Fate of Insulin-Like Growth Factors I and II Administered Orogastrically to Suckling Rats

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ABSTRACT

Milk-borne insulin-like growth factors I and II (IGF-I and -II) may be of importance in the differentiation of the gastrointestinal tract of the suckling. To test this hypothesis, 10- to 11-d-old suckling rats were given via an orogastric tube ^{125}I -IGF-I ($n = 6$) or ^{125}I -IGF-II ($n = 6$) in rat milk and killed 30 min later. The results of this study demonstrated that approximately 40% of the radioactivity administered was detected in the gastrointestinal tract for both ^{125}I -IGF-I and ^{125}I -IGF-II experiments. Gel chromatography of acid extracts of homogenates of gastrointestinal tissues and luminal contents demonstrated that a significant fraction of recovered radioactivity eluted in a position identical to "native" IGF. These findings were confirmed by subjecting similarly treated samples to high performance liquid chromatography. In addition, radioactive material recovered from M_r 7,500 fractions bound specifically to crude membrane IGF-I and -II

receptor preparations, further suggesting the preservation of biologic activity of the recovered peptides. Although skin homogenates contained large peptide fragments of ^{125}I -IGF-I, no "intact" IGF was found in the blood or other tissues. These findings suggest that milk-borne IGFs are stable in the neonatal gastrointestinal tract and remain biologically active for as long as 30 min postingestion. (*Pediatr Res* 37: 586-592, 1995)

Abbreviations

IGF, insulin-like growth factor
IGFBPs, IGF binding proteins
RP, reversed phase
HPLC, high performance liquid chromatography
TCA, trichloroacetic acid

IGFs are peptides that bear significant homology to insulin and are produced by a variety of tissues in the developing mammal (1-3). IGF-I and -II have been observed to be potent mitogens and to have insulin-like effects on cellular metabolic processes such as glucose and amino acid uptake. Because these hormones and their receptors are ubiquitous during development, it has been suggested that IGFs may play a significant role in control of growth during the perinatal period, which is a time of rapid somatic and organ growth and differentiation (1-3). Recent studies using transgenic mice that either over or under express IGF transcripts have confirmed this supposition (4, 5). Recently, significant quantities of IGFs have been documented in the milk of several species, including the human (6-8). In the gastrointestinal tissues of the newborn

rat and pig there are disparities between the relatively large rates of synthesis of IGF receptors, as measured by receptor-specific mRNA levels, and the lesser rates of synthesis of endogenous IGF (9, 10). Because of the known ease of absorption of other peptide hormones (11), and of lower digestion of proteins by the gastrointestinal tract of the newborn (12), the fate of orogastrically administered radiolabeled IGFs was determined, using the suckling rat as a model of premature newborn gastrointestinal function.

METHODS

Chemicals. Recombinant human IGF-I and -II were obtained from Kabi Pharmacia, Biosciences Laboratories, Stockholm, Sweden. Sephadex G-50 and BSA were purchased from Sigma Chemical Co., St. Louis, MO. ^{125}I iodine, used for iodination of peptides, was purchased from Amersham Corp., Arlington Heights, IL. Iodination of IGF-I used the chloramine T method (13), and IGF-II was iodinated using a peroxidase method (14). Appropriate concentrations of each hormone preparation were then made in 50 mM Tris-HCl, 0.2% BSA for dilution in rat milk. The specific activities of ^{125}I -IGF-I and ^{125}I -IGF-II were 200-400 mCi/mg and 150-500 mCi/mg,

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respectively. Both iodinated peptides were isolated using Sephadex G-25 gel chromatography and tested for specific activity using radioreceptor assays specific for type I and II receptors (see below). Rat milk of appropriate postnatal age was obtained from lactating mothers. Pups were removed 4 h before milk collection, and the mothers were then lightly anesthetized using Innovar-Vet (Pitman-Moore, Washington Crossing, NJ) (10–40 μ L/rat). Each dam was given 1.0 U oxytocin intraperitoneally and milked only once. Within 20 min of oxytocin injection, between 1.0 and 2.0 mL of milk was manually expressed into polystyrene test tubes and stored at -40°C for further use.

Animals and Experimental Design. Six suckling rats each were used for both the IGF-I and -II feeding experiments. The animals used were male rats between 10 and 11 d postnatal age, weighing 20–25 g each. Litters were culled to 10 per litter routinely on d 2. Rat pups were removed from their dams 4 h before administration of IGF and fasted after placement in a Plexiglas cage that was positioned so that one-half was situated over a heating pad at 33°C . Before administration of IGF, urination was stimulated by gentle stimulation of the anogenital region. Rats were then given 200 μ L each of a 1:1 mixture of ^{125}I -IGF-I or -II in whole rat milk using a 3.5 Fr (1.2-mm inside diameter) catheter (Sherwood Medical, St. Louis, MO) that was placed by mouth to a depth of approximately 4 cm. The rat milk-IGF mixture was incubated for 30 min at 37°C before administration. The radioactivity dose administered to each animal was estimated by sampling an aliquot before feeding and was approximately 10×10^6 cpm per animal (or 7–10 ng IGF, based on specific activities of ^{125}I -IGF-I or -II). This represents approximately 15% of the daily estimated milk-borne IGF-I intake/day, or 2% of IGF-II intake/day of suckling rats at 10 d postnatal age (15).

After 30 min, animals were decapitated while under light ether anesthesia. Trunk blood was then collected and allowed to clot for 30 min at 21°C . Radioactivity of each blood sample was measured using a gamma counter (AutoGamma, Beckman Instruments, Palo Alto, CA), and radioactivity of whole blood total volume was calculated assuming a blood volume of 7% of body weight. Serum was separated by centrifugation at 3000 rpm for 15 min, and samples stored at 70°C for later analysis.

Within 5–10 min after decapitation, the brain, liver, lungs, heart, thymus, spleen, kidneys, skin, stomach, and small intestine was removed from each animal. Care was taken to use clean instruments to prevent cross contamination of radioactivity. Each organ was immediately frozen in liquid nitrogen and radioactivity per organ assessed. For the stomach and intestine, the following modifications were made: 1) the stomach was dissected from the intestine at the pylorus, and the esophagus removed; 2) the stomach was incised longitudinally along the lesser curvature and opened; 3) the stomach luminal contents were gently scraped off, the mucosa flushed with 2.5 mL of ice-cold saline, and both content and saline wash combined as “stomach luminal content;” 4) the ice-cold saline solution was injected into the proximal small intestine and the resultant intestinal content and saline wash combined as “intestinal luminal content;” and 5) the combined contents and flushings from stomach and intestine, as well as stomach and

intestine wall, were then frozen and counted (as above). It was noted that approximately 50% of the animals had urine visible within the bladder. Urine in volumes of 0.1–0.7 mL was aspirated by syringe from these pups and counted in individual tubes.

Acid Extraction and Chromatography. Because of the presence of IGFBPs, acid extraction of tissue homogenates and subsequent chromatography was necessary to identify ^{125}I -IGFs in tissues of fed animals. Organ homogenates were prepared in 5 mL of 0.1 M acetic acid for 60 s each using a Polytron tissue homogenizer (Brinkman Instruments, Westbury, NY). In the case of luminal flushings, 0.2 M acetic acid was used to bring the final homogenate concentration to 0.1 M. Each sample was then centrifuged at 5000 rpm for 10 min, and the supernatant was stored for later analysis. In the preliminary studies, ^{125}I -IGF-I was added to similarly prepared rat liver homogenates. Supernatants removed contained 85–90% of the added radioactivity. Homogenate supernatants were stored at -70°C and later thawed for chromatography. Selected specimens (four to six specimens from each gastrointestinal tissue and luminal flushings, pooled sera, and one to two homogenates from all other tissue specimens) were studied. A 3-mL aliquot of each specimen was counted, placed over a 1×75 -cm Sephadex G-50 column (Pharmacia Biotech Inc., Piscataway, NJ), and equilibrated against 0.1 M acetic acid at a flow rate of 2.0 mL/h (1.5-mL fractions). Columns were calibrated using ^{125}I -IGF-I, and the column volumes were defined using cobalt chloride and blue dextran (Sigma Chemical Co.). Chromatograph fractions were then removed and counted in a γ counter. Radioactive peaks noted between 40 and 60% (fractions 45–55) of the column volume were lyophilized for later assessment of receptor-binding activity.

Radioactivity of tissue homogenates, blood and urine was also assessed for TCA precipitability. Equal volumes of sample and 20% TCA were added to polyethylene tubes and incubated for 30 min at 21 – 23°C . Samples were centrifuged at 3000 rpm for 15 min at 4°C . Precipitates were washed with 10% TCA and centrifuged again for 15 min. The remaining precipitates were examined for radioactivity and the amount remaining as a percent of the initial counts/min was calculated.

RP-HPLC. Fractions from Sephadex chromatography representing peaks at approximately M_r 7,500 were lyophilized and reconstituted in 500 μ L of 50 mM Tris-HCl, 0.1% BSA. Specimens from the stomach and intestinal wall homogenates, as well as those from intestinal luminal contents, were injected into the HPLC column for analysis. A linear gradient of 5–60% acetonitrile against 0.1% trifluoroacetic acid, monitored at a flow rate of 1 mL/min for 30 min (column temperature, 40°C), was used to elute the samples from a VYDAC 18 column (The Separations Groups, Hesperia, CA) (5 μ m; 25 cm $\times$ 4 mm), as described previously (16). Elution of ^{125}I radioactivity was continuously monitored by a radioactivity flow detector (Radiomatic model A250, Flow One β , Tampa, FL) and recorded on a strip chart recorder. Radiolabeled IGF-I and -II were used as controls.

Receptor-Binding Studies. Human placental membranes (IGF-I studies) and rat liver membranes (IGF-II studies) were prepared, as previously described (17, 18). Human placental

membranes were used to assess IGF-I binding to type I IGF receptors. Because of the known preponderance of type II IGF receptors in this tissue (18), rat liver membranes were used to assess IGF-II binding. Membranes (400 μ g protein/tube) were incubated overnight at 4°C with 125 I-IGF, with or without an excess of unlabeled IGF-I (20 ng/mL) or -II (200 ng/mL), in 50 mM Tris-HCl, 0.1% BSA, pH 7.4. These concentrations were based on previous observations (our unpublished studies) documenting maximal suppression of labeled IGF binding to each membrane. Lyophilized "peaks" from six previously identified Sephadex G-50 chromatography samples were reconstituted in 50 mM Tris-HCl and 0.1% BSA and incubated with or without excess unlabeled stock IGF. After incubation, specimens were centrifuged at 6500 rpm for 30 min at 4°C. The precipitates were washed with 0.75 mL Tris and recentrifuged. All precipitates were then counted and percent of binding (counts/min remaining/initial counts/min added) calculated to examine differences in the percentage of receptor-binding activity between labeled IGFs and organ homogenate "peaks."

Statistical Analysis. All data are represented as mean $\pm$ SEM. Differences between IGF-I and -II studies and in receptor-binding studies were assessed using unpaired and paired *t* tests. Correlation of TCA precipitation studies with chromatography used linear regression analyses by the least squares method.

RESULTS

Recovery of Radioactivity. Suckling rats tolerated the administration of the radiolabeled IGF-milk mixture well ($n = 6$ for 125 I-IGF-I and $n = 6$ for 125 I-IGF-II, respectively). Figure 1 depicts the recovery of radioactivity from selected organs and blood of IGF-treated rats 30 min after administration. The highest activities were observed in stomach luminal contents of animals treated with either 125 I-IGF-I or -II. Overall, gastrointestinal tract tissues, including radioactivity present in stomach

Figure 1. Distribution of radioactivity to selected organs and blood in animals fed 125 I-IGF-I or -II 30 min previously. Results have been calculated as percent counts/min recovered of dose administered. ST-L, stomach lumen; ST-W, stomach wall; INT-L, intestinal lumen; INT-W, intestinal wall; LIV, liver; BR, brain; KID, kidneys; LU, lungs; BLD, whole blood; and SK, skin. 125 I-IGF-I in light hatched bars, 125 I-IGF-II in dark hatched bars. Data are presented as mean $\pm$ SEM, * = $p < 0.05$ between IGF-I- and -II-fed rats; $n = 6$ each group.

wall and lumen and small intestinal wall and lumen, contained 42.3 ± 5.0 and $38.4 \pm 2.2\%$ of the doses of 125 I-IGF-I and -II administered, respectively. These results were not statistically different from each other. Within these tissues the only significant differences between values of animals fed 125 I-IGF-I or -II were in the stomach wall ($11.7 \pm 0.9\%$ versus $7.5 \pm 1.2\%$, $p < 0.05$, respectively). IGF-I and -II were retained in the stomach (wall + lumen) at a similar level (26–27%). For both the IGF-I and -II studies, radioactivity present in stomach wall and luminal contents represented approximately 65% of the total gastrointestinal tract radioactivity overall. In other tissues, blood and skin radioactivity represented 4–7% of counts administered, with other individual organs having $<2\%$ of the dose administered per organ. Of these, only liver radioactivities were found to be statistically different between IGF-I and -II fed animals ($1.1 \pm 0.1\%$ versus $0.7 \pm 0.1\%$, $p < 0.05$, respectively). Urine radioactivity (not depicted) was $<0.05\%$ of the administered dose in all animals from which urine could be obtained. Total radioactivity recovered per animal in the organs studied was between 45 and 65% of the doses administered for both the IGF-I and -II experiments.

Gel Chromatography Studies. Extracts of selected organ homogenates were subjected to acid gel chromatography and the resulting fractions assessed for radioactivity. In general, four to six homogenates from gastrointestinal tract tissues and luminal flushings were examined. Only two to three homogenates from other organs were examined. Figure 2 shows a typical chromatograph of stomach wall homogenate from an 125 I-IGF-I-fed animal superimposed upon a chromatograph from a 5- μ L sample of 125 I-IGF-I milk mixture used for the feeding experiments. The patterns are similar with peak activity observed between 40 and 60% of the column volume (approximate M_r of 7,500 = "7.5 K" peak). Similar elution profiles were noted when 125 I-IGF-II was mixed with milk and subjected to chromatography. Small early peaks at fraction 30

Figure 2. Chromatograph of milk 125 I-IGF-I mixture (5 μ L) superimposed upon acid extract of stomach wall homogenates from 125 I-IGF-I-fed animals. Sephadex G-50, 0.1 M acetic acid, V_o = fractions 25–32, using blue dextran. Depending upon column used, 125 I-IGF eluted between 40 and 60% of column volume (between fractions 45 and 55).

are artifactual and are also apparent in some chromatographs in Figures 3 and 4. These most likely represent iodine or small peptide fragments bound to albumin and other large molecular weight proteins. Figure 3A–D shows representative chromatographs from gastrointestinal tract tissues of ^{125}I -IGF-I fed animals. In stomach wall, stomach lumen, and intestinal lumen, significant peaks of radioactivity can be seen, eluting in a position identical with native IGF-I. However, in both intestinal lumen and wall, peaks P₁ and P₂ of radioactivity, probably representing ^{125}I and ^{125}I -peptide fragments, are also apparent. Similar findings were noted in chromatographs from gastrointestinal tissues of animals fed ^{125}I -IGF-II (Fig. 4A–D).

The percentages of radioactivity present in the peaks designated as 7.5 K versus the total radioactivity per chromatograph were then calculated for both IGF-I and -II studies. Figure 5 shows that for each tissue or luminal content examined, significantly more radioactivity was present in the 7.5 K peaks for animals fed ^{125}I -IGF-I than for animals fed ^{125}I -IGF-II. Stomach wall and luminal content homogenates from animals fed ^{125}I -IGF-I had the highest percentage of activity in the 7.5 K peaks of any group ($69.5 \pm 7.4\%$ and $62.9 \pm 6.9\%$, respectively). Intestinal wall homogenates from animals fed ^{125}I -IGF-II were the only ones devoid of activity at the 7.5 K position.

Chromatographs from serum of IGF fed animals showed radioactive peaks consistent only with ^{125}I or ^{125}I -related small peptide fragments (data not shown). This was also true for all other samples tested, except skin. Skin homogenates from ^{125}I -IGF-I fed (but not ^{125}I -IGF-II fed) animals had a peak of radioactivity near, but not identical with, native IGF-I.

TCA precipitation of homogenate aliquots was also performed as an adjunct to the chromatography studies. The

percentage of TCA precipitation per homogenate was compared with the percentage of activity found in the 7.5 K peak per sample for all gastrointestinal tract and liver samples. Linear regression analyses revealed significant correlations between TCA-precipitable radioactivity and 7.5 K peak radioactivity for both ^{125}I -IGF-I and -II studies ($r = 0.79$, $p < 0.01$; and $r = 0.85$, $p < 0.01$, respectively).

RP-HPLC Studies. Figure 6A (IGF-I) and B (IGF-II) depict the distribution of radioactivity from material taken from the M_r 7,500 peaks identified from homogenates of stomach wall and intestinal lumen and wall using simple gel chromatography. As shown by arrows in Figure 6A and B, the peak identified comigrates with that of the control native radiolabeled IGF. Intestinal wall homogenates from IGF-II-fed animals had no demonstrable activity at M_r 7,500 either by gel chromatography or HPLC.

Receptor-Binding Studies. Lyophilized fractions from the 7.5 K peaks noted in Figures 3 and 4 were reconstituted in assay buffer (as described previously in "Methods") and analyzed for their ability to bind specifically to crude membrane preparations bearing IGF receptors. Representative binding studies from stomach wall extracts from rats fed ^{125}I -IGF-I or -II are shown in Fig. 7A and B. Binding of radioactivity from the 7.5 K peak recovered from IGF-I fed animals was identical to that of ^{125}I -IGF-I (Fig. 7A) using human placental membranes. In addition, an excess of unlabeled IGF-I inhibited binding by 50% of both homogenate radioactivity and ^{125}I -IGF-I. Similar findings were observed for extracts in the IGF-II experiments using rat liver membrane receptor in the presence or absence of unlabeled IGF-II (Fig. 7B). Similar results were obtained using peaks obtained from stomach and intestinal luminal contents for IGF-I and -II experiments (not shown).

Figure 3. A–D, Representative of four to five chromatographs (Sephadex G-50, 0.1 M acetic acid) of extracts of ^{125}I -IGF-I fed rats. A, Stomach lumen; B, stomach wall; C, intestine lumen; D, intestine wall. Peaks P₁ and P₂ represent ^{125}I and ^{125}I -IGF fragments, respectively.

Figure 7. Specific binding of radioactivity from stomach wall homogenates (7.5 K peak) from ^{125}I -IGF-I (A) or -II (B) fed animals contrasted with binding of native ^{125}I -IGF-I or -II (tracer) with or without excess of unlabeled IGF-I (20 ng/mL) or -II (200 ng/mL). Asterisk denotes significant effect of the excess of cold IGF-I or -II.

high-affinity binding sites for IGF (28) and some differential binding between IGF-I and -II exists. Binding of IGF-II to IGF-binding proteins in rat milk is roughly twice as great as that of IGF-I (15). The presence of IGFBPs may also affect the kinetics of absorption and/or degradation of IGFs in the neonatal gut. However, in the doses administered in our present experiments, more than 75% of IGF administered was not bound to carrier proteins in the ingested milk-IGF mixtures.

Absorption of biologically active IGFs to organs distant to the gut could not be documented, and whether or not absorption and transport had already occurred before the conclusion of the experiments is unknown. However, the finding of large molecular weight fragments of IGF-I in neonatal rat skin is intriguing and warrants future studies, particularly in view of a previous finding of enhanced skin epithelial maturation in rat pups injected with a similar recombinant IGF-I preparation (29).

In summary, radiolabeled IGF-I and -II were delivered orogastrically with milk to the newborn rat. Both IGF-I and -II were recovered in potentially biologically active forms 30 min postingestion in the gastrointestinal luminal contents and tissues, but not from organs distant to the gut. We speculate that milk-derived IGF may serve a regulatory function to stimulate stomach and intestinal growth and differentiation in the newborn.

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