Effect of insulin and lack of effect of workload and hypoxia on protein degradation in the perfused working rat heart

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1. Protein degradation was studied in the glucose (5 mM)-perfused working rat heart preparation of Taegtmeyer, Hems & Krebs [(1980) *Biochem. J.* **186**, 701–711]. 2. The effects of cardiac workload were investigated in three different preparations: (a) control (low workload), (b) increased pressure workload (simulating conditions of aortic pressure *in vivo*) and (c) increased volume workload. There was no effect of increased workload on protein degradation in preparation (b) or (c) when compared with preparation (a). Insulin inhibited protein degradation in all three preparations. Significantly greater inhibition by insulin was observed in the increased-pressure-workload preparation (b). 3. Hypoxia was induced by the partial replacement of O₂ in the gaseous phase by N₂. Hearts maintained their cardiac output when O₂ content was decreased from 95% to 55% by volume, but the stability of the preparation was less at 50% O₂. Lactate output was significantly increased at O₂ contents of 65% or less. The rate of protein degradation was not different from control values (95% O₂) in perfusions with 65, 55 or 50% O₂. 4. We conclude that acutely increased workload or acute hypoxia does not affect protein degradation in the perfused working rat heart when cardiac output is relatively stable.

The effect of workload *in vitro* on cardiac protein degradation is contentious. Schreiber *et al.* (1973), using a perfused working guinea-pig heart preparation, were unable to detect any alteration in the rate of release of radioactivity from cardiac protein prelabelled with [¹⁴C]lysine or [¹⁴C]phenylalanine when the afterload on the left ventricle was increased by raising the aortic pressure. Such a manoeuvre increases the pressure work done by the heart. Since the left ventricular wall and septum comprise about 80% of the ventricular mass (Gibson & Harris, 1972; Caspari *et al.*, 1978), it was suggested that increased workload *in vitro* does not affect cardiac protein degradation. More recently, Morgan *et al.* (1980) have presented data which suggested that rat hearts performing external work *in vitro* exhibited a lower rate of protein degradation compared with Langendorff (1895)-perfused hearts (which do no external work). The conclusions of Morgan *et al.* (1980) were dependent on which conditions of perfusion were examined, since protein degradation was influenced by the perfusate fuel content. This subject has been reviewed by Schreiber *et al.* (1981).

Related to the subject of workload *in vitro* and cardiac protein degradation is the effect of hypoxia on cardiac protein degradation. Chua *et al.* (1979)

have shown that hypoxia inhibited protein degradation in Langendorff (1895)-perfused hearts. Anoxia or ischaemia further inhibited protein degradation. We do not know, however, of any analogous experiments in working heart preparations where the cardiac output provides a simple measure of the ability of the heart to do work. The effects of hypoxia on protein degradation are made more interesting by the proposal of Tischler (1980) that redox-state changes may affect protein degradation in the rat diaphragm (see also Tischler, 1981). He has suggested that an increased NADH/NAD⁺ ratio may be responsible for inhibition of protein degradation. Aoki *et al.* (1978) have suggested that an increase in NADH/NAD⁺ ratio in human skeletal muscle may serve to diminish nitrogen wasting. We have therefore investigated the effects of workload, insulin and hypoxia on protein degradation in the working rat heart preparation of Taegtmeyer *et al.* (1980).

Experimental

Materials

Soluble insulin from ox pancreas for injection (concn. 80 units/ml, lot A4306) was from The

Wellcome Foundation, London NW1 2BP, U.K. Chemicals were from BDH Chemicals, Dagenham, Essex RM8 1RZ, U.K. Biochemicals were from Sigma (London) Chemical Co., Poole, Dorset BH17 7NH, U.K. Radiochemicals were from Amersham International, Amersham, Bucks. HP7 9LL, U.K. Male Sprague-Dawley rats (weighing 250–300 g on arrival) were from Bantin and Kingman, Hull, Humberside HU11 4QE, U.K. They were kept for at least 48 h before use, during which time they had free access to food and water.

Heart perfusions; general comments

Working heart perfusions were carried out by the method of Taegtmeier *et al.* (1980). Our methodology has been described in detail elsewhere (Sugden & Smith, 1982b) and will only be outlined here. Hearts were initially perfused as non-recirculating Langendorff (1895) preparations with Krebs & Henseleit (1932) buffered bicarbonate saline solution supplemented with 5 mM-glucose and 0.02 mM-cycloheximide equilibrated with the gas mixtures described below. They were then switched to working preparations. The first 250 ml of perfusate pumped by the working hearts was discarded. Hearts were then perfused in a recirculating system with 120 ml of perfusate as above. In some cases, insulin (50 munits/ml) was present (see Table 1). When this was the case, insulin was also present in the Langendorff preperfusion. The aortic and coronary flows were monitored throughout the perfusion. The sum of these flows is the cardiac output. Perfusions were for 130 min (zero time is at the initiation of cardiac work). The volume of perfusate in the apparatus was measured by the dilution of 1 μ Ci of [3 H]sucrose added after 130 min perfusion (Sugden & Smith, 1982a). Ventricular dry weights were estimated by drying to constant weight over P_2O_5 *in vacuo*.

Perfusions for investigation of the effects of workload on protein degradation

For these perfusions, perfusate was equilibrated with O_2/CO_2 (19:1). The rate of flow of perfusate into the oxygenator was 130 ml/min and the rate of flow of O_2/CO_2 into the oxygenator was 600 ml/min. Three perfusion conditions were studied: (i) a control perfusion with a left-atrial filling pressure of 5 cm of water and an aortic pressure of 70 cm of water; (ii) an increased-pressure-workload perfusion with a left-atrial filling pressure of 15 cm of water and aortic pressure of 140 cm of water; (iii) an increased-volume-workload perfusion with a left-atrial filling pressure of 15 cm of water and an aortic pressure of 70 cm of water. These preparations have been described in detail elsewhere (Sugden & Smith, 1982b) and the effects of the various pressures on aortic and coronary flows, cardiac output and

hydraulic work done by the hearts are shown in Table 1. The increased-pressure-workload preparation most closely resembles the physiological condition with regard to aortic pressure.

Perfusions involving hypoxia

Hearts were perfused at a left-atrial filling pressure of 5 cm of water and an aortic pressure of 70 cm of water. Initially the perfusate was equilibrated with O_2/CO_2 (19:1). The flow rate of perfusate into the oxygenator was 85 ml/min and that of the gas was 600 ml/min. After 7 min of perfusion as a working heart preparation, the gas content was altered as appropriate to induce hypoxia. The gas contained O_2 as described in Table 2, plus 5% by volume of CO_2 , with the balance being N_2 .

Analytical methods

Protein degradation was measured by the release of phenylalanine from the heart into the perfusion medium in the presence of cycloheximide (Morgan *et al.*, 1979; Williams *et al.*, 1981). This is the simplest method of measuring protein degradation. We recognize that the method is not devoid of problems, in that there is evidence that cycloheximide may inhibit cardiac protein degradation slightly (Rannels *et al.*, 1975; Morgan *et al.*, 1979). Inhibition of protein degradation by cycloheximide has also been reported in the liver (Hershko & Tomkins, 1971; Ballard & Hopgood, 1973; Woodside, 1976). Perfusate (0.5 ml) was removed at 10 min after the start of heart work and subsequently at 20 min intervals. Samples were heated at 100°C for 1 min and stored at –20°C. Phenylalanine was assayed by the method of Rubin & Goldstein (1970) with the modifications described previously (Sugden & Smith, 1982a). Glucose was measured by the glucose oxidase method with a kit purchased from Sigma, and lactate was measured by the method of Hohorst (1963).

Statistical methods

Results are expressed as means $\pm$ S.E.M. Statistical significance was determined by a two-tailed Student's *t* test or, for ratios, a Mann-Whitney U-Wilcoxon Rank Sum *W* test. Values of $P < 0.05$ were taken as being significant. When applicable, the numbers of observations are shown in parentheses.

Results

Effects of workload and insulin on cardiac protein degradation

The time course of protein degradation (as measured by phenylalanine release) in the presence and absence of insulin in increased-pressure-workload perfusions is shown in Fig. 1. The rates of protein degradation in either condition were linear

Fig. 1. Time course of protein degradation in perfused working rat hearts

Hearts were perfused as described in the Experimental section as increased-pressure-workload preparations. Five hearts were used in perfusions in the absence (●) of insulin and four hearts were used in perfusions in the presence (■) of insulin (50 munits/ml). The value for phenylalanine release at 10 min after initiation of cardiac work was subtracted from subsequent values for each perfusion. The correlation coefficient of both lines is 0.998. The slopes in the absence and presence of insulin correspond to rates of protein degradation of 1.66 and 0.80 μmol of phenylalanine released/h per g dry wt. of ventricle respectively. The 30 min points are significantly different at $P < 0.05$, the 50 min points at $P < 0.01$ and the remaining points at $P < 0.001$.

with time. For the 10–70 min and 70–130 min intervals, hearts released 1.62 ± 0.13 and 1.46 ± 0.16 (5) μmol of phenylalanine/g dry wt. of ventricle respectively in the absence of insulin. In the presence of insulin, the values were 0.76 ± 0.05 and 0.86 ± 0.10 (4) $\mu\text{mol/g}$ respectively. The rates of protein degradation in the first and second hours of perfusion were thus not significantly different under either condition. We have also presented similar data for the control preparation (Sugden & Smith, 1982a), and similar conclusions apply to the increased-volume-workload preparation (results not shown). This result was different from those of Morgan *et al.* (1980), when there was a significant decrease in the rate of protein degradation in the second hour of perfusion. As with hearts perfused at lower pressures (Sugden & Smith, 1982a), the effects of insulin on protein degradation were significant after 30 min of perfusion at physiological aortic pressure values (Fig. 1). There is no obvious time-lag before effects of insulin were observed.

The effects of cardiac workload and insulin on

protein degradation are shown in Table 1. In the absence of insulin, there was no significant effect of increased pressure workload or increased volume workload on protein degradation. Insulin (50 munits/ml) inhibited protein degradation in all three preparations. Although such insulin concentrations are hyperphysiological, we have observed inhibition of protein degradation in control perfusions at physiological insulin concentrations (Sugden & Smith, 1982a). The effects of insulin on glucose uptake confirm our earlier findings (Sugden & Smith, 1982b). Interestingly, even when there was no effect of insulin on glucose uptake in the increased-volume-workload preparation, there was still a significant effect of the hormone on protein degradation. We also observed that the rate of protein degradation in the presence of insulin in the increased-pressure-workload preparation is significantly less ($P < 0.01$) than that for the other preparations. Furthermore, the ratio of the rates of protein degradation in the absence and presence of insulin in the increased-pressure-workload preparation was significantly less ($P < 0.01$) than the ratio for the increased-volume-workload preparation (last column, Table 1). This finding suggests that increased pressure workload may alter the response of cardiac protein degradation to insulin.

Effects of hypoxia on protein degradation in the working heart

Results are shown in Table 2. Hearts did not show any significant difference in cardiac output even when the O_2 content of the gassing mixture was decreased from 95% to 50% (v/v). There was a significantly greater decline in cardiac output over the course of the 130 min of perfusion at 50% O_2 content and also a significant decrease in the ventricular dry-/wet-weight ratio, possibly indicating hypoxia-induced oedema. However, at 55% (v/v) O_2 , performance data and dry-/wet-weight ratio were indistinguishable from those at 95% (v/v). As might be expected, at 50 or 55% (v/v) O_2 , glucose uptake (which was linear with time; results not shown) by the hearts was significantly increased, indicating a greater reliance on anaerobic glycolysis for energy production. Fig. 2 shows that, at 65, 55 and 50% (v/v) O_2 , lactate output by the hearts was significantly greater (maximum $P < 0.01$ for any given time point) than in perfusions with 95% (v/v) O_2 . The fact that there is no significant increase in glucose uptake at 65% (v/v) O_2 presumably indicates a role for cardiac glycogen in hypoxia. Although we induced relatively severe hypoxia in our working-heart preparations, there was no significant alteration in the rate of protein degradation (Table 2). This is in contrast with the results obtained by other workers in Landendorff (1895)-perfused hearts (Chua *et al.*, 1979).

Table 1. *Effects of workload and insulin on cardiac protein degradation*

Hearts were perfused as described in the Experimental section at the various filling and aortic pressures shown. Aortic flow, coronary flow and cardiac output were measured after 70min perfusion of the heart as a working preparation. Hydraulic work is the product of cardiac output after 70min perfusion and aortic pressure. The percentage decline in cardiac output is given by $100 [1 - (\text{cardiac output at 130min}/\text{cardiac output at 10min})]$. The protein-degradation ratio is the rate of protein degradation in the presence of 50munits of insulin/ml divided by that in the absence of insulin. The rat weights and the ventricular dry weights for the various experimental groups (number of observations as below) were respectively; control, 306 ± 6 g and 0.1778 ± 0.0035 g; control + insulin, 314 ± 5 g and 0.1868 ± 0.0034 g; increased pressure workload, 296 ± 9 g and 0.1776 ± 0.0061 g; increased pressure workload + insulin, 298 ± 6 g and 0.1811 ± 0.0019 g; increased volume workload, 280 ± 4 g and 0.1717 ± 0.0043 g; increased volume workload + insulin, 286 ± 8 g and 0.1797 ± 0.0038 g. Statistical significance: * $P < 0.001$ versus same preparation in absence of insulin; † $P < 0.01$ versus increased-volume-workload perfusions.

Perfusion condition	Filling pressure (cmH ₂ O)	Aortic pressure (cmH ₂ O)	No. of observations	Aortic flow (ml/min per g dry wt.)	Coronary flow (ml/min per g dry wt.)	Cardiac output (ml/min per g dry wt.)	Hydraulic work (kg·m/min per g dry wt.)	Decline in cardiac output (%)	Glucose uptake (μmol/h per g dry wt.)	Protein degradation (μmol of phenylalanine released/h per g dry wt.)	Protein degradation ratio +/− insulin
Control	5	70	21	192 ± 10	88 ± 4	285 ± 10	0.20 ± 0.01	14 ± 2	334 ± 14	1.60 ± 0.05	0.60 ± 0.02
			16	200 ± 13	84 ± 7	284 ± 12	0.20 ± 0.01	12 ± 4	504 ± 23*	0.96 ± 0.03*	
Increased pressure workload	15	140	13	170 ± 12	161 ± 8	331 ± 17	0.46 ± 0.02	26 ± 3	633 ± 18	1.45 ± 0.06	
			13	213 ± 17	156 ± 10	369 ± 21	0.52 ± 0.03	26 ± 3	798 ± 23*	0.71 ± 0.04†	0.49 ± 0.03†
Increased volume workload	15	70	10	431 ± 35	86 ± 5	516 ± 37	0.36 ± 0.03	11 ± 3	601 ± 31	1.42 ± 0.08	
			4	377 ± 34	76 ± 6	453 ± 38	0.32 ± 0.03	19 ± 3	638 ± 7	0.99 ± 0.01*	0.70 ± 0.04

Table 2. *Effects of hypoxia on cardiac protein degradation*

Hearts were perfused as 'control' preparations as described in the Experimental section at the O₂ partial pressures described in the Table. Aortic and coronary flows, cardiac output and decline in cardiac output were calculated as described in Table 1. The rat weights and ventricular dry weights for the various experimental groups (number of observations as in the Table) were: 95% O₂, 288 ± 3 g and 0.1712 ± 0.0022 g; 65% O₂, 287 ± 4 g and 0.1739 ± 0.0037 g; 55% O₂, 293 ± 4 g and 0.1750 ± 0.0039 g; 50% O₂, 293 ± 4 g and 0.1729 ± 0.0028 g. Statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus perfusion with 95% O₂ in gaseous phase.

O ₂ content in gaseous phase (% v/v)	No. of observations	Aortic flow (ml/min per g dry wt.)	Coronary flow (ml/min per g dry wt.)	Cardiac output (ml/min per g dry wt.)	Decline in cardiac output (%)	Heart dry-wt./wet-wt. ratio	Glucose uptake (μmol/h per g dry wt.)	Protein degradation (μmol of phenylalanine released/h per g dry wt.)
95	15	218 ± 16	84 ± 7	302 ± 17	10 ± 3	0.1945 ± 0.0025	297 ± 15	1.44 ± 0.05
65	8	222 ± 22	85 ± 3	306 ± 23	12 ± 4	0.1951 ± 0.0017	301 ± 22	1.34 ± 0.14
55	11	220 ± 8	86 ± 5	305 ± 11	16 ± 3	0.1928 ± 0.0017	362 ± 15**	1.36 ± 0.07
50	12	194 ± 11	82 ± 4	276 ± 11	24 ± 5*	0.1874 ± 0.0016*	458 ± 18***	1.49 ± 0.08

Fig. 2. Lactate production by hearts exposed to various O_2 partial pressures

Hearts (which were those used in experiments described in Table 2) were perfused at 'control' perfusion pressures as described in the Experimental section and Table 2. The value of lactate production at 10 min after initiation of cardiac work was subtracted from subsequent values of lactate release for each perfusion. The partial pressures of O_2 in the gaseous phase to which the hearts were exposed were: ●, 95%; ■, 65%; ▲, 55%; ○, 50%. Each point is statistically significantly different from the comparable point for the 95%- O_2 perfusion at a maximum P of <0.01.

Discussion

Effects of workload and insulin on cardiac protein degradation *in vitro*

Morgan *et al.* (1980) have shown that rat hearts perfused with 15 mM-glucose and 0.2% bovine serum albumin as working preparations at a physiological aortic pressure [in their preparation, these have a 20-gauge needle outflow resistance and a mean aortic pressure calculated by us to be about 150 cm of water] showed, over 2 h of perfusion, a significant inhibition of protein degradation compared with Langendorff (1895)-perfused hearts (which do not do any external pumping work). Increasing the mean aortic pressure to a calculated 170 cm of water (23-gauge needle outflow resistance) further decreased protein degradation. Although Morgan *et al.* (1980) do not comment, hearts working against a 23-gauge needle resistance show a significant ($P < 0.05$) decrease in the rate of protein degradation compared with hearts working against a 20-gauge needle resistance over the second hour and over the entire 2 h perfusion. There was no significant difference during the first hour. When alternative substrates (palmitate, 3-hydroxybutyrate, lactate) or

hormones (insulin, glucagon) were present in various combinations, cardiac work either decreased, did not change or perhaps even increased protein degradation compared with Langendorff (1895)-perfused hearts. We consider that a serious interpretative problem with the results of Morgan *et al.* (1980) is the non-linearity of rates of protein degradation with time. Rates of protein degradation in the second hour of perfusion ranged from 43 to 78% of those in the first hour. Rannels *et al.* (1975) and Williams *et al.* (1980) have also observed non-linear rates of protein degradation in working or Langendorff (1895)-perfused hearts in the absence or presence of insulin.

Other investigators (Schreiber *et al.*, 1973) have failed to observe any effects of cardiac work *in vitro* on the rate of release of radioactivity from guinea-pig hearts in which protein was prelabelled by injection of [^{14}C]phenylalanine or [^{14}C]lysine into the animal. The differences between these two groups of investigators could be caused by a species difference or a difference in the perfusion pressures used.

Morgan *et al.* (1980) mainly compared the rates of protein degradation in working hearts with Langendorff (1895)-perfused hearts, which do no external work. Because of the versatility of the working heart preparation of Taegtmeier *et al.* (1980), it is easy to vary filling and aortic pressures. The effects of cardiac work can thus be investigated in similarly cannulated preparations. Using this preparation, we found that protein degradation was linear with time [Fig. 1 and Sugden & Smith (1982a)] for the three perfusion conditions investigated. We measured degradation of whole cardiac protein by the release of phenylalanine into the perfusate in the presence of cycloheximide, as did Morgan *et al.* (1980). We were unable to find any evidence that acutely increased pressure or volume workload itself altered rates of cardiac protein degradation (Table 1). Increased pressure workload apparently enhanced the ability of insulin (50 units/ml) to inhibit cardiac protein degradation (Table 1). Whether this has any significance is not known, but it would be of interest to compare the effects of workload on the insulin-concentration-dependence of cardiac protein degradation. In increased-volume-workload hearts, protein degradation was inhibited by insulin even though the hormone did not stimulate glucose uptake (Table 1). In view of our earlier results (Sugden & Smith, 1982a), which indicated that the inhibitory effects of insulin on protein degradation were partially dependent on the presence of glucose, it would seem that stimulation of glucose uptake itself is not a prerequisite for this action of insulin. Glucose could thus play a 'permissive' role in this action of insulin.

In our experiments and those of Schreiber *et al.*

(1973) and Morgan *et al.* (1980), hearts were subjected to acutely increased workloads. *In vivo*, rabbit cardiac protein degradation has been studied in chronic pressure overload induced by aortic coarctation (which induces cardiac hypertrophy). Myosin degradation was increased (Morkin *et al.*, 1972), although no correction was made for the reutilization of the lysine label (Millward, 1970; Waterlow *et al.*, 1978). Interpretation would have anyway been complicated by the non-steady-state conditions of cardiac protein accumulation during hypertrophy (Zak *et al.*, 1977). Using the non-reutilizable haem label δ -amino[2,3- ^3H] β -laevulinate, Albin *et al.* (1973) have shown that, in rats, cytochrome *c* degradation is decreased in the first 3 days after aortic coarctation. Sanford *et al.* (1978) have shown a decrease in the rate of phenylalanine release in the presence of cycloheximide from hypertrophied perfused hearts of thyrotoxic rats. The effects of increased workload on protein degradation *in vivo* are thus equivocal.

Effects of hypoxia on cardiac protein degradation in vitro

Chua *et al.* (1979) showed an inhibition of protein degradation by hypoxia or anoxia in Langendorff (1895)-perfused hearts and by ischaemia in hearts perfused via the left atrium and ventricle as described by Neely *et al.* (1973) and Kao *et al.* (1976). Ten (unspecified) amino acids were present in the perfusion media. We investigated whether hypoxia decreased protein degradation without decreasing cardiac output in a working heart preparation. These studies were of interest in view of the possibility of a regulatory role of lactate or redox state in the regulation of muscle protein degradation and turnover (Rannels *et al.*, 1974; Aoki *et al.*, 1978; Chua *et al.*, 1979; Tischler, 1980; Hedden & Buse, 1982; but see Sugden & Smith, 1982a). Although we were able to stimulate cardiac lactate release dramatically by decreasing perfusate O_2 content (Fig. 2) without diminishing cardiac output, we were unable to inhibit cardiac protein degradation (Table 2). We did not measure NADH/NAD $^+$ nor intracellular lactate/pyruvate (an indirect measure of NADH/NAD $^+$; Williamson *et al.*, 1967), but hypoxia and anoxia are well known to increase both cytoplasmic and mitochondrial NADH/NAD $^+$ ratios and lactate output. Thus an increase in NADH/NAD $^+$ ratio cannot inhibit protein degradation when the heart is pumping normally. Further decrease in O_2 content below 55% decreased the stability of the working heart preparation. The inhibitory effects of anoxia on protein degradation are well established in several tissues (Simpson, 1953; Steinberg & Vaughn, 1956; Brostrom & Jeffay, 1970; Hershko & Tomkins,

1971; Poole & Wibo, 1973; Chua *et al.*, 1979; Sugden, 1980), but under such conditions the tissues are probably irreversibly damaged. We cannot thus see any role for acute hypoxia regulating cardiac protein degradation *in vivo*.

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