Photoaffinity labelling of hepatic plasma membranes suggests two classes of hepatic insulin receptor

F.J. Haynes and C.C. Yip

Department of Physiology and the Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario M5G 1L6, Canada

Summary. Photoaffinity labelling of hepatic insulin receptors revealed specifically-labelled bands of 130, 90 and 40 kDa. Endogenous protease activity in hepatic plasma membranes, as well as contaminating proteases present in preparations of clostridial collagenase, degraded some of the 130-kDa insulin-binding subunit to a 115-kDa form. However, a large proportion of the 130-kDa subunits were resistant to degradation, suggesting the presence of two classes of insulin receptor in hepatic plasma membranes. In one class the 130-kDa subunit was sensitive to proteolysis, while in the other it was not. In

contrast, the 130-kDa receptor subunits of adipose tissue were all resistant to such degradation. Scatchard analysis of control and collagenase-treated plasma membranes demonstrated that conversion of the 130-kDa subunit to a 115-kDa form did not affect the insulin-binding characteristics of the receptor. It was also apparent that insulin binds to a single class of high-affinity sites in hepatic plasma membranes.

Key words: Insulin receptor, α -subunit, insulin binding.

It is now well-established that the insulin receptor of several tissues, including liver, fat and human placenta, contains disulphide-linked glycoprotein subunits with respective molecular weights of 125–135 and 90–95 kDa [1–5]. The 125–135-kDa subunit is considered to be responsible for insulin binding [1–5] and the 90–95-kDa subunit has been characterised as a protein kinase [6]. A specifically-labelled band of 40–45 kDa has also been detected by several laboratories [1–4]. However, disagreement regarding the significance of this component [1, 4, 7] has resulted in two different models of the insulin receptor [1, 3, 4, 8]. In one model the insulin receptor is suggested to exist as a heterodimer of $(\alpha\beta)_2$ [4]. In the other model the insulin receptor is suggested to contain components of 85 kDa and 40 kDa as well as the α and β subunits [1, 8]. In addition, a specifically-labelled band of 120–125 kDa has been observed in the insulin receptor of hepatic tissue. Fehlmann et al. [9, 10] suggest that this is a degradation product of the 125–135-kDa subunit, produced by endogenous protease activity [9] and by preparations of clostridial collagenase [10]. If so, this indicates that the 125–135-kDa subunit present in hepatocytes and hepatic plasma membranes exists in a partially-degraded state. Alternatively, others have suggested that hepatic tissue contains more than one type of insulin receptor [11–14]. Thus, Donner and Yonkers

[14] concluded that the 120–125-kDa subunit represents the high-affinity binding site for insulin. The nature and role of the 120–125-kDa subunit in insulin binding therefore require further study.

We have used the technique of photoaffinity labelling to examine the relationship between the 125–135-kDa subunit and the 120–125-kDa band. The binding of insulin as influenced by the relative abundance of these two forms of subunit was analysed by Scatchard analysis.

Materials and methods

The preparation and characterization of the photoreactive derivative of insulin, N^ε-B²⁹-monoazidobenzoyl insulin (B29-MABI) used in this study have been described previously [15]. The B29-MABI photoprobe was iodinated using chloramine T [15] to a specific activity of 150 μ Ci/nmol. Bovine insulin was a gift from Connaught Laboratories Ltd (Canada). Type 1 clostridial collagenase (130 U/mg) was purchased from Worthington Diagnostic Systems Inc. (Freehold, New Jersey). Benzamidine was purchased from Aldrich Chemicals Co. Inc (Milwaukee, WI). Staphylococcus aureus V8 protease (505 U/mg) was purchased from Miles Scientific (Naperville, USA). Trypan Blue and all other enzymes and inhibitors were obtained from Sigma Chemicals Co. Inc. Leibovitz medium (L-15), Eagle's Minimum Essential Medium (Eagle's medium), penicillin, streptomycin, fetal calf serum and fungizone were purchased from GIBCO (Canada). Mono-

Fig. 1. Radioautogram showing specific labelling of subunits in the hepatocyte insulin receptor. Freshly-isolated hepatocytes were obtained by digestion with clostridial collagenase and then photolabelled and analysed by electrophoresis as described in the text. Lane 1 was obtained from cells incubated with photoprobe in the presence of excess insulin. Dots indicate the position of protein standards used to calculate apparent molecular weights. The specifically labeled bands of 130 kDa (●), 115 kDa (○) and 90 kDa (■) are indicated

iodinated A 14 insulin and α -amino isobutyric acid were obtained from Amersham (Canada). Chemicals for polyacrylamide gel electrophoresis were obtained from Bio-Rad Laboratories (Richmond, California). All other chemicals were of reagent grade.

Preparation of Hepatocytes and Plasma Membranes

Hepatocytes were isolated from male Wistar rats (Charles River, 225–275 g) by perfusion with collagenase (500 mg/l, 15 min, 37 °C) according to the method of Berry and Friend [16], except that heparin (1.5 IU/ml) was added to the preperfusion buffer. The exclusion of Trypan Blue by freshly-isolated cells was over 94%. The insulin stimulation of amino acid transport in the freshly isolated hepatocytes was measured by the method of Fehlmann et al. [17]. Insulin at 100 nmol/l was found to induce a 2-fold increase in the uptake of α -aminoisobutyric acid. Insulin at 85 nmol/l stimulated 2.5-fold the synthesis of glycogen in cultured hepatocytes as measured by the method of Nyfler et al. [18].

A crude plasma membrane fraction was prepared from rat liver by homogenizing 1 g of minced tissue in 5 ml Eagle's medium. Aliquots of 1.5 ml of homogenate were then centrifuged for 10 s at 6500 g in an Eppendorf microfuge. The supernatant was then centrifuged for 4 min at 6500 g to yield a pellet which was resuspended in 1 ml Eagle's medium by vortexing for 30 s and then centrifuged for 4 min at 6500 g. This crude pellet was then resuspended in Eagle's medium or binding buffer and used without further manipulation. Plasma membrane fractions from freshly-isolated hepatocytes were prepared in a similar fashion. Plasma membranes from rat adipocytes were prepared as previously described [19].

Photoaffinity labeling and Scatchard analysis

Cells (1×10^9 cells/l) or plasma membrane fraction (100–300 mg protein/l) were incubated for 60 min at 18 °C with the photoreactive insulin

(10–50 nmol/l) in 1 ml of Eagle's medium containing bacitracin (0.16 g/l) with or without an excess of insulin (10 μ mol/l). Photolysis was then carried out for 20 s using a focussed light source from a 100-W high-pressure mercury lamp [1]. The photolabelled samples were washed in Eagle's medium and immediately solubilized in 2% sodium dodecyl sulphate containing dithiothreitol (100 mmol/l). The binding of insulin with hepatic plasma membranes (100–200 μ g protein/0.2 ml) was carried out at pH 7.4 for 20 h at 4 °C in 0.2 ml Tris-HCl (50 mmol/l) containing 0.4 ng/ml monoiodinated A14 125 I-insulin (0.4 μ g/l), bovine serum albumin 0.1% and bacitracin 1.0 mmol/l. About 2% of the 125 I-insulin was degraded during binding as estimated by trichloroacetic acid precipitation. Binding data were analysed by Scatchard plot using the "Ligand" programme [20]. Electrophoresis in 5–10% gradient polyacrylamide slab gels, radioautography and the calculation of apparent molecular weights were carried out as previously described [1].

Results

Complete reduction of the photolabelled insulin receptor of intact hepatocytes revealed specifically-labeled subunits of 130 kDa and 90 kDa and a 115-kDa band (Fig. 1). A 40-kDa band was not clearly labelled in this particular incubation but was identified more clearly in others (Fig. 2a and b). The rôle of endogenous proteolytic activity and clostridial collagenase in the appearance of the 115-kDa band was examined in freshly-prepared hepatic plasma membranes. The 115-kDa band was barely detectable in hepatic plasma membranes kept on ice prior to photolabeling (Fig. 2a, Lane 2), but was increased if the plasma membranes were incubated at 37 °C for 15 min (Fig. 2a, Lane 4). Treatment with clostridial collagenase further increased the appearance of this band (Fig. 2a, Lane 6). This treatment did not significantly affect the labelling of the 90-kDa and 40-kDa components (Fig. 2a and b). When isolated hepatocytes were cultured for 18 h or more, photolabelling did not reveal the 115-kDa band (Fig. 2b, Lane 2). However, treatment of these cells with the preparation of clostridial collagenase used for hepatocyte isolation induced the appearance of the 115-kDa band (Fig. 2b, Lane 3). In contrast to this effect on hepatocytes, earlier reports have shown that the isolation of adipocytes using collagenase did not induce the labelling of a 115-kDa band [4, 8]. In this study we found further that treatment of adipocyte plasma membranes with collagenase similarly failed to produce a labelled 115-kDa band despite the presence of a readily-labelled 130-kDa subunit (Fig. 2c). There was no decrease in the overall photolabelling of the hepatic or adipocyte insulin receptor after treatment of the membranes with collagenase at the concentrations used in this study.

To determine the extent to which treatment with clostridial collagenase could increase the appearance of the 115-kDa band we incubated freshly-prepared hepatic plasma membranes with collagenase (50 or 500 mg/l) for increasing periods of time. After photolabelling and gel electrophoresis the labelled bands of 130, 115, 90 and 40 kDa were excised for the determination of radioactivity. The 130-kDa subunit and 115-kDa

Fig. 2A-C. Radioautograms showing the effect of endogenous protease activity and treatment with clostridial collagenase on the appearance of the 115-kDa band in the insulin receptor of hepatic and adipose tissue. **a** Freshly-prepared crude hepatic plasma membranes were photolabelled as described in the text. Prior to photolabelling the plasma membranes were either kept on ice (lanes 1-2), incubated for 15 min at 37°C (lanes 3-4), or treated with collagenase (500 mg/l) for 15 min at 37°C (lanes 5-6). The preparation was then centrifuged for 4 min at 6500 g and the pellet resuspended in fresh Eagle's medium by vortexing for 30 s. This procedure was repeated twice. **b** Freshly-isolated hepatocytes were cultured for 18 h and then photolabelled (Lanes 1 and 2). The 90-kDa band in Lane 2 was partially obscured by a contaminating radioactive spot. Lane 3 was obtained from cultured hepatocytes treated with collagenase as above and washed prior to photolabelling. The viability of the control or collagenase-treated cells was over 94% as assessed by the exclusion of Trypan Blue. **c** Adipocyte plasma membranes were prepared as outlined in the text and then photolabelled (Lanes 1 and 2). The faint band of 100 kDa in Lane 2 was non-specific since it was also present in Lane 1. Lane 3 was obtained from adipocyte plasma membranes treated with collagenase as above prior to photolabelling. After photolabelling, samples were analysed by gel electrophoresis as described in the text. Lanes marked INS (+) were obtained from samples incubated with photoprobe in the presence of excess insulin. Dots mark the position of protein standards used to calculate apparent molecular weights. The specifically-labeled bands of 130 kDa (●), 115 kDa (○), 90 kDa (■) and 40 kDa (□) are indicated

band together accounted for more than 85% of the total radioactivity specifically associated with the bands. In control samples kept on ice before photolabelling the ratio of radioactivity in the 115-kDa band to that in the 130-kDa band was 5:95. Incubation of the plasma membrane for 15-40 min at 37°C without collagenase increased this ratio to 21:79. Treatment with collagenase 50 mg/l for 40 min, or with 500 mg/l for 15-40 min, changed this ratio maximally to 36:64 (Fig. 3). To investigate the possibility that this maximal limit resulted from inactivation of some component(s) of the clostridial collagenase during the incubation, experiments were carried out in which, after incubation for 20 min with up to 300 mg collagenase per litre, a second aliquot of enzyme preparation (up to 300 mg/l) was

added and the incubation continued for a further 20 min. Under these conditions the plasma membranes was exposed to up to 600 mg collagenase per litre for 40 min at 37°C. Once again the ratio of radioactivity specifically associated with the 115-kDa band and 130-kDa subunit remained unchanged at 36:64%.

We found that both the endogenous generation of the 115-kDa band and the effect of treatment with clostridial collagenase were Ca^{++} dependent, inhibited completely by benzamidine (10 mmol/l) and by 3 molar equivalents of α_1 antitrypsin [21], but not by soybean trypsin inhibitor (SBTI) nor by α_2 macroglobulin, a specific inhibitor of collagenolytic enzymes [22]. The activity in the collagenase preparation responsible for the increased detection of the 115-kDa band was heat-labile

Fig. 3. The effect of collagenase concentration and duration of treatment on the limited increase in the appearance of the 115-kDa band detected in hepatic plasma membranes. Before photolabelling, crude hepatic plasma membranes were incubated with collagenase (0, 50 or 500 mg/l) for the various periods of time at 37°C. After photolabelling, the membranes were analysed by gel electrophoresis as described in the text. The specifically-labelled bands were then excised for determination of radioactivity. The specific radioactivity associated with the 130-kDa subunit and the 115-kDa band was then expressed as a percentage of the total radioactivity of both bands. Except where (*n*) is indicated, the data represent the mean of three experiments. One standard deviation is indicated by the vertical bar. * indicates a significant difference from samples incubated at 37°C without collagenase ($p > 0.05$; one-way analysis of variance). 15/40 indicates that similar results were obtained after 15 or 40 min of incubation

and non-dialysable. These findings demonstrated that some contaminating components (probably heat-labile proteases) in the enzyme preparation, and not the collagenolytic enzymes themselves, were responsible for inducing the increased appearance of the labelled 115-kDa band.

It was possible that the increased electrophoretic mobility of hepatic 115-kDa band was due to the deglycosylation of some of the 130-kDa subunits. However, we found that treatment of the labelled receptor with neuraminidase increased the electrophoretic mobility of both bands to the same extent. This finding indicates that both were glycoproteins containing sialic acid. It also suggests that the 115-kDa glycoprotein band in liver was distinct from the non-glycosylated 115-kDa subunit detected in insulin receptors present in brain tissue [23, 24].

Fig. 4. Radioautogram showing the result of in-gel digestion of the labelled 130-kDa subunit and 115-kDa band obtained from hepatic insulin receptor. Freshly-prepared hepatic plasma membranes were treated with collagenase (500 mg/l, 15 min, 37°C) and then photolabelled and analysed by gel electrophoresis as described in the text. The labelled 130-kDa and 115-kDa bands were then excised and inserted into a 10–15% gradient slab gel for in-gel proteolysis with chymotrypsin (CT), elastase (E'ASE) and staphylococcus aureus V8 protease (V8). Lanes 1 and 2 were obtained from undigested 130-kDa and 115-kDa bands respectively. Lanes 3–8 were obtained following in-gel digestion of the 130-kDa band (O) and 115-kDa band (●) as indicated

The possibility that the hepatic 115-kDa band was produced by proteolytic “nicking” of the 130-kDa subunit was examined indirectly by the in-gel digestion method of Cleveland [25]. Utilizing three different enzymes, a high degree of similarity was observed between the peptide bands generated from the 130-kDa subunit and from the 115-kDa band (Fig. 4).

The effect of the relative abundance of the 115-kDa band and the binding of insulin to the hepatic insulin receptor was examined in hepatic plasma membranes prepared from freshly isolated hepatocytes and hepatic membranes prepared from freshly-homogenized liver. In either case the plasma membranes were prepared using ice-cold Eagle's medium and kept on ice. The insulin receptors in these two preparations displayed marked differences in their proportions of photolabelled 130-kDa subunit and 115-kDa band (Fig. 5a). In

Fig. 5A and B. The effect of partially degrading the hepatic 130-kDa subunit to the 115-kDa form on the binding of insulin to the insulin receptor. **a** Radioautogram showing the different amounts of 130-kDa subunit and 115-kDa band present in plasma membranes obtained from freshly-homogenised liver (lanes 1–4), and from freshly isolated hepatocytes (lanes 5–8). In each case samples were incubated with the photoprobe for 1 h at 18°C (Lanes 1, 2, 5 and 6) and for 24 h at 4°C (Lanes 3, 4, 7 and 8) to determine the stability of 130-kDa subunit during the binding study. Following photolabelling, samples were analysed by gel electrophoresis as described in the text. Lanes marked COLD IN (+) were obtained from samples incubated in the presence of excess insulin. Dots show the location of protein standards. The specifically-labelled bands of 130 kDa (●), 115 kDa (○) and 90 kDa (□) are shown. **b** Scatchard analysis of insulin binding to crude plasma membranes obtained from freshly-homogenised liver and freshly-isolated hepatocytes. Plasma membranes from each preparation were incubated with A14 moniodoinsulin in Tris-BSA buffer, pH 7.4 as described in the text. Top, the displacement curve obtained from both preparations assayed in duplicate is shown and is representative of the results of three separate experiments. Bottom, the Scatchard plots derived from these data. ---○--- Hepatic plasma membranes; —■— hepatocyte plasma membranes

the plasma membranes prepared from hepatocytes the labelled 130-kDa subunit and 115-kDa band were in a ratio of 64:36, while in that prepared from liver the ratio was 95:5. In each preparation the relative amounts of the labelled 130-kDa subunit and 115-kDa band remained the same when photolabelling was carried out for either 60 min at 18°C or 24 h at 4°C (Fig. 5a). In both preparations Scatchard analysis demonstrated a single class of high-affinity binding sites (Fig. 5b). In plasma membranes from hepatocytes the binding association (K_a) was $(1.0 \pm 0.1) \times 10^9 \text{ mole}^{-1}$, while in that from homogenized liver the K_a was $1.1 \pm 0.1 \times 10^9 \text{ mole}^{-1}$ (mean $\pm$ SEM). In the plasma membranes from hepatocytes the binding capacity (R_0) was $0.29 \pm 0.01 \text{ nmol/g protein}$ and in the preparation from homogenized liver the R_0 was $0.26 \pm 0.02 \text{ nmol/g protein}$. Thus, the insulin binding properties of these two plasma membrane preparations were identical.

Discussion

In this study, in addition to the expected receptor components of 130 kDa, 90 kDa and 40 kDa [1–8], a band of 115 kDa was detected in the insulin receptor of both intact hepatocytes and hepatic plasma membranes (Fig. 1 and 2). Respectively, the 115-kDa band and the 130-kDa subunit detected here are equivalent to the 120–125-kDa band and 125–135-kDa subunit reported by others [9, 10, 14]. Analysis of the peptides from the proteolytic digestion of these bands (Fig. 4) suggests that the 115-kDa band may be derived from the 130-kDa subunit. This supports the suggestion by others that the appearance of the 115-kDa band is the result of proteolytic “nicking” of the 130-kDa subunit [9, 10]. Additional evidence for this process is provided by the demonstration that labelled 130-kDa subunit can be converted to a 115-kDa form in hepatic plasma mem-

branes [14]. Our results also confirm the suggestion by others that treatment of hepatic plasma membranes with clostridial collagenase [10], and endogenous proteolytic activity in hepatic plasma membranes [9] can degrade the 130-kDa subunit to a 115-kDa form. We demonstrated further that collagenase treatment of intact hepatocytes also degrades the 130-kDa subunit to a 115-kDa form. Our results also showed that this effect of clostridial collagenase was due to contaminating protease activity in the enzyme preparation rather than the collagenolytic enzymes themselves.

However, the effect of collagenase preparation on the hepatic insulin receptor had a limited, maximal extent, indicated by the inability of exhaustive treatment with the enzyme preparation to degrade all of the 130-kDa subunit. Thus, over 60% of the 130-kDa subunits were resistant to degradation to a 115-kDa form despite a 10-fold increase in the amount of collagenase used (Fig. 3). This was not due to the inactivation of the contaminating proteolytic activity, since identical results were obtained when aliquots of fresh collagenase were added for a second incubation. One possible explanation for this observation is that hepatic plasma membranes contain two classes of insulin receptor, the 130-kDa subunit being susceptible to proteolytic nicking in one but not in the other. It is noteworthy that insulin receptors in hepatic plasma membranes could also be separated into two populations based on their hydrophobic/hydrophilic properties [11] or on their binding of insulin analogues [12, 13]. However, the relationship between our findings and these other observations is uncertain.

In contrast, adipocyte plasma membranes seem to contain only one class of insulin receptor. Thus, photolabelling of adipocytes isolated using the same collagenase preparation and photoprobe failed to reveal a 115-kDa band [8]. In addition, the treatment of adipocyte plasma membranes with this preparation was unable to generate the appearance of the 115-kDa band (Fig. 2c). Similarly, other investigators have observed that the 130-kDa subunit in insulin receptor purified from IM-9 lymphocytes or placental membranes was totally insensitive to degradation by preparations of clostridial collagenase [26]. Consequently, in contrast to the insulin receptor of hepatic tissue, that of adipose tissue, placenta and IM-9 lymphocytes appears to be of a class whose 130-kDa subunit is insensitive to the proteolytic activity present in preparations of collagenase. These possible tissue differences in the proteolytic sensitivity of the 130-kDa subunit are in keeping with the findings by other investigators that there are structural differences between the insulin receptors in hepatic, adipose and placental tissue [27–30]. The different proteolytic sensitivities may reflect alterations in the orientation of the receptor within the plasma membranes or in the organisation of subunits within the receptor.

The existence of two different receptor classes may have a role in the regulation and function of the hepatic

insulin receptor. It has been reported by others that a limited proportion of the 130-kDa subunit in the insulin receptors of both adipose [31, 32] and hepatic [9, 10, 14] tissue may undergo conversion in the plasma membranes to a 115-kDa form. In adipocytes this process is associated with internalisation [31, 32]. Studies of receptor recycling in hepatocytes have also demonstrated that while the 130-kDa subunit was recycled back to the plasma membranes, the 115-kDa form was not [10]. Therefore, the two classes of the insulin receptor may enable intracellular organelles to sort out those receptors destined for degradation from those destined for recycling [33].

In this study, we have observed that insulin binding by hepatic insulin receptors was not affected by the relative proportion of the 130-kDa and 115-kDa forms of the 130-kDa subunit (Fig. 5). This suggests that conversion of the 130-kDa subunit to the 115-kDa form involves a region of the subunit not involved in insulin binding. Thus, these two forms of the insulin binding subunit do not represent high- and low-affinity conformations of the insulin receptor as has been previously suggested [14]. This earlier study was based upon the increased appearance of the 115-kDa form of the 130-kDa subunit during the dissociation of insulin from the receptor, rather than upon analysis of binding characteristics. In view of our present findings this increased appearance of the 115-kDa form was likely to be due to the endogenous proteolytic degradation of the 130-kDa subunit during the period of dissociation. Our results also support the findings by others that there is a single class of high-affinity binding sites on the insulin receptor [34–38].

In summary, photoaffinity labelling of insulin receptors present in hepatic plasma membranes demonstrated that endogenous protease activity and proteolytic activity contaminating commercial preparations of clostridial collagenase were able to degrade the 130-kDa subunit of the insulin receptor to a 115-kDa form. However, such degradation was restricted to only a limited proportion of the insulin receptors, raising the possibility of the presence of two classes of insulin receptor in hepatic plasma membranes. One class of receptors is characterised by a 130-kDa subunit which can be proteolytically converted to a 115-kDa form. The other class is characterised by receptors with a 130-kDa subunit resistant to this effect. The degradation of the 130-kDa subunit did not alter the binding characteristics of the hepatic insulin receptor which exhibited a single class of high affinity sites.

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Dr. C. C. Yip
Banting and Best Department of Medical Research
University of Toronto
112 College Street
Toronto, Ontario, M5G 1L6
Canada