Reprod Nutr Dev (1996) 36, 513-521

Original article

Insulin receptors: the binding capacity and localization in the digestive tract during the rabbits neonatal period

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(Received 26 January 1996; accepted 27 July 1996)

Summary — The aim of this study was to localize and measure the receptor-binding capacity for insulin in the stomach, duodenum, ileum, jejunum, caecum, colon and in isolated hepatocytes in 48 rabbits aged 1–7, 14 and 21 days. Two methods were employed: radioimmunoassay (RIA) and receptor-binding assay (RBA). Isolated rabbit hepatocytes were used to estimate the maximal binding (B_o). Insulin (INS) concentrations measured by RIA-INS test were highest in the colostrum (701 μU/mL as 100%) of the does, and in the blood (82 μU/mL) of newborn rabbits during the first day postpartum. The B_o of colostral insulin for isolated hepatocytes was 556 μU/mL, which corresponded to 79.1% of the total binding as assessed by the RIA-INS test. The B_o for insulin binding in the different parts of the digestive tract tested was the highest on the first day (mean 8.0 fM/g) and the lowest on the 6th day postnatally (mean 4.9 fM/g; $P < 0.01$). The receptor capacity for insulin varied in relation to the different parts of the digestive tract, and with the age of each individual rabbit.

insulin / receptor / localization, digestive tract / newborn rabbits / colostrum / hepatocytes

Résumé — Récepteurs insuliniques : capacité de liaison et localisation dans le tube digestif du lapin nouveau-né. *Le but de cette étude était de localiser et de mesurer la capacité de liaison des récepteurs à l'insuline dans l'estomac, le duodénum, l'iléon, le jéjunum, le cæcum, le côlon et dans des hépatocytes isolés chez 48 lapereaux âgés de 1, 7, 14 et 21 jours. Deux méthodes ont été employées : radio immunologie (Ria) et liaison au récepteur (RBA). Des hépatocytes isolés de lapin ont été utilisés pour évaluer la capacité maximale de liaison B_o. Les concentrations en insuline mesurées par le test Ria-INS étaient plus élevées dans le colostrum (701 μU/mL) des mères et dans le sang (82 μU/mL) des nouveau-nés pendant le premier jour postpartum. La valeur B_o de l'insuline de colostrum pour les hépatocytes isolés était de 556 μU/mL correspondant à 79,1 % de la liaison totale mesurée par le test Ria-INS. La valeur B_o de liaison de l'insuline dans les différents segments du tube digestif était la plus élevée le premier jour (moyenne de 8,0 fM/g) et la plus faible le 6e jour après la naissance (moyenne de 4,9 fM/g ; $p < 0,01$). La capacité de liaison des récepteurs pour l'insuline variait en fonction du site intestinal et de l'âge de l'animal.*

insuline / récepteur / localisation / tube digestif / lapin nouveau-né / colostrum / hépatocyte

INTRODUCTION

The physiological actions of the digestive tract are continously regulated by more than 70 different hormones and hormone-like peptides (Glass, 1980; Tsung-Min Lin, 1980; Buts et al, 1990; Konturek, 1990).

During the last decade, studies on the action of insulin and its binding by the receptors present in the digestive tract (DT), mainly in rats, but also in some other mammals, have established the view that insulin is one of the hormones providing the strongest stimulation of DT development (Pierce et al, 1964; Forgue-Lafitte et al, 1980; Shulman, 1990; Read et al, 1991; Baumrucker et al, 1993; Cheatham and Kahn, 1995).

The occurrence of high concentrations of insulin and other growth factors in milk suggests that they may be important for the growth of offspring. Growth factors may play a vital role in the proliferation and maturation of the gut without necessarily entering the circulation, although they may cross the intestinal wall to regulate the development of other organs (Read et al, 1984).

Cevreska et al (1975) was probably the first to describe a high concentration of insulin in the colostrum of women. A few years later, the presence of insulin in colostrum, 30 to 40 times higher than in blood, was described in women (Kulski and Hartmann, 1983; Read et al, 1984); cows (Malven et al, 1987); women, cows and sows (Nowak and Nowak, 1989); and in sheep (Falconer et al, 1984; Nowak et al, 1994).

The transfer of insulin from the ingested colostrum into the blood of newborns during the first 25 h of life was observed in piglets (Asplund et al, 1962; Nowak, 1989), calves (Pierce et al, 1964) and newborn infants (Koldovsky, 1989).

The binding of insulin and glucagon by receptors and the effect of these hormones on the motility, secretion and development of the digestive tract was described in rats (Forgue-Lafitte et al, 1980; Cooke et al, 1986; Koldovsky, 1989; Buts et al, 1990; Baumrucker et al, 1993); rat fetuses (Sodoyez-Goffaux et al, 1985); piglets (Ito et al, 1987); minature pigs (Shulman, 1990); dogs, humans, chickens, rats, cats, sheep, guinea pigs (Tsung-Min Lin, 1980); and in human infants, rats, ovines, bovines, pigs and calves (Koldovsky, 1989).

Up to now, no studies have been carried out evaluating the insulin concentration in the colostrum and milk of rabbit does, or on the presence of insulin receptors in the digestive tract during the neonatal period. The biological activity of colostral insulin assessed by its binding capacity to rabbit hepatocytes is also not known.

The aim of this study was to measure the maximal binding of ^{125}I insulin (B_0) by the anticipated receptors for insulin in the parietal cells of the stomach, duodenum, jejunum, ileum, caecum and colon. This binding is considered indicative of the biological activity manifested by colostral insulin and/or its localization within the digestive tract of the developing, neonatal rabbit.

MATERIALS AND METHODS

In this study, 48 New Zealand White rabbits were used. They were divided into nine groups of different ages: 1–7, 14 and 21 days. During the postnatal period the animals were kept with their mothers and were fed exclusively on the mammary secretion of their does. It was observed that rabbits, after 14 to 21 days of age, tried to take the fodder of the doe.

On the day of the experiment the animals were anaesthesized by intraperitoneal (ip) injection of Vetbutal (Polfa) at a dose of 36 mg/kg. Under anaesthesia, 5 mL of blood was taken from each animal by heart puncture to determine the insulin (INS) concentration by the radioimmunoassay (RIA)-INS test.

The liver was perfused in situ under sterile conditions and without recirculation, with 50 mL of calcium-free buffered, cooled saline 4 °C pH 7.4

(first step perfusion) at a rate of 10 mL/min through an inserted catheter to the portal vein (inlet) and to the anterior and posterior vena cava, terminating above the branch to the right kidney (outlet) (Berry et al, 1991). The opening of the abdominal cavity, the cannulation of veins and the one-step perfusion lasted up to 10 min.

The liver including the catheters was removed from the abdomen and put into a beaker and perfused again (second step) with 150 mL of buffered saline solution (pH 7.4), containing 15 mg of collagenase (type IV, Sigma) and 1 mM Ca^{2+} (Sigma) (Seglen, 1976; Gill and Hart, 1980; Berry et al, 1991). The second step perfusion lasted 30 min at 30 °C, pH 7.4, at a rate of 15 mL/min, with recirculation. This medium, initially equilibrated to pH 7.4 at 30 °C in a water bath, under carbogen (a mixture of O_2 and CO_2, 95:5) was under pH meter control with the addition of 0.001 phenol red. The perfusion fluid contained 15 mM of glucose. At the end of the digestion period, the liver was freed of ligaments and mesentery, weighed and combed with a stainless steel dog comb (3 mm between teeth) (Seglen, 1976; Berry et al, 1991). The tissue had the consistency of a very concentrated homogenate. This 'parenchymal paste' was gently passed through a progressive series of steelon meshes (200, 100, 50 μm pore-size) into a 250 mL beaker with about 50 mL of Williams Medium E (Berry et al, 1991) at about 4 °C, and centrifuged at 40 x *g*, 10 min, 4 °C. Streptomycin (50 μg/mL; Polfa, Poland) and Traskolan (500 KIU/mL; Jelfa, Poland) were added to this washing medium. The suspension was carefully mixed and centrifuged twice at 50 x *g*, 10 min, 4 °C. The washing procedure ensured that the damaged and nonparenchymal cells were removed. After the last centrifugation, the supernatant was discarded and a cell pellet of 0.5 g samples was put into centrifuge tubes (4 mL each) with Williams Medium E (1.0 mL). The density of the isolated hepatocytes in the cell pellets obtained by this method was similar to the density of hepatocytes obtained from the liver of other species in sheep (Gill and Hart, 1980); mice, hamsters and rabbits (Maslansky and Williams, 1982); and rats (Berry et al, 1991).

Radioimmunoassay

The concentration of insulin in the plasma of the newborn rabbits and in the colostrum infranatant samples was determined by the method of Yallow and Berson (1960) using RIA-INS kits (Świerk, Poland) with modifications introduced by Nowak et al (1994).

Colostrum samples preparation

Colostrum samples were taken from lactating rabbits at 0700 hours, into the tubes containing Traskolan (Jelfa, Poland; 10 μL/mL of sample) and streptomycin (Polfa, Poland).

The high concentrations of insulin in the colostrum required dilution (1:50 to 1:10 v/v) with phosphate buffer (RIA-INS assay buffer 0.04 M, pH 7.4, containing 1.0 g bovine serum alumin [BSA, Sigma] and Thiomersal [BDH, UK] per liter). After acidification of the diluted mammary gland secretion samples with acetic acid (POCH, Poland) under pH meter control to pH 4.6 (casein isoelectric point), and centrifugation at 2 500 x *g* for 15 min at 0 °C, the fat layer and casein pellets were discarded and the infranatant (colostrum whey) samples were collected and kept either for immediate determination of insulin or stored at –20 °C until use. The insulin concentrations were measured in 0.2 mL colostrum infranatant solution using the RIA-INS kits mentioned earlier (Nowak et al, 1994).

Colostral insulin concentration measured by receptor-binding assay (RBA)

i) hepatocyte pellet 0.5 g;

ii) ^{125}I insulin (OPiDI, Świerk, Poland) in 0.5 mL buffered saline solution (pH 7.4), the concentration of 19.9 fM = 115.62 pg = 2.948 μU;

iii) colostrum infranatant solution, 0.2 mL containing streptomycin (50 μg; Polfa, Poland);

iv) Williams Medium E (Sigma), 0.8 mL to a final volume of 2.0 mL, containing Traskolan (500 KIU/mL; Jelfa, Poland). This proteinase inhibitor is similar to Trasylol (Sigma).

The sample for non-specific binding (NSB) of ^{125}I insulin

i) hepatocyte pellets 0.5 g;

ii) ^{125}I insulin in 0.5 mL buffered saline solution (pH 7.4);

iii) colostral infranatant solution 0.2 mL;

iv) a 50 μg excess of mono component (MC) insulin porcine (NOVO) in 0.8 mL Williams Medium E to a final volume of 2.0 mL.

Hormone-receptor dissociation

The dissociation of insulin from its hepatocyte receptors was accomplished by incubating the cells with ^{125}I insulin for 40 min. Excess native hormone was then added to the incubation tubes and the reaction was stopped at various intervals (Gill and Hart, 1980).

The extent to which the ^{125}I insulin was degraded during incubation with hepatocytes was assessed according to Gill and Hart (1980) by examining the activity of the incubated supernatant fractions obtained after a binding assay, assessed by determining the quantity of radioactivity which was precipitated with 10% trichloroacetic acid (TCA) in the same supernatant fractions. Degradation was approximately 40% for insulin after incubation for 40 min. In the presence of streptomycin and Traskolan this degradation was reduced to 15%; streptomycin and Traskolan were thus used routinely in all assays.

Incubation conditions

RBA specific and non-specific binding were determined by simultaneous incubation in duplicate tubes mixed at intervals of 10 min, at 30 °C, 25 min, pH 7.4. After incubation, the tubes were centrifuged (2 000 x *g* for 10 min at 4 °C), and the supernatant fractions aspirated and discarded. The radioactivity of the cell pellets was measured on a gamma counter with a counting efficiency of about 76% and a counting error of 2% (Gill and Hart, 1980).

Preliminary assessment of the tracer

After precipitation of the ^{125}I insulin by 10% TCA (POCH, Poland), 94% of the sample was regarded as being suitable for binding studies and for standard curve preparation. Besides, the NSB for this tracer used for standard curve was 5.6% and therefore was recognized as pure enough.

Calculation of B_o

The quantity of insulin specifically bound to the hepatocytes was obtained by subtracting the NSB from the total binding. It was expressed as fM/g of hepatocyte pellets initially present in the tube, in the absence of MC insulin porcine standard according to Gill and Hart (1980).

The preparation of the digestive tract samples

After the first step of the liver perfusion, the total digestive tract was removed and rinsed with cold saline solution (4 °C) through a catheter attached to the duodenum. The stomach was washed separately and then the mesentery was stripped off (Shulman, 1990). The digestive tract was divided into six segments: the stomach, duodenum, jejunum, ileum, caecum and colon. From each segment, four parts were weighed (0.5 g each). Each segment was opened lengthwise, gently blotted and everted. The serosa and mucosa were saved. These parts (0.5 g each) were submitted to incubation (25 min, 30 °C, pH 7.4).

Sample contents: the digestive tract fragment 0.5 g, ^{125}I insulin in buffered saline solution 0.5 mL, pH 7.4 and Williams Medium E 2.0 mL in duplicate.

NSB sample contents: the digestive tract fragment 0.5 g, ^{125}I insulin in solution as above, MC insulin, porcine (as standard) of 50 μg/0.5 mL buffered saline solution and Williams Medium E 0.5 mL in duplicate. The quantity of ^{125}I insulin specifically bound to the intestinal cells on the different parts of the DT was obtained after subtracting the NSB from the total binding and was expressed in fM/g of intestinal segments.

Calculations and statistics

All results are given as means ± SD. Differences between means were tested for statistical signif-

icance using the Student's *t*-test and by ANOVA. Differences were considered significant for $P < 0.05$ (Tashman and Lamborn, 1979).

RESULTS

The isolated hepatocytes were considered viable from their morphological appearance under microscope and their ability to exclude more than 95% of the vital dye (Trypan blue, Sigma). The number of parenchymal cells per cc of fresh liver tissue was about 70%.

It was found that the incubation of the hepatocytes with ^{125}I insulin (115.6 pg) and the doubling of dilutions of native MC insulin resulted in progressive displacement of labelled hormone from the receptors. The specific binding of hormone to receptor was half-maximal after 15 min. B_0 was obtained after 30 min. Optimum specific binding was recorded at 30 °C. The optimum pH was between 7 to 8. The mixing time and volume of each sample were stable because increasing time and reducing the incubation medium enhanced specific binding as already described by Gill and Hart (1980).

The mean values of the insulin concentrations in the rabbit doe colostrum samples assessed by RIA was, on average, 701 μU

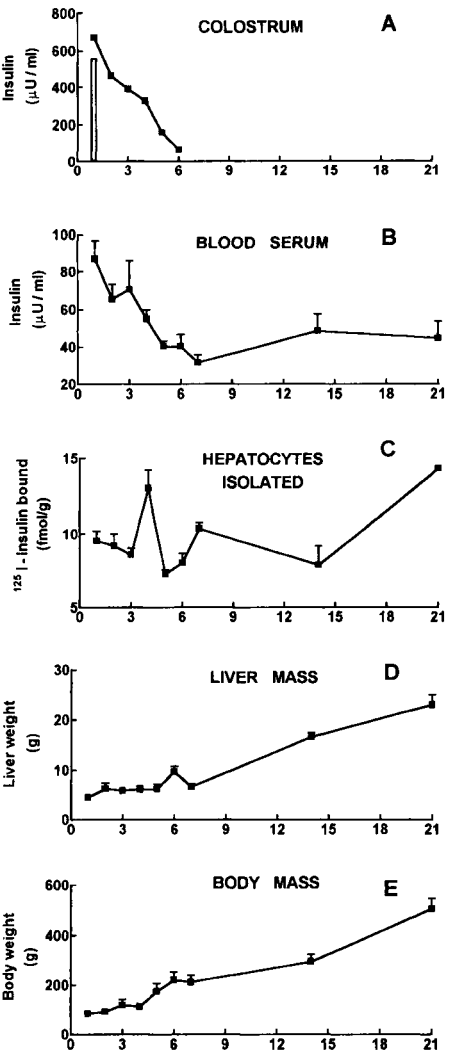

Fig 1. A: The insulin concentration in the colostrum samples (taken during the days after parturition) of rabbit does, measured by means of radioimmunoassay (RIA) and the colostral insulin (the same samples of colostrum) specifically bound by the receptors of rabbits' isolated hepatocytes by means of receptor-binding assay (RBA). The results were expressed as the maximal binding in percent of the total insulin concentration measured by means of RIA, on the first day only (open bar) of lactation. **B:** Insulin concentrations (mean values) in newborn rabbit serum decreased and a comparison of the levels between days 1 and 6 was statistically significant ($P < 0.001$). **C:** ^{125}I insulin specifically bound by the receptors present in newborn rabbit isolated hepatocytes, by means of RBA. A statistically significant result. **D:** The increase in liver mass in newborn rabbits during the experimental period, compared to the value on the first day was significant ($P < 0.001$). **E:** The increase in body mass of newborn rabbits during the experimental period (21 days) compared to the valve on the first day was significant ($P < 0.001$).

(n = 10) on the day of parturition (considered as 100%) and then successively decreased to a level of 29 ± 1.7 μU/mL on day 6 postpartum ($P < 0.01$) (fig 1A).

Plasma insulin level in newborn rabbits (first day of life) was, on average, 82 ± 1.9 μU/mL (fig 1B) ($P < 0.01$). The insulin concentrations in the colostrum, on day of parturition, was much higher than in the blood of newborn rabbits.

The biological ability of the colostral insulin to bind with the receptors of the isolated rabbit hepatocytes, using the RBA method, reached 556 μU/mL and this value represented 79.1% of the total insulin concentration determined by the RIA-INS test (fig 1A, open bar).

A statistically significant increase in insulin-binding ability ($P < 0.02$) of the receptors of isolated hepatocytes was found on day 4 after birth (fig 1C).

The liver and the body mass showed a progressive increase during the period of 21 days of the newborn rabbit's life. The increment of increase, when compared to the results of the first day, was significant ($P < 0.001$; fig 1D and E, respectively).

The insulin binding by the receptors (expressed as B_0 in fM/g of the tissue) decreased with age in every segment of the digestive tract (markedly on day 6; fig 2A–F). Statistical differences were as follows: colon ($P < 0.01$; fig 2F), caecum ($P < 0.05$; fig 2E), ileum ($P < 0.001$; fig 2D), jejunum ($P < 0.001$; fig 2C), duodenum ($P < 0.01$; fig 2B) and stomach (not significant; fig 2A).

This study provides evidence for the presence of receptors for insulin in all segments (with the exception of the stomach) of the rabbit digestive tract tested, from the age of 1 to 21 days of life, as evidenced by the RBA test. The binding reaction between insulin and receptor was saturable and reversible.

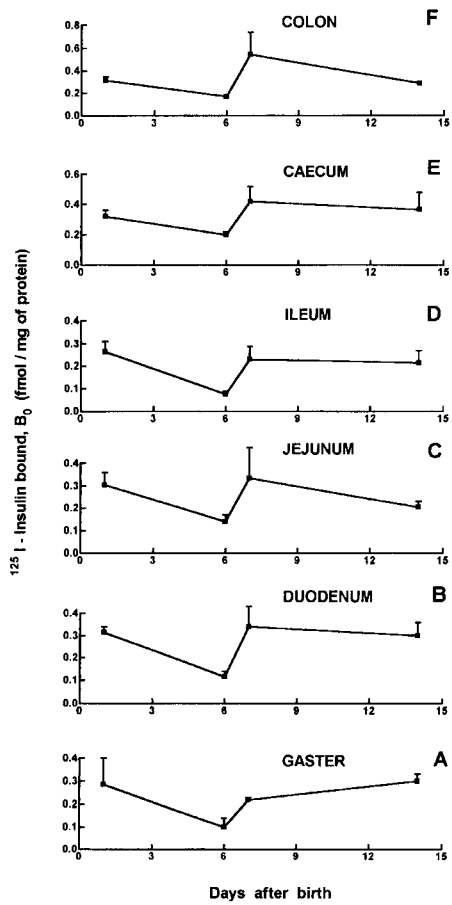

Fig 2. Specific ^{125}I insulin binding by the receptors present in the parietal cells of every part of the digestive tract tested (mentioned later) by means of receptor-binding assay (RBA) expressed as B_0 in fmol/g of tissue (wet weight). The amount of insulin binding clearly decreased on day 6 in comparison with the results of the first day for newborn rabbits. Statistical differences for these decreases (except of the stomach) were significant. **A**: In the stomach (NS); **B**: in the duodenum ($P < 0.01$); **C**: in the jejunum ($P < 0.001$); D: in the ileum ($P < 0.001$); **E**: in the caecum ($P < 0.05$); **F**: in the colon ($P < 0.01$).

DISCUSSION

According to many authors, insulin, similar to EFG, IGF-I and IGF-II, is a potent stimulator of intestinal growth and development in newborn infants and other mammals and has been found in mammary secretions by various authors (Forgue-Lafitte et al, 1980; Maslansky and Williams, 1982; Read et al, 1984; Cooke et al, 1986; Malven et al, 1987; Koldowsky, 1989; Buts et al, 1990; Schams, 1990; Read et al, 1991; Baumrucker et al, 1993; Grosvenor et al, 1993; Nowak, 1993; Nowak et al, 1994). Their results encouraged us to undertake this study to locate receptors for insulin in the digestive tract of neonatal rabbits.

It was assumed that each growth factor found in mammary secretions had a specific receptor in the DT in order to be able to exert a physiological action upon the DT of sucklings. Studies on the influence of insulin and its binding to DT receptors have already been conducted in mice, hamsters, rats, piglets, sheep and calves (Pierce et al, 1964; Gill and Hart, 1980; Forgue-Lafitte et al, 1980; Maslansky and Williams, 1982; Read, 1988; Buts et al, 1990; Baumrucker et al, 1993) but not in rabbits.

Different methods were employed in the DT sample preparation (Read et al, 1986; Buts et al, 1990; Shulman, 1990). In our experiment intact intestinal segments were weighted and used according to Berry et al (1991).

The insulin concentration was high in the colostrum of rabbit does on the first day postpartum and in the blood of newborn rabbits on the first day of life. It decreased on day 6 to basal levels. This could indicate that the increased insulin level in the blood of nonfasted newborn rabbits is dependent on the high concentration of insulin transferred from the DT into the blood as well as to the stimulatory effect of the nutritional ingredients of colostrum such as proteins, fat and carbohydrates towards the secretion of endogenous insulin. In fact, the transfer of colostral insulin from the digestive tract into the systemic blood was observed in piglets (Asplund et al, 1962; Nowak, 1989, 1990), infants and rats (Koldovsky, 1989).

Colostral insulin concentrations averaging 701 μU/mL on day 1 postpartum, as measured by RIA, were considered as 100%. The bioactivity of the colostral insulin measured by RBA (556 μU/mL) was about 80% of that insulin measured by RIA. This difference, suggesting a loss of bioactivity, could be the result of the enzymatic degradation of ^{125}I insulin bound to the receptors, in spite of the fact that all samples for insulin measurement (RIA and RBA) were protected by streptomycin and proteases inhibitor (Traskolan) during the sample preparation. This is in agreement with the observation that bound insulin is a substrate for insulin degradation by intact hepatocytes (Dial et al, 1977). The degradation of bound MC insulin occurs at a rate that is almost 25 times faster than that of the free MC insulin. The interaction of insulin with purified liver plasma membranes involves two processes: binding and degradation. These processes can be physically separated and shown to occur independently (Levey, 1976; Dial et al, 1977).

A trial of colostral insulin separation and the purification on a Sephadex G-50 column in human milk to identify the material, assayed by radioreceptor and immunoassay, was performed. Insulin recovered from the column had 90% of the activity measured by RBA or RIA (Read et al, 1984). This result may reflect differences among species or may indicate a need for specific methodologies.

The reasons for the decrease in ^{125}I insulin binding by the parietal cell receptors present in the DT could be the result of the rapid growth and digestive tract maturation during the neonatal period and the likely

simultaneous diminution of colostral insulin content during early lactation of rabbit does.

The presence of insulin receptors in the digestive tract (and liver) of newborn rabbits allows us to assume that colostral insulin is a potent stimulator and that it can influence the development of the digestive tract and probably all target tissues during the early postnatal period.

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