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

Michael J. FISHER,*†‡ Alan J. DICKSON* and Christopher I. POGSON*§
*Department of Biochemistry and Molecular Biology, University of Manchester, Oxford Road, Manchester, M13 9PL, U.K., and †Department of Biochemistry, University of Liverpool, P.O. Box 147, L69 3BX, U.K.

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]:

\[ \text{L-Phenylalanine} + \text{tetrahydropteridine} + O_2 \rightarrow \text{L-tyrosine} + \text{dihydropteridine} + \text{H}_2\text{O} \]

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 hydroxylation 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. Dibutyryl 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) 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-

† To whom correspondence and reprint requests should be sent, at Liverpool.
§ Present address: Biochemistry Department, Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, U.K.
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 × 10⁶ d.p.m./ml. Hormones and other effectors were added 2 min before phenylalanine substrate unless otherwise stated. Incubations were terminated by addition of 0.2 ml of 2M-HClO₄. Flux through both phenylalanine hydroxylase and homogentisate oxidase was measured by determination of H₂O release as described in Fisher & Pogson (1984a).

In phosphorylation experiments liver cells were incubated with [³²P]P at a final radiochemical concentration of approx. 100 μCi/ml. Hormones and other effectors were added after 60–90 min, by which time both the [γ-³²P]ATP specific radioactivity and the [³²P]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 Pi and albumin. Cells were then separated from medium by rapid centrifugation and resuspended in a buffer containing 250 mM-sucrose, 2 mM-sodium phosphate, 5 mM-EDTA, 100 mM-NaF, 0.5 mM-phenylmethanesulphonyl fluoride, leupeptin (20 μg/ml), pepstatin (2 μg/ml) and chymostatin (2 μg/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 [³²P]-labelled phenylalanine hydroxylase.

**Quantification of the phosphate content of phenylalanine hydroxylase**

³²P-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 Mr. The number of mol of phosphate per mol of subunit was obtained from the ³²P content of immunoprecipitates (account was taken of non-specific ³²P contamination, by determination of ³²P radioactivity of immunoprecipitates of unlabelled purified enzyme from ³²P-labelled cell extracts previously depleted of ³²P-labelled hydroxylase) and the specific radioactivity of [γ-³²P]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. 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 μM-insulin. Phenylalanine hydroxylation flux (a) and homogentisate oxidation flux (b) were then determined. Results are means ± S.E.M. for three different liver cell preparations, except for incubations with 1 mM-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.
Hormonal control of phenylalanine hydroxylation

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

<table>
<thead>
<tr>
<th>Condition</th>
<th>No insulin</th>
<th>+ insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.22 ± 0.04</td>
<td>0.27 ± 0.03</td>
</tr>
<tr>
<td>1 nm-Glucagon</td>
<td>0.37 ± 0.04*</td>
<td>0.26 ± 0.02*</td>
</tr>
<tr>
<td>0.1 μM-Glucagon</td>
<td>0.44 ± 0.05*</td>
<td>0.38 ± 0.05</td>
</tr>
</tbody>
</table>

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 μM-insulin. The phosphorylation state of the enzyme was then determined. Results are means ± 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.

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 μM) resulted in a significant decrease in the stimulation of phosphorylation brought about by 1 nm-, but not by 0.1 μM-, glucagon. Insulin alone had no effect on the enzyme phosphorylation status.

Insulin-like action of vanadate on phenylalanine metabolism

Orthovanadate (VO₄³⁻) 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 μM-Na₂VO₄, a concentration that was without any adverse effects on cellular integrity, as judged by ATP content (results not shown). This pre-treatment decreased

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 μM) on basal flux or flux stimulated by a maximally effective concentration of glucagon (0.1 μM). 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 (decycling); 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 (Dickis et al., 1983).

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., 1983).

The above data were obtained from experiments in
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±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; †P < 0.05 versus control; other differences not significant.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Phosphorylation state (mol of P/mol of subunit)</th>
<th>Hydroxylation flux (mmol/h per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.24±0.02</td>
<td>5.73±0.19</td>
</tr>
<tr>
<td>1 nM-Glucagon</td>
<td>0.39±0.05†</td>
<td>9.25±0.47†</td>
</tr>
<tr>
<td>10 μM-Vanadate</td>
<td>0.30±0.03</td>
<td>5.93±0.24</td>
</tr>
<tr>
<td>1 nM-Glucagon+</td>
<td>0.22±0.06*</td>
<td>6.50±0.51*</td>
</tr>
<tr>
<td>10 μM-vanadate</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 μM-noradrenaline, 141±7; 10 μM-Na3VO4, 106±1; 10 μM-noradrenaline plus 10 μM-Na3VO4, 131±13 (μmol basal flux; means±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 μM-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 report that phenylalanine hydroxylase purified from liver is a substrate for cyclic GMP-dependent protein kinase (Dokseland 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 (Rapport et al., 1982) failed to enhance hydroxylation flux (results not shown).
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 μM) 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++-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.

REFERENCES

Received 22 September 1986; accepted 17 November 1986