Time Dependent Effect of Indomethacin on the Stimulation of Protein Synthesis in Isolated Rabbit Muscle by Insulin

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Insulin (100 \mu U/ml) stimulated protein synthesis and PGF$_2 \alpha$ release in isolated rabbit muscle, but had little effect on the rate of protein degradation. The effect of insulin persisted for at least 5 h after removal of the hormone. Indomethacin, added at the start of the incubation, inhibited the stimulatory effect of insulin on protein synthesis and PGF$_2 \alpha$ release, but did not block the binding of iodinated insulin. When added 2 h after insulin, indomethacin did not inhibit the stimulation of protein synthesis but completely inhibited the increase in PGF$_2 \alpha$ release. The results suggest that the stimulation of protein synthesis by insulin is mediated by metabolites of membrane phospholipids but that these changes are involved during the phase of response that immediately follows the binding of insulin to its receptor.

INTRODUCTION

The stimulation of skeletal muscle protein synthesis by insulin has been shown to be accompanied by an increase in the release of prostaglandin (PG)F$_2 \alpha$ and to be sensitive to inhibition by drugs that inhibit PG synthesis such as indomethacin, both in vitro (Reeds and Palmer, 1983) and in the whole animal (Reeds et al., 1985). Changes in PG metabolism have also been implicated in the control by insulin of lipolysis (Lambert and Jacquemin, 1980) and pyruvate dehydrogenase activity (Begum et al., 1985).

Insulin also induces changes in membrane potential (Zierler and Rogus, 1981) and alters membrane phospholipid metabolism, increasing inositol phospholipid and
phosphatidic acid content and turnover in adipocytes (Farese et al., 1984, 1985). It has been proposed that metabolites of the phospholipid components of the plasma membrane, including diacylglycerols (Nishizuka, 1984) and phosphoinositol 4,5 bisphosphate (Berridge, 1984) may act as second messengers in the action of insulin.

In the present paper we demonstrate that the addition of the cyclo-oxygenase inhibitor indomethacin to isolated muscles only inhibits the protein stimulatory effect of insulin when added at the start of the incubation, and suggest that the mediation of the action of insulin by metabolites of arachidonic acid may occur only at an early stage of the response.

EXPERIMENTAL

Materials

L-\([2,6^3\text{H}]\)phenylalanine and \([^{125}\text{I}]\)iodinated insulin were purchased from Amersham International (Amersham, Bucks., UK). Actrapid monoclonical pig insulin was obtained from Farillon (Romford, Essex, UK). Indomethacin was purchased from Sigma Chemical Co. (Poole, Dorset, UK). \([^{3}\text{H}]\)PGF$_{2\alpha}$ and \([^{125}\text{I}]\)PGE$_2$ radioimmunoassay kits were purchased from Sterani Research Ltd (St Albans, Herts, UK) and N.E.N. (Southampton, UK) respectively. All other chemicals were purchased either from Sigma Chemical Co. or from B.D.H. (Poole, Dorset, UK).

Tissues and Incubation Conditions

Forelimb digit extensor muscles were removed intact from rabbits (700–900 g) which had been fasted for 18 h before being killed. The muscles were incubated under a constant tension of 10 g (Palmer et al., 1981), for periods ranging from 1.5–6.5 h. The medium used in all incubations contained 40 $\mu$M tyrosine, but was otherwise as described previously (Reeds et al., 1980). Insulin was added to the appropriate incubation vessels (capacity approximately 2 ml) from a stock solution in medium, prepared daily, to achieve a concentration of 100–120 $\mu$U/ml. In some experiments, insulin was present only during the first hour of the incubation, after which time the medium was removed, the muscles were washed twice in fresh, insulin-free medium, and the incubation was continued for a further 1–5 h in the absence of the hormone. The efficiency of the removal of the free insulin was determined by radioimmunoassay. Medium from muscles as described above had the same insulin concentration as medium from muscles incubated in insulin-free medium for 4 h (<6 $\mu$U ml). Indomethacin was added to the appropriate incubations, to a final concentration of 50 $\mu$M, from a stock solution in ethanol.

Protein Synthesis and Tyrosine Release

Protein synthesis was measured over the final 30 min of the incubation by replacing the medium with fresh medium of a similar composition but containing 1.5 mM phenylalanine and \([^{3}\text{H}]\)phenylalanine (final specific radioactivity 2000
dpm/nmol). Thirty minutes later the muscles were removed, rinsed in ice cold NaCl (9 g/l) and blotted dry. Subsequent treatment of the muscles to determine the specific radioactivity of protein bound phenylalanine has been described previously (Palmer et al., 1981). Tyrosine release was measured over the period preceding the addition of [3H]phenylalanine by the method of Waalkes and Udenfriend (1957) modified as described previously (Smith et al., 1983). PG release from the muscles was also measured during this preliminary period of incubation by radioimmunoassays using ~0.05–0.3 ml medium.

**Effect of Indomethacin on [125I] Insulin Binding**

Muscles were incubated for 30 min in the presence of 100 μU insulin/ml. Each incubation vessel contained in addition 0.15 μCi iodinated insulin. After 30 min the labelled medium was removed, the muscles were washed twice (30 s) with unlabelled medium and incubated for a further 15 min in unlabelled medium containing 5 mU insulin/ml to remove non-specifically bound insulin. The effect of indomethacin (50 μM) added throughout the labelling period was examined by determining the amount of reversibly and irreversibly bound [125I] by γ counting (Philips PW4580 counter). Muscles were then homogenised in 70% ethanol, centrifuged (3000 × g for 5 min) and the precipitate was redissolved in 0.3 M-NaOH (3 ml) prior to recounting to determine the proportion of the total counts in the supernatant and the protein pellet.

**Calculation of Rates of Protein Synthesis and Degradation**

Rates of protein synthesis were calculated as described previously (Smith et al., 1983). Rates of protein degradation were calculated as the algebraic sum of the rate of protein synthesis and the rate of release of tyrosine, both expressed as fractional rates, i.e. the proportion of total protein mass synthesised per day and the proportion of total protein bound tyrosine released into the medium per day. We have shown previously that incubation in a medium containing 40 μM tyrosine does not alter the tissue-free tyrosine concentration, and that insulin (100 μU/ml) has no effect on tissue-free tyrosine (Palmer et al., 1985).

**RESULTS AND DISCUSSION**

The data presented here confirm previous observations (Reeds and Palmer, 1983) that insulin stimulates both protein synthesis and PGF2α release, and that the PG synthesis inhibitor indomethacin, whilst having no effect on control muscles, inhibits these stimulatory effects of insulin when added at the start of the incubations (Table 1).

The effect of insulin persists after the removal of the hormone since muscles incubated in the presence of insulin for one hour and subsequently maintained for up to five hours in insulin-free medium showed rates of protein synthesis that were at all time points significantly above the stable rate in control muscles incubated without insulin (Fig. 1). However there was a reduction with time in the response, so that five hours
after insulin withdrawal the stimulation in the rate of protein synthesis had fallen by half, to 30% above the control value.

The previous suggestion, based on the indirect evidence of a lack of effect on insulin-stimulated hexose transport, that indomethacin was unlikely to be acting merely by inhibiting the binding of insulin to its receptor (Reeds and Palmer, 1983) is also confirmed by the lack of effect of indomethacin on the binding of iodinated insulin (Table 2).

Calculated rates of protein degradation were generally unaffected by either insulin or indomethacin, the small increase effected by insulin in the rate of protein degradation (+11%) was not significant (Table 1). However, insulin did stimulate PGE2 release by 70%, from 3.8 ± 0.6 pg PGE2 mg⁻¹ h⁻¹ in the control muscles to 6.4 ± 1.6 pg PGE2 mg⁻¹ h⁻¹ in the muscles incubated with insulin (means of 6 observations ± SEM). The lack of a significant effect on protein degradation contrasts with reports that PGE2 stimulated protein degradation in isolated rat muscles (Rodemann et al., 1982), and that both Interleukin-1 (Baracos et al., 1983) and E. coli endotoxin (Fagan and Goldberg, 1985) stimulated protein degradation and PGE2 release, these actions also being blocked by indomethacin. Thus, while there appears

Table 1. Effect of insulin (INS; 10 μU/ml) and of indomethacin (IND; 50 μM) added at different times on rates of protein synthesis and degradation and PGE2 release in isolated rabbit muscles

<table>
<thead>
<tr>
<th>Addition to medium</th>
<th>Rate of protein synthesis ($k_s$, %d⁻¹)</th>
<th>Rate of protein degradation ($k_d$, %d⁻¹)</th>
<th>PGE2 release (pg mg⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-2 h</td>
<td>2-4 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None*</td>
<td>None</td>
<td>1.49 ± 0.12</td>
<td>4.86 ± 0.54</td>
</tr>
<tr>
<td>IND</td>
<td>IND</td>
<td>1.51 ± 0.18</td>
<td>4.88 ± 0.61</td>
</tr>
<tr>
<td>INS</td>
<td>INS</td>
<td>2.39 ± 0.20***</td>
<td>5.39 ± 0.61</td>
</tr>
<tr>
<td>IND + INS</td>
<td>IND + INS</td>
<td>1.77 ± 0.17††</td>
<td>5.32 ± 0.51</td>
</tr>
<tr>
<td>INS</td>
<td>IND + INS</td>
<td>2.44 ± 2.4***</td>
<td>5.14 ± 0.61</td>
</tr>
</tbody>
</table>

* PGE2 release measured over a 4 h period.
† Not application, see Table 3.
Significantly different from control (no additions). **P < 0.005. ***P < 0.001.
Significantly different from INS alone. ++P < 0.005. +++P < 0.001.
Table 2. Effect of indomethacin (50 μM) on the binding of [125I]insulin in isolated rabbit muscle

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>+ Indomethacin</th>
</tr>
</thead>
<tbody>
<tr>
<td>μU bound/g muscle</td>
<td>106 ± 8.5</td>
<td>115 ± 6.1</td>
</tr>
<tr>
<td>μU bound to precipitate/g</td>
<td>69 ± 6.9</td>
<td>78 ± 6.0</td>
</tr>
<tr>
<td>Reversibly bound μU/g</td>
<td>174 ± 27</td>
<td>195 ± 20</td>
</tr>
</tbody>
</table>

Results are expressed as μU bound, calculated from the specific radioactivity of [125I]-insulin in the incubation medium (195 cpm/μU). Values are means ±SEM for 6 observations; there are no statistically significant differences.

Table 3. Release of PGF_{2α} from muscles incubated with insulin (INS; 100 μU/ml) in the 2 h before and 2 h after the addition of indomethacin (IND; 50 μM)

<table>
<thead>
<tr>
<th></th>
<th>+ INS 0–4 h</th>
<th>+ IND 2–4 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGF_{2α} release (% control value)</td>
<td>+ 100 ± 16</td>
<td>+ 107 ± 17</td>
</tr>
<tr>
<td>0–2 h</td>
<td>+ 86 ± 11</td>
<td>− 16 ± 9</td>
</tr>
</tbody>
</table>

Values are expressed relative to the release of PGF_{2α} in control muscles (no additions) for the corresponding periods and are means ±SEM* for 8 estimations. Significantly different from corresponding control values. **P < 0.005.
observations have been reported recently on the effect of indomethacin on the stimulation of protein degradation in muscles taken from animals treated with E. coli endotoxin (Fagan and Goldberg, 1985). In these experiments, indomethacin administered to rats before an injection of endotoxin inhibited the development of an elevated rate of muscle protein degradation, but the administration of indomethacin in vitro to muscles from rats pretreated with endotoxin did not reduce the elevated rate of protein degradation.

These results suggest that an early response to insulin and other extracellular factors such as E. coli endotoxin is the activation of a chain of events which are sensitive to inhibition by indomethacin in the early stages and thus may stem initially from an increase in prostaglandin synthesis. Subsequently the stimulation of cellular metabolism, including polypeptide translation is insensitive to cyclooxygenase inhibitors.

The changes effected by insulin in the plasma membrane appear to stem initially from an increase in the release of arachidonic acid and its metabolism to prostaglandins, including PGF$_{2\alpha}$, since the inhibition of these events at an early stage of incubation inhibits the ability of insulin to stimulate protein synthesis. A role for prostaglandins as mediators in other cellular processes influenced by insulin has also been proposed. Thus the action of insulin lipolysis has been associated with alterations in prostaglandin metabolism (Lambert and Jacquemin, 1980), and the ability of insulin to stimulate the activation of pyruvate dehydrogenase is also blocked by indomethacin (Begum et al., 1985). Suggestions regarding a possible cascade of events whereby prostaglandins might influence the rate of protein synthesis remain largely speculative. Recent work has suggested that changes in ribosome protein phosphorylation, mediated by protease activated kinase II (PAK II) may be involved in the action of insulin (Perisic and Traugh, 1983). It is possible that PGF$_{2\alpha}$ may act by stimulating protein kinase C either directly (McPhail et al., 1984) or via phospholipase C activation (Siess et al., 1983) and that this activates a membrane protease, leading to PAK II release and ribosomal activation.

REFERENCES

Insulin, Indomethacin and Protein Synthesis