The effects of glucose, acetate, lactate and insulin on protein degradation in the perfused rat heart

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Rat hearts were perfused as working preparations by the method of Taegtmeier, Hems & Krebs [(1980) *Biochem. J.* **186**, 701–711]. In the presence of glucose, insulin significantly inhibited protein degradation at concentrations as low as 50 μ units/ml. Acetate or lactate, when present either as sole fuel for contraction or in combination with glucose, did not inhibit protein degradation. Insulin inhibition of protein degradation was decreased with either lactate or acetate as sole fuel. We suggest that the inhibition of protein degradation occurs over the normal range of plasma concentrations of insulin present *in vivo* and that the presence of glucose may be at least in part necessary for this effect of insulin.

Insulin is known to inhibit protein degradation in the perfused rat heart (Rannels *et al.*, 1975). However, the concentration requirement for insulin was high (200 μ units/ml of perfusate) before significant inhibition was observed. The physiological importance of this effect is thus dubious, because in man the plasma concentration of insulin rarely rises to such a high value (Hales & Randle, 1963; Nikkilä *et al.*, 1965; Welborn *et al.*, 1966; Cerasi *et al.*, 1973). We therefore re-investigated the effects of insulin concentration on protein degradation in the perfused working rat heart. We also investigated the effects of acetate and lactate on protein degradation. These fuels have been reported to inhibit protein degradation in the perfused heart (Chua *et al.*, 1979a,b). Because the heart will function normally with either lactate or acetate as fuel, it was decided to investigate whether insulin would inhibit protein degradation in the absence of exogenous glucose. The results are given below.

Experimental

Materials

Soluble insulin from ox pancreas for injection (concn. 80 international units/ml; lot A6306) was obtained from The Wellcome Foundation, London N.W.1, U.K. Chemicals were from BDH Chemicals, Enfield, Middx., U.K. Biochemicals were from Sigma Chemical Co., Poole, Dorset, U.K. Lactic acid was purchased as the L(–)-isomer and all references to lactate mean this isomer. Radio-

chemicals were from Amersham International, Amersham, Bucks., U.K. Male Sprague–Dawley rats (weighing 250–300 g on arrival) were from Bantin and Kingman, Hull, Yorkshire, U.K. They were kept for at least 48 h before use, during which time they had free access to food and water unless otherwise stated.

Heart perfusions

Hearts were removed from animals that had been anaesthetized and heparinized as described by Taegtmeier *et al.* (1980). They were perfused with Krebs & Henseleit (1932) bicarbonate-buffered saline solution at a filling pressure of 5 cmH₂O and an aortic pressure of 70 cmH₂O as described by Taegtmeier *et al.* (1980). The perfusion media were supplemented with 0.02 mM-cycloheximide, insulin and fuel substrates as appropriate. Lactate and acetate were added as their sodium salts prepared by neutralization of the free acids to pH 7.4 with AristaR-grade NaOH. For perfusions with acetate, AristaR-grade acetic acid was used. The NaCl concentration in the perfusate was decreased appropriately to maintain constant Na⁺ concentration in perfusions with lactate or acetate. Hearts were initially perfused as Langendorff (1895) preparations and switched to working preparations as quickly as possible (about 5 min) by cannulation of the pulmonary vein. After the change from the Langendorff perfusion to the working perfusion, a volume (250 ml) of perfusate was passed through the heart and discarded before switching to a re-

circulating perfusion with 100–120 ml of perfusate (see legends to the Table and Figures). The pre-perfusion and the test-perfusion media were always identical with one another. Cardiac output (sum of coronary and aortic flows) was monitored throughout the perfusions to ensure that the hearts were pumping satisfactorily. Perfusions were for 130 min, at which time cardiac output had fallen to about 90% of the value at 10 min. Because of the change from Langendorff (1895) perfusion to working perfusion and because the volume of perfusate discarded in the pre-perfusion was not precisely known, the exact volume of perfusate remaining in the apparatus at the end of the perfusion was estimated by an isotope-dilution technique. A volume (0.5 ml) of [^3H]sucrose (2 $\mu\text{Ci/ml}$) was added after 130 min perfusion and allowed to equilibrate for 10 min. A volume of perfusate (3 ml) was removed and the radioactivity therein was measured by liquid-scintillation spectrometry with the use of 10 ml of PCS Phase Combining System (BDH Chemicals) as a fluor. Results were corrected for quenching by the external standard method. Heart dry weights were determined by removing the atria and other attached tissue and subsequent drying of the ventricles to constant weight over P_2O_5 *in vacuo*. The atria etc. comprised 6% of the heart dry weight or wet weight. Thus the procedure of removal of the atria before measurement of the dry weight will not substantially affect the rates of protein degradation presented below. The manoeuvre was performed merely to assist removal of the heart from the cannulae.

Measurement of protein degradation

Because phenylalanine is poorly metabolized by the perfused heart (Williams *et al.*, 1981), the rate of protein degradation was measured by the rate of release of phenylalanine into the perfusate in the presence of cycloheximide (Morgan *et al.*, 1979). Samples (0.5 ml) were removed at 20 min intervals, heated at 100°C for 1 min and stored at -20°C. Results represent the release of phenylalanine from the whole heart. Because the heart is not a homogeneous organ, we cannot exclude the possibility that some areas contribute disproportionately for their mass to the release of phenylalanine.

Assay of metabolites

Phenylalanine in the centrifuged samples was assayed by the method of Rubin & Goldstein (1970), except that commercially available *Escherichia coli* aminoacyl-tRNA synthetase and tRNA were used and the incubation was terminated by immersion of the filter-paper squares in 5% (w/v) trichloroacetic acid at 0°C. The paper squares were subsequently washed in 5% (w/v) trichloroacetic acid (three changes) at 4°C as described by Corbin

& Reimann (1974). The second wash was usually for 1 h, the remainder for 30 min. By use of prolonged washing intervals, the variation of the blank radioactivity with phenylalanine concentration (Rubin & Goldstein, 1970) was avoided. The paper squares were rinsed in methanol and then diethyl ether, and dried under a hair-dryer. Their radioactivities were counted in a toluene-based scintillant [5 g of 2,5-diphenyloxazole/litre and 0.1 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre]. Standards and blanks containing known amounts of phenylalanine in the appropriate Krebs & Henseleit (1932) perfusion media were run simultaneously. By this technique, as little as 50 pmol of phenylalanine could be measured. Whole-heart phenylalanine contents were measured by extracting the hearts with 2 vol. of 5% (v/v) perchloric acid (70%; BDH Chemicals), removal of precipitated protein by centrifugation and subsequent neutralization of the supernatant with KOH. After centrifugation, phenylalanine contents of the supernatants were measured as above.

Glucose was measured by using a glucose oxidase kit purchased from Sigma Chemical Co., and lactate by the method of Hohorst (1963). Initial rates of lactate release are quoted in the present paper.

Statistical methods

Results are expressed as means $\pm$ S.E.M. with the numbers of observations in parentheses. Statistical significance was determined by a two-tailed Student's *t* test. Values of $P < 0.05$ were taken as being significantly different.

Results

Effects of insulin concentration on protein degradation

The protein degradation rate in this perfused working rat heart preparation was linear with time over the perfusion period (Fig. 1). This is in contrast with some previous results (e.g. Rannels *et al.*, 1975; Morgan *et al.*, 1980; Williams *et al.*, 1980), when a large decrease in the rate of protein degradation during the second hour of perfusion was observed in either Langendorff (1895) perfused hearts or working (Morgan *et al.*, 1980) perfused hearts. Statistically significant differences between the rates of phenylalanine release in perfusions with and without insulin were seen by 30 min (Fig. 1).

The release of phenylalanine into the perfusate represents net degradation of heart protein. Heart phenylalanine contents were unchanged during perfusions. The phenylalanine contents of hearts were 92 ± 12 nmol/g wet wt. of tissue (three observations) at the start of the working perfusion and 87 ± 23 nmol/g wet wt. of tissue (three obser-

Fig. 1. Time course of protein degradation in the perfused heart

Hearts were perfused with 100 ml of Krebs & Henseleit (1932) bicarbonate-buffered saline containing 5 mM-glucose and 0.02 mM-cycloheximide as described in the Experimental section in the absence (●) or in the presence (▲) of maximally effective concentrations of insulin (1–10 munits of insulin/ml). Each line was constructed from eight experiments. The hearts perfused without insulin weighed 0.972 ± 0.021 g (range 0.869–1.034 g) when wet and 0.187 ± 0.002 g (range 0.175–0.197 g) when dried. They were from rats weighing 291 ± 4 g (range 275–305 g). The hearts perfused with insulin weighed 0.902 ± 0.018 g (range 0.834–0.976 g) when wet and 0.171 ± 0.004 g (range 0.158–0.191 g) when dried. They were from rats weighing 294 ± 12 g (range 250–310 g). The amount of phenylalanine present in the perfusate at 10 min was subtracted from the subsequent values. With the exception of the points at 30 min, which are significantly different at $P < 0.05$, at each given time point pair the rate of protein degradation with and without insulin is significantly different at $P < 0.001$. Where error bars are not shown, they are obscured by the symbols. The slopes correspond to a rate of phenylalanine release of $1.68 \mu\text{mol/h}$ per g dry wt. in the absence of insulin and $0.86 \mu\text{mol/h}$ per g dry wt. in the presence of insulin.

The effect of insulin concentration on protein degradation is shown in Fig. 2(a). Significant inhibition of protein degradation is observed at an added insulin concentration of $50 \mu\text{units/ml}$. Perfusions were performed in the absence of any added protein in the perfusate. Insulin concentrations in the perfusates may hence be overestimates, because of the known tendency of insulin to bind to glassware. Alternatively, sufficient protein may be released from the heart during perfusion to prevent this. [Viable tissue preparations release small quantities of protein over prolonged incubation periods, presumably because of cell damage (see, e.g., Hearse *et al.*, 1975; Sugden, 1980).]

Because a knowledge of precise insulin concentrations in the perfusates during the perfusions was unavailable, the effects of insulin concentration on other insulin-regulated processes were compared with the effect of the hormone on protein degradation (see Figs. 2b and 2c). Glucose uptake by hearts was significantly stimulated at insulin concentrations of 25 – $50 \mu\text{units/ml}$, and lactate release was stimulated significantly at $50 \mu\text{units/ml}$. All three processes thus exhibit similar insulin-dose-response curves. It should be noted that the presence of insulin in the perfusate did not affect the extent of inhibition of cardiac protein synthesis by cycloheximide, which was always about 95% (results not shown).

Effects of glucose, lactate and acetate on protein degradation

Because insulin produces a marked stimulation of lactate release from the perfused working rat heart and because it has been reported that lactate inhibits protein degradation in this organ (Chua *et al.*, 1979a; Crie & Wildenthal, 1980), it seemed possible that insulin might inhibit protein degradation by stimulating intracellular lactate production, which is reflected by an increased rate of release of lactate into the perfusate. Hearts were therefore perfused with glucose, lactate or acetate, to investigate this hypothesis. There were no large variations in cardiac output under the different perfusion conditions (Table 1). Protein degradation is not significantly different when hearts were perfused with 5 mM-glucose, 10 mM-lactate or 10 mM-acetate (to produce media approximately equal to those containing 5 mM-glucose in terms of fuel energy available per ml of medium, assuming complete oxidation of fuels utilized by the hearts). Perfusion with acetate essentially completely inhibited lactate release. Perfusions of hearts with higher concentrations of substrates or with substrate pairs did not inhibit protein degradation (Table 1). Perfusion of hearts with 20 mM-glucose plus 50 mM-lactate (cf. Chua *et al.*, 1979a) did not show any significant alteration

ations) after 130 min perfusion. These values were not corrected for phenylalanine concentration in the extracellular space, which was negligible compared with the intracellular phenylalanine concentration (less than 2%) even after 130 min perfusion.

Fig. 2. Effects of insulin concentration on protein degradation, glucose uptake and lactate output in the perfused heart. Hearts were perfused as described in Fig. 1 legend and the Experimental section. Rats were starved during the night before the experiments. The body weights were 304 ± 4 g (36), range 270–345 g. The heart wet weights were 0.962 ± 0.015 g (36), range 0.768–1.224 g. The dry weights were 0.181 ± 0.002 g (36), range 0.152–0.223 g. The effects of insulin concentration on protein degradation (a), glucose uptake (b) and lactate output (c) are shown. Statistical significance: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ versus zero added insulin for 3–12 observations at each point.

in the rate of protein degradation compared with perfusions with 20 mM-glucose. Although addition of insulin (50 munits/ml) or 10 mM-acetate to perfusion media containing 5 mM-glucose significantly increased lactate production [221 ± 22 (8) or 160 ± 20 (6) versus 58 ± 11 (12) $\mu\text{mol/g dry wt.}$; $P < 0.001$], only insulin decreased protein degradation. There is thus no evidence (a) that insulin may inhibit protein degradation by increasing intra-

cellular lactate concentrations, or (b) that lactate or acetate inhibit protein degradation in this working heart preparation. Moreover, it is unlikely that insulin could inhibit protein degradation by inducing a fall in intracellular pH by stimulation of lactic acid production (a decrease in perfusate pH decreases protein degradation; see Chua *et al.*, 1979a; Crie & Wildenthal, 1980), because insulin does not decrease cardiac intracellular pH *in vitro* (Bailey *et al.*, 1982).

Table 1. *Effects of glucose, acetate and lactate on protein degradation in the absence or in the presence of insulin*
Hearts were perfused with 120 ml of Krebs & Henseleit (1932) bicarbonate-buffered saline containing 0.02 mM-cycloheximide and substrates indicated as described in the Experimental section. The body weights of the rats were 315 ± 2 g (91), range 255–345 g. The heart wet weights were 0.979 ± 0.008 g (91), range 0.824–1.139 g. The dry weights were 0.180 ± 0.001 g (91), range 0.146–0.214 g. Insulin concentration was 50 munits/ml. Statistical significance: * $P < 0.01$ and ** $P < 0.001$ versus rate of protein degradation in the absence of insulin. For statistical analyses in the last column: † $P < 0.01$ and †† $P < 0.001$ versus the ratio of the rates of protein degradation with and without insulin for perfusions with 5 mM-glucose (line 1, last column).

Substrate(s)	–Insulin		+Insulin		Protein degradation ratio +/– insulin (%)
	Cardiac output at 70 min (ml/min per g dry wt. of heart)	Protein degradation (μ mol of phenylalanine released/h per g dry wt. of heart)	Cardiac output at 70 min (ml/min per g dry wt. of heart)	Protein degradation (μ mol of phenylalanine released/h per g dry wt. of heart)	
Glucose (5 mM)	268 ± 14	1.49 ± 0.04 (9)	264 ± 13	$1.00 \pm 0.04^{**}$ (8)	67 ± 3
Glucose (20 mM)	284 ± 23	1.74 ± 0.09 (5)	310 ± 17	$1.08 \pm 0.09^*$ (3)	62 ± 5
Glucose (5 mM) + acetate (10 mM)	260 ± 19	1.71 ± 0.06 (6)	254 ± 27	$1.06 \pm 0.07^{**}$ (4)	62 ± 3
Glucose (20 mM) + lactate (50 mM)	282 ± 18	1.84 ± 0.12 (3)	—	—	—
Acetate (10 mM)	267 ± 11	1.62 ± 0.06 (13)	262 ± 11	$1.30 \pm 0.05^{**}$ (13)	$80 \pm 3^\dagger$
Lactate (10 mM)	281 ± 22	1.55 ± 0.08 (8)	320 ± 14	1.41 ± 0.08 (8)	$91 \pm 5^{\dagger\dagger}$
Lactate (50 mM)	274 ± 7	1.99 ± 0.13 (6)	257 ± 8	1.72 ± 0.09 (5)	$87 \pm 6^\dagger$

Effect of insulin on protein degradation in the presence of non-carbohydrate fuels

Insulin decreased protein degradation by 33–38% whenever glucose was present (Table 1). In the presence of lactate alone, protein degradation was only slightly (and insignificantly) inhibited by insulin. In the presence of acetate, insulin significantly inhibited protein degradation compared with perfusions with acetate in the absence of insulin ($P < 0.001$). The ratio of the rates of degradation with and without insulin was significantly less in perfusions with 5 mM-glucose compared with those with 10 mM-acetate, 10 mM-lactate or 50 mM-lactate ($P < 0.01$). This raises the possibility that the presence of glucose is necessary for the full expression of insulin-dependent inhibition of protein degradation.

Discussion

Many of the findings in the present study apparently contradict those of earlier investigations. In view of this, methodological differences between ourselves and other workers are discussed here. Most workers have used Langendorff (1895) perfused heart preparations. We used a working heart preparation. Although it has been suggested that heart work may inhibit protein degradation (Morgan *et al.*, 1980), the subject remains contentious (Schreiber *et al.*, 1973, 1981).

We demonstrate (Fig. 2) that the inhibition of protein degradation in the perfused working rat heart exhibited a similar insulin-concentration-dependence to that observed with other insulin-affected processes (stimulation of glucose uptake and lactate release).

The concentration at which insulin significantly inhibits protein degradation (50 μ units/ml) is within the range found in plasma of man after a glucose load (Hales & Randle, 1963; Nikkilä *et al.*, 1965; Welborn *et al.*, 1966; Cerasi *et al.*, 1973). Notwithstanding interpretative problems (e.g. the species source of insulin, insulin binding by apparatus glassware, a lack of knowledge of the precise insulin concentration throughout the perfusion period), we consider that insulin may be important in the regulation of protein degradation in the heart *in vivo*. We also consider that we have avoided many of the interpretative problems by comparing the concentration-dependence of insulin inhibition of protein degradation with other insulin-affected processes. In our hands cardiac protein degradation is more sensitive to insulin than observed by Rannels *et al.* (1975). They perfused hearts from overnight-starved animals by the Langendorff (1895) method with perfusates containing 15 mM-glucose, normal plasma concentrations of all amino acids except phenylalanine, 4% (w/v) bovine serum albumin and 0.02 mM-cycloheximide. These workers also took statistical significance as being established at $P < 0.01$. The insulin concentration at which they therefore observed significant effects on protein degradation was 200 μ units/ml. However, the rate of protein degradation that they observed at an insulin concentration of 100 μ units/ml was not significantly different at $P < 0.05$ from the rate observed in the absence of insulin. It remains possible that some of the reasons for the differences between our results and those of Rannels *et al.* (1975) could be ascribed to differences in methodology. It is unlikely that the presence of albumin explains these differences,

because albumin would be expected to prevent binding of insulin to the apparatus glassware, thereby increasing the apparent sensitivity of protein degradation to the hormone. Although cycloheximide has been reported to inhibit protein degradation in liver (Hershko & Tomkins, 1971) and heart (Rannels *et al.*, 1975), it was present in our perfusion medium and that of Rannels *et al.* (1975). Since we suggest (Table 1) that the effects of insulin on protein degradation are at least partially dependent on the presence of glucose in the perfusion medium, and since heart work is known to increase the maximal velocity of glucose transport (Neely *et al.*, 1967; Neely & Morgan, 1974), it remains possible that the perfusion method may affect the insulin-sensitivity of protein degradation. The presence of amino acids and higher (15 mM) glucose concentrations in the perfusion medium could also affect insulin-sensitivity of protein degradation. We have not fully explored all these possibilities.

We were unable to detect any inhibition of protein degradation by lactate in the working heart in the absence or in the presence of glucose (Table 1). In Langendorff (1895) perfused hearts, Chua *et al.* (1979a) showed that protein degradation was inhibited by 25 mM-lactate over a range of perfusate pH (6.8–7.4). Their perfusion media included 15 mM-glucose and plasma concentrations of ten amino acids. They used overnight-starved animals. We have been unable to detect any differences in rates of protein degradation between hearts from fed or overnight-starved animals (results not shown). In contrast with Chua *et al.* (1979a), Crie & Wildenthal (1980), also using Langendorff perfused hearts from mature rats, observed inhibition of protein degradation by lactate only at pH 6.8 and not at pH 7.4. These latter workers did not include amino acids in their perfusion media but did include insulin and glucose. Conditions may therefore have been unfavourable for the detection of effects of lactate because of the presence of insulin and glucose in the perfusate. We were also unable to detect any inhibition of protein degradation by acetate, in contrast with Chua *et al.* (1979b). However, our methodology differed from that of Chua *et al.* (1979b) in that they used Langendorff (1895) preparations and included amino acids in their perfusion media. It is not known whether the absence of amino acids from our perfusion media might account for the differences between our results and those of other workers.

The inhibitory effects of insulin on protein degradation appear to have a requirement for the presence of glucose. Inhibition by insulin is decreased when acetate or lactate is the fuel for cardiac contraction. Protein degradation may thus differ from protein synthesis in this regard, since Chain & Sender (1973) have shown stimulation of protein

synthesis by insulin in hearts perfused with glucose-free solutions.

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