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INSULIN ACTION IN EARLY EMBRYONIC LIFE: ANTI-INSULIN RECEPTOR ANTIBODIES RETARD CHICKEN EMBRYO GROWTH BUT NOT MUSCLE DIFFERENTIATION IN VIVO

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Insulin receptors are present in chicken embryos at day 2 of development and insulin stimulates embryonic growth and differentiation. Most important, anti-insulin antibodies cause either death or developmental retardation in chicken embryos of that age. To determine if the embryo's endogenous insulin acts through its own receptor, we compared the effects of anti-insulin antibodies to the effects of anti-insulin receptor antibodies on growth and differentiation indexes in the chicken embryo. While the anti-insulin antibody caused a dose-dependent decrease in growth parameters like weight, total protein, DNA, RNA, total creatine kinase activity and a marker of differentiation, the creatine kinase-MB, the anti-insulin receptor antibody decreased all parameters except the creatine kinase-MB. Many, but not all, of the effects of insulin in early embryos, thus, are mediated through the insulin receptor.

Insulin appears to have a physiological function in early chicken embryo development. The hormone is found in the egg (1,2) and embryo (2); exogenous insulin accelerates development while anti-insulin antibodies retard growth and differentiation (3); and insulin receptors are present in multiple tissues throughout organogenesis (4,5,6). However, chicken embryos have also insulin-like growth factor I (IGF I) receptors which could mediate some of the effects of insulin, particularly on growth (7). To evaluate if insulin receptors are functional in chicken embryos after 2 days of development (≈20 somites) we have studied the effects of a polyclonal anti-insulin receptor antibody (B-10) upon

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embryonic growth. The parameters measured were embryo weight, total protein, DNA and RNA and the ubiquitous enzyme creatine kinase. In addition, the proportion of creatine kinase-MB isozyme, which at this age starts being expressed in more differentiated tissues, particularly muscle (8), was used as a marker of differentiation. If the embryo's own insulin was acting through insulin receptors the blockade with anti-receptor antibodies was expected to produce similar effects to those obtained when anti-insulin antibodies were applied.

MATERIALS AND METHODS

Chicken embryos,antibodies, and peptides: Fertilized white Leghorn eggs were incubated at 38°C for two days, staged (9) and injected as described previously (3). Anti-insulin antiserum (lot #624, a polyclonal anti-pork insulin antiserum raised in guinea-pig) and control guinea pig serum were affinity chromatographed on a Protein-A column. The eluted anti-insulin IgG, had an insulin binding capacity of 15μg/ml. Anti-insulin receptor antiserum was from a patient (B-10) with extreme insulin resistance and autoantibodies against the insulin receptor. Serum IgG was obtained by chromatography on a Protein-A column and further purified on an insulin-Sepharose affinity column (3) to eliminate a small population of contaminating anti-insulin antibodies. Normal human IgG was used as control for these experiments. Porcine insulin (Elanco Products, Inc.) and human recombinant IGF I (Amgen Biologicals) were dissolved in 0.01N HCl.

Evaluation of embryos: At days 3, 4 and 5 of development, embryos were staged under a stereomicroscope and dead embryos were discarded. Live embryos were dissected, weighed and stored at -70°C until biochemical parameters were measured. Embryos in each group were homogenized in 50 mM Tris-HCl as described (3) and the concentration of protein (10), total DNA and RNA (11) and triglycerides (12) was determined. Creatine kinase activity was measured spectrophotometrically (13) after addition of 0.12 nM diadenosine pentaphosphate. The separation of the isozymes BB and MB was accomplished by FPLC as shown in Figure 1.

RESULTS

The chick embryo culture *in ovo* is a useful system to analyze the receptor-mediated stimulatory effects of insulin and insulin-related peptides in early development (12). In this report we show that chicken embryos treated at day 2 with an anti-insulin receptor antibody were morphologically and biochemically retarded. At day 4 there was macroscopic growth retardation of embryos treated with anti-receptor IgG compared to controls (9% vs. 1%, $p<0.05$). In

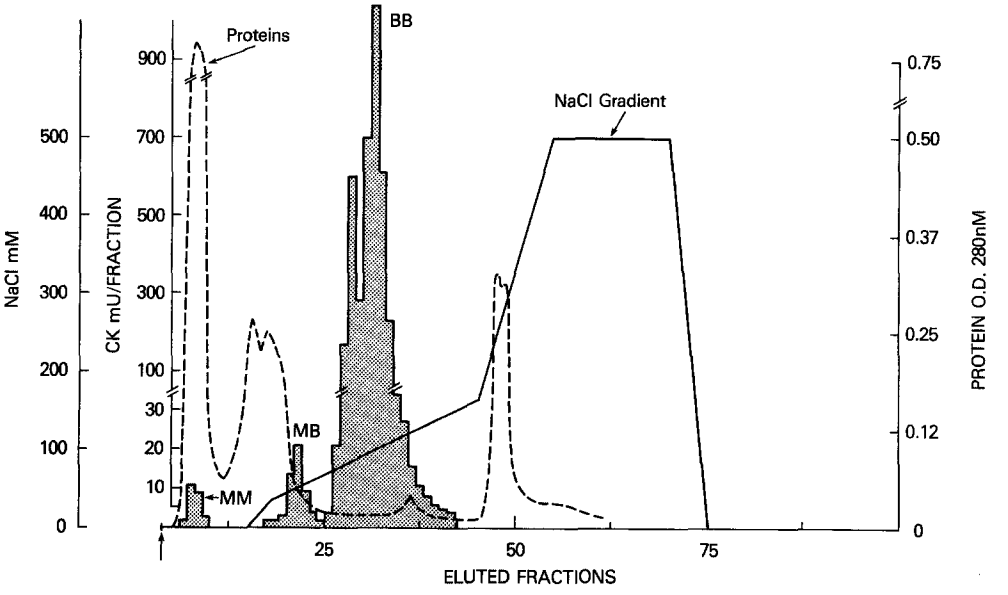

Figure 1. Separation of creatine kinase isozymes by FPLC (Fast Protein Liquid Chromatography) using an anion exchange mono Q HR 5/5 column. Samples of day 4 embryo homogenates were filtered and 80 fractions (0.4 ml/fraction) were eluted with a linear 0-175 mM NaCl gradient (in 50 mM Tris-HCl, pH 7.5). Creatine kinase activity was measured in individual fractions, and the peaks corresponding to the isozyme-MB (elution at 55 mM Na+) and isozyme-BB (elution at 100 mM Na+) were calculated. Total proteins eluted were monitored by UV absorbtion at 280 nm.

addition, a decrease in weight, total protein and total creatine kinase activity occurred at days 3 and 4 of development (Figure 2). By day 5 values tended to return to normal. To evaluate differentiation, we separated the isozymes of creatine kinase by a highly sensitive FPLC system which allowed measurements of the low proportion of MB isozyme (also called "transitional") in the presence of large amounts of BB isozyme (also called "embryonic") (Figure 1). The creatine kinase-MB did not change in day 4 and day 5 embryos after treatment with the anti-insulin receptor IgG, (day 3 was not tested), in contrast with the significant decrease observed with anti-insulin antibodies in our earlier work (3).

Since day 4 was the most sensitive time-point, the effects of the anti-insulin receptor antibody were analyzed further by comparison with the effects of an anti-insulin antibody (Figure 3). All the parameters measured were decreased in a dose-dependent fashion by the

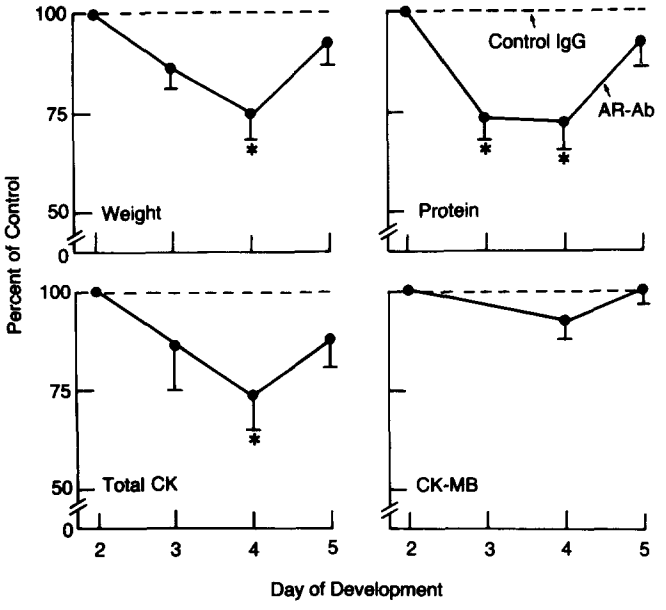

Figure 2. Effects of anti-insulin receptor antibodies on chicken embryos injected at day 2. Groups of 10 embryos received 400 μg of either anti-insulin receptor IgG (AR-Ab) or control human IgG. Live embryos at days 3, 4 and 5 were weighed, homogenized and their content of protein, total creatine kinase activity (total CK) and creatine kinase-MB isozyme (CK-MB) was determined. The results are the values of 3 to 6 experiments at each age and are expressed relative to the values in control groups (100%). The difference between treated and control groups was analyzed by Student's t-test for paired samples (* = $p < 0.05$).

anti-insulin antibody. The anti-insulin receptor antibody was similarly devastating for embryo development except that there was no change in the creatine kinase-MB. Both antibodies, the anti-insulin receptor to a greater extent, also decreased the triglycerides content of day 4 embryos (results not shown).

DISCUSSION

There are at least two cell surface receptors known, insulin and IGF I receptors, capable of mediating insulin action. In some cells *in vitro* it is the insulin receptor which mediates the growth promoting effects of insulin (14) while in others is the IGF I receptor (15). Since we have previously reported that IGF I binding is higher than insulin binding in chicken embryos at days 2 and 3 of development (16) it was intriguing to know 1) if insulin receptors

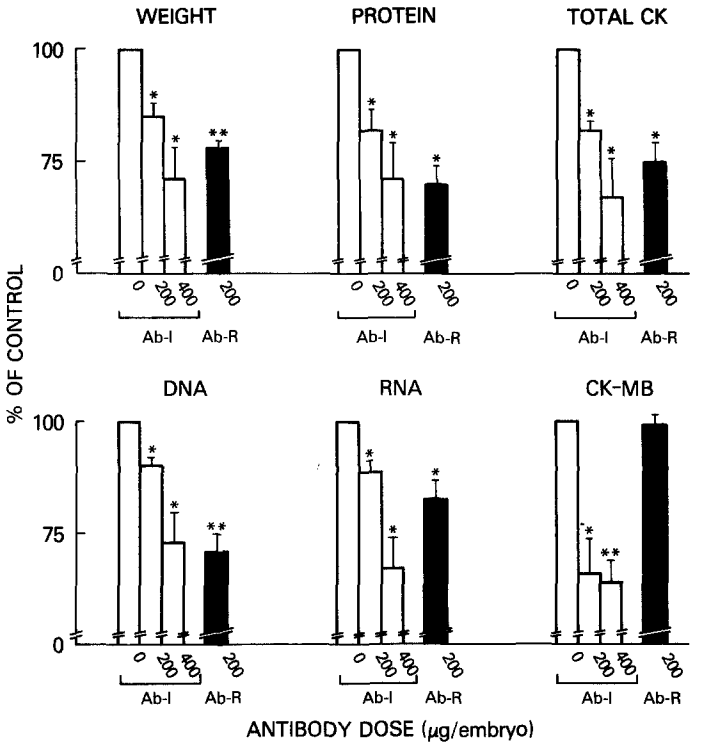

Figure 3. Biochemical effects of anti-insulin antibody (Ab-I) and anti-insulin receptor antibody (Ab-R) on day 4 embryos. Materials (100 μl containing 200 or 400 μg IgG) were applied to embryos at day 2 along with groups injected with control IgG (100% value, "0" bar). Live embryos on day 4 were processed as in Figure 2. Differences between treated and control groups were analyzed by Student's t-test for paired samples (*= $p<0.05$, **= $p<0.005$).

were functional in vivo at this early stage of embryogenesis, and 2) if insulin effects on development were mediated exclusively by this receptor.

Anti-receptor antibodies developed by patients with autoimmunity are powerful tools to study insulin receptor function. The antiserum B-10 blocks predominantly insulin receptors, although in chicken brain membranes is also able to block, slightly, the binding of IGFs to IGF I receptors (17). In the present study, both the anti-insulin antibody and the anti-insulin receptor antibody decreased overall development and growth indexes. This confirms that indeed the insulin receptors are active in vivo in early embryos and that the embryo's endogenous insulin is acting physiologically through its own receptor in many pathways. By contrast, the marker

of muscle differentiation creatine kinase-MB was only decreased by the anti-insulin antibody but not by the anti-receptor antibody. This result indicates that endogenous insulin is probably important for muscle differentiation in vivo, but rather than using the insulin receptor it may influence that process through an alternative receptor, perhaps IGF I receptor. When we added insulin or IGF I (100 ng/embryo) to the anti-receptor antibody treated embryos, there was a clear stimulation of creatine kinase-MB activity, more effective with IGF I than insulin (results not shown), further suggesting that another receptor was available to mediate the effect. An alternative, less likely, explanation for the lack of decrease in the creatine kinase-MB would be that the anti-receptor antibody had an acute insulin-mimicking effect (18) followed by a slower blocking effect. In this way, the net value of creatine kinase-MB could appear as unchanged over the long experimental period. This mechanism would support, as well, the existance of active insulin effector pathways in the embryo.

In conclusion, even if insulin binding in multiple chicken embryo tissues during organogenesis is less prominent than IGF I binding, insulin receptors are functionally relevant for early normal development.

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