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INDOMETHACIN INHIBITS ENDOCYTOSIS AND DEGRADATION OF INSULIN

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SUMMARY : Indomethacin inhibits autophosphorylation of the insulin receptor. The lack of a consensus as to whether phosphorylation of the insulin receptor is necessary for its endocytosis prompted an investigation into the effects of indomethacin on the uptake and subcellular processing of insulin in the perfused rat liver. Indomethacin did not affect total uptake of insulin by the liver, but there was a concentration dependent inhibition of transfer from the plasma membrane to the endosomes (1mM, 32% inhibition; 5mM, 90% inhibition). Compartmental analysis showed the endocytic rate constant to be inhibited by 82% at 5mM indomethacin (0.0084 to 0.0015 sec^{-1}). The similarity between the level of inhibition of autophosphorylation and the inhibition of endocytosis suggest that phosphorylation of the receptor is necessary for endocytosis. Indomethacin at 5mM completely abolished efflux of insulin degradation products from the perfused liver, suggesting that internalisation is an absolute requirement for degradation.

For a large number of receptors, interaction with the appropriate ligand is followed by internalisation of the receptor-ligand complex into the cell via the process known as receptor-mediated endocytosis [1,2]. In the case of receptors such as the insulin receptor, the initial binding of insulin activates a tyrosine kinase, which catalyses an auto-phosphorylation of the beta subunit [3]. There is considerable debate concerning the role of autophosphorylation in the various mechanisms of insulin action [4,5] and also as to whether phosphorylation of the receptor is a pre-requisite for endocytosis of the receptor-ligand complex. [6-8].

It has been shown that indomethacin, more commonly known as an inhibitor of cyclo-oxygenase [9], is also an effective inhibitor of phosphorylation of solubilised adipocyte insulin receptor [10]. It was also demonstrated that several insulin-sensitive metabolic events were affected by indomethacin.

In the present study we have used analytical subcellular fractionation techniques combined with compartmental analysis of the perfused rat liver to investigate the effects of indomethacin on the receptor-mediated uptake and endocytosis of insulin.

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MATERIALS AND METHODS

Acetylsalicylic acid, indomethacin and bovine serum albumin (BSA) were obtained from Sigma Chemical Co Ltd (Poole, Dorset, UK). Indomethacin was dissolved in a small quantity of dimethyl-sulphoxide, and was slowly added to the appropriate buffer. [^{125}I-Tyrosine A14]-insulin was prepared and purified as previously described [11] from a zinc-stabilised porcine insulin (Monotard MC Novo, Basingstoke, UK). Other reagents were of Analar grade and obtained from BDH Ltd (Poole, Dorset, UK).

Isolated liver perfusion: Rat liver perfusion was carried out as previously described [12] via a fixed head apparatus, with a Krebs-Henseleit bicarbonate buffer containing D-glucose (5mM) and bovine serum albumin (0.5%w/v) at pH 7.4. The liver was pre-perfused for 10min with either buffer alone or buffer containing indomethacin or acetylsalicylate. This was followed by a 2min perfusion of the same buffer containing radio-iodinated insulin (1.85 kBq/ml) plus carrier insulin (1nM). Perfusion was then continued in the absence of ligand for up to 20 min. Where examination of the perfusate for insulin degradation products was required, the efferent perfusate was collected over 12sec intervals from the start of perfusion with the radiolabelled ligand. Trichloroacetic acid (0.5ml, 20% w/v) was added to 0.5 ml of each fraction, and following 15 min on ice, the sample was centrifuged. Both supernatant and pellet were counted for radioactivity. At the end of the perfusion the liver was removed and used either to prepare endocytic vesicles or was subjected to analytical subcellular fractionation.

This perfusion procedure was modified to accommodate a technique for compartmental analysis, in which the processing of insulin could be followed at early time points. After the period of pre-perfusion, a bolus (0.5ml) of radiolabelled insulin (1×10^5Bq + 1nM native insulin) with ^{51}Cr-EDTA (2×10^4Bq) as an extracellular marker was injected into the perfusion line. The perfusate was then collected over 1s intervals for a total of 100s. The perfusate supply was then instantaneously switched to a Krebs buffer, the pH of which had been adjusted to 4.0 with HCl. The perfusate was collected over 1s intervals as before, for a further 100s. This latter procedure causes the non-endocytosed insulin remaining on the sinusoidal plasma membrane to be dissociated and facilitates the compartmental analysis in addition to determining the quantity of insulin endocytosed by the liver.

Subcellular Fractionation: Liver (2g) was removed, homogenised, and fractionated on a sucrose density gradient in a Sorval vertical pocket rotor as previously described [13]. Subcellular distributions were calculated as described by Smith *et al.* [14], for radiolabel and for organelle marker enzymes.

Compartmental Analysis: Compartmental analysis was carried out as described previously [15]. Computation was performed on a SUN-4 system, running ASSESSOR, an in-house numerical equation solver and optimiser.

RESULTS

Effect of indomethacin on the uptake and subcellular distribution of insulin.

The effect of indomethacin on uptake of insulin by the liver is shown in Table 1, where uptake by the homogenate is compared with uptake into an endosomal vesicle fraction. There is no significant difference in the total uptake into the liver at either concentration of indomethacin. However, 1mM indomethacin significantly reduces transfer into the endosomal fraction, and 5mM indomethacin almost completely

TABLE 1. Effect of indomethacin on the uptake of insulin in the perfused rat liver

	n	Uptake into homogenate (%)	Uptake into Endosomes (%)
Control	8	32.4 ± 5.3	11.3 ± 1.0
1mM Indomethacin	4	33.1 ± 5.9	*7.8 ± 1.6
5mM Indomethacin	3	34.6 ± 3.6	*1.6 ± 2.2

The uptake into the liver homogenate and the endosomal fractions are expressed as a percentage of the total recovered radioactivity (perfusate + liver) and are given as the mean ± one standard deviation. * = $p < 0.01$.

inhibits transfer. Acetylsalicylate (5mM), an inhibitor of cyclo-oxygenase [9], is without effect on either uptake or transfer of insulin (Results not shown).

The results were confirmed by analytical subcellular fractionation of rat liver perfused with radiolabelled insulin in the presence of indomethacin. In the control perfusion (Figure 1, top left), the main peak of activity is found in the endosomal region (ρ=1.12 g.cm^{-3}) with only a small amount remaining associated with the plasma membrane (ρ=1.18 g.cm^{-3}). However, when the liver is perfused with radiolabelled insulin in the presence of 5mM indomethacin (Figure 1, top right), very little insulin is seen in the

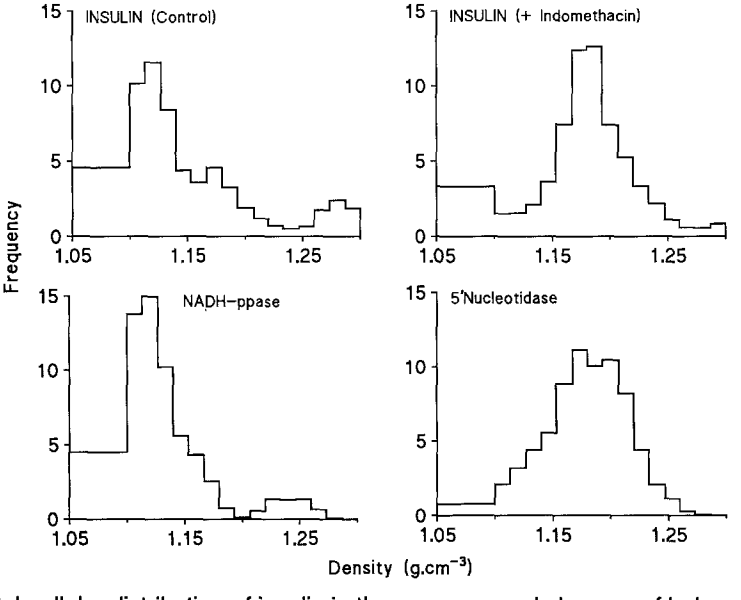


Fig.1. Subcellular distribution of insulin in the presence and absence of indomethacin. Rat liver was perfused with radiolabelled insulin in the presence and absence of 5mM indomethacin as described in Methods. The figure shows the frequency-density distribution of labelled insulin and the marker enzymes 5'nucleotidase (plasma membrane) and latent-NADH pyrophosphatase (endosomes). The histograms represent the mean of 5 experiments for the control and 3 experiments in the presence of indomethacin.

endosomal region. The majority of the label is now associated with the plasma membrane.

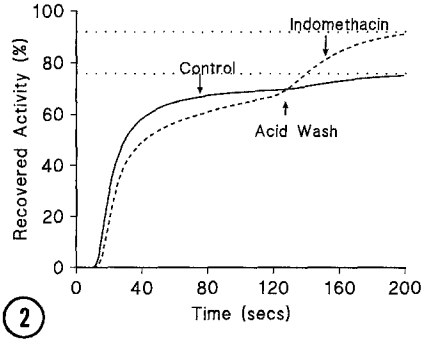
To confirm the retention of receptor-bound insulin at the plasma membrane, the perfusion was modified to include an acidic wash as described in Methods. The time course of appearance of radioactivity in the efferent perfusate is shown in Figure 2. In both the control and indomethacin perfusions approx. 30% of the bolus is associated with the liver before the acid wash. In the control perfusion, the acid wash releases approx 5% of the total bolus indicating that 25% has been endocytosed. In the presence of indomethacin however, 24% of the total counts in the bolus are released by the acid wash.

Compartmental Analysis.

Compartmental analysis according to the scheme shown in Figure 3, of the efferent perfusate data in Figure 2, gives estimates for the rate constants governing uptake and internalisation (Table 2). The most significant change is in the rate constant for endocytosis, k_3, which is decreased by a factor of 5.5 in the presence of indomethacin.

Subcellular processing of insulin.

Following receptor-mediated endocytosis, the majority of the internalised insulin undergoes degradation within the endosomal compartment [16,17] the degradation products returning to the portal circulation [18]. In order to examine the possible effects of indomethacin on the subcellular processing of insulin, the time course of



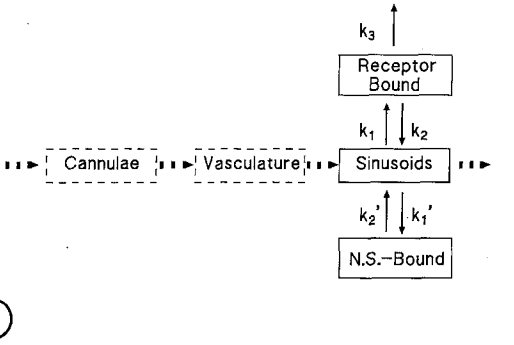


Fig.2. Effect of indomethacin on appearance of radiolabelled insulin in the efferent perfusate. The figure shows the time-course for the appearance of the radioactivity in the efferent perfusate for a control perfusion (solid line) and a perfusion in the presence of 5mM indomethacin (dashed line). The afferent perfusate was switched to an acidic medium at 100s, the effects on the appearance of radioactivity in the perfusate being seen at 120s as indicated. 100% represents the total counts recovered in the liver and the perfusate.

Fig. 3. Schematic model for compartmental analysis. The cannulae and vasculature are composite time delays and compartmental volumes, the sinusoidal volume is a single formal compartment. Time delays and volumes were estimated from the dilution curves of ^{51}Cr-EDTA. k_1' and k_2' are first order rate constants describing a non-specific binding component, k_1 and k_2 are the pseudo-first order and first order rate constants for association and dissociation of insulin from the plasma membrane receptors, and k_3 is the first order rate constant for endocytosis.

TABLE 2. Comparison of the rate constants from compartmental analysis

	k_1'	k_2'	k_1	k_2	k_3
Control n = 6	0.360 ±0.144	0.198 ±0.086	0.129 ±0.051	0.0052 ±0.0011	0.0084 ±0.0012
Indomethacin n = 4	0.098 ±0.028	0.221 ±0.039	0.092 ±0.036	0.0076 ±0.0013	0.0015 ±0.0006
p (students t)	>0.05	>0.05	>0.05	<0.05	<0.001

Results are mean ± SD.

the perfusion was extended to investigate the appearance of insulin degradation products in the efferent perfusate. Figure 4 compares the appearance of trichloroacetic acid-precipitable radioactivity (intact insulin) and trichloroacetic acid-soluble radioactivity (degraded insulin) in the perfusate in the presence and absence of 5mM indomethacin. In the control experiment (Figure 4A) trichloroacetic acid-soluble insulin degradation products begin to appear after 4 min and reach a maximum at 8-10 min. However, in the presence of 5mM indomethacin (Figure 4B), efflux of insulin degradation products is virtually abolished.

DISCUSSION

Indomethacin is known primarily as an anti-inflammatory agent which acts by inhibiting prostaglandin synthesis [9]. However, reports of indomethacin's ability to inhibit autophosphorylation of the insulin receptor[10], suggested the possibility of using indomethacin to investigate the importance of receptor phosphorylation in endocytosis of the insulin receptor.

Malchoff *et al.*[10] found that 1mM indomethacin produced approximately 35% inhibition of receptor phosphorylation. In the study reported here, both 1 and 5 mM indomethacin produced no significant effect on total insulin uptake in the perfused

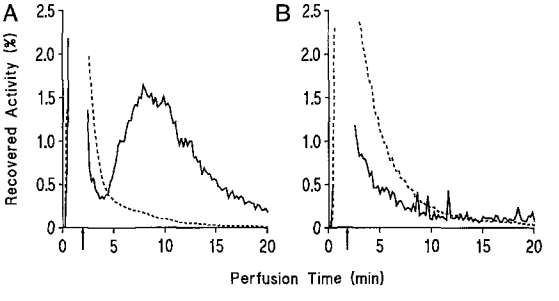


Fig. 4. Effect of indomethacin on the appearance of insulin degradation products in the efferent perfusate. The percentage of recovered trichloroacetic acid-soluble (solid line) and trichloroacetic acid-precipitable (dashed line) radioactivity is plotted against perfusion time for (A) control and (B) indomethacin (5mM) perfusions. The end of the radiolabelled insulin pulse at 2 min is indicated by the arrow on the abscissa.

liver, but resulted in 32% and 90 % inhibition of transfer respectively into an endosomal fraction. The similarity between the level of inhibition of autophosphorylation and the inhibition of transfer at 1mM indomethacin, suggest that the phosphorylation state of the receptor may indeed be important in this process. The subcellular fractionation experiments confirmed that the effect of indomethacin was to block transfer of insulin from the plasma membrane to the endosomes; at 5mM indomethacin virtually all the radiolabelled insulin is associated with the plasma membrane region of the gradient. Compartmental analysis showed that, as expected, indomethacin was not affecting the initial interaction of insulin with its receptor, the rate constant for association being unchanged from the control. There is a small but significant ($p < 0.05$) increase in the value for the rate constant for dissociation of insulin from the plasma membrane. However, there was a highly significant ($p < 0.001$) decrease in the endocytosis rate constant, there being an 82% inhibition of k_3 with 5mM indomethacin. The lack of any observable effect of acetylsalicylate on the internalisation of insulin suggests that the effect of indomethacin is not being mediated by inhibition of cyclo-oxygenase.

At present there is no consensus as to whether phosphorylation of the insulin receptor is necessary for internalisation of the insulin-receptor complex. Backer *et al.* [8] have produced evidence to suggest that autophosphorylation is not necessary for endocytosis, whereas McClain *et al.*[6], and Gherzi *et al.*[7], suggest that phosphorylation is a necessary pre-requisite. The results presented here argue for the involvement of auto-phosphorylation in the endocytosis of the insulin receptor. There has been much speculation as to whether internalisation of insulin is necessary for degradation of the hormone [19]. The present study demonstrates the absence of degradation, as judged by the criterion of trichloroacetic acid-precipitability, when the ligand is trapped at the plasma membrane. However, recent experiments have suggested that the insulin degrading ability is very closely associated with the insulin receptor [18]. If this is the case then it is also possible that degradation may be linked to the phosphorylation state of the receptor. The present experiments do not show whether phosphorylation of the receptor directly influences degradation since occupied receptors that are internalised may well be phosphorylated.

The concentrations of indomethacin used in this study are considerably higher than might be expected to be seen in the plasma (10 μM) following oral administration of indomethacin for medical purposes. Thus it is unlikely that inhibition of insulin endocytosis by the liver would result from pharmacological concentrations of indomethacin, particularly since about 90% of indomethacin in the blood is bound to plasma proteins [20]. There are reports that acetylsalicylate can accentuate both basal and stimulated insulin response in normal man and patients with non-insulin dependent diabetes mellitus [21]. It has been assumed that this effect is mediated via inhibition of prostaglandin synthesis and therefore inhibition of insulin clearance

by indomethacin as reported in this study would probably augment this response. The interaction of ligands with their receptors is responsible for producing many key metabolic events. However, many of the subsequent events, particularly the mechanisms underlying receptor-mediated endocytosis are as yet poorly understood. Insight into biological mechanisms often results from the application of specific inhibitors of a process, however in the case of endocytosis, suitable specific inhibitors are not readily available. Metabolic inhibitors such as DNP can affect endocytosis [22], but of the few specific inhibitors of endocytosis, phenylarsine oxide is the most commonly used [23]. However, in our hands, phenylarsine oxide has not produced consistent results in the perfused liver. The effects presented here, of the inhibition by indomethacin of ligand internalisation, introduce a new tool to the study of endocytic mechanisms.

REFERENCES

1. Willingham, M.C and Pastan, I. (1984) Rec. Prog. Horm. Res. 40, 569-581.
2. Bergeron, J.J.M., Cruz, J., Kahn, M.N. and Posner, B.I. (1985) Ann. Rev. Physiol. 47, 383-403.
3. Kasuga, M., Zick, Y., Blithe, D.L., Crettez, M. and Kahn, C.R. (1982) Nature, 298, 667-669.
4. Denton, R.M. (1986) Adv. Cyc. Nucl. Res. 20, 293-341.
5. Goldfine, I.D. (1987) Endocrine Rev. 8, 235-255.
6. McClain, D.A., Maegawa, H., Lee, J., Dull, T.J., Ulrich, A., Olefsky, J.M., (1987) J. Biol. Chem. 262, 14663-14671.
7. Gherzi, R., Sesta, G., Andraghetti, G., De Pirro, R., Lauro, R., Adezati, L., Cordera, R., (1989) J. Biol. Chem. 264, 8627-8635.
8. Backer, J.M., Kahn, C.R., White,M.F., (1989) Proc. Natl. Acad. Sci. USA 86, 3209-3213.
9. Flower, R.J., (1974) Pharm. Rev. 26, 33-41.
10. Malchoff, D.D., Messina, J,L,. Gordon, V., Tamura, S., Larner, J., (1985) Mol. Cell. Biochem. 69, 83-91.
11. Rideout, J.M., Smith, G.D., Lim, C.K., Peters, T.J., (1985) Biochem. Soc. Trans. 13, 1225-1226.
12. Christensen, J.R., Smith, G.D., Peters, T.J., (1985) Cell Biochem. Funct. 3, 13-19.
13. Smith, G.D. and Peters, T.J. (1980) Eur. J. Biochem. 104, 305-311.
14. Smith,G.D., Osterloh,K.R., Peters, T.J., (1980) Anal.Biochem. 160, 17-23.
15. Hammond, B.J., Christensen, J.R and Smith, G.D. (1990) J. Theoret. Biol. in press.
16. Pease, R.J., Smith, G.D., Peters, T.J., (1985) Biochem. J. 228, 137-146.
17. Doherty, J.J., Kay, D.G., Lai, W.H., Posner, B.I. and Bergeron, J.J.M. (1990) J. Cell Biol. 110, 35-42.
18. Smith, G.D., Christensen, J.R., Rideout, J.M., Peters, T.J., (1989) Eur.J.Biochem. 181, 287-294.
19. Duckworth, W.C. (1988) Endocrine Rev. 9, 319-345.
20. Goueli, S.A. and Ahmed, K. (1980) Nature, 287, 171-172.
21. Robertson, R.P. and Chen, M. (1977) J. Clin. Invest. 60, 747-753.
22. McClain, D.A. and Olefsky, J.M. (1988) Diabetes, 37, 806-815.
23. Sonne, O. (1988) J. Biochem. 103, 348-353.