} (1 μ M) was simultaneously combined at 75 min. This is shown in a single experiment which was representative of two experiments ($\bullet$). The absence of proteolytic inhibitory effect due to Zn^{2+} infusion (1 μ M) without Phe-Arg-Arg ($\blacksquare$) is shown in (a) and (b) as results averaged from three separate parallel experiments. (b) Effect of increasing the background perfusate Zn^{2+} concentrations on the proteolytic inhibitory action of half-maximally (0.1 μ M) and maximally effective (10 μ M) Phe-Arg-Arg concentrations. The time course and potency of maximally effective Phe-Arg-Arg (10 μ M) ($\blacktriangle$) was essentially the same under background Zn^{2+} (20 nM) or physiological Zn^{2+} (1 μ M) conditions (see Figure 1b and text). (c) Effect of prior 3 h perfusion with $CaNa_2EDTA$ (2 μ M) on the proteolytic inhibitory action of a submaximal concentration of Phe-Arg-Arg (1 μ M). The time courses and potencies of Phe-Arg-Arg (1 μ M) are shown under background perfusate Zn^{2+} (20 nM) conditions ($\blacktriangle$) and after a 3 h preliminary perfusion with $CaNa_2EDTA$ (2 μ M) ($\bullet$). The 0.5 h average delay in onset of action with $CaNa_2EDTA$ ($\pm$ S.D.) and decreased potency were highly significant as compared with controls in the presence of background Zn^{2+} ($P < 0.01$, $n = 3$). The absence of any proteolytic inhibitory effect during infusion of $CaNa_2EDTA$ alone at zero time is shown by results averaged from three separate parallel experiments ($\blacksquare$).

from 3 to 6 h as described in greater detail previously (Thorne and Lockwood 1991, 1993).

Insulin-mimetic action of Phe-Arg-Arg in the presence of background perfusate Zn^{2+}

Infusion of Phe-Arg-Arg at 10 μ M or more caused a maximal inhibition of $[^3H]$ leucine release of $38 \pm 5\%$ S.D. with a 1–1.5 h time course, which is similar to that described previously for the maximally effective concentration of insulin (5 nM) [Figures 1(b), 2 and 3]. The Phe-Arg-Arg concentration producing a half-maximal effect at 90 min was approx. 50 nM under background

Zn^{2+} (20 nM) conditions (Figure 1b). The inhibitory actions of maximal levels of insulin or Phe-Arg-Arg were not spontaneously reversed for at least 2 h after termination of drug exposure.

After $38 \pm 5\%$ inhibition of proteolysis with Phe-Arg-Arg, the subsequent combined infusion of either maximally effective levels of chloroquine (30 μ M), phenformin (2 μ M) or insulin (5 nM) did not cause appreciable additive inhibition (Figures 2a–c). After infusion of a maximally effective concentration of insulin (5 nM), the subsequent infusion of the maximal level of Phe-Arg-Arg (10 μ M) did not cause appreciable additive inhibition (results not shown).

Infusion of maximally effective amounts of isoprenaline

(0.5 μM) caused $32 \pm 2\%$ proteolytic inhibition either before or after 35–40% lysosomal inhibition with chloroquine or insulin (Lockwood, 1988). Alpha-1 adrenergic agonists inhibit 35% of proteolysis non-additively with β -agonist (Thorne and Lockwood, 1993). The proteolytic inhibitory effect of isoprenaline was additive to the prior inhibitory effect of Phe-Arg-Arg (Figure 2c). Thus, Phe-Arg-Arg inhibits lysosomal proteolysis but not the non-lysosomal adrenergic-responsive proteolytic process.

Effects of perfusate Zn^{2+} supplementation and CaNa_2EDTA addition on the insulin-mimetic potency of Phe-Arg-Arg

The background Zn^{2+} content of the perfusate was determined previously, using atomic absorption, to be approx. 20 nM. This background level was derived, at least 90%, from the residual Zn^{2+} of the BSA, the remainder was from the salts of the Krebs–Henseleit buffer (Thorne and Lockwood, 1991). As discussed previously the perfusate contains the major Zn^{2+} -buffering components of serum: citrate, complete amino acids and serum albumin (Glusker, 1980; Thorne and Lockwood, 1991; and reviewed in Cunnane, 1988). Because there is a large excess of Zn^{2+} -buffering components the addition of 1 μM Zn^{2+} does not exceed the physiological free- Zn^{2+} concentration of serum (Cunnane, 1988; Mills, 1989).

Infusion of physiological levels of Zn^{2+} (1 μM) concurrent with a submaximal concentration of Phe-Arg-Arg (5 nM) caused an increase in potency and acceleration of the time course of Phe-Arg-Arg action [Figure 1(b) and Figure 3(a–c)]. At maximally effective concentrations the onset of action of Phe-Arg-Arg or insulin lagged behind the infusion time by less than 2 min under either background or physiological Zn^{2+} (1 μM) conditions. However, at the lowest effective Phe-Arg-Arg concentration (5 nM) the onset of action lagged behind the initiation of exposure by approx. 15 min under routine conditions in the presence of background perfusate Zn^{2+} (Figure 3a). Addition of physiological levels of Zn^{2+} (1 μM) to minimally effective Phe-Arg-Arg (5 nM) caused a prompt acceleration in its action within 2 min (Figure 3a). The action of half maximally effective Phe-Arg-Arg (0.1 μM) under background perfusate Zn^{2+} (20 nM) conditions was also promptly enhanced by infusion of physiological levels of Zn^{2+} (1 μM) (Figure 3b). However, it is possible that this Phe-Arg-Arg concentration (Figure 3b) would eventually proceed to a maximal degree of inhibition under background Zn^{2+} conditions at exposure times longer than 2 h. Indeed, a precise distinction between the effects of added Zn^{2+} on acceleration of the Phe-Arg-Arg time course and the increase in final potency was difficult to determine, as previously described for both insulin and phenformin (Thorne and Lockwood, 1991). In a similar manner to insulin and phenformin, the action of a maximally effective Phe-Arg-Arg concentration (10 μM) was not appreciably changed by addition of physiological levels of Zn^{2+} (1 μM) to background Zn^{2+} (20 nM) (Figure 3b and see Thorne and Lockwood, 1991).

The prior perfusion of hearts for 3 h in the presence of CaNa_2EDTA (2 μM) caused both a delay of action and decreased potency of submaximal levels of Phe-Arg-Arg (1 μM) (Figure 3c). Without prior chelation the onset of action of a 1 μM Phe-Arg-Arg concentration was less than 2 min after infusion (Figure 3c). However, after 3 h of CaNa_2EDTA exposure (2 μM) the onset of Phe-Arg-Arg (1 μM) action was delayed to approx. 30 min. The potency and time course of Phe-Arg-Arg (1 μM) was promptly restored by infusion of a 1.5 μM concentration of non-chelated Zn^{2+} , i.e. 2.0 μM CaNa_2EDTA and 3.5 μM added Zn^{2+} (Figure 3c).

Human therapeutic concentrations of CaNa_2EDTA are far

higher than these minimally effective micromolar levels; and present conditions were selected in order to minimize toxicity and permit accurate addition of a slight physiological excess of Zn^{2+} in combination with minimal levels of CaNa_2EDTA . Although not rigorously studied, the prior perfusion of hearts with 2.0 μM CaNa_2EDTA for only 30 min, instead of 3 h, caused little delay in action or decrease in the potency of insulin (Thorne and Lockwood, 1991) or Phe-Arg-Arg (1 μM) (results not shown). Therefore, the present results require a preliminary exposure to impermeant chelator of several hours duration at this low chelator concentration. Higher CaNa_2EDTA concentrations were not investigated.

The 50%-effective Phe-Arg-Arg concentration at 90 min (50 nM) was decreased or increased by approx. 10-fold by Zn^{2+} supplementation or Zn^{2+} chelation respectively (Figure 1b). However, this change in concentration–response relationship was not further characterized at low agonist concentrations (5–10 nM) due to the difficulty in clearly distinguishing between changes in time course and changes in steady-state potency.

DISCUSSION

Zinc might influence the action of insulin or Phe-Arg-Arg at several sites, i.e. receptor binding, receptor aggregation, kinase or phosphatase activity, agonist dissociation and/or degradation, post-receptor signals, or effectors (Thorne and Lockwood, 1991). The source of Zn^{2+} influencing the action of insulin or Phe-Arg-Arg *in vivo* could be intracellular, extracellular, or both. The active Zn^{2+} could be bound to agonist, receptor, or some unknown Zn^{2+} -interactive substance modifying hormone action at a receptor or post-receptor level. It is conceivable that Zn^{2+} might simultaneously increase hormone–receptor-binding affinity and decrease intralysosomal hormone dissociation, degradation and receptor recycling (Arquilla et al., 1978). Involvement of cycling receptor-bound Zn^{2+} or other intracellular Zn^{2+} is suggested by the failure of CaNa_2EDTA (2 μM) to interfere with the action of insulin (Thorne and Lockwood, 1991) or Phe-Arg-Arg (see above) after a preliminary exposure time of only 30 min instead of 3 h.

Because supraphysiological Zn^{2+} concentrations are insulin mimetic alone, the structural requirements for a Zn^{2+} -binding, insulin-mimetic substance might not be highly specific. The number of Zn^{2+} ions complexed with biguanides or Phe-Arg-Arg is unknown, as are the multimeric aggregation states of the complexes. Insulin contains multiple Zn^{2+} ions bound to its surface with unknown affinities; and both biguanide drugs and Phe-Arg-Arg might complex multiple Zn^{2+} ions. In theory, the affinities of multiple Zn^{2+} ions bound to an insulin-mimetic or interactive substance need not be high in order to cause an increased Zn^{2+} activity at the unknown active site(s) responding to slightly supraphysiological Zn^{2+} concentrations alone. Moreover, it is possible that Phe-Arg-Arg might not be the most potent vicinal Arg-Arg-containing peptide; and other Zn^{2+} -binding peptides without arginine might also be effective.

There are no obvious structural similarities in the primary sequence of insulin and Phe-Arg-Arg, although the proinsulin-connecting peptide contains an Arg-Arg site. Inhibition of lysosomal proteolysis can also be caused by insulin-like growth factors I and II. The present myocardial action of insulin is apparently caused via the insulin receptor as shown by the sensitivity of proteolysis to inhibition at very low (50 pM) insulin levels (Thorne and Lockwood, 1991). However, if Phe-Arg-Arg currently acts via receptor agonism, the exact identity of such receptors is uncertain (Cascieri et al., 1989).

Although it has been suggested that Zn^{2+} might modulate

several endocrine systems, the exact relationships between intra- and/or extra-cellular Zn^{2+} levels and hormone actions remains unknown (Emdin et al., 1980; Vander et al., 1983; Reeves and O'Dell, 1985; Park et al., 1986; Cunningham et al., 1990; Faure et al., 1991; and reviewed in Cunnane, 1988). Non-bound Zn^{2+} levels could be modulated in a disproportionate relationship to total Zn^{2+} by many metabolites and peptides which can bind Zn^{2+} (Glusker, 1980; Hamer, 1986; Vallee and Auld, 1990). Thus, the search for endogenous and pharmacological insulin-mimetic agents or response modifiers should include consideration of Zn^{2+} -binding substances that might increase or decrease effective Zn^{2+} activity.

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