Untoward effects of pharmacological doses of insulin in early chick embryos: through which receptor are they mediated?

F. de Pablo, E. Hernández, F. Collia and J. A. Gómez

Servicios de Endocrinología y Bioquímica, Hospital de la Santa Cruz y San Pablo, Barcelona, Spain

Summary. The teratogenic effect of insulin in early vertebrate embryos is controversial and the mechanisms involved are unknown. We studied the effects of pharmacological doses of insulin in chick embryos during the period of differentiation. We compared the effects of insulin with two proinsulins, desoctapeptide-insulin and multiplication-stimulating activity, peptides that have little insulin-like metabolic activity while they have significant growth effects. Chick embryos at 46 h of development were injected with the different peptides. At 96 h the mortality and abnormal growth elicited by the peptides were dose-dependent. Considering the indices of lethality (LD_{50}) and affected embryos (ED_{50}) as 100% for insulin, proinsulin was 59–66% as potent as insulin, desoctapeptide-

insulin 2–6% and multiplication-stimulating activity 176–204%. In the surviving embryos, insulin (5 μ g, decreased DNA, RNA and protein content by 49%, 40% and 48% respectively compared with controls. The effects of insulin were not corrected by simultaneous glucose injections. These data suggest that insulin, at pharmacological doses, interferes with embryo development through a non-metabolic pathway, probably via a growth-type receptor.

Key words: Insulin, proinsulin, insulin-like growth factors, multiplication-stimulating activity, insulin effects on growth, embryogenesis, chick embryo.

Infants of diabetic mothers have complications of late development, such as fetal macrosomia and neonatal hypoglycaemia, clearly related to maternal hyperglycaemia and fetal hyperinsulinaemia [1, 2]. Early developmental abnormalities typically result in high incidence of fetal waste and congenital malformations, the cause of which is controversial and probably multifactorial [3–6]. The demonstration that exogenous insulin is transferred from mother to fetus, carried by anti-insulin antibodies [7], reopens the debate whether hyperinsulinaemia in the early embryo could partially derive from maternal insulin and in some cases, be harmful to the embryo in the initial weeks of gestation.

Almost four decades ago, insulin at high doses was shown to produce malformations and death in chick embryos [8, 9]. However, the mechanism underlying these teratogenic effects has not been elucidated [10, 11]. The broad spectrum of insulin physiological effects includes acute well-known metabolic effects, and insulin stimulation of growth in vitro [12] and in a few animal models [13, 14]. In recent years, effects of insulin in cell differentiation have been demonstrated also [15–18].

We have proposed that insulin may play a role not yet recognized in early differentiation of vertebrate em-

bryos. Insulin is present in chicken eggs and chick embryos at 2 days of development [19] and specific insulin binding is demonstrable in 3-day embryos [20]. In the chick both insulin and insulin receptors are widespread at 4 days of embryogenesis.

The objective of the present study was to evaluate the effects of very high doses of insulin and insulin analogues (proinsulin, desoctapeptide-insulin and multiplication-stimulation activity peptide), in the microenvironment of the embryo. This initial approach may help us to understand better the role of insulin, insulin-related peptides and receptors in normal and abnormal embryogenesis.

Materials and methods

Chick embryos and injection procedure

Fertilized eggs from White Leghorn chickens (Granja Gibert, Cambrils, Tarragona, Spain) were incubated in a forced draft egg incubator at $38 \pm 0.5^\circ\text{C}$ and 70–80% relative humidity. Forty-eight hours after the beginning of incubation (day 2), eggs were transilluminated to locate the air chamber and embryonic area. A hole was drilled into the air space to release the pressure inside the egg, and 1 cm^2 of shell over the embryo was removed [21]. Only embryos between stages 11–14 of development (Hamburger and Hamilton classification) [22], verified

Fig. 1. Mortality and morbidity in chick embryos produced by the different peptides. Embryos were injected at 2 days of development and the percentages of deaths (■) and deaths plus abnormalities (□) were evaluated at 4 days. Histograms represent the mean of eight experiments for insulin (total number of embryos (n = 333), four for proinsulin (n = 156), two for desoctapeptide-insulin (DOP) (n = 63) and four for multiplication stimulating activity peptide (MSA) (n = 126). The vertical line on each bar indicates SEM. * indicates the lowest dose of peptide significantly different from control ($p < 0.05$), for deaths plus abnormalities. The mortality dose-response curves fitted in all cases to logarithmic curves with "r" values of 0.61 for MSA ($p < 0.02$), 0.91 for insulin ($p < 0.0005$), 0.94 for proinsulin ($p < 0.0005$), and 0.79 for DOP ($p < 0.05$).

through the shell's window under a stereomicroscope (Bauch and Lomb, Rochester, New York, USA), were used. Peptides (50 µl) were injected using an insulin syringe (B-D Plastipak, Rochelle Park, New Jersey, USA). Substances were gently released on the dorsal surface of the embryo, covering the entire embryonic area. Although the distribution of the injected peptides was not assessed, preliminary ink injections showed that a volume of 50 µl appeared to remain at the site of injection when the eggs were kept in the horizontal position for the remaining period of study. The window was sealed with adhesive tape, and the eggs were returned to the incubator for 48 h until day 4 of development. Groups of approximately ten embryos were injected with each test dose, unless specified otherwise. In each experiment, five to eight doses of the peptides were tested, at least three of which were with insulin (as an internal control). When biochemical parameters were determined, embryos surviving after injection of each dose were pooled; therefore, the number of embryos used varied with the peptide dose and lethal potency.

Polypeptides and controls

Porcine insulin was purchased from Elanco Products, Indianapolis, Indiana, USA (lot ODY 44C). A stock solution of 1 mg/ml in 0.01 N HCl was neutralized with concentrated NaOH, filtered through a Millipore (0.22 µm low absorption, Millipore Corporation, Bedford, Massachusetts, USA) and kept at -20°C . Further dilutions in NaCl (155 mmol/l) were prepared the day before the injections. Bovine insulin was purchased from Eli Lilly, Indianapolis, Indiana, USA. Porcine proinsulin (lot 615-70 N -279-1) and bovine proinsulin (lot 615-2H2-62) were a gift from Dr R. E. Chance (Lilly Research Laboratories). Desoctapeptide - insulin (DOP) from porcine origin (lot 681) was provided by the National Pituitary Agency, Baltimore, Maryland, USA.

All peptides were prepared as described for insulin. The effects of proinsulin were assessed using two different species, porcine and bovine. The bovine proinsulin was used within a few weeks after reconstitution of a lyophilized preparation, while the pork preparation had been stored at -20°C for several months after dilution. It is important to note that conversion to insulin could be favoured under the latter conditions. Both proinsulins, however, produced almost identical effects, so that a significant contribution due to increased insulin contamination appeared unlikely.

Multiplication-stimulating activity peptide (MSA, rat insulin-like growth factor II) was purchased from Collaborative Research, Waltham, Massachusetts, USA. Each 10 µg vial (lot 82-235), con-

tained 100 µg of sodium acetate and was kept lyophilized at 4°C until used. Dilutions were made with filtered NaCl (155 mmol/l).

Analytical grade HCl and sodium acetate were injected as control solutions, in appropriate concentration to match the highest content of the peptide doses in the experimental groups (0.005 N HCl and 1 mg/ml sodium acetate). Bovine serum albumin (BSA, Sigma, St Louis, Missouri, USA) was diluted in NaCl (155 mmol/l). IgG was prepared from normal (Miles Research Laboratories, Elkhart, Indiana, USA) and anti-insulin (lot 624, Department of Pharmacology, University of Indiana) guinea-pig sera. Adsorption of these sera to and elution from a protein-A sepharose column allowed recoveries of 4-6 mg/ml IgG. Insulin depletion experiments were carried out by incubating 1 ml of normal or anti-insulin IgG with 20 µg porcine insulin (final concentration 1 µg/50 µl) for 48 h at 4°C . This mixture was centrifuged at 1500 g for 20 min and 50 µl of the supernatant was injected into the embryos.

Embryo mortality and morbidity evaluation

Embryos were evaluated at 96 h of development. At this stage the area vasculosa and heart beat were evident macroscopically. When the heart beat was not verified under a stereomicroscope (Bauch & Lomb), the embryo was considered dead and was discarded. The live embryos were examined to assess the stage of development, stages 23-24 (Hamburger and Hamilton Classification) being considered normal. Embryos less developed (stages 22 or lower) and embryos with macroscopic abnormalities were considered abnormal (stereomicroscopic evaluation revealed mostly microphthalmia, body oedema, malformed limbs or tail). The live embryos, normal and abnormal, were dissected from the membranes, washed in saline and kept at -70°C for biochemical determinations.

The LD_{50} was defined as the dose of each peptide that killed 50% of the treated embryos and ED_{50} the dose that caused 50% affected embryos (abnormal + dead). Both parameters were calculated graphically from the dose-response curves and also by microcomputer analysis using a curve-fitting program. The fitness to logarithmic curves was evaluated by calculating the correlation coefficient "r" and the "t" value for r. The relative potency of each peptide interfering with development was established by comparison with pork insulin. The dose of porcine insulin which caused 50% mortality was divided by the corresponding value of the other peptides, and multiplied by 100. A similar method was applied to the ED_{50} values. Differences between the effects of peptides and controls were analyzed using Student's unpaired t-test.

Fig. 2. Comparison of dose-response curves for insulin (●—●), proinsulin (○—○), desoctapeptide-insulin (DOP) (△—△) and multiplication-stimulating activity peptide (MSA) (▲—▲). Percentage of total affected embryos are based on the same data as Figure 1 after subtraction of non-specific effects. The data fitted to logarithmic curves with "r" values of 0.75 for MSA ($p < 0.005$), 0.89 for insulin ($p < 0.005$), 0.95 for proinsulin ($p < 0.0005$), and 0.81 for DOP ($p < 0.02$)

Fig. 3. The influence of glucose on mortality caused by insulin in embryos. Increasing doses of insulin were injected at 2 days of development together with 1 (■) or 10 mg (▨) of D-glucose. Insulin alone (□) and D-glucose alone (▨ 10 mg or ■ 1 mg) were also injected into control groups. The bars represent the mean of two to three experiments (total number of embryos = 180) and the vertical line on each bar the SEM. There was no significant difference between the mortality effects of 1 mg of glucose plus insulin and 10 mg glucose plus insulin. Compared to insulin alone, at a dose of 0.1 μg, both (■) and (▨) were significantly more lethal ($p < 0.05$)

Biochemical determinations

The surviving embryos in each group were thawed on ice, weighed and homogenized in four volumes of cold phosphate buffered saline (pH 7.5) twice for 30 s using a homogenizer (Ultra-Turrax, Janke & Kunkel, Staufen i. Breisgau, FRG). The homogenate was centrifuged at 800 g for 10 min at 4°C and the supernatant was used for determination. Proteins were determined by the method of Bradford [23]. A simplified micromethod using ethidium bromide was carried out to measure DNA and RNA [24]. Standard DNA from calf thymus, type II, and protease, type XIV, were purchased from Sigma and RNA-ase, type A, from Boehringer, Mannheim, FRG.

Results

Abnormal development in insulin-treated embryos

Increasing doses of porcine insulin (0.1–25 μg/embryo) were injected into chick embryos at 2 days of development. Lethality and abnormal or retarded growth in embryos evaluated at 4 days were dose-dependent

Table 1. Indices of lethality (LD_{50}) and affected embryos (ED_{50})

	LD_{50}		ED_{50}	
	(μg/embryo)	(%)	(μg/embryo)	(%)
Insulin (pork and beef)	0.7	100	0.4	100
Proinsulin (pork and beef)	1.2	59	0.6	66
DOP (pork)	38	2	7	6
MSA	0.4	176	0.2	204

Percentages expressed as relative potency with respect to insulin, based on dose-response curves without correcting for the non-specific effects

Table 2. DNA, RNA and protein content in embryos after insulin, proinsulin or control HCl injections

Injection (μg/embryo)	DNA (μg/embryo)	RNA (μg/embryo)	Protein (μg/embryo)	Number of pooled embryos
Experiment 1				
Insulin 0.1	15.2	129	896	7
1	9.3	111	758	3
5	7.7	75	577	2
Proinsulin 0.1	11.7	120	1287	8
1	9.0	156	1530	8
5	9.3	94	1038	9
Control	14.9	118	928	3
Experiment 2				
Insulin 0.1	12.8	160	664	3
1	6.7	63	630	2
5	6.4	62	620	1
Proinsulin 0.1	11.5	116	1092	4
1	10.0	124	1060	3
5	4.0	8	160	1
Control	14.2	161	1376	5

Groups of six to 12 embryos were injected with each dose of peptide; only embryos surviving after injections were pooled for determinations

(Fig. 1). Bovine insulin injected in the same dose-range gave effects almost identical to those of pork insulin (mean $\pm$ SEM dead embryos: $30 \pm 7\%$ with 0.1 μg of bovine insulin, $58 \pm 4\%$ with 1 μg and $90 \pm 7\%$ with 25 μg). Control groups injected with HCl, 1–100 μg BSA or 100–500 μg guinea-pig IgG showed low non-specific abnormalities; in all cases $< 30\%$ of embryos were affected. The lethality induced by insulin was appreciably reduced when insulin was immunoprecipitated by anti-insulin antibodies but not by normal guinea-pig IgG. When a dose of 1 μg insulin was pre-incubated with anti-insulin IgG, the mean percentage of deaths in two experiments was 12.5%. When insulin was pre-incubated with control IgG, 54% of embryos were dead.

Relative potency of insulin related peptides

Doses of proinsulin from 0.1 to 50 μg/embryo also produced high lethality and abnormal embryo growth,

Fig.4. DNA, RNA, and protein content in surviving embryos at 4 days of development. Treatment at day 2 was insulin (●—●), insulin + 10 mg glucose (▲—▲), or MSA (□····□). In each case the value is expressed as a percentage of the content in embryos treated with the low dose (0.1 µg/embryo) of the respective peptide. The values shown represent the mean $\pm$ SEM of two to four determinations in pools of surviving embryos from different experiments (approximate total number of embryos = 210). * indicates the lowest dose of peptide causing a significant change ($p < 0.05$)

even though its metabolic potency is only 2–3% that of insulin [25]. Two lots of proinsulin, porcine and bovine, were equally potent. DOP was tested up to 25 µg/embryo. A significant percentage of altered embryo development followed ($53 \pm 9\%$ deaths with a 25 µg dose, $p < 0.05$). In vitro, however, DOP has only approximately 0.4% metabolic potency, but 30% growth potency of insulin [25]. MSA (0.05–5 µg/embryo) was even more potent than insulin in affecting embryogenesis, although its insulin-like metabolic effects were 0.2% compared with insulin [25] (Fig. 1). Figure 2 compares the untoward effects of insulin and related peptides upon embryogenesis. LD₅₀ and ED₅₀ were calculated for all peptides and the order of potency was: MSA > insulin > proinsulin > DOP (Fig. 2 and Table 1). MSA was only 85% pure, according to the manufacturer and each 10 µg vial also contained 100 µg sodium acetate. For control purposes, an appropriate concentration of sodium acetate was injected demonstrating a low non-specific effect. In an additional single experiment “MSA-peak II” (kindly supplied by Dr. M. M. Rechler, National Institutes of Health Bethesda, Maryland) was injected. The lethal effect was only 50% as high as with MSA. The reason for this discrepancy was not immediately clear and more experiments with MSA peak II are necessary.

Influence of glucose on the effects of insulin

D-glucose administered to the embryos simultaneously with insulin did not decrease the abnormalities (data not shown) or lethality caused by insulin (Fig. 3). Further, when the same doses of D-glucose (1 or 10 mg/embryo) were injected alone, lethality was also evident, with approximately 40% of embryos affected.

DNA, RNA and protein content of embryos

In embryos surviving injection with 5 µg of insulin, the contents of DNA, RNA and protein were reduced by 49%, 40% and 48%, respectively, when compared with the group injected with 0.1 µg insulin (Table 2, Fig. 4). The effect of insulin was not corrected by the simultaneous addition of D-glucose. On the contrary, D-glucose (10 mg) added to insulin further decreased these values. Injection of proinsulin (5 µg/embryo) similarly decreased DNA by 43%, RNA by 58% and protein by 53% compared with the 0.1 µg proinsulin dose (Table 2). Interestingly, MSA at 5 µg/embryo increased the DNA content of the embryos approximately 2.5-fold (247%), with a small increase in RNA (154%) and negligible change in total protein (113%).

Discussion

Insulin has been reported to be a teratogen for young vertebrate embryos [11, 26, 27]. However, neither the mechanism of action nor the physiological role of insulin in early embryogenesis have been elucidated. Pharmacological doses of insulin have been shown to elicit classic effects later in embryogenesis. For example, hypoglycaemia was induced by exogenous insulin in chick embryos after day 6 of development [28]. Our characterization of mortality and morbidity caused by pharmacological doses of insulin and related peptides, in the most active period of differentiation in the chick, supports the hypothesis of a potential action of insulin on early developmental events in vivo; i.e. pharmacological doses of insulin are harmful for embryo development but this, of course, does not mean that insulin in physiological doses is harmful. It clearly indicates that the pathways of insulin action are functional in early embryogenesis.

We have extended Landauer's pioneer experiments on the teratogenicity of insulin in chick embryos [8] by using purified insulin of two different species. Both porcine and bovine insulins gave essentially identical results. That the effects of insulin were not merely due to its proteic nature was further substantiated by injecting other proteins, such as bovine serum albumen and normal guinea-pig IgG, which had little effect. Proinsulin and MSA relative potencies interfering with embryo growth were comparable to their potencies in other growth bioassays.

There is evidence from many systems that insulin and proinsulin cross-react significantly with receptors for the structurally related insulin-like growth factors, while the latter react weakly with the insulin receptor [29]. Studies in vitro on the growth and differentiation-promoting effects of insulin indicate that insulin generally acts through a growth receptor instead of through the insulin receptor [18, 29, 30], with a few exceptions [31]. We postulate that the pharmacological effects of

insulin in early chick embryos may be mediated by an insulin-like growth factor receptor similar to that described in older embryo fibroblasts [32]. Preliminary experiments have confirmed that such receptors are already present at day 2 of development in the chick [33]. Although indirect, our data support this hypothesis: (a) the order of potencies of the peptides altering embryo development was MSA > insulin > proinsulin > DOP. This is the order of affinity of these peptides for binding to insulin-like growth factor receptors *in vitro*, and it is very different from that for the insulin receptor. In addition, it parallels their potencies to elicit biological effects through the insulin-like growth factor receptors [29]. (b) the dose-range and potencies of insulin, proinsulin and MSA in our system are comparable to their potencies in other growth bioassays [34, 35], and compatible with a growth-receptor mediated effect. (c) that insulin's teratogenic effect on embryos was due to low glucose supply is unlikely. Glucose content (approximately 150 mg) in egg albumen [36] is readily available to the embryo in this type of *in ovo* culture. The addition of 1 or 10 mg of D-glucose plus insulin did not reverse the deleterious effects of insulin but, in fact, increased the percentage of affected embryos. (d) the decrease in DNA, RNA and protein content in the surviving embryos treated with insulin could reflect arrested cell multiplication or a decrease in nucleic acid synthesis, suggestive of a growth rather than a metabolic effect of insulin. This decrease also was induced by high doses of proinsulin. In contrast to most reports describing the stimulatory effects of insulin in protein, DNA synthesis and ultimately cell growth, there have been other demonstrations of inhibitory effects of insulin at high concentrations, in acellular systems [37], melanoma cells [38] and also cultured chick embryos [39]. A hypothetical mechanism by which insulin, proinsulin and DOP in vast doses could reduce DNA, RNA and protein synthesis could be the competitive inhibition of binding of endogenous growth factors in the embryo.

MSA lethality, on the other hand, can be interpreted as embryo death raised by a growth stimulus inappropriately high for the specific developmental stage. Nevertheless, it is paradoxical that changes in DNA and RNA in the embryos are divergent, if insulin appears to act through the same receptor as MSA interfering with embryogenesis. This probably reflects complex interactions between insulin and insulin-like growth factor receptors, which require further experiments *in vitro*.

The other possibilities, that the injected peptides act through the insulin receptor or through both insulin and insulin-like growth factor receptors, could still be true if proinsulin and DOP have much longer half-life than insulin in the embryo environment. In this case, they would appear to exert a greater potency with respect to insulin when acting through the insulin receptor. But, in fact, in our previous work we were not able to demonstrate the presence of insulin receptors at 2 days of development in the chick. On the other hand, our data

confirm earlier findings where changes in glucose were not considered to play an important role in insulin-induced malformations [11, 27], and also reflect that glucose itself may be toxic for developing embryos. This latter aspect has been known for several years [36], and although it is not the focus of our work, it has relevant implications for the abnormal development of infants of diabetic mothers.

We postulated a developmental role for insulin in embryogenesis after showing that insulin and insulin receptors were present very early in chick embryos. We now suggest that untoward pharmacological effects of insulin, during the period of differentiation of the chick, are probably mediated by insulin binding to or modulation of insulin-like growth factor receptors. We have not yet proved that insulin has any physiological role in development of vertebrate embryos, an aspect that certainly deserves future investigation.

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Dr. Flora de Pablo
Servicio de Endocrinología
Hospital de la Santa Cruz y San Pablo
Ave. S. Antonio M. Claret, 167
Barcelona - 25
Spain