Effect of Short-Term Fasting/Refeeding on Epidermal Growth Factor Content in the Gastrointestinal Tract of Suckling Rats (43332)

J. GRIMES,* P. SCHAUDIES,† D. DAVIS,* C. WILLIAMS,* B. J. CURRY,† M. D. WALKER,† AND O. KOLDOVSKÝ*,[1]

Departments of Pediatrics and Physiology and Children's Research Center, The University of Arizona, Tucson, Arizona 85724 and Department of Nephrology,† Walter Reed Army Institute of Research, Washington, DC 20307-5100*

Abstract. **Epidermal growth factor (EGF) is trophic for varying regions of the developing gastrointestinal tract (GIT) of suckling rats. The presence of large amounts of EGF in milk from various species, combined with low production of EGF by suckling animals, led to speculation that milk is a major source of EGF for suckling rats. We report that short-term fasting (8 hr) of 12-day-old suckling rats resulted in a significant decrease in the levels of immunoreactive EGF (irEGF) in the GIT. Pups refed by lactating mothers for 1 to 4 hr exhibited an increase in irEGF to original levels, whereas pups fed a rat milk substitute by gastric gavage did not have an increase in irEGF content. The irEGF levels in the GIT of pups that were manually fed normal rat milk, or rat milk substitute supplemented with EGF, returned to the prefasted levels. Fasted suckling rats refed 2 ml of rat milk in 2 h exhibited significantly higher level of irEGF in the GIT than did those refed with 0.5 ml in 45 min. Since rat milk irEGF exists in three distinct forms (A, B, and C; C is equal to authentic submandibular gland EGF, the irEGF forms in the GIT were characterized by native polyacrylamide gel electrophoresis. In the stomach luminal contents of the fed suckling rats, only the larger form, Peak B, was observed. Both the luminal content and the mucosa scrapings of all other segments of all groups contained only Form D (comigrating with desarginyl EGF), a metabolic derivative of EGF. All forms were immunoreactive, exhibited receptor binding, and stimulated DNA synthesis in growth-arrested fibroblasts. The rapid changes in EGF within the GIT of suckling rats suggest the EGF can acutely modify some GIT functions of suckling rats.**

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In adult mammals, epidermal growth factor (EGF) is produced mainly in the submandibular and Brunner's glands and kidneys (1–3). The production of EGF in suckling mammals is considered to be very low. Popliker *et al.* (4) reported the absence of EGF mRNA in suckling mice; others described only very small amounts of EGF present in the submandibular glands of suckling rodents (1, 5–7). EGF is known to play an important regulatory role in mammals; many studies suggest that EGF is a trophic factor for various regions of the developing gastrointestinal tract (8–17) and the liver (18, 19).

The presence of considerable amounts of EGF in the milk of various species (20), together with the known low proteolytic activity in the gastrointestinal tract of newborn mammals (21), led to speculation that milk may be a major source of EGF for the suckling mammal (20). In the preceding studies, we have shown that ^{125}I-mEGF is absorbed from the gastrointestinal tract of suckling rats and delivered to several peripheral organs in forms that are immunoreactive and bind to EGF receptors (22). Characterization of the differences between mouse and rat EGF (rEGF), the availability of rEGF, and the development of a homologous radioimmunoassay (23) enabled us to demonstrate that the small intestine of suckling rats contains amounts of immunoreactive EGF (irEGF) that are much higher than those in adult rats (24). Furthermore, we have found that in contrast to adult rats, overnight (18 hr) fasting in suckling rats led to a considerable decrease of

[1] To whom correspondence and requests for reprints should be addressed at Department of Pediatrics, University of Arizona, 1501 N. Campbell Ave., Tucson, AZ 85724.

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irEGF levels in the small intestine; these values were lower than those in adult rats.

Experiments reported in this communication were, therefore, designed to characterize the time dependency of the disappearance of irEGF from the gastrointestinal tract of suckling rats and time and dose dependency of EGF reappearance. To demonstrate that the appearance of irEGF depends on the intake of irEGF in the diet, we fed rats a rat milk substitute (RMS) (25) containing either no irEGF or one to which rat submandibular gland EGF was added. Since our recent study has shown the presence of EGF in rat milk in three distinct immunoreactive, receptor active, and biologically active forms (6), we studied irEGF forms in the gastrointestinal tract of suckling rats in which the oral intake of irEGF was manipulated. Part of the data were presented at the Society of Pediatric Research in 1990 (26).

Materials and Methods

Rats. Suckling Sprague-Dawley rats born in our animal colony, culled on Day 2 postnatally to 10 per litter, were used on Day 12. Two periods of fasting were used—2 and 8 hr. In all experiments in which the pups were without the mother, they were kept in plastic cages with bedding (Aspen Sannichips; P. J. Murphy Forest Products, Montvilla, NJ). These cages were placed with half of their bottom on electric heating pads to help the pups keep their normal body temperature (27). Since the 2-hr period gave irregular responses of irEGF levels, we restricted our fasting studies to 8 hr only. Animals originating from the same litters were always assigned to at least three groups. The groups were as follows: (i) SUC, animals were taken directly from the mother (n/group = 7); (ii) FAST, animals were fasted 8 hr (n = 20); (iii) RESU, animals were fasted 8 hrs and then they suckled a rat mother between 60 and 240 min (n = 11) (The mother had not been used previously in any experiments to ensure a normal milk supply. Their sucklings were removed 6 hr before using them in this experiment). The last four groups after 8 hr of fasting were fed a rat milk or a rat milk substitute via an orogastric tube (6). (iv) RMS, animals were fed a rat milk substitute of 0.5 ml or 2 ml for 45 or 120 min. Since EGF data were similar, both groups were combined (n = 8). Lo-M, rats were fed 0.5 ml of rat milk and sacrificed 45 min later (n = 4). (vi) Hi-M, rats were fed 2.0 ml of rat milk and sacrificed 120 min later (n = 5). (vii) EGFRMS, rats were fed 2.0 ml of RMS (divided into four doses) and sacrificed 120 min later (n = 5). All pups were kept in plastic cages and placed in a 38°C infant incubator during the experiments to enable them to maintain normal body temperature.

The pooled rat milk sample (see below) and the RMS with and without added rat EGF were assayed by radioimmunoassay (RIA) for irEGF (see below) and the following amounts of EGF were detected: 40 ng/ml, 3 ng/ml, and 29 ng/ml, respectively; the EGF milk value agrees very well with our previous report (6). Sex and body weight (at the beginning of the experiment, after fasting, and after eventual refeeding) were recorded. Urination and defecation was stimulated by gentle stroking of the anogenital area with a wet, warm, soft paper tissue before weighing. The animals were sacrificed by decapitation; the stomach and small intestine were then quickly taken out. The small intestine was separated into duodenum (from pylorus to the ligamentum of Treitz) and three segments of equal length, referred to as proximal, middle, and distal third. Each segment was flushed with cold double-distilled water (2 ml per segment) into plastic tubes. The tissues were then split open and the mucosa was scraped into a previously weighed tube using a metal spatula. All tubes were reweighed and stored at −20°C before further processing. Within 2 weeks, tissues were homogenized in 10 vol of 0.05 M acetic acid using a Polytron (Speed 8; Brinkman, Rexdal, Ontario, Canada) for 30 sec and then spun at 106,000g at 4°C for 30 min; the supernatant was lyophilized for RIA determination. The volume of the material flushed from the digestive tract was measured in volumetric test tubes to the nearest 0.1 ml and 0.25 M acetic acid was added to a final concentration of 0.05 M. The mixture was homogenized for 10 to 15 sec and centrifuged as above. Recovery of immunoreactive rat EGF by this procedure was determined to be in excess of 80% for all tissues by the addition of ^{125}I-rEGF prior to the initial homogenization. Flushes and tissues were analyzed for EGF by RIA.

Collection of Rat Milk. Lactating Sprague-Dawley rats (Day 12 of lactation) were separated from their pups 4-6 h prior to milking, anesthetized intramuscularly with 200 μl/kg of Inovar-Vet (Pitman Moore, Inc., Washington Crossing, NJ), and subsequently injected intraperitoneally with 2 units of oxytocin (Sigma Chemical Co., St. Louis, MO). Each nipple was manually massaged and the milk was expressed directly into Eppendorf tubes. Care was taken not to injure the nipples. Each nipple was expressed two to three times, and approximately 2–4 ml of milk were obtained from each dam. Each lactating rat was used as a milk donor only once and retired from breeding. Rat milk from several mothers was then pooled, frozen in aliquots, and used after gentle thawing at 37°C in the experiments.

Preparation of Submandibular rEGF. Rat EGF was isolated from the submandibular glands of adult male rats and purified rEGF was radioiodinated using a chloramine-T method to a sp act of 150–200 μCi/μg, as described previously (23). Concentrations of standard rEGF were determined using an extinction coefficient A_{280} of 1.0 for a 1% solution in water. Antiserum

P-1 was generated in rabbits as described previously (23) and purity of antigen was >95%, as determined by gel electrophoresis and amino acid analysis.

Radioimmunoassay. EGF equivalents were determined using a homologous RIA (23). The assay has a sensitivity of 25 pg submandibular rEGF per tube at an antibody titer of 1/1,500,000. Experimental samples, or known amounts of purified rEGF, were added to tubes containing 10,000 cpm of ^{125}I-rEGF and RIA buffer, and then vortexed. Antiserum P-1 was added to a final dilution of 1/1,500,000 and was followed by vortexing (final volume, 0.5 ml). Tubes were incubated at room temperature for 1 hr and was followed by a 16-hr incubation at 4°C. One-half milliliter of 20% human plasma (citrate anticoagulated) in RIA buffer was added to each tube and was followed by the addition of 1.0 ml of 20% polyethylene glycol 8,000 mol wt (w/v in water). The tubes were vortexed and incubated on ice for 30 min. Following centrifugation at 2,000*g* for 15 min at 4°C, the supernatants were aspirated and the pellets were monitored for ^{125}I using a LKB-1442 compugamma counter equipped with an RIA calculating program. All samples were assayed in duplicate. All duplicate RIA values agreed within less than 10%.

Polyacrylamide Gel Electrophoresis. EGF was extracted from tissues, luminal contents, and milk, as described above. The extract was affinity purified and subjected to nondenaturing polyacrylamide gel electrophoresis to determine the rates of migration of the immunoreactive species by the method of Davis (28), with slight modifications as described (23). Preparation of desarginyl standard rEGF was conducted by incubation with carboxypeptidase B, as described by Schaudies *et al.* (23). Following electrophoresis, lanes were separated and cut into 3.0-mm slices, which was followed by overnight extraction in 1.0 ml of RIA buffer. Each extract was assayed for immunoreactive material by RIA, as above, in duplicates using 350 μl of extract. A standard rEGF (2 ng) was run in a separate lane on each gel.

A statistical evaluation was performed using analysis of variance followed by Fisher PLSD (statistical program Statview for Macintosh computers; Abacus Concepts, Inc., Berkeley, CA); a *P*-value of <0.05 was considered statistically significant.

Results

General Description. The average body weight of fed, suckling, 12-day-old rats used in this study was 29.9 ± 0.35 g (mean ± SE, $n = 60$). After 8 hr of fasting, their body weight decreased to 28.5 ± 0.35 g ($n = 53$); the average body weight loss was 1.23 ± 0.06 g ($n = 53$). The increase in body weight following refeeding is shown in Table I. Rats fed 0.5 ml of RMS and the Lo-M group exhibited a small increase in body weight corresponding to the smaller amount of RMS or milk fed as compared with all other groups receiving a larger volume of milk or RMS. The RESU group exhibited similar body weight gains as those of rats fed orogastrically 2-ml vol.

Table I. Body Weight Gains in Fasted Rats after Refeeding

Group[a]	Volume given (ml)	*n*	Mean ± SE (g)	Note[b]	EGF[c]
RESU		11	1.47 ± 0.21		
RMS	0.5	3	0.30 ± 0.06	a	1.5
RMS	2.0	5	1.74 ± 0.05	b, c	6.0
Lo-M	0.5	4	0.55 ± 0.14	a	20.0
Hi-M	2.0	5	1.56 ± 0.13	b, c	80.0
RMSEGF	2.0	4	1.94 ± 0.13	b, c	58.0

[a] Group names are defined in Materials and Methods.
[b] a, statistically significant difference from the RESU; b, statistically significant difference from the RMS 0.5; and c, statistically significant difference from the Lo-M group (analysis of variance; $P < 0.0001$).
[c] Calculated amount of irEGF given orogastrically.

irEGF in the Gastrointestinal Tract: Quantitative Studies. *Content in the lumen.* Results are summarized in Table II (stomach and duodenum) and Figure 1A (jejunoileum). Eight-hour fasting led to a considerable decrease (<70%) of irEGF in the lumen of the entire gastrointestinal tract (except the duodenum, in which the total amounts were also the smallest). Pups that were returned to lactating mothers for a period lasting between 60 and 240 min exhibited a return to prefasting values. The values of pups refed by gastric gavage with 0.5 ml of RMS (0.5 ml for 45 min or 2 ml for 2 hr) did not differ from those of fasting controls. Rats refed with milk or RMS supplemented with EGF exhibited a significant increase in EGF content in the lumen; the exception was ileum of the Lo-M group (in the duodenum, the only significant change (increase) was seen in the Hi-M group). A high rat milk dose (2 ml, 2 hr) caused a significantly higher increase than did the low dose (0.5 ml, 45 min), with the exception of duodenum (see Table II).

Content in the wall. Results are summarized in Table II (stomach and duodenum) and Figure 1B (jejunoileum). The contents in the stomach wall and duodenal mucosa were very small in comparison to that in the jejunoileum mucosa. Interestingly enough, in all groups refed after fasting, the values in the stomach wall were significantly lower. No significant changes were seen in the duodenal mucosa among the groups; changes in jejunoileal mucosa paralleled those in the jejunoileal lumen.

irEGF in the Gastrointestinal Tract: Qualitative Studies. In agreement with our previous report (6), rat milk exhibited the presence of three distinct immunoreactive forms (A, B, and C). Figure 2 demonstrates that in the stomach luminal content of fed suckling rats taken directly from the mother, only the larger form

Table II. irEGF (Mean ± SE; ng/organ) in the Stomach Wall and Lumen and the Duodenal Mucosa and Lumen

Groups[a]	*n*	Stomach		Duodenum	
		Wall	Lumen	Mucosa	Lumen
SUC	7	0.244 ± 0.098	6.010 ± 0.422[b]	0.283 ± 0.087	0.292 ± 0.153
FAST	20	0.414 ± 0.053	1.324 ± 0.241	0.065 ± 0.020	0.165 ± 0.068
RESU	11	0.142 ± 0.065[b]	6.284 ± 0.536[b]	0.059 ± 0.042	0.312 ± 0.068
RMS	8	0.102 ± 0.029[b]	1.117 ± 0.169	0.109 ± 0.078	0.074 ± 0.030
Lo-M	4	0.112 ± 0.066[b]	2.668 ± 0.166	0.000 ± 0.000	0.279 ± 0.113
Hi-M	5	0.138 ± 0.017[b]	4.960 ± 0.326[b,c]	0.234 ± 0.090[c]	1.198 ± 0.354[b,c]
EGFRMS	5	0.180 ± 0.020[b]	14.31 ± 0.853[b]	0.738 ± 0.738	0.310 ± 0.070
ANOVA (*P*)		0.0014	0.0001	NS[d]	0.001

[a] Group names are defined in Materials and Methods.
[b] Statistically significant difference from FAST group.
[c] Statistically significant difference between Lo-M and Hi-M groups.
[d] NS, not significant.

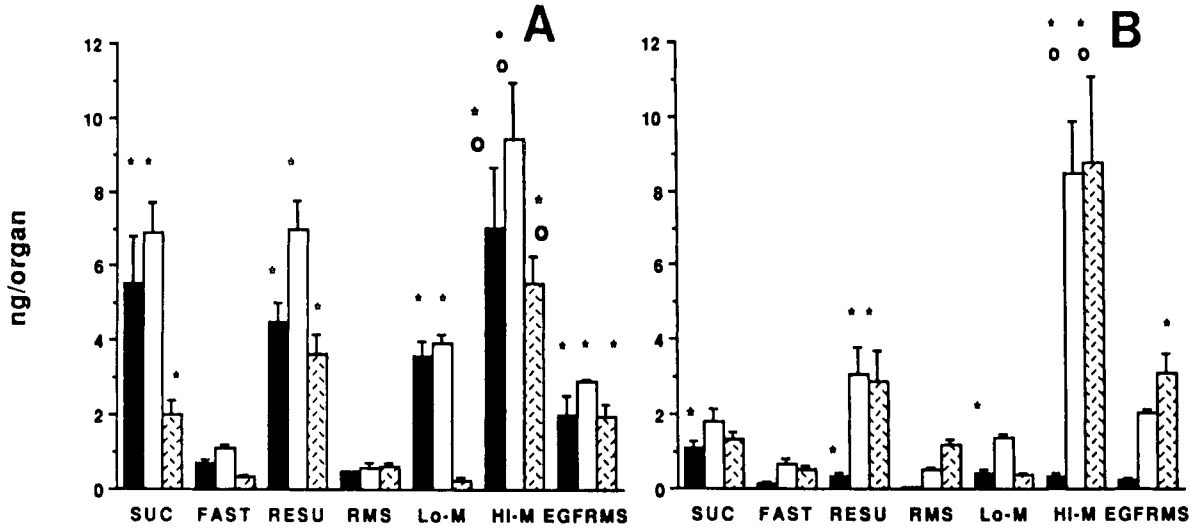

Figure 1. Effect of orogastric intake on amount of irEGF detected in the (A) lumen and (B) mucosa of jejunoileum of suckling rats. Vertical bars denote mean ± SE. SUC, animals taken directly from the mother (n = 7); FAST, fasted 8 hr (n = 20) (all other animals were fasted 8 hr and then refed); RESU, suckling a rat mother between 60 and 120 min (n = 11); RMS, fed rat milk substitute, 0.5 ml or 2 ml for 45 or 120 min (since EGF data were similar, both groups were combined) (n = 8); Lo-M, rats fed 0.5 ml of rat milk and sacrificed 45 min later (n = 4); Hi-M, rats fed 2.0 ml of rat milk and sacrificed 120 min later (n = 5); and EGFRMS, rats fed 2.0 ml of RMS and sacrificed 120 min later (n = 5). Full, open, and hatched bars: jejunum, midjejunum, and ileum, respectively. Significant differences: *, from fasted rats; O, between Lo-M and Hi-M.

(Peak B) was found. The luminal content and mucosa of the small intestine contained in all segments only Form D comigrating with desarginyl EGF (6). In agreement with the preceding study (6), all forms (A, B, C, and D) exhibited EGF receptor binding and stimulated DNA synthesis in growth-arrested fibroblasts. In all treatment groups, similar electrophoretic patterns were seen, as in control-fed suckling rats; an exception was the content of stomach lumen in the 8-hr fasting group. In this case, Form B represented 40% and newly appearing authentic rEGF (Form C) and desarginyl (Form D) represented another 40% and 20% of the total immunoreactive material, respectively, indicating partial proteolysis in the stomach of the sucklings.

Discussion

This is the first study that explores the fate of native EGF after orogastric administration in the gastrointestinal tract of suckling rats. Previous studies demonstrating the "survival" of orogastrically administered EGF in suckling rats followed the fate of ^{125}I-labeled mouse EGF. Our present results demonstrated two major facts: (i) the dependency of EGF content in the gastrointestinal tract on EGF intake, and (ii) qualitative changes of milk-borne EGF in the gastrointestinal tract of the suckling rats.

It is noteworthy that the amount of irEGF present in the lumen of the entire gastrointestinal tract of fed rat (20.8 ± 2.0 ng) corresponds to that present in 0.5 ml of rat milk (40 ng/ml); the entire gastrointestinal mucosa contained the equivalent of about 0.15 ml of milk. Short-term fasting (2 hr) resulted in some significant decrease of EGF content, but the results were inconsistent, thus, the 2-hour fasting period could not be reliably used as a start for the refeeding studies.

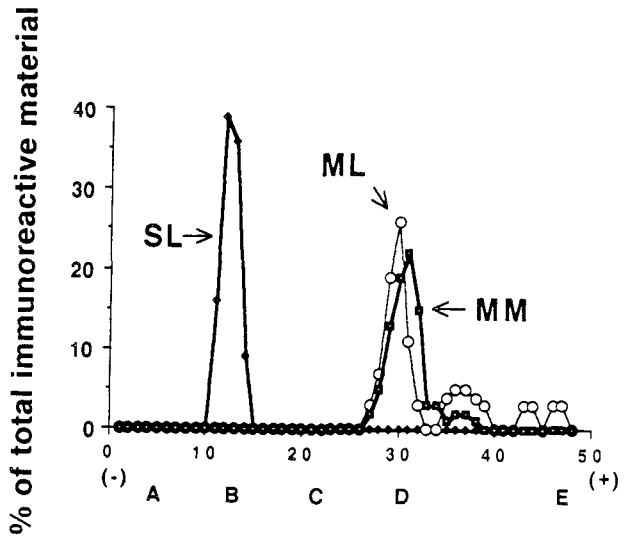

Figure 2. Native polyacrylamide gel electrophoresis of extracts of stomach luminal content (SL), middle segment luminal content (ML), and mucosa (MM). Vertical axis, percentage of the total immunoreactive material recovered from the gel per slice. Horizontal axis, number of slices. Direction of migration is from left to right. A and B, the migration rate of larger forms of EGF present in milk; C, the migration rate of submandibular rEGF (SG-E); D, the migration rate of desarginyl EGF (dE); E the location of the dye front. *Note:* Results obtained with preparations of duodenum, jejunum, and ileum segments were similar to those of the midjejunum.

Longer fasting (8 hr) led to a considerable decrease; values of the irEGF at this time period were similar to those reported earlier after an 18-hr fast (24). The increase in EGF content after refeeding did not depend on caloric intake, since RMS (containing only a little EGF immunoreactive material) led to no changes; only rat milk and RMS supplemented with EGF evoked the expected increase in the gastrointestinal EGF content. Comparison of the levels in the Hi-M and Lo-M groups showed that the changes in irEGF values were dose dependent. We have no explanation for the unexpected lower values in the stomach wall in all refed groups. The higher content of irEGF in the EGFRMS group, as compared with the Hi-M group, was also unexpected. Semi-quantitive observations showed larger amounts of "milky" material to be found in the stomach of the EGFRMS group, thus indicating delayed stomach emptying. The higher irEGF values in the stomach were then accompanied by lower irEGF values in the jejunoileal lumen and mucosa. Further studies are needed to evaluate this phenomenon. Nevertheless, we consider noteworthy that although the total amount of irEGF detected in the entire gastrointestinal tract in the EGFRMS group was about 25% lower than that in the corresponding amount of milk (27.7 ± 1.7 ng vs 37 ± 2.3 ng), it was the same when corrected per the amount of irEGF detected in rat milk (40 ng/ml) and EGFRMS (29 mg/ml), respectively.

The other important aspect of this study is that the EGF present in milk in different forms (6, 29) than those in the submandibulary glands (6, 22, 29) undergoes further processing in the gastrointestinal tract of suckling rats. The form detected in the stomach is larger than the submandibular gland EGF, whereas in the intestinal lumen and mucosa, the smallest form (mainly the desarginyl form of EGF) is found. All these forms (6) are immunoreactive, bind to receptors, and exhibit a biological activity in a bioassay. Further studies are needed to characterize the significance of the gastrointestinal processing of milk-borne EGF.

We consider important the fact that the EGF content in the small mucosa responds in a rapid manner to the oral intake; therefore, we postulate that EGF can acutely modify some gastrointestinal functions of suckling rats.

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