

# Effects of Anti-Insulin Receptor Antibodies (AIRA) on Downregulation and Turnover of Insulin Receptors on Cultured Hepatocytes

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## SUMMARY

We have studied the effects of two polyclonal anti-insulin receptor antibodies (AIRA) on insulin receptor downregulation and turnover in rat hepatocytes in primary culture. Downregulation was determined by measurement of insulin binding after acid washing of cells to remove AIRA. Insulin receptor turnover was estimated by measurement of insulin binding after inhibition of synthesis of functional receptors with tunicamycin (0.5  $\mu\text{g}/\text{ml}$ ). Exposure of hepatocytes to AIRA (both sera were of comparable effectiveness) resulted in progressive, time- and dose-dependent losses of insulin binding (maximal loss was about 55% after 24 h of incubation with AIRA diluted 1:25). Cycloheximide (100  $\mu\text{M}$ ) prevented AIRA-mediated downregulation.

The  $t_{1/2}$  of disappearance of cell surface insulin binding capacity determined with tunicamycin was 8.0 h. Addition of insulin (1000 ng/ml) or AIRA to tunicamycin reduced the  $t_{1/2}$  to 2.6 h (insulin), 2.2 h (patient B10), and 2.0 h (patient 1). These data suggest that (1) AIRA downregulated insulin receptors on cultured hepatocytes by accelerating their rate of disappearance, (2) inhibition of protein synthesis prevented AIRA-mediated downregulation, and (3) downregulation by AIRA of insulin binding may be partially responsible for the desensitization of target cells to some of the insulin-like actions of these autoantibodies. *DIABETES* 1986; 35:28-32.

**P**rolonged elevation of plasma insulin concentration reduces insulin cell surface binding sites.<sup>1</sup> This action has been termed "receptor downregulation" and is believed to be an important protective mechanism against the effects of sustained hyperinsulinemia. AIRA can mimic many insulin actions,<sup>2-7</sup> including downre-

gulation of insulin receptors. This has been shown in IM-9 lymphoblastoid cells and in 3T3-L1 fatty fibroblasts.<sup>8-10</sup> Both cell lines are, however, not classic insulin targets. Moreover, their mechanism of downregulation appears to be atypical. In IM-9 cells, insulin receptors have been shown to be discharged into the outside medium,<sup>11,12</sup> while in 3T3-L1 cells, insulin-mediated receptor downregulation has been difficult to demonstrate.<sup>13,14</sup> We have therefore studied the effects of two polyclonal AIRA on insulin receptor downregulation in rat hepatocytes. In addition, we have explored the mechanism by which AIRA affect downregulation with tunicamycin, an antibiotic that blocks the appearance of new insulin receptors on the cell surface,<sup>15,16</sup> and with cycloheximide, a potent inhibitor of protein synthesis. Hepatocytes, grown in primary culture, were used because they are important target cells for insulin and because they downregulate insulin receptors at physiologic insulin concentrations<sup>17</sup> primarily via internalization.<sup>18,19</sup>

## MATERIALS AND METHODS

**AIRA sera.** AIRA sera were obtained from two patients with acanthosis nigricans and type B insulin resistance (patients 1 and B10). Clinical details of these patients have been published.<sup>20,21</sup> Their IgG concentrations were 1.4 and 1.8 mg/ml of plasma, respectively.<sup>22,23</sup> Since both sera contained high concentrations of insulin, they and the control sera were treated with charcoal to remove insulin.<sup>24</sup> Insulin concentrations after charcoal treatment were  $<5 \mu\text{U}/\text{ml}$  for both sera. Insulin binding antibodies, determined according to Gerbitz and Kemmler,<sup>24</sup> were undetectable in sera from patient 1 or B10 (diluted 1:100).

**Isolation and culture of hepatocytes.** Hepatocytes were isolated by *in situ* collagenase digestion of livers from male, Sprague-Dawley, fed rats (300-350 g). Primary cultures of rat hepatocytes were prepared as described.<sup>20</sup>

**Preincubation with AIRA, insulin, tunicamycin, or cycloheximide.** Hepatocytes in monolayer (about  $2 \times 10^6$  cells/flask) were preincubated with AIRA or normal serum (final dilution 1:25 or 1:100 in minimal essential medium

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(MEM, final volume 2.5 ml) for 0.5, 2, 6, and 24 h at 37°C. After preincubation, the medium was aspirated and the cells washed four times with ice-cold Krebs-Ringer bicarbonate (KRB) buffer.

In the "acid wash" experiments, preincubations were performed as described above, after which the medium was replaced with ice-cold glycine buffer (glycine 50 mM, KCl 5 mM, and NaCl 73 mM, pH 3.5) for 3 min. Thereafter, cells were washed four times with KRB buffer.

In the tunicamycin experiments, hepatocytes (conditions as above) were preincubated either with tunicamycin alone (0.5  $\mu$ g/ml, Sigma Chemical Co., St. Louis, Missouri), or with tunicamycin plus AIRA serum (1:25), or with tunicamycin plus insulin (1000 ng/ml) for 1, 2, 3, 4, 5, and 6 h at 37°C. In the cycloheximide experiments, hepatocytes were preincubated for 24 h at 37°C either with cycloheximide (100  $\mu$ M) alone or with cycloheximide plus patient serum (1:25). Thereafter, cells were acid washed as described above. Least-squares regression analysis was used to determine the half-life of insulin binding capacity.

**Insulin binding studies.** After preincubation and washing (pH 7.4 or 3.5), cells were incubated with  $^{125}$ I-insulin (0.3 ng, sp. act. 100–150 mCi/mg) in KRB buffer containing 1% BSA in the absence and presence of excess unlabeled insulin (10,000 ng/ml) for 1 h at 20°C. The binding in the presence of 10,000 ng/ml of insulin was usually <1.2%, was regarded as nonspecific, and was subtracted from total binding.

## RESULTS

**Effects of AIRA on insulin binding.**  $^{125}$ I-insulin binding decreased slightly, from  $10.0 \pm 0.3\%$  after 0.5 h to  $8.6 \pm 0.6\%$  after 24 h of preincubation with control serum. Preincubation with either of the two AIRA sera (diluted 1:25) reduced insulin binding by about 80% after 0.5 h (from  $10.0 \pm 0.3\%$  to  $2.4 \pm 0.1\%$  and  $1.7 \pm 0.1\%$ , respectively) and by >90% after 2 h (from  $8.9 \pm 0.4\%$  to  $0.9 \pm 0.1\%$  and  $0.5 \pm 0.1\%$ , respectively; Figure 1, upper panel). The rapid decrease in binding could have been the result of AIRA preventing insulin from gaining access to its receptors and/or disappearance of insulin binding sites from the cell surfaces (downregulation). To distinguish between these two possibilities, hepatocytes were incubated for 0.5, 2, 6, and 24 h with AIRA sera. The antibodies were then washed off the cells with an acidic buffer, after which insulin binding was determined. The acid washing per se had no effect on  $^{125}$ I-insulin binding, as binding was the same after neutral or acid washing when hepatocytes were incubated with control serum (Figure 1). Moreover, acid washing was able to remove AIRA completely, as insulin binding could be restored fully after short (0.5 h) incubations with AIRA sera (diluted 1:100) followed by acid washing (data not shown). Longer incubations with AIRA sera, however, revealed a progressive decrease in insulin binding (after acid washing), which fell by about 35% (from  $9.0 \pm 0.1\%$  to  $6.1 \pm 0.4\%$  and  $5.6 \pm 0.4\%$ ) after 6 h and by about 55% (from  $8.5 \pm 0.2\%$  to  $4.2 \pm 0.3\%$  and  $3.8 \pm 0.1\%$ ) after 24 h (Figure 1, lower panel).

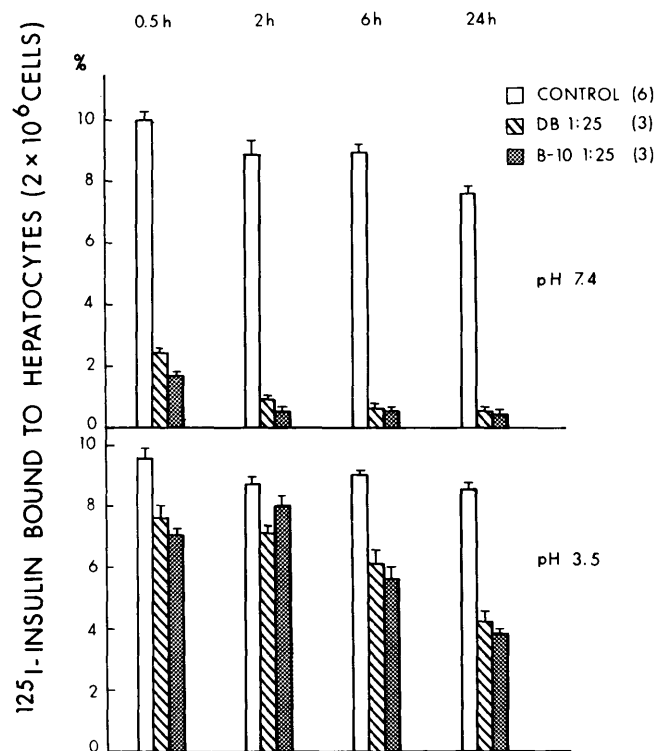
The AIRA-mediated downregulation was dose dependent, as AIRA sera diluted 1:25 were almost twice as effective as AIRA sera diluted 1:100.

To determine whether the observed loss of binding was due to a loss of binding sites or a decrease in receptor affinity,

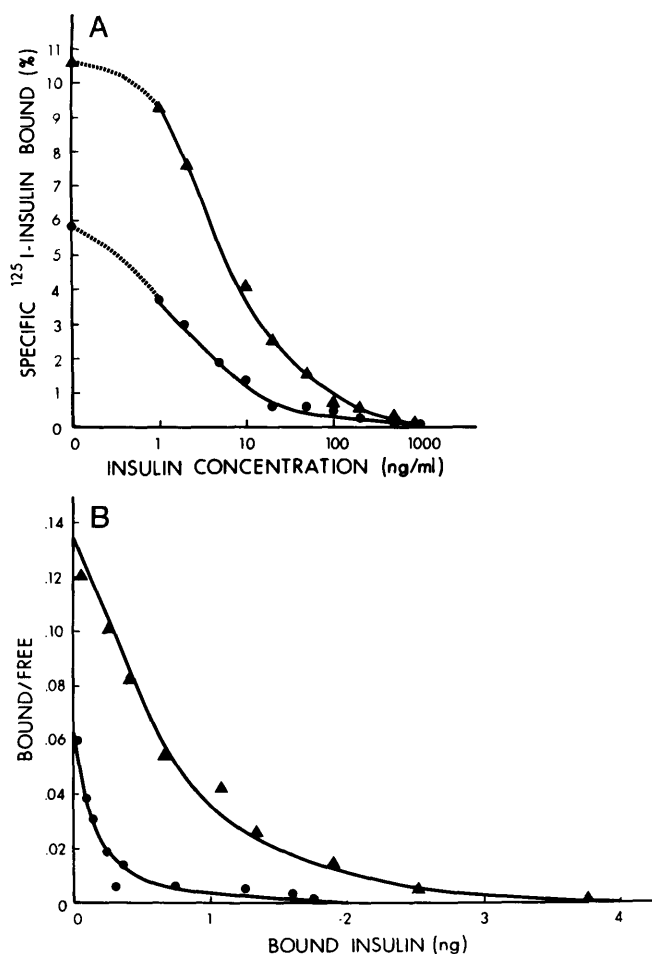
hepatocytes were preincubated for 24 h with serum from patient 1 or with control serum (both diluted 1:25) and then acid washed. Insulin binding was determined without and with unlabeled insulin in concentrations from 1 to 10,000 ng/ml. Scatchard analysis of the data obtained indicated that the decrease in insulin binding was due mainly to a decrease in receptor number (from about  $210 \times 10^3$  to  $101 \times 10^3$  per cell) with little or no change in receptor affinity (Figure 2).

To determine the influence of protein synthesis on AIRA-mediated receptor downregulation, we have used cycloheximide, a potent inhibitor of protein synthesis. After 24 h of incubation with AIRA serum from patient 1 (diluted 1:25), hepatocyte insulin binding decreased from  $10.5 \pm 0.7\%$  to  $5.2 \pm 0.4\%$ . Addition of cycloheximide (100  $\mu$ M) to the incubation medium completely prevented this decrease (insulin binding was  $10.0 \pm 0.5\%$  after 24 h of incubation with patient 1 serum and cycloheximide).

**Effects of AIRA or insulin on receptor turnover (Figure 3).** To determine whether the AIRA-induced decrease in cell surface insulin binding capacity was caused by an increase in the rate of receptor disappearance or a decrease in the rate of receptor appearance, hepatocytes were incubated with tunicamycin, an antibiotic that blocks appearance of new functional receptors on the cell surface.<sup>15,16</sup> When cells were incubated with tunicamycin alone, insulin binding capacity



**FIGURE 1.** Effects of AIRA on insulin binding capacity. Hepatocytes ( $2 \times 10^6$  cells/flask) were incubated with AIRA or control serum (final dilution 1:25) in minimal essential medium (MEM, final volume 2.5 ml) for 0.5, 2, 6, and 24 h at 37°C. After preincubation, the MEM was aspirated and the cells were either washed four times with cold KRB buffer (pH 7.4, upper panel) or the medium was replaced with cold glycine buffer (pH 3.5) for 3 min, after which the cells were washed four times with cold KRB buffer (lower panel). Thereafter, insulin binding was determined. Shown are means  $\pm$  SEM of three experiments with each of the two AIRA sera and six experiments with control serum.



**FIGURE 2.** Effect of AIRA and acid washing on insulin binding. Panel A, cultured rat hepatocytes were preincubated with control serum (●, 1:25) or patient 1 serum (▲, 1:25) for 24 h. Thereafter, cells were washed once with glycine buffer (pH 3.5), then washed four times with KRB buffer (pH 7.4) and incubated for 1 h with  $^{125}\text{I}$ -insulin in the presence of increasing concentrations of cold insulin. Panel B shows a Scatchard plot of the same data. All values are the mean of triplicate determinations.

decreased with a  $t_{1/2}$  of 8 h ( $r = 0.76$ ,  $P < 0.05$ ). Addition of AIRA serum (1:25) to tunicamycin shortened the  $t_{1/2}$  from 8.0 to 2.0 (patient 1,  $r = 0.94$ ,  $P < 0.001$ ) and 2.2 (patient B10,  $r = 0.93$ ,  $P < 0.01$ ). When cells were incubated with tunicamycin and insulin (1000 ng/ml), insulin binding capacity decreased with a  $t_{1/2}$  of 2.6 h ( $r = 0.84$ ,  $P < 0.02$ ). This was not significantly different from the  $t_{1/2}$  obtained with tunicamycin plus AIRA, but it was significantly different from the  $t_{1/2}$  obtained with tunicamycin alone ( $P < 0.01$  by analysis of variance).

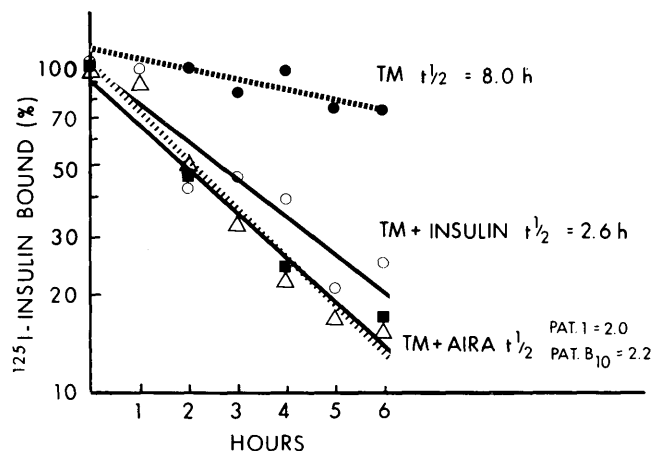
#### DISCUSSION

Both AIRA sera (diluted 1:25) reduced insulin binding to hepatocytes by >90% within 2 h of incubation, confirming earlier reports.<sup>20,21</sup> The inability to bind insulin did not, however, reflect absence of binding sites but was caused primarily by blockade of binding sites by AIRA. To gain information on changes in cell surface binding capacity it was necessary to remove the AIRA. This was accomplished by washing the cells with an acidic buffer, which dissociated the AIRA-insulin receptors complex. That AIRA was removed completely was suggested by the observation that exposure

of hepatocytes to AIRA (diluted 1:100) for 0.5 h reduced insulin binding by about 50%, while the cells regained their capacity to bind insulin normally after acid washing.

Using the acid wash procedure, we found that exposure of hepatocytes to AIRA resulted in a slow but progressive decrease in cell surface insulin binding capacity. The effects of the two AIRA sera were comparable and were time and dose dependent. After 2, 6, and 24 h of incubation, about 15%, 35%, and 55%, respectively, of insulin binding capacity had disappeared (Figure 1). AIRA diluted 1:25 were approximately twice as effective as AIRA diluted 1:100.

To explore the mechanism by which AIRA promoted downregulation, we have used tunicamycin and cycloheximide. Tunicamycin is an antibiotic that inhibits the addition of core oligosaccharides during the synthesis of N-linked glycoproteins.<sup>15</sup> Insulin receptors are glycoproteins,<sup>16,25</sup> and their carbohydrate components have been shown to be important for insulin binding.<sup>25,26</sup> In several cell types, including hepatocytes, tunicamycin has been demonstrated to prevent the formation of functional insulin receptors while not interfering with insulin degradation.<sup>15,16,27,28</sup> When cultured hepatocytes were incubated with tunicamycin (0.5  $\mu\text{g/ml}$ ), insulin binding capacity decreased with a  $t_{1/2}$  of 8 h. This is in agreement with  $t_{1/2}$  values ranging from 7.5 to 15 h reported by others with various cell lines (hepatocytes, IM-9, and 3T3-L1 cells) and different techniques, including heavy isotope labeling of cells during receptor synthesis,<sup>19,27</sup> biosynthetic and surface labeling of cells followed by immunoprecipitation,<sup>8,9</sup> and tunicamycin blockade of receptor synthesis.<sup>16</sup> Addition of insulin (1000 ng/ml) or of serum from patient 1 or B10 (diluted 1:25) to tunicamycin reduced the  $t_{1/2}$  to 2.6, 2.0, and 2.2 h, respectively. These findings suggested that AIRA, similar to insulin,<sup>8,29,30</sup> stimulated receptor downregulation in hepatocytes by accelerating their rate of disappearance. It could be argued that interference of tunicamycin with insulin receptor recycling<sup>31</sup> could have influenced the rate of receptor disappearance. The finding that the disappearance rates for insulin binding sites were the same whether measured with



**FIGURE 3.** Effect of AIRA sera and insulin on half-life of disappearance of insulin binding sites. Hepatocytes ( $2 \times 10^6$  cells/flask) were preincubated for intervals ranging from 1 to 6 h with tunicamycin (0.5  $\mu\text{g/ml}$ , solid circles, broken line), with tunicamycin plus insulin (1000 ng/ml, open circles, solid line), with tunicamycin plus patient 1 serum (diluted 1:25, open triangle, broken line), or with tunicamycin plus patient B10 serum (diluted 1:25, solid squares, solid line). Cells were then acid washed and insulin binding determined.

tunicamycin (this study) or by direct receptor labeling<sup>8,9,19,27,30</sup> indicated, however, that tunicamycin had no effect on receptor recycling.

Cycloheximide at a concentration (100  $\mu$ M) known to inhibit protein synthesis completely abolished downregulation by AIRA. Similar results have been reported by others using IM-9 lymphoblastoid cells, rat hepatoma cells, and fetal hepatocytes.<sup>28,32,33</sup>

It was noteworthy that the effect of AIRA on downregulation persisted for at least 24 h. In contrast, all other acute AIRA-mediated insulin-like activities disappear after much shorter (1–6 h) exposure of target cells to the autoantibodies, a phenomenon that has been termed desensitization.<sup>34</sup> The mechanism for desensitization is not known. It is tempting to speculate that it may be caused, at least in part, by downregulation of insulin binding sites. Although far from proven, this theory is supported by several *in vivo* and *in vitro* observations showing that AIRA-mediated insulin-like actions decreased with diminishing insulin binding capacity. For instance, dilute serum from patient 1 reduced *in vitro* hepatocyte insulin binding capacity to <50% after 24 h of incubation (this study). Undiluted serum and longer incubation may have resulted in even greater loss of binding. As shown previously *in vivo*, her AIRA produced insulin-like actions that were equivalent to about 10% of maximal insulin action.<sup>20</sup> Grunfeld found that prolonged exposure (7 h) of 3T3-L1 cells *in vitro* to a monoclonal or a polyclonal AIRA reduced insulin binding as well as glucose transport and oxidation.<sup>10,35</sup> Others, however, found that downregulation of insulin receptors in cultured rat hepatoma cells<sup>36</sup> or in cultured hepatocytes<sup>37</sup> could not account for complete desensitization and concluded that desensitization was a result of postreceptor events.

Our own studies, as well as other data in the literature, are compatible with the concept that AIRA cause severe insulin resistance by interfering with binding of endogenous or exogenous insulin to its receptors. This action occurs rapidly and can be demonstrated even before desensitization has taken place. Glucose intolerance or diabetes occur only after target cell desensitization has reduced the intrinsic insulin-like activity of the autoantibodies to low levels. According to this concept, insufficient desensitization would result in continuous insulin-like activity and hypoglycemia. Severe hypoglycemia has been reported in several patients with type B severe insulin resistance,<sup>38</sup> but its cause has remained enigmatic.

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