Distinction between major chloroquine-inhibitable and adrenergic-responsive pathways of protein degradation and their relation to tissue ATP content in the Langendorff isolated perfused rat heart

Thomas D. LOCKWOOD

Department of Pharmacology and Toxicology, School of Medicine, Wright State University, Dayton, OH 45435, U.S.A.

In the Langendorff isolated perfused rat heart, 36 % of total basal protein degradation was inhibited by the lysosomal inhibitor chloroquine (30 μ M), after elimination of rapid turnover proteins during a 3 h preliminary degradation period. Prior inhibition of degradation with chloroquine was additive to the 30 % inhibition caused by simultaneous infusion of 50–200 nM-isoprenaline. This additivity suggests that the adrenergic-controlled process is independent of the lysosomal degradative pathway. After discontinuation of drug infusions, the isoprenaline-inhibited degradation rate returned to the previous baseline; however, the chloroquine-inhibited degradation rate transiently exceeded the previous baseline. NaN_3 (0.3 mM) caused a decrease of left-ventricular myocardial ATP content of approx. 60 % at 14 min and extreme impairment of contractile function; however, the total lysosomal and non-lysosomal protein degradation was not changed at this time. Conversely, left-ventricular tissue ATP content was not changed during proteolytic inhibition by 10 nM-isoprenaline or 10 μ M-chloroquine at 14 min. The results indicate that depletion of myocardial energy stores in this preparation is neither necessary nor sufficient to cause inhibition of the total of lysosomal and non-lysosomal protein degradation.

INTRODUCTION

Bulk cell protein degradation takes place by independent lysosomal and non-lysosomal pathways, three of which have been experimentally distinguished thus far (reviewed by Dean, 1980). In brief observations of total cell protein degradation, the initial release of amino acids is derived largely from a subclass of rapidly degraded proteins reported to be degraded by a non-lysosomal mechanism (Warburton & Poole, 1977). After elimination of rapid turnover proteins during a 3 h preliminary degradation period, 35–40 % of the amino acid release from remaining cell protein can be inhibited by chloroquine or other inhibitors of the lysosomal pathway (reviewed by Dean, 1980; Seglen, 1982). Such partition of lysosomal and non-lysosomal proteolysis has been found in many cell types, including cardiac and skeletal muscle, although the exact kinetics of degradation appear to depend on nutritional, hormonal or growth conditions (Lockwood *et al.*, 1982a).

In the absence of rapid-turnover proteins, a major proteolytic process of myocardium is inversely coordinated with β -adrenergic-receptor-mediated changes in contractility (Lockwood *et al.*, 1982b; Lockwood, 1985a,b); however, the intracellular degradative site and controlling factors have not been described. The β -receptor agonist isoprenaline (isoproterenol) can cause depletion of energy stores under some conditions of perfusion (Rannels *et al.*, 1980; Chua *et al.*, 1982; Morgan, 1982; Gordon *et al.*, 1985); and glycolytic or respiratory inhibitors can cause a decrease in protein degradation in cultured cells (Warburton & Poole, 1977; Hershko & Ciechanover, 1982; Hershko, 1985; Goldberg, 1985; Gronastajski *et al.*, 1985; reviewed by Khairallah *et al.*, 1985). However, it has not been established for any cultured cell type whether the minimal possible energy availability attained *in vivo* can suffice to

maintain protein degradation. Conversely, it is also unknown whether a decrease in cell energy availability necessarily accompanies all conditions *in vivo* under which major proteolytic pathways change.

A method has been developed by which minute-to-minute percentage changes in myocardial protein degradation can be compared with simultaneous changes in right-intraventricular contractile function in the Langendorff perfused rat heart, by using non-recirculating perfusion (Lockwood, 1985b). The present paper distinguishes chloroquine-sensitive degradative processes from isoprenaline-sensitive degradation, and also characterizes the response of both to depletion of high-energy phosphates by mitochondrial poisons.

METHODS

Myocardial proteins were biosynthetically labelled *in vitro* by infusion of [^3H]leucine for 0.5 h as previously described (Lockwood, 1985a,b). Then 4 mM-leucine (non-radioactive) was added as an inhibitor of re-incorporation of radiolabelled degradation product. A 3 h preliminary perfusion period preceded the experimentally observed degradation period from 3 to 4.5 h after termination of labelling. Experimental solutes were infused into the non-recirculating flow of perfusate (35 °C) in 100–1000-fold concentrated solutions. The effluent perfusate was continuously collected with a fraction collector at 1 min intervals, or as described. The minute-to-minute percentage changes from the baseline rate of [^3H]leucine release were compared with the simultaneously recorded measurement of right-intraventricular pressure, as described below. Although degradation is not directly linked to contractile function, severely dysrhythmic hearts exhibited suppressed or

fluctuating baseline rates of leucine release; such hearts were not employed (Lockwood, 1985b).

ATP determination

The luciferin-luciferase assay kit (Sigma) routinely employed for cultured cells was adapted to the determination of ATP in small ventricular biopsies so as to employ each heart as its own control before and after experimental treatments (reviewed by De Luca & McElroy, 1978). For each determination, three biopsies (each approx. 50 mg) were taken 0.25 cm apart from the left ventricle, blotted, weighed and swirled in 0.2 ml of 10% (w/v) trichloroacetic acid (-1°C) within 5 s. After addition of 50 ml of water, tissues were extensively homogenized and diluted to the final ATP-assay concentrations with 0.01% trichloroacetic acid. The final trichloroacetic acid concentration in the assay was within 0.001% of this diluent acid concentration; such concentration did not interfere with the assay (see Gronastajski *et al.*, 1985). Luminescence of disodium ATP standards (Sigma) in 0.01% trichloroacetic acid, tissue homogenates and enzyme-substrate blanks were determined in parallel, in quadruplicates, with a photomultiplier tube apparatus (constructed by Dr. Minocher Reporter, Battelle-Kettering Institute, Yellow Springs, OH, U.S.A.) and monitored continuously on a strip-chart recorder. The luminescence 1 min after initiation of the reaction was directly proportional to ATP standards and dilutions of tissue homogenates in the assay range of 10 pmol of ATP.

RESULTS

Determination of percentage inhibition of protein degradation by chloroquine, by using extrapolation of a declining baseline of leucine release in a single perfused heart

From 3 to 5 h after a brief 0.5 h period of biosynthetic labelling, the progress of the basal rate of release of [^3H]leucine from the myocardium is described by a curvilinear declining baseline (Lockwood, 1985b; Figs. 1–3). This 3–5 h time constraint of the present method results from the necessity of a 3 h preliminary perfusion period (see the Introduction), and the 5 h duration of optimal function of the preparation (Lockwood, 1985b). The exact baseline characteristics at any time after labelling depend on such multiple variables as the duration of the labelling period and the time of the preceding degradation period, as well as the obvious technical variability. However, a percentage change in basal control degradation rate of a single preparation can be readily observed by using each preparation as its own individual control.

As illustrated in Fig. 1, at 4–5 h after labelling the baseline slope and curvature are sufficiently small so that the control degradation rate is closely approximated by linear extrapolation of the previous basal trace 20–30 min after infusion of the inhibitor. The percentage inhibition of degradation was essentially maximal after 20–30 min exposure to 20 μM -chloroquine (Figs. 1–3, and results not shown), which is similar to the concentration previously reported to be maximal in a variety of other cell types (reviewed by Seglen, 1982; Goldberg *et al.*, 1982). In four successive experiments the mean

Fig. 1. Inhibition of protein degradation in the presence of chloroquine at 4.5 h after labelling

The preliminary degradation period was 4.5 h before zero time. The single experiment shown is representative of four separate hearts. In each experiment, the linear regression of the 12 1 min measurements of leucine release (c.p.m./min) before zero time was obtained in arbitrary units by least-squares analysis, and the resulting traces were normalized at zero time (i.e. S.D. = 0). The mean equation of these four baselines (-12 min to 0 time) was extrapolated to 30 min (broken trace); the S.D. of experimental points about the mean of baselines was less than those indicated at -12 and 30 min. Also shown at 30 min is the mean $\pm$ S.D. of the measured chloroquine-inhibited leucine release; the $36 \pm 5\%$ inhibition is significant at $P < 0.001$. The maximal possible errors due to non-linearity of the extrapolated baseline and change in slope after inhibition are obviously small under these conditions.

percentage steady-state inhibition of degradation under 30 μM -chloroquine was $36 \pm 5\%$ (S.D.) as observed at 4.5 h (Fig. 1).

Leupeptin and some other lysosomotropic agents are peptide derivatives (Seglen, 1982), which were found to permeate the present preparation so slowly as to prevent accurate extrapolation of the baseline, as shown in Fig. 1. Nonetheless, in two experiments, 2 h of infusion of 40 μM -leupeptin caused an approx. 30–40% inhibition of degradation; and the simultaneous infusion of 30 μM -chloroquine produced an additional inhibition of less than 3% (results not shown). This suggests that the known common inhibitory effect of chloroquine and 40 μM -leupeptin was essentially maximal with chloroquine alone.

Absence of effect of prior inhibition of degradation by chloroquine on the adrenergic-responsive pathway

The prior inhibition of degradation by maximal concentrations of chloroquine did not alter the time course or magnitude of the subsequent inhibition of degradation caused by sub-maximal or near-maximal concentrations of isoprenaline infused simultaneously with chloroquine (Fig. 2). The previously reported inhibition of degradation by a maximal isoprenaline concentration (0.5 μM) alone is $29 \pm 3\%$ (S.D.) (Lockwood, 1985b). Because this maximal isoprenaline concentration did not alone cause inhibition of degradation in excess of

Fig. 2. Absence of effect of prior infusion of chloroquine on the reversible adrenergic inhibition of protein degradation

The labelling procedure and 3 h preliminary perfusion, including 4 mM-leucine, were as described in the text. However, at 3 h the routine perfusate was changed to one containing 20 μ M-cycloheximide and 11 mM-glucose in the absence of any amino acids. A further 1 h preliminary period preceded the observation period, which began 4 h after labelling. In this particular experiment, the actual curvature of the baseline degradation rate cannot be accurately extrapolated over this extended time period (see the text and Fig. 1); however, the indicated inhibition of degradation caused by chloroquine is within the variability of the $36 \pm 5\%$ (S.D.) mean inhibition described in Fig. 1. The submaximal 22% inhibition of leucine release shown at 10 min was similar to the $23 \pm 3\%$ (S.D.) mean inhibition previously described with 50 nM-isoprenaline (Lockwood, 1985b). The chloroquine concentration was increased gradually over the first 4 min of the indicated infusion period to prevent arrhythmia. Abbreviation: ISO, isoprenaline.

36% in more than five separate experiments (Lockwood, 1985b), the two agents cause additive inhibitory effects greater than those of either alone, at a probability level of $P < 0.005$.

Reversal of inhibition of degradation on discontinuation of isoprenaline and chloroquine

The isoprenaline-inhibited degradation rate returned to the prior baseline by the same percentage as the previous inhibition several minutes after discontinuation of a 10 min exposure (Fig. 2) or a 1 h exposure (Lockwood, 1985b). In contrast, the chloroquine-inhibited degradation rate began to increase approx. 5 min after drug discontinuation, and reached a variable transient peak, approximately twice the previous baseline, within 0.5–1 h (Fig. 3). This slightly sub-maximal 45 min chloroquine (15 μ M) exposure was selected to minimize injury under conditions used in Fig. 3, and to permit partial reversal of chloroquine effects during these experimental time constraints. The reversibility of higher and longer chloroquine exposures was not characterized, nor was the time integral of the rate of leucine release after chloroquine infusion.

Fig. 3. Protein degradation after discontinuation of chloroquine infusion

Conditions were as described in Fig. 1, except that a 3 h preliminary perfusion time preceded the indicated zero time. A corresponding control baseline rate of leucine release from a separate heart during this time period is shown in the inset, as previously described (Lockwood, 1985b). Although the exact curvature and slope of individual control traces varied considerably (see the text), the continuous contour shown over this time period was observed in more than ten unexposed hearts (results not shown). In more than 30 consecutive experiments the reversal of the adrenergic-inhibited degradative rate exceeded the previous baseline by less than 7% for 1 h after discontinuation of isoprenaline (Lockwood, 1985b, and results not shown). Therefore the excess degradation after discontinuation of chloroquine shown in this single experiment differs from the average of 30 separate experiments after discontinuation of isoprenaline at $P < 0.001$ (Student's *t* test). The experiment shown is also representative of two separate chloroquine-treated hearts (results not shown).

Absence of effect on total lysosomal and non-lysosomal protein degradation of inhibition of mitochondrial function with NaN_3 and 2,4-dinitrophenol

Infusion of the reversible cytochrome oxidase inhibitor NaN_3 causes severe depletion of myocardial ATP and phosphocreatine stores within approx. 10 min (reviewed by Wilson & Maria, 1978; Morgan, 1982). In the experiment of Fig. 4, infusion of 0.15 mM- NaN_3 caused irregular rhythm beginning after 10 min of exposure. Shown at 12 min in Fig. 4 are irregular peak systolic pressures, variable heart rate and the impaired relaxation that are characteristic of the energy-depleted myocardium. The inhibitor was discontinued at these indications of sub-lethal energy depletion at 12 min, and the contractile function largely recovered by 18 min (Fig. 4).

A second infusion of 0.3 mM- NaN_3 at 20 min caused near-cessation of contraction by 30 min. In the present preparation, the relationship between changes in right-intraventricular pressure and corresponding changes in wall tension is uncertain; however, in Fig. 4 the elevation of the diastolic ventricular pressure above zero, beginning at 22 min, is associated with slight visible distension and intraventricular fluid accumulation after compromise of ventricular contractile function. Thus both systolic and

Fig. 4. Effect of NaN_3 on myocardial contractile function, and absence of effect on protein degradation

A 3.5 h preliminary degradation period preceded the measurement. All control parameters were stable and similar to those shown at zero time for more than the preceding 0.5 h. At the times indicated by the arrows, the right-intraventricular traces from the identical experiment are shown at a chart speed of 50 mm/s, movement leftward: 100 mmHg pressure and 2 s of movement are indicated. The heart rate was irregular from 11 to 15 min. During the time period in which the systolic pressure is indicated by the dashed trace, contractile function was nearly stopped; however, occasional contractions continued as illustrated at 38 min. The basal rate of protein degradation remained unchanged throughout this experiment. Protein degradation was also insignificantly changed in two other experiments at the level of experimental precision ($P < 0.01$, two-sided Student's t test).

diastolic pressures were elevated in compromised hearts under infusion of 0.3 mM- NaN_3 as shown from 25 to 50 min in Fig. 4. After discontinuation of NaN_3 infusion at 43 min, ventricular function partially returned by 50 min, thereby decreasing the diastolic pressure over the

Table 1. Change in left-ventricular ATP content in the presence of isoprenaline, chloroquine and NaN_3

Ventricular biopsies were taken 5 min before the beginning of drug infusions. Tissues were processed in parallel as described in the text, thereby using each heart as its own control. The function of the preparation in the presence of isoprenaline and NaN_3 is described in Lockwood (1985b) and in the text. Chloroquine (10 μM) caused less than a 5% decrease in heart rate and peak right-ventricular pressure at 15 min, and less than an 8% decrease at 30 min in any of these preparations. The ATP content was significantly different from controls only under NaN_3 (* $P < 0.05$, Student's t test). Results are means $\pm$ S.D. for n samples.

Experimental condition	ATP ($\mu\text{mol/g}$ wet wt.)
Control	4.9 ± 0.7 ($n = 8$)
10 nM-Isoprenaline (14 min)	5.1 ± 0.7 ($n = 4$)
10 μM -Chloroquine (30 min)	4.7 ± 0.4 ($n = 4$)
0.3 mM- NaN_3 (14 min)	2.1 ± 0.3 ($n = 3^*$)

time period shown (Fig. 4, 50 min). At the time of severe reversible functional compromise, the tissue remained intact, as evidenced by the observation that the radioactivity released contained less than 1% trichloroacetic acid-precipitable protein from injured or dead cells during the experiment of Fig. 4.

Despite severe impairment of contractile function under presumptive depletion of high-energy phosphate stores, the total bulk protein degradation was essentially unchanged during the time periods shown in Fig. 4. The slight transient fluctuations in leucine release shown at 8 and 28 min can accompany any experimental manipulation that causes marked alteration of mechanical function; these were not reproducible. In three separate experiments, infusion of 2,4-dinitrophenol (30 μM) (reviewed by Hunter, 1955) also caused severe reversible ventricular functional impairment in approx. 12–15 min; however, protein degradation was unchanged at this time ($P < 0.05$, two-sided Student t test).

Effect of isoprenaline, chloroquine and NaN_3 on left-ventricular ATP content

Although isoprenaline at 10 nM inhibited protein degradation by 20% within 5–7 min (see Lockwood, 1985b), this concentration did not significantly change tissue ATP content under the present conditions (Table 1). A chloroquine concentration of 10 μM inhibited degradation by at least 20% (Fig. 3) in less than 20 min; however, this concentration did not significantly decrease tissue ATP content during this time period (Table 1).

Although NaN_3 at 0.3 mM did not cause sustained change in protein degradation during the time period illustrated in Fig. 4, this concentration caused an approx. 60% decrease in ATP content in separate experiments (Table 1).

DISCUSSION

Independence of the chloroquine-sensitive and adrenergic-responsive degradative pathways

The strict interpretation of the present results is limited to the conclusion that chloroquine and isoprenaline additively inhibit proteolytic processes; how-

ever, the known locus and mechanism of chloroquine action also strongly suggest that these processes represent distinct lysosomal and non-lysosomal pathways. Chloroquine raises intralysosomal pH and inhibits some proteinase activity directly (Seglen, 1982). As indicated in several studies, chloroquine produces a minimum of 80–95 % inhibition of the degradation of specific membrane-associated proteins that are known to be degraded largely by the lysosomal pathway (Libby *et al.*, 1980, and literature reviewed therein). The 5–20 % of remaining degradation of these membrane proteins in the presence of chloroquine almost certainly includes at least some amount of non-lysosomal degradation, owing to disruption of swollen vesicles in cell culture. Thus it seems likely that chloroquine inhibits an approximate minimum of 90 % of intravesicular lysosomal degradation in diverse cell types. It is hypothetically conceivable that isoprenaline could control a step in the lysosomal process that is proximal to the incomplete intravesicular inhibition caused by chloroquine (i.e. change in protein acquisition rate or substrate properties, or lysosomal membrane properties). However, if the 30 % of basal myocardial degradation that is adrenergic-responsive were simultaneously part of the 36 % of basal degradation that is lysosomal, then at least some diminution of the adrenergic-responsive inhibition would be expected after only partial inhibition of the intralysosomal process; this was not observed at sub-maximal or near-maximal isoprenaline concentrations (Fig. 2).

Reversal of the isoprenaline- and chloroquine-inhibited protein degradation

Under non-recirculating perfusion at 8 ml/min, the exchange of intracellular leucine pools is largely complete in approx. 1 min (Lockwood, 1985b, and literature reviewed therein). Therefore the excess leucine release after discontinuation of chloroquine infusion (Fig. 3) is presumably attributable to compensatory degradation of the retained intravesicular peptides that continue to accumulate during inhibition of proteolysis. Accordingly, the exact kinetics of leucine release after discontinuation of intravesicular inhibitors might depend on such combined factors as the duration of prior substrate acquisition, reversibility of any proteinase inhibition, drug compartmentation, binding, ionization, membrane permeability etc. Moreover, vesicular function and integrity might be altered irreversibly in association with eventual cell injury at longer or higher chloroquine exposures than those investigated here. Regardless, the contrasting precise reversal of the isoprenaline-inhibited proteolysis after 1 h (Lockwood, 1985b) is consistent with the possibility that partially degraded peptide intermediates or sequestered intravesicular proteins do not continue to accumulate during adrenergic inhibition.

Described separately are myofibrillar structural changes that suggest a non-vesicular site of at least some adrenergic-responsive degradation and/or disassembly (Hull & Lockwood, 1986), although the mechanism is far from clear.

Maintenance of a constant myocardial degradative rate during maximal physiological decreases in high-energy phosphate content

Mean control values of ATP content per wet weight of tissue were lower than values found by other methods by approx. 10 % (reviewed by Jennings & Reimer, 1981).

One possible cause was a correspondingly greater percentage of surface perfusate fluid that survived the necessarily rapid blotting procedure in small biopsies as compared with larger tissue samples. However, the amount of ATP lost between excision of the biopsy and fixation of the tissues would seem to be negligible, based on the known time course of decrease in ATP during the first minutes of ischaemia in non-working myocardium (reviewed by Jennings & Reimer, 1981).

After elimination of rapid-turnover proteins, the sustained inhibitory effect of a submaximal concentration of β -agonist on protein degradation (Lockwood, 1985a,b) need not be simultaneously accompanied by decrease in myocardial ATP content (Table 1). Conversely, a decrease in ATP is not sufficient to change simultaneously total lysosomal and non-lysosomal protein degradation at those extents of ATP depletion that cause cessation of contractile function (Fig. 4, Table 1). Thus changes in ATP and the presently observed major pathways of protein degradation are not necessarily linked simultaneously under these experimental conditions. Tissue ATP contents under higher or longer exposures to isoprenaline or chloroquine were not investigated; however, the eventual toxicities of these agents suggests that they might indeed cause a later ATP decrease.

After adrenergic-receptor occupancy, many aspects of myocardial metabolism are integrated with mechanical function by the complex signal network referred to collectively as contractility controls, without necessary depletion of high-energy phosphates (Will-Sahab & Krause, 1980; DeMaille & Pechere, 1983; Koretsky *et al.*, 1983; Berridge, 1984; Lockwood, 1985a; Carafoli, 1987). Nonetheless, the present results do not suggest that either of the major proteolytic processes themselves or their controlling factors are independent of some unknown energy requirement. Moreover, the effect of prolonged energy depletion on proteolysis was not investigated. Indeed, if protein degradation is simultaneously influenced by the intracellular signals that also influence contractility, then it is conceivable that the adrenergic control of myocardial protein degradation might somehow involve several known energy-requiring processes (Snowdowne *et al.*, 1985; Carafoli, 1987); however, such requirements appear to be satisfied by the minimal amounts of ATP necessary to sustain contraction during the presently observed times.

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