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The effect of indomethacin on the stimulation of protein synthesis by insulin in young post-absorptive rats

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Groups of young rats (100g body wt.) were starved from 23:00 to 11:00h. The animals were then infused intravenously with diluent or insulin at three different doses to achieve plasma insulin concentrations of 20, 50 and 150 μunits/ml. Before the start of the infusion, animals received a single intravenous injection of indomethacin (250 μg) or diluent. After 20min of infusion, the rats were injected with a large amount of labelled phenylalanine and were killed 10min later. Insulin produced a dose-dependent decrease in plasma glucose and a dose-dependent rise in protein synthesis in cardiac, gastrocnemius, plantaris and soleus muscles. Protein synthesis in the liver was unaffected by insulin. Indomethacin had no effect on plasma glucose concentrations, but blocked the insulin-induced rise in protein synthesis in cardiac, gastrocnemius and plantaris, but not in soleus muscle. The hormone also increased the plasma concentration of prostaglandin E_2 and of prostaglandins $F_{2\alpha}$ and E_2 in gastrocnemius and plantaris muscle. The results show close similarities to previous observations with isolated rabbit muscles *in vitro* and suggest that the involvement of arachidonic acid metabolism in the action of insulin on protein synthesis is of physiological significance.

Effects of insulin on muscle protein anabolism have been known for many years (Manchester & Young, 1961; Wool *et al.*, 1968; Manchester, 1970), yet it is only relatively recently that a direct and rapid effect of a physiological concentration of insulin on muscle protein synthesis has been demonstrated (Frayn & Maycock, 1979; Stirewalt & Low, 1983; Garlick *et al.*, 1983). Furthermore, although changes in muscle protein synthesis have been shown to be brought about by changes in the rate of initiation of peptide-bond synthesis (Jefferson *et al.*, 1977; Jefferson, 1980), the nature of the link between the binding of insulin to specific receptors on the cell surface and the change in ribosomal function that occurs has remained obscure. As insulin exerts these effects only in intact cells, presumably some 'second messenger' is involved.

Evidence has implicated changes in arachidonic acid metabolism to $PGF_{2\alpha}$ in the control of muscle protein turnover (Rodemann *et al.*, 1982; Smith *et al.*, 1983; Goldberg *et al.*, 1984). Thus indomethacin, an anti-inflammatory drug which acts by inhibiting the first enzyme on the pathway of arachidonic acid metabolism to prostaglandins (Irvine, 1982), blocks the stimulation of muscle protein synthesis by mechanical forces (Smith *et al.*, 1983; Palmer *et al.*, 1983) and insulin (Reeds & Palmer, 1983) *in vitro*, and both these stimuli increase prostaglandin release by isolated muscles. In addition the inhibition of protein synthesis by dexamethasone is associated with a decrease in $PGF_{2\alpha}$ release (Reeds & Palmer, 1984). It has been proposed therefore that this pathway is a common link between hormonal changes in the extracellular environment and ribosomal activity (Reeds & Palmer, 1984).

The evidence for this proposition has been based entirely on studies with isolated muscles *in vitro*. Although the use of this experimental approach is of great importance to the study of metabolic control, there are examples in the literature in which hypotheses developed on the basis of experiments *in vitro* have not proved to be readily transferrable to the intact animal. For example, leucine is able to stimulate muscle protein synthe-

Abbreviation used: PG, prostaglandin.

sis *in vitro* (Fulks *et al.*, 1975; Buse & Reid,1975), but the injection of this amino acid appears not to lead to an acute stimulation of muscle protein synthesis *in vivo* (McNurlan *et al.*, 1982). It is important therefore to test whether the role of arachidonic acid metabolism in the control of muscle protein synthesis, proposed on the basis of experiments *in vitro*, is applicable in the whole animal. This paper reports the results of such an investigation in which the action of the inhibitor of prostaglandin synthesis, indomethacin, on insulin-stimulated protein synthesis has been studied *in vivo*.

Materials and methods

Materials

L-Phenylalanine, indomethacin and the reagents for the phenylalanine assay were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). The kits for the assay of PGE_2 were obtained from New England Nuclear (Dreieich, W. Germany) and those for the assay of $PGF_{2\alpha}$ from Travenol Laboratories (Thetford, Norfolk, U.K.). L-[2,6-^{3}H]Phenylalanine and [2-^{14}C]indomethacin were purchased from Amersham International (Amersham, Bucks., U.K.). Monocomponent zinc-free pig insulin and insulin diluent were purchased from Farillon Ltd. (Romford, Essex, U.K.). NE 265 scintillant was bought from Nuclear Enterprises (Edinburgh, Scotland, U.K.). All other reagents were the products of BDH (Poole, Dorset, U.K.).

Animals

Male hooded Lister rats of the Rowett strain were weaned at 19 days after birth and housed singly in cages with a wire-mesh floor in a room with a 12h-light/12h-dark cycle (lights on at 06:00h). Animals were weighed daily and were divided, six per group, into eight experimental groups of equal mean weight. Experiments were performed on 3 consecutive days with two rats from each group per day, starting with the heavier animals on day 1. The animals had free access to a commercial pelleted diet (Oxoid; Herbert C. Styles, Bewdley, Worcs., U.K.) until 23:00h on the night before they were killed when the feed was removed. Injections were made between 10:30h and 13:00h.

The procedure for cannulating a tail vein and for injection and infusion has been described previously (Garlick *et al.*, 1983). After cannulation each animal was injected with either 0.2ml of ethanol (5.0%, w/v, in water) or with 0.2ml of 5% ethanol containing 250μg of indomethacin. The indomethacin was injected as a suspension prepared, a few seconds before injection, by adding 25μl of a solution of the drug (20mg/ml, in ethanol) to 0.4ml of water. After 5min the catheter was connected to a syringe, and insulin diluent or insulin solution was infused at a rate of 0.74ml/h. Then 20min later the infusion line was disconnected and [^{3}H]phenylalanine [150μmol and 50μCi (185MBq)/100g body wt.] was injected as described previously (Garlick *et al.*, 1983). After exactly 10min further infusion, the cannula was removed and the animal decapitated. Blood was drained for 10s into a tube containing dry heparin, and both hind limbs were removed, skinned and immediately immersed in ice/water (0.5min after death). The abdominal cavity was opened and a portion of liver (0.8min) and the whole heart (1.1min) were removed, cooled rapidly in ice/water and frozen in liquid N_2. The gastrocnemius, plantaris and soleus muscles were dissected out and also frozen in liquid N_2. All tissue samples were stored at -20°C until analysed. Within 10s of removal, 100μl of blood was mixed with ice-cold ethyl acetate. The remaining blood was placed on ice, centrifuged, and samples of the plasma were taken for assay of glucose and insulin.

Analytical procedures

Preparation of the tissue for analysis and measurement of RNA, protein and the specific radioactivity of free and protein-bound phenylalanine have been described previously (Garlick *et al.*, 1980; McNurlan *et al.*, 1982). Plasma glucose was measured as described by Trinder (1969), and plasma insulin by radioimmunoassay (Basset & Thorburn, 1971) with human insulin as the standard.

The rate of protein synthesis was calculated as in previous publications (Garlick *et al.*, 1983), the times of incorporation being 10min plus the time between death and the cooling of the tissue (see above). The rate of protein synthesis per unit of RNA (k_{RNA} in Tables 2 and 3) was calculated by dividing the fractional rate of protein synthesis by the RNA/protein ratio.

For measurement of blood PGE_2 the ice-cold ethyl acetate extract was dried under N_2 and reconstituted in 0.2M-Tris/HCl buffer (pH7.4) containing 0.1% gelatin. Tissue prostaglandins were extracted from deep-frozen powders by placing the powder in a polypropylene tube and mixing it with 1ml of water and 3ml of propan-2-ol/ethyl acetate/0.2M-HCl (3:3:1, by vol.) at 4°C. Extraction was performed by adding three glass beads and mixing the sample vigorously on a vortex mixer. The mixture was cooled in solid CO_2 and then re-mixed. A further 2ml of ethyl acetate and 3ml of water were then added, and the mixture was re-mixed, stored on ice for 10min and then centrifuged (1500*g*, 10min). Then 3ml of the

organic phase was removed, evaporated to dryness under a stream of N_2 and reconstituted in the appropriate buffer (0.2M-Tris/HCl buffer, pH7.4, containing 0.1% gelatin). The aqueous phase was treated with 1ml of 2M-$HClO_4$, and after centrifugation (as above) the pellet was processed for analysis of protein and RNA. The concentrations of PGE_2 (in muscle and blood) and $PGF_{2\alpha}$ in muscle were measured by radioimmunoassay using, without modification, commercially available kits.

The results were evaluated statistically by analysis of variance, means being compared by two-tailed *t* tests, by using the pooled estimate of variance.

Results

Over the 40min period covered by the experiments, the concentration of indomethacin in plasma fell steadily from 30μM 1min after injection to 11μM 40min later (Fig. 1). The average concentration was 15μM. There was a slower equilibrium of the drug into the muscles, with soleus showing a somewhat more rapid equilibration than plantaris. Over the 40min period, and assuming that the extracellular space was 17% of fresh weight, the average 'intracellular' concentration of indomethacin was very low in both muscles, about 1μM.

Infusion of insulin at three different rates produced a dose-dependent increase in the plasma insulin concentration and a corresponding decrease in plasma glucose (Table 1), as described previously (Garlick *et al.*, 1983). The injection of indomethacin had no effect on either insulin or glucose concentrations at any rate of insulin infusion, compared with uninjected animals. The blood concentration of PGE_2 was significantly decreased in the animals that received indomethacin; 7 of the 24 animals that received the drug had no detectable blood PGE_2. The infusion of insulin at the two higher doses (groups 5 and 7) significantly ($P<0.05$) elevated the blood concentrations of PGE_2, and this increase was reversed by the presence of indomethacin.

The infusion of insulin at the two higher doses significantly elevated the rate of protein synthesis in all three leg muscles, but the lower dose, which gave a plasma insulin concentration of 20μunits/ml, had, as observed previously (Garlick *et al.*, 1983), no effect. The soleus muscle showed a smaller proportional rise in protein synthesis than did either the gastrocnemius or plantaris muscles (Table 2). In the animals that were not infused with insulin, the rate of protein synthesis per unit of RNA was significantly higher in the soleus than in the other leg muscles, but this difference disappeared when insulin was infused at the two higher doses. In gastrocnemius and plantaris muscles the

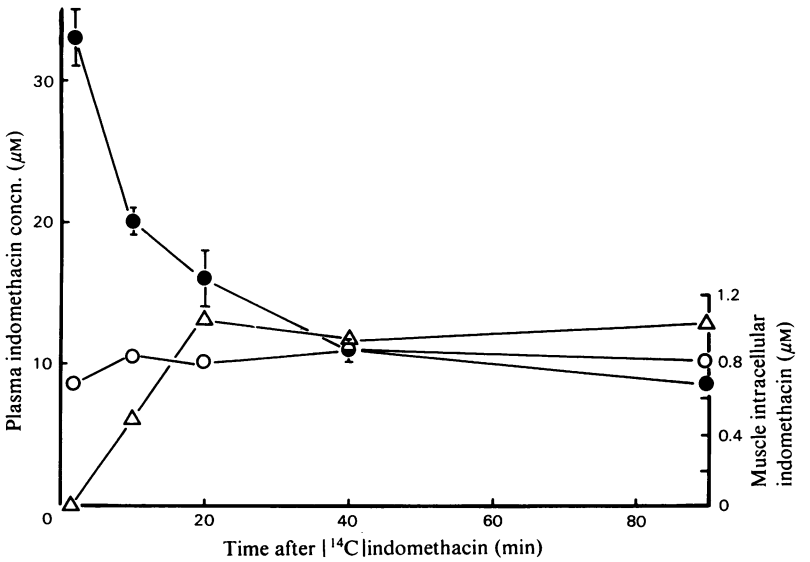

Fig. 1. *Changes in plasma and muscle indomethacin after a single injection of* [^{14}C]*indomethacin*
Young post-absorptive rats were injected with 2μCi (74kBq) of [^{14}C]indomethacin (sp. radioactivity 8μCi/mg). At various times after injection, groups of four animals were killed, and samples of blood (●) and of plantaris (△) and soleus (○) muscles were obtained. These were homogenized in 9vol. of 95% ethanol and radioactivity in a sample was measured. For the two muscles the S.E.M. is smaller than the diameter of the symbol.

Table 1 *Body weight, plasma glucose and insulin and blood PGE_2 concentrations in young post-absorptive rats*
The effect of intravenous insulin infusion at three rates, preceded by a single injection of indomethacin or vehicle (5% ethanol). Mean values are given, together with a common estimate of variance (residual standard deviation; RSD) for six rats/group.

Group	Insulin (munits/h)	Indomethacin (μg)	Body wt. (g)	Plasma glucose (mM)	Plasma insulin (μunits/ml)	Blood PGE_2 (pg/ml)
1[a]	0	0	97	7.00	9	5.8
2[a]	0	250	98	7.11	6	1.8*,†
3	50	0	96	5.39	18	8.2
4	50	250	98	5.06	20	2.4††
5	100	0	96	3.44	53	10.8*
6	100	250	94	3.22	52	3.0†††
7	200	0	93	2.22	>140	9.7*
8	200	250	98	2.61	>140	2.3†††
RSD			9	0.35	10	2.4

[a] These groups received insulin diluent (0.74ml/h).
* $P<0.05$ with respect to group 1.
† $P<0.05$, †† $P<0.01$, ††† $P<0.001$ with respect to non-indomethacin control.

Table 2. *Protein synthesis in three leg muscles of young post-absorptive rats: effects of insulin infusion and indomethacin*
Mean values for k_s (%/day) and k_{RNA} (g of protein/day per g of RNA) are given together with a common estimate of variance (residual standard deviation; RSD) for six animals per group. See Table 1 for details of groups

	Protein synthesis					
	Gastrocnemius		Plantaris		Soleus	
Group	k_s	k_{RNA}	k_s	k_{RNA}	k_s	k_{RNA}
1	9.72	11.98	9.85	11.56	17.40‡	14.23‡
2	9.50	11.55	9.43	10.27	17.80	15.88
3	9.93	12.47	11.20	12.26	17.80	14.90
4	9.27	12.26	10.53	11.04	17.70	15.83
5	11.13*	14.75*	14.42**	14.69**	19.60	17.03*
6	9.88†	12.44†	13.01	11.94†	21.15*	16.69*
7	12.28**	16.42**	15.64**	15.78**	19.65	16.78*
8	10.60	12.99††	13.04†	11.08††	20.50*	17.02*
RSD	1.24	1.84	1.96	1.93	1.94	2.06

* $P<0.05$, ** $P<0.01$ with respect to group 1.
† $P<0.05$, †† $P<0.01$ with respect to non-indomethacin control.
‡Significantly higher than values in other muscles.

injection of indomethacin significantly inhibited the effect of insulin on protein synthesis, but the drug had no effect in controls or at the lowest rate of insulin infusion. In the soleus muscle indomethacin had no effect on the rate of protein synthesis either under basal or under insulin-stimulated conditions. The response of protein synthesis in the heart (Table 3) to both insulin and indomethacin was similar in magnitude to that of gastrocnemius and plantaris muscles, but the rate of protein synthesis in the liver was completely insensitive to a short period without food, to insulin or to indomethacin.

The effect of insulin infusion of PG concentrations in muscle was examined in separate groups of rats that were infused with insulin diluent or with insulin at the highest rate (200munits/h). Four groups each of four animals were killed after 20min, corresponding to the start of the measurement of protein synthesis in Table 1, and four further animals received 150μmol of unlabelled phenylalanine and were killed after 30min of insulin infusion.

There were marked between-muscle differences in the concentrations of both prostaglandins, those in the soleus muscle being almost 10-fold higher than in the gastrocnemious (Table 4). In gastrocnemius and plantaris muscles the infusion of insulin was associated with an increase in the concentrations of both $PGF_{2\alpha}$ and PGE_2 whether the animals had received phenylalanine or not. This was not the case in the soleus, where insulin had no

Table 3. *Protein synthesis in the whole heart and liver of young post-absorptive rats: effects of insulin and indomethacin*
Mean values for k_s (%/day) and k_{RNA} (g of protein/day per g of RNA) together with a common estimate of variance (residual standard deviation; RSD) for six animals per group. See Table 1 for details of groups

	Protein synthesis			
	Heart		Liver	
Group	k_s	k_{RNA}	k_s	k_{RNA}
1	17.12	10.20	97.7	17.92
2	16.52	9.42	94.7	16.55
3	18.48	11.73	100.3	18.46
4	18.08	10.82	101.6	16.59
5	20.23**	12.39*	98.0	16.59
6	18.02†	10.71	102.1	17.25
7	21.35**	13.53**	100.5	17.03
8	18.05††	10.67††	93.6	16.81
RSD	1.45	1.78	18.5	2.69

*$P<0.05$, **$P<0.01$ with respect to group 1.
†$P<0.05$, ††$P<0.01$ with respect to non-indomethacin group.

significant effect on the concentration of either prostaglandin. The injection of indomethacin prevented the insulin-induced rise in PG concentrations, but had no effect on the basal concentrations in either the gastrocnemius or the plantaris. By contrast, in soleus indomethacin significantly decreased the concentration of both PGs in the control animals.

Discussion

The present results obtained in intact rats, and with lower concentrations of indomethacin, closely parallel our previous observations *in vitro* (Reeds & Palmer, 1983). Thus indomethacin (15μM) largely inhibited the effect of an infusion of insulin on protein synthesis in gastrocnemius, plantaris and cardiac muscle, and the infusion of insulin significantly increased the concentrations of both $PGF_{2\alpha}$ and PGE_2 in gastrocnemius and plantaris. On the other hand, indomethacin had no effect on muscle protein synthesis in animals that received no insulin, nor did the drug inhibit the ability of insulin to lower blood glucose, confirming a lack of effect of the drug on insulin-stimulated hexose transport *in vitro* (Reeds & Palmer, 1983). Thus it appears unlikely that indomethacin was merely having a toxic action, or that it interfered with the binding of insulin to its receptors.

Although the injection of indomethacin prevented the insulin-induced rise in muscle PG concentrations, the drug had no effect on their concentration in the control animals. These results suggest that *in vivo*, as well as *in vitro* (Palmer *et al.*, 1983; Reeds & Palmer, 1983), elevated rates of PG synthesis in muscle are more sensitive than the basal rate to indomethacin. However, it seems unlikely that the failure of indomethacin to lower basal PG concentrations was due to a failure of the assay, i.e. an inability to detect a fall in PG concentrations when they are already low, as in separate experiments (Reeds & Palmer, 1984) dexamethasone added *in vitro* decreased basal PG release. On the other hand, in the soleus, a muscle in which the 'basal' concentrations of $PGF_{2\alpha}$ and PGE_2 are high, indomethacin was effective in lowering the concentration of both PGs in control and insulin-treated animals.

The results that were obtained in soleus were interesting in other respects. In this muscle insulin increased the rate of protein synthesis, but did not increase the concentration of the PGs. Although this is consistent with the lack of effect of indomethacin on insulin-stimulated protein synthesis in this muscle, this result also suggests that other factors can stimulate protein synthesis in skeletal muscle and that all do not act through changes in prostaglandin synthesis. Thus, although there is evidence that $PGF_{2\alpha}$ stimulates protein synthesis in the soleus (Rodemann & Goldberg, 1982), the present results suggest that this is not the mechanism whereby insulin acts in this muscle. It is possible that protein synthesis in soleus is more sensitive to the supply of extracellular substrates (Buse, 1981) and that insulin stimulates protein synthesis in this muscle primarily by enhancing substrate transport. This would be consistent with the poor effect of indomethacin on insulin-stimulated hexose transport (Reeds & Palmer, 1983) and on plasma glucose (Table 1).

In soleus it was also noteworthy that in the control animals the rate of protein synthesis per

Table 4. *Prostaglandin $F_{2\alpha}$ and E_2 concentrations in the leg muscles of young post-absorptive rats: effects of insulin infusion and injection of indomethacin*
Mean values are shown with RSD for four animals per group.

Group	Insulin (munits/h)	Indomethacin (μg)	Body wt. (g)	Plasma glucose (mM)	Prostaglandin concn. (pg/mg of protein)					
					Gastrocnemius		Plantaris		Soleus	
					$PGF_{2\alpha}$	PGE_2	$PGF_{2\alpha}$	PGE_2	$PGF_{2\alpha}$	PGE_2
9	0	0	98	6.50	18	9.6	97	27.6	194	56.3
10	0	250	100	6.27	15	7.2	80	21.5	56**	30.1***
11	200	0	98	2.33	43**	19.9*	136*	38.7*	167	46.0
12	200	250	99	2.56	17†	7.9††	90†	24.9†	110	31.6
13[a]	200	0	98	2.44	33*	13.2	134*	36.2*	186	53.1
RSD			5	0.55	10	4.7	26	6.7	40	7.1

[a] This group received in addition 150 μmol of L-phenylalanine 10 min before death.
* $P<0.05$, ** $P<0.01$, *** $P<0.001$ with respect to group 9.
† $P<0.05$, †† $P<0.01$ with respect to non-indomethacin control.

unit of RNA (k_{RNA}) was significantly higher than in the other muscles, confirming previous observations of the relative insensitivity of protein synthesis in soleus to starvation (Frayn & Maycock, 1979; Buse, 1981; Preedy & Garlick, 1983). Furthermore, as insulin equalized k_{RNA} in the three leg muscles, the proportional rise in k_{RNA} in the soleus (+17%) was lower than in gastrocnemius (+37%) and plantaris (+36%). It appeared then that, again in keeping with previous observations in perfused rat hemicorpus (Preedy & Garlick, 1983) and in isolated muscles (Frayn & Maycock, 1979), protein synthesis in soleus muscle was less sensitive to insulin as well as to a short period without food. Protein synthesis in the soleus is also less sensitive to experimental diabetes (Flaim *et al.*, 1980; Pain *et al.*, 1983).

It is generally assumed that insulin stimulates muscle protein synthesis while simultaneously decreasing protein degradation (Fulks *et al.*, 1975; Jefferson, 1980). If changes in prostaglandin concentrations were also involved in this action of insulin, the hormone should have lowered the concentration of PGE_2, as this compound has been shown to stimulate protein degradation (Rodemann & Goldberg, 1982; Rodemann *et al.*, 1982). In fact PGE_2 concentrations were elevated in the muscles of insulin-treated rats, and this observation is therefore incompatible with a role for PGE_2 in the insulin-induced suppression of muscle protein degradation. However, there is little information on the acute effects of insulin on muscle protein degradation in the whole animal, but it is doubtful whether physiological concentrations of insulin suppress this process. Much of the evidence has been derived from experiments with isolated muscles and, although the presence of insulin at high concentrations (> 1 munits/ml) will decrease protein degradation, we have been unable to find evidence that insulin concentrations even as low as 100 μunits/ml (which undoubtedly stimulate protein synthesis *in vitro*) have any effect on protein degradation (Jefferson *et al.*, 1977; Frayn & Maycock, 1979; Stirewalt & Low, 1983). Whether this apparent difference in the dose–response of protein synthesis and protein degradation to insulin *in vitro* is also applicable *in vivo* will remain unanswered until methods are devised for assessing acute changes in muscle protein degradation in the whole animal.

The present results show that insulin stimulation of protein synthesis is sensitive to indomethacin in a number of muscles, but not in soleus. The results suggest that stimulation of arachidonic acid metabolism may be closely involved in the generation of intracellular mediators of insulin's action on protein synthesis. The close similarity of these results, obtained in the whole animal, to those

obtained with isolated muscles from another species suggests that the role of prostaglandin metabolism in the control of muscle protein synthesis (Rodemann & Goldberg, 1982; Smith *et al.*, 1983) and specifically in the insulin-mediated changes (Reeds & Palmer, 1983) is of physiological significance.

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