The role of insulin in the modulation of glucagon-dependent control of phenylalanine hydroxylation in isolated liver cells

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1. The stimulation of phenylalanine hydroxylation in isolated liver cells by sub-maximally effective concentrations of glucagon ($< 0.1 \mu\text{M}$) is antagonized by insulin (0.1 nM – $0.1 \mu\text{M}$). 2. This phenomenon is a consequence of a decrease in the glucagon-stimulated phosphorylation of phenylalanine hydroxylase from liver cells incubated in the presence of insulin. 3. The impact of insulin on the phosphorylation state and activity of the hydroxylase is mimicked by incubation of liver cells in the presence of orthovanadate ($10 \mu\text{M}$). 4. A series of cyclic AMP and cyclic GMP analogues enhanced phenylalanine hydroxylation: in each case insulin diminished the stimulation of flux. 5. These results are discussed in the light of the characteristics of insulin action on other metabolic processes.

INTRODUCTION

The initial step of phenylalanine degradation in the liver involves hydroxylation to tyrosine. This is catalysed by the enzyme phenylalanine hydroxylase [L-phenylalanine, tetrahydropteridine: oxygen oxidoreductase (4-hydroxylating); EC 1.14.16.1]:

The activity of phenylalanine hydroxylase is controlled by reversible phosphorylation *in vivo* (Donlon & Kaufman, 1978) and in isolated liver cells (Abita *et al.*, 1980; Fisher & Pogson, 1984a). Glucagon and α -adrenergic agents stimulate phenylalanine hydroxylation through the action of cyclic AMP-dependent and Ca^{2+} -dependent protein kinases respectively (Doskeland *et al.*, 1984; Fisher *et al.*, 1984).

There is conflicting evidence for a role of insulin in the control of hydroxylase activity. Tourian (1975) has reported that insulin increases enzyme activity in hepatoma cells by a cycloheximide-sensitive mechanism; however, Sorimachi *et al.* (1981) were unable to demonstrate such an effect. Experimentally induced diabetes in rats is associated with a significant increase in metabolic flux through the phenylalanine-catabolic pathway in isolated liver cells (Carr & Pogson, 1981). An increase in immunoreactive hydroxylase activity in liver cell extracts is also observed (Donlon & Beirne, 1982; Howard & Donlon, 1985; Santana *et al.*, 1985). Changes in the control of phenylalanine hydroxylase phosphorylation state may also accompany the onset of diabetes (Donlon & Beirne, 1982; Stanley *et al.*, 1985). These observations are suggestive of a role for insulin in the modulation of the phosphorylation state of phenylalanine hydroxylase under physiological conditions.

The studies described in the present paper were undertaken in order to clarify the nature of the contribution of insulin to the control of phenylalanine

hydroxylase activity and phosphorylation state in isolated liver cells. Portions of this work have appeared in preliminary form (Fisher *et al.*, 1986a; Pogson *et al.*, 1986).

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats weighing 180–220 g were used throughout. Animals were fed *ad libitum* [Labsure Animal Diet (CRM); C. Hill Group, Poole, Dorset, U.K.].

Chemicals

Reagents and radiochemicals were obtained from the sources given previously (Fisher & Pogson, 1984a), with the following additions. Dibutyl cyclic AMP, 8-bromo cyclic AMP, 8-bromo cyclic GMP and 8-(4-chlorophenylthio) cyclic AMP were obtained from Boehringer Corp. (London). Isobutylmethylxanthine, sodium orthovanadate and sodium nitroprusside were obtained from Sigma. Monocomponent pig glucagon was a gift from Dr. W. Bromer (Eli Lilly, Indianapolis, IN, U.S.A.), and monocomponent bovine insulin was a gift from the Wellcome Foundation, Dartford, Kent, U.K. All other chemicals were of the purest grade available from standard suppliers.

Preparation and incubation of liver cells

Liver cells were prepared as described previously (Elliott *et al.*, 1976). For metabolic flux experiments, cells were incubated in Krebs–Henseleit (1932) medium. For phosphorylation experiments, low-phosphate (0.4 mM-P_i) Krebs–Henseleit incubation medium was used. In all cases the incubation medium was supplemented with 2.5 mM-CaCl_2 , 2% (w/v) bovine serum albumin and lactate/pyruvate (9:1; final concn. 10 mM). Incubation volumes were 2 ml throughout. In all experi-

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ments, metabolic integrity was assessed by measurement of cellular ATP content (Dickson & Pogson, 1977).

In flux experiments L-[4-³H]phenylalanine was added to a final concentration of 0.05 mM and a specific radioactivity of 6.0×10^5 d.p.m./ml. Hormones and

Fig. 1. Influence of insulin on glucagon-stimulated phenylalanine hydroxylation and homogentisate oxidation in isolated liver cells

Liver cells were isolated and incubated as described in the Materials and methods section. Cells were incubated with the indicated concentration of glucagon in the presence (○) or absence (●) of $0.1 \mu\text{M}$ -insulin. Phenylalanine hydroxylation flux (a) and homogentisate oxidation flux (b) were then determined. Results are means $\pm$ S.E.M. for three different liver cell preparations, except for incubations with 1 nM -glucagon, where seven different preparations were used. The basal hydroxylation flux was 5.76 ± 0.19 ($n = 13$) nmol/h per mg. The basal homogentisate oxidation flux was 1.59 ± 0.10 ($n = 13$) nmol/h per mg. The significance of differences between means was assessed by Student's *t* test: * $P < 0.05$ for glucagon only versus glucagon plus insulin; other differences not significant.

other effectors were added 2 min before phenylalanine substrate unless otherwise stated. Incubations were terminated by addition of 0.2 ml of 2M-HClO_4 . Flux through both phenylalanine hydroxylase and homogentisate oxidase was measured by determination of $^3\text{H}_2\text{O}$ release as described in Fisher & Pogson (1984b).

In phosphorylation experiments liver cells were incubated with [^{32}P]P_i at a final radiochemical concentration of approx. $100 \mu\text{Ci/ml}$. Hormones and other effectors were added after 60–90 min, by which time both the [γ - ^{32}P]ATP specific radioactivity and the [^{32}P]phosphate content of phenylalanine hydroxylase had attained a steady state (Fisher & Pogson, 1984a). Incubations were terminated by dilution with a 4-fold excess of ice-cold incubation medium without P_i and albumin. Cells were then separated from medium by rapid centrifugation and resuspended in a buffer containing 250 mM-sucrose, 20 mM-Tris/HCl, 2 mM-sodium phosphate, 5 mM-EDTA, 100 mM-NaF, 0.5 mM-phenylmethanesulphonyl fluoride, leupeptin ($20 \mu\text{g/ml}$), pepstatin ($2 \mu\text{g/ml}$) and chymostatin ($2 \mu\text{g/ml}$), pH 7.4. Cells were broken by three cycles of freezing in liquid N₂ and thawing in water at 37°C ; the final extract was centrifuged at $12000 g$ for 2 min at room temperature and then at $100000 g$ for 10 min (Beckman Airfuge) at 4°C before use for immunoprecipitation of ^{32}P -labelled phenylalanine hydroxylase.

Quantification of the phosphate content of phenylalanine hydroxylase

^{32}P -labelled phenylalanine hydroxylase was immunoprecipitated from cell extracts as described previously (Fisher & Pogson, 1984a). Resulting immunoprecipitates were resuspended in 0.05 ml of a buffer containing 12 mM-sodium phosphate, 1% (w/v) sodium deoxycholate, 1% (w/v) SDS and 0.1% (v/v) Triton X-100, pH 7.4. After centrifugation at $12000 g$ for 5 min through a two-step sucrose gradient (0.15 ml of buffer containing 1 M-sucrose under 0.2 ml of buffer containing 0.5 M-sucrose), precipitates were resuspended in 0.05 ml of buffer and re-centrifuged. This washing procedure was repeated once, then immunoprecipitates were solubilized in NCS before counting for radioactivity in 2 ml of PCS scintillator cocktail.

The phosphate content of the hydroxylase was assessed as described in Fisher & Pogson (1984a). Briefly, the number of moles of enzyme subunit per immunoprecipitate was calculated from the antibody titre, the enzyme specific activity and the subunit M_r . The number of mol of phosphate per mol of subunit was obtained from the ^{32}P content of immunoprecipitates (account was taken of non-specific ^{32}P contamination, by determination of ^{32}P radioactivity of immunoprecipitates of unlabelled purified enzyme from ^{32}P -labelled cell extracts previously depleted of ^{32}P -labelled hydroxylase) and the specific radioactivity of [γ - ^{32}P]ATP. The latter was assessed essentially as described by Hawkins *et al.* (1983) and was found to be unaffected by the addition of hormones or effectors.

RESULTS AND DISCUSSION

Impact of insulin on glucagon-stimulated phenylalanine metabolism

Incubation of isolated liver cells with a series of concentrations of glucagon results in a dose-dependent

Fig. 2. Dose-dependent effect of insulin on phenylalanine hydroxylation in isolated liver cells

Liver cells were isolated and incubated as described in the Materials and methods section. Cells were incubated in the presence of 1 nM-glucagon and the indicated concentration of insulin. Phenylalanine hydroxylation flux was then determined. Results are the means $\pm$ S.E.M. for three or four different liver cell preparations. The significance of differences between means was assessed by Student's *t* test: *P < 0.05 for glucagon only versus glucagon plus insulin; other differences not significant.

enhancement of phenylalanine hydroxylation flux (Fisher & Pogson, 1984a). The influence of insulin on this phenomenon is shown in Fig. 1(a). As previously observed (Fisher & Pogson, 1984a), there was no significant effect of insulin (0.1 μ M) on basal flux or flux stimulated by a maximally effective concentration of glucagon (0.1 μ M). However, enhancement of flux by sub-maximally effective concentrations of glucagon (0.1–10 nM) was significantly diminished by the simultaneous addition of insulin. This effect was also reflected, though to a lesser extent, in decreased flux through the homogentisate oxidase [homogentisate: oxygen 1,2-oxidoreductase (deacylizing); EC 1.13.11.5]-catalysed step of phenylalanine degradation (see Fig. 1b). This observation is consistent with the notion that the major controlling influence on homogentisate oxidation is variation in the provision of substrate, a direct consequence of alterations in phenylalanine hydroxylation flux (Dickson *et al.*, 1986).

Insulin increased the concentration of glucagon required to produce a half-maximal effect on hydroxylation flux by approx. 5-fold, from 0.6 to 3 nM. This is of a similar magnitude to the impact of insulin on glucagon-stimulated glycogenolysis (Blackmore *et al.*, 1979), gluconeogenesis and inhibition of pyruvate kinase activity (Pilkis *et al.*, 1983).

Fig. 2 shows the effect of varying the concentration of insulin, added concurrently with 1 nM-glucagon, on phenylalanine hydroxylation in isolated liver cells. Insulin was maximally effective at 10–100 nM; however, concentrations as low as 0.1 nM resulted in a significant decrease in flux. This is of a similar order of magnitude to the effectiveness of insulin in the modulation of other metabolic processes, e.g. glucagon-stimulated glycogenolysis (Beebe *et al.*, 1985).

The above data were obtained from experiments in

Table 1. Influence of insulin on the phosphorylation state of phenylalanine hydroxylase in isolated liver cells

Liver cells were isolated and incubated as described in the Materials and methods section. Cells were incubated with the indicated concentration of glucagon in the absence or presence of 0.1 μ M-insulin. The phosphorylation state of the enzyme was then determined. Results are means $\pm$ S.E.M. for three different liver cell preparations. The significance of differences between means was assessed by Student's *t* test: *P < 0.05 for absence of insulin versus presence of insulin); †*P* < 0.05 versus corresponding control; other differences not significant.

Condition	Phosphorylation state (mol of P/mol of subunit)	
	No insulin	+ insulin
Control	0.22 $\pm$ 0.04	0.27 $\pm$ 0.03
1 nM-Glucagon	0.37 $\pm$ 0.04†	0.26 $\pm$ 0.02*
0.1 μ M-Glucagon	0.44 $\pm$ 0.05†	0.38 $\pm$ 0.05

which insulin and glucagon were added simultaneously to incubations. Other workers have shown that hormone-responsiveness of liver cell metabolism may be acutely dependent on the sequence and timing of hormone additions (Heyworth *et al.*, 1983). In the present work, insulin addition up to 30 min before glucagon was effective; however, its effect was maximal when added 0–5 min before glucagon (results not shown).

Control of phenylalanine hydroxylase phosphorylation state

The close correlation between the phosphorylation state of phenylalanine hydroxylase and phenylalanine hydroxylation in liver cells (Fisher & Pogson, 1985) indicates that insulin may exert its influence on hydroxylation flux by causing a decrease in the phosphate content of the enzyme.

This is confirmed by the data presented in Table 1. Simultaneous addition of insulin (0.1 μ M) resulted in a significant decrease in the stimulation of phosphorylation brought about by 1 nM-, but not by 0.1 μ M-, glucagon. Insulin alone had no effect on the enzyme phosphorylation status.

Insulin-like action of vanadate on phenylalanine metabolism

Orthovanadate (VO_4^{3-}) has been shown to mimic a variety of insulin-dependent processes in intact cells. For example, glycogen synthase is activated by incubation of fat-cells with orthovanadate (Tamura *et al.*, 1983, 1984). Similarly, lipogenesis in isolated liver cells is increased (Castro *et al.*, 1984). These phenomena probably reflect an effect of orthovanadate on the phosphorylation state of the insulin receptor (Swarup *et al.*, 1982; Tamura *et al.*, 1983, 1984). The influence of orthovanadate on phenylalanine hydroxylation flux and hydroxylase phosphorylation state was therefore investigated.

Table 2 shows the effect of preincubation of liver cells for 30 min (to allow uptake of the anion) in the presence of 10 μ M- Na_2VO_4 , a concentration that was without any adverse effects on cellular integrity, as judged by ATP content (results not shown). This pre-treatment decreased

Table 2. Influence of orthovanadate on the metabolic flux through, and phosphorylation state of, phenylalanine hydroxylase in isolated liver cells

Liver cells were isolated and incubated as described in the Materials and methods section. Cells were incubated in the presence or absence of orthovanadate for 30 min and then with glucagon for 5 min as indicated. Hydroxylation flux and enzyme phosphorylation state were then determined. Results are means $\pm$ S.E.M. for three or four different liver cell preparations. The significance of differences between means was assessed by Student's *t* test: *P < 0.05; for absence of vanadate versus presence of vanadate; †*P* < 0.05 versus control; other differences not significant.

Condition	Phosphorylation state (mol of P/mol of subunit)	Hydroxylation flux (nmol/h per mg)
Control	0.24 $\pm$ 0.02	5.73 $\pm$ 0.19
1 nM-Glucagon	0.39 $\pm$ 0.05†	9.25 $\pm$ 0.47†
10 μ M-Vanadate	0.30 $\pm$ 0.03	5.93 $\pm$ 0.24
1 nM-Glucagon + 10 μ M-vanadate	0.22 $\pm$ 0.06*	6.50 $\pm$ 0.51*

both the hydroxylation flux and the phosphorylation of the enzyme in the presence of 1 nM-glucagon. Basal metabolic flux and enzymic phosphorylation were unaffected. These data resemble those obtained with insulin and are consistent with the suggested effect of this anion on insulin receptor activity.

Contrasting with the action of insulin (Santana *et al.* 1985), Na₃VO₄ did not modulate the α -adrenergic stimulation of phenylalanine hydroxylation [10 μ M-noradrenaline, 141 $\pm$ 7; 10 μ M-Na₃VO₄, 106 $\pm$ 1; 10 μ M-noradrenaline plus 10 μ M-Na₃VO₄, 131 $\pm$ 13 (% of basal flux; means $\pm$ S.E.M. for three different liver cell preparations)]. These data may reflect the reduction of orthovanadate to the vanadyl state (VO²⁺) in the presence of noradrenaline (Cantley *et al.*, 1978); the vanadyl ion is without effect on insulin receptor activity.

Insulin and the stimulation of phenylalanine hydroxylation by cyclic nucleotide analogues

Insulin may exert its effect on the phosphorylation state of phenylalanine hydroxylase by modulating the activity of a variety of components of the glucagon-stimulated cyclic AMP cascade system. Thus insulin has been reported to inhibit liver cell membrane adenylate cyclase activity (Heyworth & Houslay, 1983) and increase cyclic AMP phosphodiesterase activity (Heyworth *et al.*, 1983, 1984; Benelli *et al.*, 1986).

The role of insulin-mediated alterations in cyclic AMP metabolism in the control of phenylalanine hydroxylase activity were assessed by using membrane-permeant phosphodiesterase-resistant analogues of cyclic AMP. Table 3 shows the influence of cyclic nucleotide analogues, with and without 0.1 μ M-insulin, on phenylalanine hydroxylation in isolated liver cells. The concentrations of analogue chosen were such that flux increases of a similar magnitude to the insulin-suppressible glucagon responses were obtained. Also included in Table 3 are data from experiments involving 8-bromo cyclic GMP: this was used in the light of a

Table 3. Influence of insulin on cyclic-nucleotide-stimulated phenylalanine hydroxylation in isolated liver cells

Liver cells were isolated and incubated as described in the Materials and methods section. Cells were incubated in the presence of the indicated cyclic nucleotide analogue in the presence or absence of 0.1 μ M-insulin. Phenylalanine hydroxylation flux was then determined. The basal hydroxylation flux was 5.84 $\pm$ 0.23 (*n* = 5) nmol/h per mg. Results are the means $\pm$ S.E.M. for three different liver cell preparations. The significance of differences between means was assessed by Student's *t* test: *P < 0.05 for analogue only versus analogue plus insulin).

Condition	Hydroxylation flux (as % of basal flux)	
	No insulin	+ insulin
5 μ M-Dibutyl cyclic AMP	163 $\pm$ 5	131 $\pm$ 5*
1 μ M-8-(4-Chlorophenylthio) cyclic AMP	183 $\pm$ 5	154 $\pm$ 6*
1 μ M-8-Bromo cyclic AMP	130 $\pm$ 1	90 $\pm$ 10*
0.1 mM-8-Bromo cyclic GMP	131 $\pm$ 4	96 $\pm$ 8*

Fig. 3. Dose-dependent effects of bromo cyclic AMP and bromo cyclic GMP on phenylalanine hydroxylation in isolated liver cells

Liver cells were isolated and incubated as described in the Materials and methods section. Cells were incubated in the presence of the indicated concentration of 8-bromo cyclic AMP (●) or 8-bromo cyclic GMP (○). Phenylalanine hydroxylation flux was then determined. Results are the means $\pm$ S.E.M. for three or five different liver cell preparations.

report that phenylalanine hydroxylase purified from liver is a substrate for cyclic GMP-dependent protein kinase (Dokeland *et al.*, 1984). The present data are consistent with this suggestion; however, it is clear that the cyclic GMP derivative is substantially less potent in stimulating hydroxylation flux than the corresponding cyclic AMP derivative (see Fig. 3). Also, concentrations of sodium nitroprusside up to 20-fold greater than those known to stimulate cyclic GMP-dependent protein phosphorylation in smooth muscle (Rapoport *et al.*, 1982) failed to enhance hydroxylation flux (results not shown). These

results indicate that cyclic GMP-dependent control of the hydroxylase is unlikely to be of significance *in vivo*.

The data in Table 3 show that stimulation of flux by cyclic nucleotide analogues is significantly decreased in the presence of insulin. The magnitude and insulin-sensitivity of flux responses are similar whether the phosphodiesterase inhibitor isobutylmethylxanthine ($0.1\ \mu\text{M}$) is present in the incubation medium or not (results not shown).

One group (Beebe *et al.*, 1985) has suggested that insulin modulation of cyclic-AMP-analogue-stimulated processes may be explained solely on the basis of phosphodiesterase activation and subsequent slow, but possibly significant, hydrolysis of the analogue. In contrast, similar experimental findings concerning the inhibition by insulin of cyclic AMP-stimulated glycogenolysis in liver cells (Gabbay & Lardy, 1984) and the anti-lipolytic effect of insulin on fat-cells (Gabbay & Lardy, 1985) have been interpreted as indicative of a modulation of cyclic AMP-dependent protein kinase activity, independent of changes in cyclic AMP concentration. Other workers, however, have suggested that increases in protein phosphatase activity may be significant in the insulin modulation of cyclic AMP analogue action in both liver cells (Marks & Parker Botelho, 1986) and fat-cells (Londos *et al.*, 1985). In this context, it is noteworthy that the action of insulin on glucagon-stimulated phosphorylation of phenylalanine hydroxylase is mimicked by exposure of liver cells to polyamines (Fisher *et al.*, 1986b). This may reflect a direct effect of polyamines on the activity of protein phosphatase 2A towards phosphorylated phenylalanine hydroxylase, as has been demonstrated *in vitro* (Tung *et al.*, 1985). A relationship, if any, between polyamine-dependent control of phosphatase activity and insulin action remains to be established. However, the observation that insulin antagonizes both cyclic-nucleotide-dependent and Ca^{2+} -dependent (Santana *et al.*, 1985) regulation of the hydroxylase is not inconsistent with modulation of phosphatase action.

Concluding remarks

The results presented in this paper show that insulin has a significant role to play in the hormonal control of phenylalanine hydroxylase phosphorylation state in isolated liver cells. The findings suggest that the blood [insulin]/[glucagon] ratio is a key regulator of the phenylalanine hydroxylating activity of the liver *in vivo*. This is consistent with the suggestion that alteration of enzyme phosphorylation state may occur in diabetes (see the Introduction). The characteristics of the insulin effect on phenylalanine hydroxylation are broadly similar to those of other insulin-sensitive metabolic processes. This is perhaps not surprising, in view of the fact that the same protein kinases and protein phosphatases are involved in many different metabolic processes (Cohen, 1985). Through such a regulatory system different metabolic processes can be co-ordinated and integrated. Thus, through alterations in the [insulin]/[glucagon] ratio phenylalanine metabolism, ultimately a gluconeogenic process, can be co-ordinated with protein and carbohydrate metabolism as a whole.

We thank the Medical Research Council for financial support.

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Received 22 September 1986; accepted 17 November 1986