Effects of insulin, biguanide antihyperglycaemic agents and β -adrenergic agonists on pathways of myocardial proteolysis

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Pathways of bulk protein degradation controlled by insulin and isoprenaline (isoproterenol) were distinguished in Langendorff-perfused rat hearts. Proteins were biosynthetically labelled *in vitro* with [3 H]leucine, followed by addition of 2 mM non-radioactive leucine to competitively prevent reincorporation. Rapidly degraded proteins were eliminated during a 3 h preliminary perfusion period without insulin. One third of bulk myocardial protein degradation was inhibited by isoprenaline as described previously. An insulin concentration of 5 nM maximally inhibited proteolysis, beginning within 2 min. Inhibition reached 32 % within 1.25 h and 35 % after 1.5 h. The minimum effective insulin concentration was approx. 10–50 pM, which caused 10–20 % inhibition. Following 3 h of perfusion without insulin, the lysosomal inhibitor, chloroquine (30 μ M), inhibited 38 % of bulk degradation. The 35 % proteolytic inhibition caused by insulin was followed by very little further inhibition on subsequent concurrent infusion of chloroquine, i.e. the inhibitory effects of insulin and chloroquine were not additive. In contrast, prior inhibition of lysosomal proteolysis by insulin or chloroquine did not prevent the subsequent additive inhibition caused by isoprenaline. Insulin and β -agonists additively inhibited approx. two-thirds of bulk degradation. The biguanide antihyperglycaemic agent phenformin (2 μ M) inhibited 35 % of bulk degradation, beginning at 2 min and reaching a near maximum at approx. 1.25–1.5 h. Following inhibition of proteolysis with phenformin (20 μ M), subsequent infusion of chloroquine (30 μ M) produced only a slight additional inhibition. Following inhibition of 35 % of degradation by 1.5 h of perfusion with insulin (5 nM), subsequent exposure to phenformin (2 μ M) produced only a slight additional inhibition which did not exceed 38 % of basal proteolysis. Thus insulin and phenformin both inhibit lysosomal proteolysis; however, the adrenergic-responsive pathway is distinct.

INTRODUCTION

Bulk protein degradation is mediated by distinct lysosomal and non-lysosomal pathways. After elimination of the rapidly degraded subclass of proteins over 3 h, one third of the remaining myocardial proteolysis is co-ordinated with contractility by reciprocal minute-to-minute responses of both processes to β -adrenergic receptor occupancy (Lockwood, 1985a,b). Approx. 35–40 % of bulk proteolysis can be inhibited by the lysosomal inhibitor chloroquine; however, such prior lysosomal inhibition does not eliminate β -adrenergic inhibition of proteolysis (Lockwood, 1988). The additivity of the proteolysis inhibitions caused by chloroquine and β -adrenergic agonist suggest that the β -adrenergic process is non-lysosomal.

Proteolytic inhibition is a fundamental action of insulin (Miller, 1962), in virtually all target tissues (Pozefsky *et al.*, 1969; Wildenthal, 1972; Amenta *et al.*, 1977; Frayn & Maycock, 1979; Goldberg *et al.*, 1980; Sugden & Smith, 1982; Clark & Mitch, 1983; Tsalikan *et al.*, 1984; Fukagawa *et al.*, 1985; Garlick *et al.*, 1985; Millward *et al.*, 1985; Tessari *et al.*, 1986; Gelfand & Barret, 1987; Smith, 1988); experimentation with lysosomal agents suggest that the vesicular system is involved (reviewed by Curfman *et al.*, 1980; Goldberg *et al.*, 1982). Various agents are used to lower blood glucose in diabetes; however, their effects on protein degradation have not

been described. In contrast with other classes of antihyperglycaemic agents, the biguanides do not act by stimulating insulin release from the pancreas. It has been suggested that the biguanide mechanism might include inhibition of intestinal glucose absorption, increase of insulin binding to its receptor, or a direct insulin-mimetic action on target tissues (reviewed and discussed by Hermann, 1979; Stern & Junien, 1981; Lord *et al.*, 1985; Purrello *et al.*, 1987; Bailey, 1988).

The present studies describe, first, the combined additive inhibitory effects of simultaneous insulin and β -agonist treatment on bulk myocardial proteolytic pathways. Secondly, an insulin-mimetic action of biguanide agents on bulk proteolysis was characterized in the absence of extracellular insulin. Thirdly, the proteolytic process inhibited by biguanides was identified as the lysosomal pathway.

MATERIALS

Perfusion conditions

Hearts from 200 g rats were perfused under low pressure of approx. 40 mmHg (5.33 kPa) at a constant flow rate of 7.6 ml/min with Krebs–Henseleit buffer containing 0.2 % BSA (Lockwood, 1985b). All measurements were from monitored hearts with a regular rhythm throughout unless indicated. Nutrients routinely included

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were 11 mM-glucose and the physiological concentrations of 0.1 mM-pyruvate, 0.3 mM-lactate and 0.1 mM-citrate. Complete amino acids were added at physiological concentrations for the rat as reviewed and described previously (Lockwood, 1985b).

Labelling of proteins and measurement of protein degradation

Myocardial proteins were biosynthetically labelled *in vitro* by infusion of [4,5- ^3H]leucine for 10 min (Lockwood, 1985b). After labelling, 2 mM non-radioactive leucine was added as a competitive inhibitor of reincorporation. Alternatively, 20 μM -cycloheximide has previously been substituted for leucine to prevent reincorporation of radiolabelled degradation products in experiments reviewed below (see Lockwood, 1988). After termination of labelling, a 3 h preliminary degradation period preceded the observation period (between 3 and 5.5 h after labelling) in order to eliminate the rapidly degraded subclass of proteins. The total effluent perfusate was collected continuously with a fraction collector, and trichloroacetic acid-soluble radioactivity was determined.

The baseline of [^3H]leucine release is curvilinear when represented in rectilinear co-ordinates (illustrated in Lockwood, 1985b, 1988) or semilogarithmic co-ordinates (Fig. 1). However, inflection points and changes in slope are more readily observed in semilogarithmic dimensions as shown in Fig. 1. The measured baseline values of [^3H]leucine release versus time between 3 and 4 h of perfusion were fitted and extrapolated over the following period of 4–5.5 h with high precision ($r \sim 0.95$) using a Sigma Plot program (Jandel Scientific) (see below).

Effects of insulin, phenformin and isoprenaline on myocardial contractile function

Under the conditions described, the preparation routinely maintained rhythmic contraction-relaxation for at least 7 h as described previously (Lockwood, 1985b, 1988). During this time neither insulin nor phenformin produced an observable effect on heart rate, peak right-ventricular systolic pressure or rhythm in the experiments described (results not shown). Isoprenaline (isoprotenerol) increased heart rate and peak right-ventricular systolic pressure as illustrated previously (Lockwood, 1985b). The contractile stimulation caused by 0.5 μM -isoprenaline was identical with or without 5 nM-insulin in the 3 h preliminary perfusion (results not shown).

Materials used have been described previously (Lockwood, 1985b, 1988). Bovine crystalline insulin (0.5% zinc; 25 international units/mg) and drugs were from Sigma.

RESULTS

Measurement of percentage change in protein degradation by extrapolation of a declining baseline of [^3H]leucine release

The curvilinear declining baseline of [^3H]leucine release shown in Fig. 1 is expected for the degradation of a heterogeneous population of proteins with diverse degradation rates. The degradation rate is 4.5%/h at 3 h and 3%/h at 6 h owing to the progressive elimination of more rapidly degraded proteins; however, these values theoretically depend upon the exact labelling protocol (Lockwood, 1985b). Untreated controls demonstrated

Fig. 1. Additive inhibitory effects of insulin and isoprenaline on proteolysis

The time corresponding to 4 h after initial labelling with [^3H]leucine is shown; zero time was preceded by a 3 h preliminary perfusion without insulin. The trace from 0 to 55 min was computer-fitted and extrapolated ($r = 0.94$) (■). The mean inhibitory effects of insulin (●) and isoprenaline (ISO; ○) when infused separately are shown ($\pm$ S.D.; $n = 4$) as described in the legend to Fig. 2 and in Lockwood (1985b, 1988). These separate hormonal inhibitory effects correspond to the percentage difference between the extrapolated baseline and the measured values of [^3H]leucine release represented as A minus B. The additive inhibitions caused by concurrent insulin and isoprenaline are shown as A minus C. The final values shown represent steady-state inhibitions of proteolysis as shown in Figs. 2–4 and in Lockwood (1985b, 1988). The combined inhibition given by A minus C was significantly greater than the mean effects of either agent alone ($P < 0.01$). The mean combined inhibition was $63 \pm 5\%$ (S.D., $n = 4$) (△).

that the extrapolation of the baseline measured between 3 and 4 h after labelling does indeed describe the uninhibited release of [^3H]leucine over the following period from 4 to 5.5 h to within a few per cent. The progress of the percentage difference between the extrapolated control baseline and the inhibited [^3H]leucine release is shown in Figs. 2–4. The time point corresponding to 4 h after initial labelling is indicated in each Figure. In Figs. 3 and 4, the uninhibited basal degradation is represented as the normalized 100% value of the fitted curvilinear baseline; only 30 min of the measured 60 min control period is shown. The percentage proteolytic inhibition is calculated as the percentage difference between the extrapolated control baseline and the measured value of [^3H]leucine release after the attainment of a constant percentage inhibition (compare Figs. 1 and 3). A particular percentage inhibition of protein degradation should theoretically cause an identical sustained percentage decrease in the baseline at any time, independent of the baseline slope, provided that the conditions controlling degradation are stable before and after steady-state inhibition (illustrated in Lockwood, 1985b, 1988, and below).

Effect of insulin on protein degradation

After 3–4 h of insulin-free perfusion, infusion of the maximal endogenous insulin concentration of 5 nM

Fig. 2. Effect of insulin concentration and perfusion time on inhibition of proteolysis

Proteolytic inhibition ($\pm$ S.D., $n = 3-12$) was measured as shown in Figs. 1, 3 and 4. Insulin infusion at zero time corresponds to 4 h after labelling (see Figs. 1, 3 and 4 and the text). Both traces shown were fitted by inspection, as biased by the larger number of determinations at 10^{-10} and 10^{-9} M. As represented by the broken trace, the exact characteristics of the relationship near 10^{-11} M are uncertain due to a time lag of 5–10 min at lowest effective concentrations. Inhibitions caused by concentrations above 10^{-9} M were not significantly greater than that at 10^{-9} M ($P < 0.05$). An insulin concentration of 10^{-10} M corresponds to 15 μ units/ml. At 5×10^{-9} M-insulin, the inhibition was $35 \pm 4.3\%$ when measured at 1.5 h.

resulted in inhibition of proteolysis within 2 min. Inhibition reached $32 \pm 4.5\%$ (S.D.) after 1.25 h and a constant value of $35 \pm 4.3\%$ after 1.5 h (Figs. 1–4). Supramaximal 0.1 μ M-insulin produced the same time course and percentage inhibition of protein degradation as caused by the maximal physiological 5 nM-insulin concentration (Figs. 1–3). The minimally effective insulin concentration was between 10 and 50 pM; however, the exact threshold was difficult to determine due to a 5–10 min time lag in the onset of action at minimally effective exposure (Fig. 2). Upon discontinuation of insulin infusion, the time course of reversal of proteolytic inhibition appeared to be longer than 1 h; reversal was not characterized further.

Additive inhibitory effects of insulin and isoprenaline on protein degradation

As described previously, isoprenaline (0.5 μ M) caused a sustained $29 \pm 3\%$ inhibition of proteolysis within 5–10 min on addition at any time between 3 and 5 h after the start of insulin-free perfusion (Lockwood, 1985b). After maximal inhibition of proteolysis by insulin, the concurrent infusion of isoprenaline (0.5 μ M) at 4.5 h (1 h after insulin addition) caused an additive inhibition that was similar to the effect of isoprenaline alone (Fig. 1). The sum of the inhibitions caused by insulin and isoprenaline represented nearly two-thirds of the baseline degradation rate.

Effect of chloroquine on protein degradation

Under the conditions described, chloroquine caused a maximal inhibition of proteolysis of $38 \pm 5\%$ at 30 μ M within 45 min of perfusion (Fig. 3). Chloroquine has been described previously to cause a similar $36 \pm 5\%$ inhibition of proteolysis when amino acids, citrate, pyruvate and lactate were not included and when cycloheximide was employed to prevent reincorporation of [3 H]leucine (Lockwood, 1988).

Fig. 3. Non-additive effects of supraphysiological insulin and chloroquine on myocardial protein degradation

Methods were as described in Fig. 1 legend; each point shown at 15 min intervals is representative of ten measurements (not shown). The measured values of the curvilinear baseline have been normalized to the 100% control degradation rate. The entire 1 h control trace measured at 1.5 min intervals between 3 and 4 h after labelling was fitted and extrapolated as in Fig. 1; however, only a 30 min period of control measurement prior to zero time is shown ('4 h' refers to time after labelling). The mean effect of maximally effective chloroquine concentration (30 μ M) ($\pm$ S.D., $n = 4$) is shown (●). Regardless of the order of infusion, the combined inhibitory effects of insulin and chloroquine are not significantly greater than when either agent was infused alone.

Fig. 4. Effects of biguanidine antihyperglycaemic agents on protein degradation

(a) Effect of phenformin on protein degradation. Methods were as described in the legend to Fig. 3. The mean inhibition ($\pm$ S.D., $n = 3$) produced by 20 μM -phenformin is indicated ($\circ$), as are the mean inhibitions of two separate hearts exposed to 2 μM ($\square$), and two hearts exposed to 0.2 μM ($\triangle$), which were nearly identical. Concentrations of phenformin used: $\blacktriangle$, 0.2 μM ; $\blacksquare$, 2 μM ; $\bullet$, 20 μM , followed by 50 μM after 75 min. (b) Effect on protein degradation of phenformin followed by combined phenformin and chloroquine. The mean inhibition of $38 \pm 5\%$ (S.D., $n = 4$) caused by chloroquine (30 μM) ($\blacktriangle$) was not significantly different from the effect of combined phenformin (20 μM) and chloroquine ($\bullet$). (c) Effect on protein degradation of insulin followed by combined insulin and phenformin. Following insulin exposure (5 nM), phenformin (2 μM) was infused concurrently. The mean ($\pm$ S.D., $n = 4$) inhibition caused by insulin alone is indicated ($\circ$). (d) Effect on protein degradation of phenformin followed by combined phenformin and insulin. The order of infusion shown in (c) was reversed. In parts (c) and (d), the combined inhibition caused by insulin and phenformin was not significantly greater than the maximal effect of either alone. In all parts, '4 h' refers to the time after labelling with [^3H]leucine.

Non-additive inhibitory effects of insulin and chloroquine on protein degradation

Following the 35% inhibition of proteolysis by supra-physiological insulin concentration (0.1 μM), chloroquine (30 μM) produced only a slight inhibition of several per cent (Figs. 3a and 3b). The combined effect of 5 nM-insulin and 30 μM -chloroquine did not exceed 38% in three separate experiments (results not shown). Because the combined inhibitions due to chloroquine and insulin are not appreciably greater than the mean of either agent alone, the combined inhibitions are only very slightly additive (not significantly different). Nonetheless, the maximal 35% inhibition produced by insulin alone at 1.5 h was consistently several per cent less than the 38% inhibition caused by chloroquine; this small difference is readily observed using each preparation as its own internal control (Figs. 3a and 3b).

After 1 h of exposure to chloroquine (40 μM), the concurrent infusion of supra-physiological insulin

(0.1 μM) caused little additional inhibition of proteolysis (Fig. 3b). Thus chloroquine and insulin did not produce appreciable additive proteolytic inhibition, regardless of the order of perfusion.

Inhibitory effects of the biguanides phenformin and metformin on protein degradation

Phenformin (20 μM) inhibited proteolysis by $35 \pm 4\%$, beginning within 2 min of the start of perfusion and reaching a near maximum in 1.25–1.5 h (Fig. 4a). Lower phenformin concentrations of 2 μM and 0.2 μM produced nearly the same maximum inhibition as 20 μM , although more slowly (Fig. 4a). Phenformin binds to perfusate albumin only very slightly (Schafer, 1983). An increase in the phenformin concentration to 50 μM did not produce proteolytic inhibition appreciably greater than that produced by 20 μM (Fig. 4a). In two experiments, therapeutic concentrations of the less potent biguanide antihyperglycaemic agent metformin (30 μM ; see the Discus-

sion section), also produced an inhibitory effect on proteolysis ($P < 0.01$) (results not shown).

Non-additive inhibitory effects of phenformin and chloroquine on protein degradation

Following prior inhibition of proteolysis with maximally effective chloroquine concentration ($30 \mu\text{M}$), simultaneous infusion of phenformin ($20 \mu\text{M}$) produced only a slight additional inhibition (Fig. 4b) (not significantly different).

Non-additive inhibitory effects of insulin and phenformin on protein degradation

Following prior inhibition of proteolysis with maximally effective insulin concentration (5 nM), simultaneous infusion of phenformin ($2 \mu\text{M}$) produced only a slight additional proteolytic inhibition (Fig. 4c) (not significantly different). Insulin and phenformin also produced no appreciable additive inhibition of proteolysis when the order of infusion was reversed (Fig. 4d).

DISCUSSION

Distinctions between insulin-responsive and β -agonist-responsive proteolytic processes

Results suggest that a major fraction of myocardial proteolysis is co-ordinated with systemic functional demand on a minute-to-minute basis by β -agonists, whereas a distinct major proteolytic process is co-ordinated with systemic insulin regulation over a period of hours. A large number of additional factors interact with the insulin and/or adrenergic-related hormone systems at some level; accordingly, many factors might exert some influence over the bulk proteolytic rate via these pathways, e.g. thyroxine, glucocorticoids, glucagon, etc. Moreover, the experimental distinction between pathways does not imply that they are entirely independent of common signals, particularly at submaximal hormone concentrations or longer exposure times. The identity of labelled substrates is unknown (Lowell *et al.*, 1986; Smith & Sugden, 1986); however, the presently observed 38% proteolytic inhibition caused by chloroquine is similar to the sustained effect of lysosomal agents in cultured myocardium as measured by different methods (Curfman *et al.*, 1980, and reviewed therein).

Insulin-mimetic inhibitory effect of biguanides on lysosomal proteolysis

After 4 h of insulin deprivation, the heart preparation is sensitive to sub-physiological insulin concentrations of approx. 10 pM (Fig. 2); thus the inhibitory effect of endogenous insulin appears to have been largely eliminated. It is possible that the surface receptor number becomes 'up-regulated' in the prolonged absence of insulin. Regardless, biguanides act directly to fully mimic the proteolytic inhibitory effect of insulin in the absence of extracellular hormone. The concentrations of phenformin and metformin employed to lower blood glucose are approx. 1 and $30 \mu\text{M}$ respectively (Schafer, 1983); thus an inhibitory effect of these agents on proteolysis occurs at or below their effective insulin-mimetic concentrations. It remains unknown whether the inhibitory effect of biguanides on proteolysis is exerted at a receptor or a postreceptor level.

Experimental intervention in bulk proteolysis

The results demonstrate the experimental inhibition of two thirds of bulk proteolysis with systemically non-injurious synthetic agents. Both insulin and β -agonists are clearly hypertrophic to myocardium when either agent is studied alone; their combined simultaneous effect is unknown. The simultaneous control of proteolysis by these two complex hormone systems could be a major contributing factor in the normal and pathogenic regulation of myocardial protein mass. Counterparts of these two pathways under presumably different regulation in non-cardiac tissues are as yet unknown, however conceivable they appear (Yang & McElligott, 1989).

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