Human plasma fractions inhibit the action of insulin on adipocytes and somatomedin on cartilage

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Summary. Since human immunoglobulins exert an insulin-like stimulatory effect on adipocyte lipogenesis at concentrations markedly lower than those found in vivo, and since human serum or plasma are only mildly stimulatory, we predicted that human serum probably contains an inhibitor of adipocyte lipogenesis. Supernatant preparations, obtained from the precipitation of immunoglobulins from plasma in 2.5 mol/l ammonium sulphate, were extensively dialysed and tested for their activity on bioassay systems commonly used for measuring insulin. The supernatants produced a marked inhibition of basal and insulin- or IgG-stimulated lipogenesis and glucose oxidation by adipocytes at protein concentrations of 10 mg/l. The supernatants were further purified through ul-

trafiltration to demonstrate two main inhibitory fractions, 10 to 30 K and 30 to 50 K, which again produced marked inhibition of basal and insulin- or IgG-stimulated adipocyte lipogenesis and glucose oxidation. These fractions were then tested for basal and serum somatomedin-stimulated ^{35}S sulphate uptake by porcine cartilage: both basal and serum somatomedin-stimulated ^{35}S uptake were significantly inhibited ($p < 0.01$). Therefore, normal human serum contains at least two peptides which are markedly inhibitory to glucose metabolism and insulin action on adipocytes and ^{35}S transport and somatomedin action on cartilage.

Key words: Insulin inhibitors, somatomedin inhibitors.

We have recently demonstrated that human immunoglobulins IgG [1] and IgM [2] exert an insulin-like stimulatory effect upon lipogenesis and glucose oxidation by rat and human [3] adipocytes. This effect is mediated through the Fc moiety [3, 4] of the IgG molecule, and is not neutralised by anti-insulin antisera. This effect of Ig must therefore contribute to the non-suppressible insulin-like activity of serum/plasma. In fact, we have observed that serum non-suppressible insulin-like activity attributable to a high molecular weight fraction (NSILP) may be antigenically similar to IgG [5]. Since this potent stimulatory effect of immunoglobulins is exerted at very low concentrations when compared with those present in plasma, and since plasma itself has only a marginal stimulatory effect upon adipocyte metabolism, we postulated the presence in plasma of an inhibitor of immunoglobulin stimulated adipocyte lipogenesis [1, 3]. Our preliminary observations did, indeed, reveal the presence of such an inhibitor [1]. We have now expanded our investigations to attempt to define the nature of this inhibitor. The experiments described in this paper show that there are probably two inhibitors in human plasma which are active not only against the stimulatory effect of insulin and immunoglobulins on adipocytes, but also on that of serum somatomedin on ^{35}S uptake by cartilage.

Materials and methods

Analytical grade sodium chloride, sodium hydroxide, sodium hydrogen carbonate, potassium chloride, calcium chloride, sodium dihy-

drogen phosphate, glucose, triton X-100 (scintillation grade), toluene, hyamine-10x hydroxide (scintillation grade), methanol and sulphuric acid were obtained from British Drug House (BDH, Enfield, UK). D-[3- ^3H] glucose and D-[U- ^{14}C] glucose were obtained from Amersham International (Amersham, UK). Bovine serum albumin fraction V was obtained from Armour Pharmaceuticals (Eastbourne, UK). Collagenase from clostridium histolyticum (specific activity 131 U/mg) was obtained from Millipore Corporation (Bedford, NJ, USA). Epididymal fat pads were obtained from caesarian-delivered male Sprague-Dawley rats, weighing 100–180 g and kept on an ad libitum diet (Grain Harvesters Brand 41B). Amicon ultrafiltration membranes were obtained from Amicon Limited (Stonehouse, Glous., UK).

Costal cartilage was removed from the sternal ends of the lowest ribs of freshly killed pigs in an abattoir and transported in isotonic saline (4°C) to the laboratory. Discs were cut from the cartilage and used in the somatomedin bioassay the same afternoon.

Preparation of the inhibitory fraction from serum

Supernatants were obtained from normal human serum by precipitation of serum globulins in 2.5 mol/l ammonium sulphate followed by 10-min centrifugation at 1500 g. The supernatants were dialysed extensively for 72 h against water. The protein content of dialysed supernatants was then measured by the method of Lowry et al. [6]. These fractions were assayed for their inhibitory activity on adipocyte lipogenesis and [^{14}C] glucose oxidation to CO_2 as described below.

Further fractionation of these supernatant preparations was carried out through sequential filtration on ultrafiltration membranes (Amicon) with nominal molecular weight cut-offs: – 1, 5, 10, 30, 50, 100, and 500 K.

Each fraction thus obtained was assayed for its action on adipocytes and ^{35}S uptake by cartilage. Initial preparatory experiments were carried out at room temperature, but later it was found that there was some loss of biological activity of these inhibitor fractions at room temperature. Further preparatory work was carried out at 4°C.

The biological activity of these supernatant preparations was tested in the following biological systems: (1) adipocyte lipogenesis; (2)

Table 1. The effect of serum inhibitor on rat adipocyte lipogenesis in the presence of either insulin or IgG

Insulin concentration mU/l	Insulin alone	Insulin + inhibitor 10 mg/l	IgG concentration mg/l	IgG alone	IgG + inhibitor 10 mg/l
0	100	75 ± 8 ^a	0	100	41 ± 5 ^a
5	215 ± 44	196 ± 7	50	152 ± 8	44 ± 5 ^b
10	500 ± 28	200 ± 8 ^b	100	182 ± 10	75 ± 8 ^b
20	1054 ± 40	360 ± 12 ^b	200	817 ± 13	163 ± 4 ^b

Results are expressed as percentage stimulation over basal ± SEM. Basal counts: 257 cpm (= 100%); non-specific background counts: 87 cpm; $n = 6$. ^a $p < 0.01$ when compared with basals; ^b $p < 0.01$ when compared with the corresponding dose of insulin or IgG

glucose oxidation to CO₂ by adipocytes; and (3) ³⁵S-sulphate uptake by porcine cartilage. In the first two systems, the effect of the inhibitor fraction was tested against the basal and insulin-stimulated activity. In system (3), the effect of the inhibitor was tested against basal and serum somatomedin-stimulated activity.

Studies on rat adipocytes

Rat adipocytes were prepared from epididymal fat pads by collagenase digestion in Krebs-Ringer bicarbonate buffer, pH 7.4, containing 1% albumin [7]. The cells were gently but extensively washed and resuspended in the same buffer. Adipocyte lipogenesis was assayed by the method described by Moody et al. [8] as adapted by us [9]. [U-¹⁴C]-glucose oxidation to [¹⁴C]-CO₂ was measured as described by us previously [10]. All incubations with insulin-inhibitory fractions, with and without insulin, were carried out by incubating the fractions with adipocytes for the entire incubation period of 120 min.

The effect of heat treatment on inhibitor preparations was assessed by incubating these preparations for 20 min at 60 °C and 80 °C respectively in a shaking water bath. These treated preparations were assayed by the rat lipogenesis bioassay.

Porcine cartilage ³⁵S-sulphate uptake

The effect of the supernatants on ³⁵S-sulphate uptake by porcine cartilage was tested in the presence and absence of serum somatomedin activity as described by Spencer and Taylor [11] and as used to test the effect of other agents on this system [12]. All incubations of cartilage with inhibitory fractions were carried out for the entire incubation period of 24 h, as previously described [11].

Statistical analysis and expression of results

Results were analysed using paired Student's t-tests (two-tailed), and are all expressed as % change over basal (100%).

The absolute basal counts per minute are shown with each Table and Figure. A minimum of 4 experiments was carried out for each Table or Figure shown. Each Table or Figure shows the results obtained from one experiment only so that the values shown are directly comparable.

Results

Supernatants obtained following ammonium sulphate precipitation of serum and prolonged dialysis against water caused a marked inhibition of basal and insulin-stimulated lipogenesis. A similar inhibition of [U-¹⁴C]-glucose oxidation to [¹⁴C]-CO₂ was observed. Heat treatment of the supernatant decreased the inhibitory activity observed over basal and insulin-stimulated lipogenesis. This preparation was not tested for its effect on ³⁵S-sulphate uptake by cartilage.

Table 2. Effect of serum inhibitor on the oxidation of [U-¹⁴C]-glucose to [¹⁴C]-CO₂ by rat adipocytes

Test	Percentage stimulation + SEM
Insulin alone	
5 mU/l	153 ± 11
10 mU/l	161 ± 28
20 mU/l	177 ± 5
Serum inhibitor alone	
1 mg/l	55 ± 7 ^a
2 mg/l	19 ± 5 ^a
Serum inhibitor + 10 mU l ⁻¹ insulin	
1 mg/l + 10 mU/l insulin	96 ± 28 ^b
2 mg/l + 10 mU/l insulin	18 ± 4 ^b

^a $p < 0.01$ when compared with controls; ^b $p < 0.001$ when compared with response to 10 mU/l insulin

Results are expressed as % change over basal. Basal counts: 340 cpm (= 100%). Non-specific background counts: 45 cpm. $n = 6$

Table 3. Effect of heat treatment on serum inhibitor activity in basal and insulin-stimulated lipogenesis.

Insulin concentration (mU/l)	Insulin alone	Insulin + serum inhibitor (1 mg/l)	Insulin + serum inhibitor (60 °C)	Insulin + serum inhibitor (80 °C)
0	100	0	91 ± 21	-
5	188 ± 53	108 ± 70	114 ± 71	242 ± 45
10	222 ± 70	102 ± 16	191 ± 19	267 ± 54

Results are expressed as percentage stimulation over basal ± SEM. Basal counts: 80 cpm (= 100%); $n = 5$

Further purification of the inhibitor using ultrafiltration membranes showed that two major molecular fractions, 10–30 K and 30–50 K were markedly inhibitory to adipocyte lipogenesis. Minimal inhibition was also observed with a fraction of <10 K (data not shown). Fractions of >50 K were consistently stimulatory in the adipocyte lipogenesis assay (data not shown).

Each of the fractions inhibitory in the adipocyte assays, 10–30 K and 30–50 K, were also assayed for their action on ³⁵S-sulphate uptake by porcine cartilage. Both fractions inhibited the basal and serum stimulated ³⁵S-uptake by cartilage markedly in a dose-related fashion (Figs. 1, 2).

Incubation of the crude inhibitory fraction at 60 °C resulted in over 75% loss of inhibitory activity whilst that at 80 °C resulted in its total loss.

Table 4. Inhibition of basal and insulin stimulated-lipogenesis in rat adipocytes by serum inhibitor fractions 10–30 kilodaltons (A) and 30–50 kilodaltons (B)

Test	A Percentage stimulation + SEM (basal counts: 270 cpm = 100%) n = 6	B Percentage stimulation + SEM (basal counts: 250 cpm = 100%) n = 6
Insulin		
1.25 mU/l	226 ± 28	117 ± 16
2.50 mU/l	264 ± 37	139 ± 7
5.00 mU/l	358 ± 38	222 ± 19
10.00 mU/l	448 ± 34	323 ± 16
20.00 mU/l	531 ± 27	350 ± 25
Fractions		
0.5 mg/l	88 ± 15	62 ± 16 ^a
1.0 mg/l	72 ± 18 ^a	24 ± 2 ^a
2.0 mg/l	0 ^a	20 ± 4 ^a
3.0 mg/l	0 ^a	1 ± 3 ^a
Fractions + 2.5 mU/l insulin		
0.5 mg/l	193 ± 17 ^b	103 ± 13 ^b
1.0 mg/l	84 ± 15 ^b	69 ± 6 ^b
2.0 mg/l	0	25 ± 5 ^b
3.0 mg/l	0	1 ± 3 ^b

^a $p < 0.01$ when compared with basal; ^b $p < 0.01$ when compared with corresponding dose of insulin

Discussion

The data presented show a marked antagonistic effect of the inhibitor preparations on basal and insulin- or IgG-stimulated lipogenesis. A similar potent inhibitory effect was observed on the oxidation of glucose to CO₂ by adipocyte suspensions. Clearly, therefore, plasma/serum contains potent inhibitors of adipocyte metabolism; these inhibitors may have a modulatory effect upon the metabolic actions of insulin and immunoglobulins in vivo. The two active inhibitory fractions, 10–30 K and 30–50 K also inhibited ³⁵S uptake markedly. This inhibition was evident with and without the addition of the standard human serum which has a stimulatory effect on this preparation due to its intrinsic somatomedin content.

It is noteworthy that an inhibitor of somatomedin action has previously been recognised in sera of rats rendered experimentally diabetic [13]: this inhibitor is a protein which neutralises the biological effect of non-suppressible insulin-like activity-S (NSILA-S). It has been suggested that this protein inhibits plasma NSILA-S by binding to it. The large molecular weight of this NSILA-S binding protein complex prevents the transcapillary passage of NSILA-S and this is thought to protect the putative target tissues from an effect of NSILA-S in vivo. The inhibitory effect of this carrier protein was also observed when assessing the activity of NSILA-S in vitro; this was caused by inhibiting binding of NSILA-S to perfused rat heart. This carrier protein does not, however, inhibit the stimulatory effect of insu-

Fig. 1. The inhibitory effect of plasma fractions 10–30 K (●—●) and 30–50 K (□—□) on ³⁵S uptake by porcine cartilage. Serial concentrations of inhibitor used were derived from a parent solution containing 3 mg/l protein. Dilutions were carried out with Ham's F₁₂ medium. For 10–30 K plasma fraction assays, all values of 6% concentration and above were significantly inhibitory when compared with basal values ($p < 0.01$). For 30–50 K plasma fraction assays all values of 3% and above were significantly inhibitory ($p < 0.01$) when compared with basal values; $n = 5$

Fig. 2. The inhibitory effect of plasma fractions 10–30 K (●—●) and 30–50 K (□—□) on ³⁵S uptake by porcine cartilage stimulated by normal human serum (○—○). Protein concentrations of parent solutions of inhibitor fractions: 3 mg/l. Normal human serum and inhibitory fractions were used in dilutions from 6% to 50% in Ham's F₁₂ medium. Four experiments were performed with 4 separate sets of inhibitory fractions. For 10–30 K, plasma fraction assay values at 6, 12 and 25% concentrations were significantly ($p < 0.05$) inhibitory, and at 50% concentration ($p < 0.01$), inhibition was also achieved when compared with normal human serum. For 30–50 K plasma fraction assays, the 6% concentration was significantly inhibitory ($p < 0.05$) and the 12, 25 and 50% concentrations were also significantly inhibitory ($p < 0.01$) when compared with normal human serum; $n = 5$

lin on myocardial glucose uptake, and exerted no effect on the binding of insulin to perfused rat heart.

In contrast to these observations, our inhibitor preparations were equally effective in inhibiting: (1) basal

and insulin- and IgG-stimulated adipocyte metabolism and (2) basal and serum somatomedin-stimulated ^{35}S sulphate uptake by cartilage. Therefore, it is not likely that these inhibitory fractions are specific binding proteins. No comment, however, is possible regarding the association/binding of these inhibitors to any of these stimulators in circulation. Ultrafiltration experiments show that either there are two molecular species (10–30 K and 30–50 K) of inhibitor. Whereas the two fractions are equipotent on the rat adipocyte lipogenesis assay, the higher molecular weight fraction (30–50 K) is more potent in the cartilage bioassay. This difference in inhibitory potency is evident against both basal and human serum-stimulated ^{35}S uptake by cartilage (Figs. 1, 2). Phillips and co-workers [14] have recently isolated three molecular fractions from sera of diabetic rats which are inhibitory to ^{35}S sulphate uptake by cartilage under basal conditions and following somatomedin stimulation. Whereas one of our inhibitors may be related to their 24 K inhibitor, their other two inhibitors of molecular weights of 250 K and 940 K are not likely to be related to the two main adipocyte/cartilage inhibitors described by us.

The inhibitors described by us inhibit both basal and insulin/IgG-stimulated adipocyte metabolism. Since insulin and IgG stimulate adipocyte metabolism through different receptors, our inhibitory fractions interfere with insulin/IgG effects on adipocytes at a post-receptor level. Furthermore, these inhibitory fractions have profound effects on two different target organs, the adipose tissue and the cartilage. This suggests that the inhibitors may have a general toxic effect, or that they affect a key pathway involved in the mediation of hormonal effects on various tissues: more definite comments on the mechanism of action are not possible at the present stage. Our preparatory techniques indicate that these inhibitors are not fatty acids, since they were not dialysed out even after 72–96 h of continuous dialysis against water. The inhibitors appear to be heat labile proteins which lose over 75% of their biological activity at 60 °C and all of it at 80 °C. These inhibitors are not related to synalbumin antagonist of insulin described by Vallance-Owen [15, 16], since (1) they are not associated with albumin on ultrafiltration (molecular weight of inhibitors is 10–50 K, whereas that of albumin is > 70 K); (2) synalbumin antagonist has no effect on adipose tissue; and (3) synalbumin antagonist is found only in sera of diabetic patients.

The biological significance of these inhibitors is not clear at present and requires further elucidation. Whether the deficiency of such inhibitors may result in hypersensitivity or hyper-responsiveness to insulin *in vivo*, or that their excess would result in insulin resistance, is an area which requires critical evaluation. Similarly, these inhibitors may modulate sensitivity to somatomedins and thus affect growth processes. Meuli et al. [13] have suggested that their carrier protein may be

responsible for 'protecting tissues' from uncontrolled activity of NSILA; our inhibitor may be responsible for a similar effect.

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Received: 29 November 1985
and in revised form: 3 April 1986

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