Hormone Regulation of Choline Uptake and Incorporation in Mouse Mammary Gland Explants

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Choline is a nutrient in milk that is essential for the nourishment and growth of newborns. In rat milk, choline is present in concentrations that are more than an order of magnitude higher than in maternal serum. Using cultured mammary tissues taken from 12–14-day pregnant mice, the effects of the three primary lactogenic hormones—prolactin (PRL), insulin (I), and cortisol (H)—on choline uptake and incorporation into lipids were determined. By itself or in the presence of H and/or PRL, I was the only hormone that increased the accumulation of choline in aqueous tissue fractions. In contrast, PRL, when present with I plus H, was the only hormone that stimulated the incorporation of choline into the lipid fraction of tissues. Choline uptake was found to be sodium and time dependent; maximum distribution ratios >18 were achieved after a 6-hr uptake time. In kinetic studies the apparent K_m for choline uptake was calculated to be ~2.7 m*M*, whereas the V_{max} was 7.4 m*M* intracellular water per 30 mins. These results suggest the existence of a sodium-dependent active transporter for choline in the mouse mammary gland that is specifically stimulated by I. PRL, in contrast, only stimulates the incorporation of choline into lipids. Exp Biol Med 229:323–326, 2004

Key words: prolactin; cortisol; insulin; mammary gland; choline

Milk is composed of a plethora of nutrients that are required for the nourishment and growth of newborns. Some of these nutrients are assembled in the alveolar epithelial cells of the mammary gland and subsequently secreted into the alveolar lumen. Other milk constituents are directly taken up into the alveolar lumen from the maternal plasma by various transport processes. Choline is an essential component in milk that is taken up by both processes (1–4). The choline contained in milk is used by the newborn for the formation of acetylcholine as well as phosphatidylcholine, sphingomyolin, and choline plasmalogens, all of which are constituents of cellular membranes. A majority of the choline in milk is secreted in the form of phosphatidylcholine, whereas ~10% of choline in rat's milk appears as free choline (2). The free choline concentration in rat milk is more than an order of magnitude higher than in the maternal plasma, which suggests that choline is taken into the alveolar lumen via an active transport mechanism (2); further studies have suggested that the choline transporter is sodium dependent (2).

Three lactogenic hormones—insulin (I), cortisol (H), and prolactin (PRL)—are required to stimulate milk product synthesis in cultured mouse mammary tissue (5). Using this model system, we aimed to determine and characterize the effects of these hormones on choline uptake and incorporation.

Materials and Methods

Midpregnant (12–14 days of pregnancy) Swiss-Webster mice were used in all experiments: they were purchased from Harlan Laboratories (Indianapolis, IN). Ovine PRL (National Institutes of Health PS-19) was a gift from the National Institutes of Health. Other substances were purchased from the following sources: H from Charles Pfizer (New York, NY); choline chloride, Tris, Hanks's balanced salt solution (HBSS), and medium 199-Earle's salts from Sigma Chemical Co. (St. Louis, MO); [^{3}H]OH, [^{3}H]-inulin, and [^{3}H]-choline from New England Nuclear (Boston, MA); and porcine insulin, penicillin, and streptomyocin from Eli Lilly (Indianapolis, IN).

Explants of mouse mammary tissues were prepared and cultured as described elsewhere (5). The explants were cultured on siliconized lens paper floating on 6 ml of medium 199-Earle's salts. In specified experiments hormones were present at the following concentrations: 1 μg/ml I and 10^{-7} *M* or 1 μg/ml PRL; all incubations were carried out in 60 × 15-mm petri dishes maintained at 37°C in an atmosphere of 95% O_2–5% CO_2. In experiments where the effects of hormones

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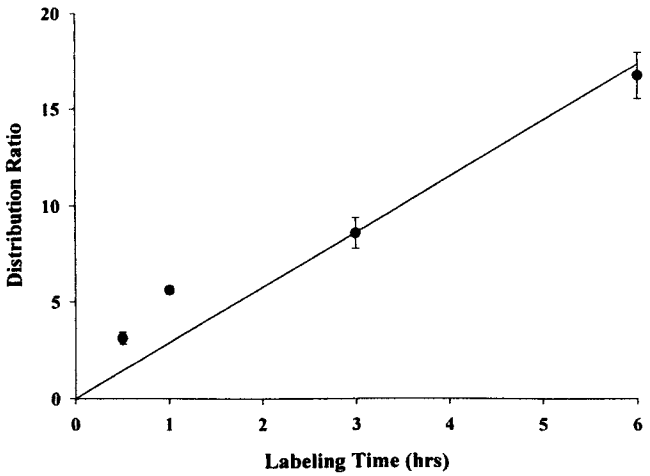

Figure 1. Time course for [^{3}H]-choline accumulation in cultured mammary gland explants. Explants were cultured for the times indicated with 0.5 μCi/ml [^{3}H]-choline (0.1 μ*M*) in HBSS. The intracellular accumulation of [^{3}H]-choline in a 5% TCA-soluble tissue fraction is expressed as a distribution ratio of the mean ± SE of six observations.

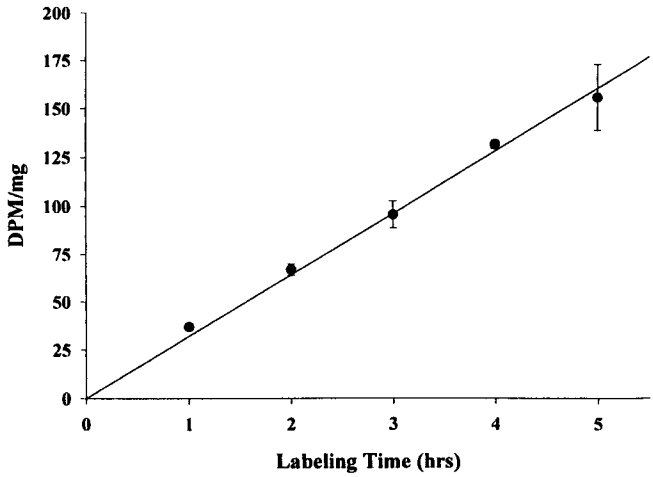

Figure 2. Time course for [^{3}H]-choline incorporation into lipids in cultured mammary gland explants. Tissues were treated as described in Figure 1, except that the incorporation of [^{3}H]-choline into the lipid fraction was determined. Results are the mean ± SE of six observations.

on [^{3}H]-choline uptake and incorporation were to be determined, the tissues were transferred to vessels that contained [^{3}H]-choline (0.5 μCi/ml, 0.1 μ*M*–5 m*M* in 6 ml of HBSS); incubations were carried out for specified times in a rotary water bath at 37°C (120 cycles/min). The tissues were then weighed and homogenized in 4 ml of 5% trichloroacetic acid (TCA). One-milliliter aliquots of the TCA homogenates were used to assess the extent of [^{3}H]-choline incorporation into lipids (6). The remaining homogenates were centrifuged at 2000 *g* for 10 mins. Radioactivity in 1-ml aliquots of the TCA-soluble fraction was determined by liquid scintillation techniques. The intracellular accumulation of radiolabeled, unincorporated [^{3}H]-choline was calculated by subtracting the amount of radiolabel in the extracellular space from the total TCA-soluble radioactivity in the tissue homogenates (5). For these calculations, the total water content (51.0%) and extracellular space (24.6%) were determined by the volume of distribution of [^{3}H]OH and [^{3}H]inulin (1 m*M*), respectively, in tissues maintained in culture for 24 hrs. In time-course studies, equilibrium was achieved with [^{3}H]OH and [^{3}H]inulin by 15 mins after their addition. Results of the choline uptake studies are expressed as a distribution ratio of the intracellular specific activity divided by the extracellular specific activity of the radiolabeled choline. The results of the lipid incorporation studies are expressed as DPM/mg wet weight of tissues.

Statistical comparisons were made with Student's *t* test when two means were compared or an analysis of variance followed by Dunnet's test for multiple comparisons. Means were considered to be significantly different (*) when $P < 0.05$. Results are expressed as the mean ± standard error (SE). In each experiment, tissues from 16–20 animals were pooled; each experiment was repeated at least 2 times.

Results

Figures 1 and 2 show the time courses over a 6-hr period for the uptake of [^{3}H]-choline and its incorporation into lipids; both time courses are linear. A distribution ratio >18 was achieved during a 6-hr uptake period (Fig. 1); this is compatible with the suggestion that choline is taken up into alveolar epithelial cells via an active transport mechanism.

The experiments in Figures 3 and 4 were done to assess the effects of lactogenic hormones on choline uptake and incorporation into lipids in mammary tissues. Of the three lactogenic hormones tested, only I stimulated the accumulation of [^{3}H]-choline into the TCA-soluble fraction of tissue homogenates; H and PRL, by themselves, were without effect (Fig. 3). In the lipid-incorporation studies, the only hormone combination that increased [^{3}H]-choline into lipids was PRL with H and I; none of the lactogenic hormones alone or in combinations of two hormones had an effect on the incorporation of [^{3}H]-choline into lipids (Fig. 4).

In a kinetic study (Fig. 5) the apparent K_m of [^{3}H]-choline accumulation in the TCA-soluble fraction was calculated to be ~2.7 m*M*, whereas the V_{max} was 7.4 m*M* intracellular water/30 mins. The sodium dependence of [^{3}H]-choline uptake and incorporation into lipids was determined in the experiments presented in Figures 6 and 7. In these experiments, Tris was substituted for sodium to maintain the proper osmolality in the sodium-free medium. Clearly, the absence of sodium decreased both [^{3}H]-choline uptake as well as its incorporation into lipids (Fig. 7). The magnitude of the prolactin effect on [^{3}H]-choline incorporation, however, was maintained in the absence of sodium (Fig. 7).

Discussion

Milk is made up of a plethora of substances that are present within relatively confined concentrations. Some of

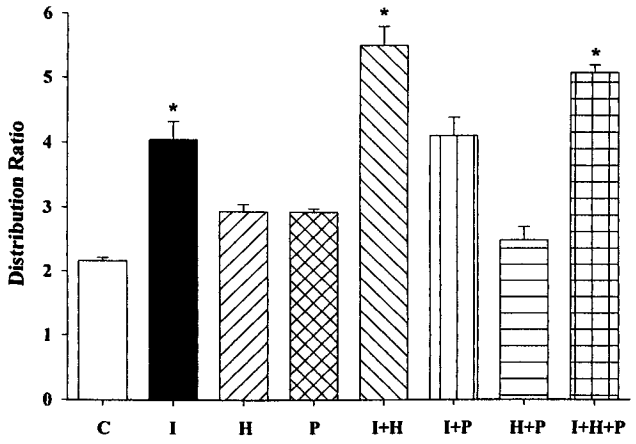

Figure 3. Effect of lactogenic hormones on [^{3}H]-choline uptake into cultured mammary gland explants. Tissues were cultured for 1 day with medium that contained no hormones. Culture was then continued for 1 day with no hormones (C) or with I (1 μg/ml), H (10^{-7} *M*), PRL (1 μg/ml), and all combinations of these hormones. After a 30-min incubation with 0.5 μCi/ml [^{3}H]-choline (0.1 μ*M*) in HBSS, the intracellular accumulation in a 5% TCA-soluble tissue fraction was calculated and expressed as a distribution ratio. Numbers represent the mean ± SE of six observations. *Greater than control with $P < 0.05$.

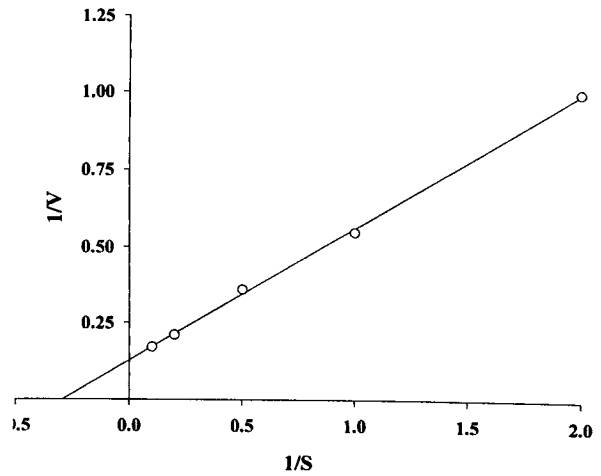

Figure 5. Line weaver Burke Plot for [^{3}H]-choline uptake in cultured mammary gland explants. Tissues were treated with I + H or I + H + PRL, as described in Figure 4. The tissues were then incubated for 1 hr with 0.5 μCi/ml [^{3}H]-choline at the concentrations indicated. The reciprocals of the substrate concentrations and intracellular choline accumulations were then plotted. Data from the two treatment combinations were not different and were therefore pooled. The SE of each number ($n = 8$) was <5% of the mean.

these substances—such as lactose, the caseins, α-lactalbumin, and the triglycerides—are synthesized from precursors taken up from the maternal plasma. Other substances are taken up into milk via special transport processes present in the alveolar epithelial cells of the mammary gland; examples of these types of milk components include sodium, potassium, chloride, phosphate, iodide, calcium, citrate, choline, carnitine, glucose, amino acids, and pyrimidine nucleosides (7–11). Specific transporters for choline have been identified in isolated epithelial cells taken from rat mammary glands (2). These transporters were reported to be sodium dependent, with an apparent K_m of ~0.035 m*M*. In other tissues, such as the intestine, choline is transported via a sodium-independent mechanism (12). Our results clearly support those of earlier studies in which sodium-dependent choline transporters were identified in rat mammary epithelial cells. The apparent K_m for the choline transporter, however, was quite different in our studies (2.7 m*M*) than in earlier studies (0.035 m*M*). The model systems employed (explants vs. isolated cells), the species used (mice vs. rats), and/or the physiological state of mammary cell source (midpregnant vs. lactating animals) may all have contributed to the appreciable difference in K_m values. It has been pointed out by Millar *et al.* (13) that tissue explants have a much larger extracellular space and, consequently, larger

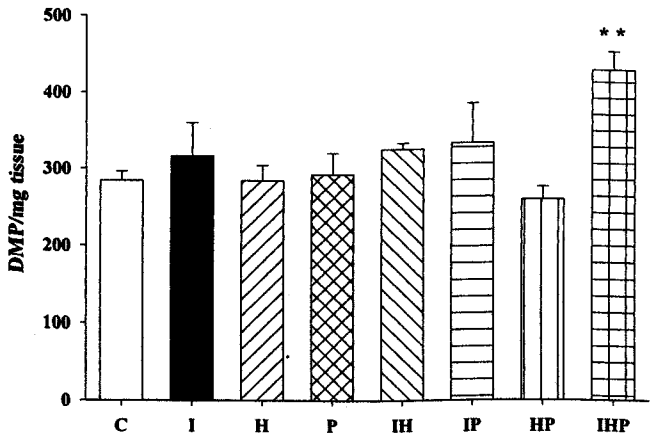

Figure 4. Effect of lactogenic hormones on the incorporation of [^{3}H]-choline into lipids. Experimental details are the same as described in Figure 3. Numbers represent the mean ± SE of six observations. *Greater than controls with $P < 0.05$.

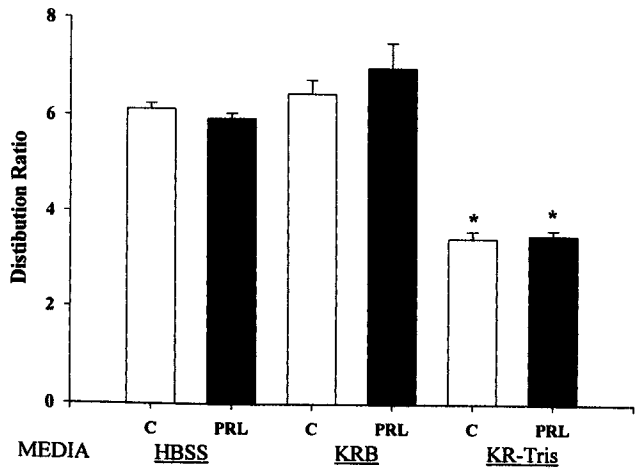

Figure 6. Sodium dependence of [^{3}H]-choline uptake into cultured mammary gland explants. Mammary tissues were cultured for 1 day with I + H. Incubation was then continued for 24 hrs with or without PRL. During a subsequent 30-min incubation, [^{3}H]-choline was present in HBSS, Kreb's Ringer bicarbonate buffer (KRB, containing sodium), or KRB that contained Tris substituted for the sodium chloride (KR-Tris). Results are expressed as the mean ± SE of six observations. *Less than comparable control with $P < 0.05$.

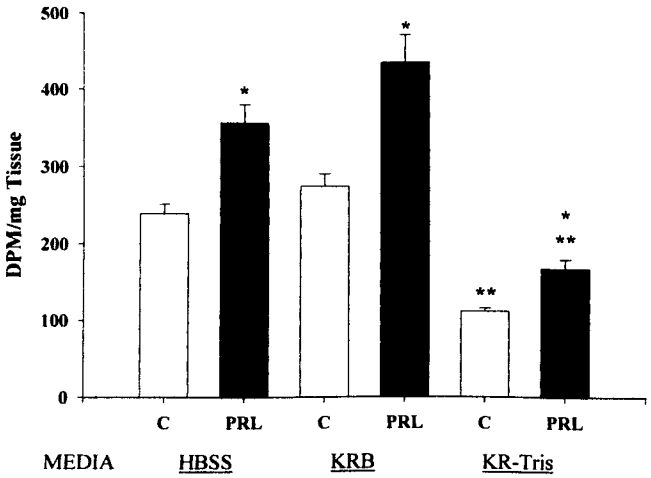

Figure 7. Sodium dependence of [^{3}H]-choline incorporation into lipids. Experimental details are the same as described in Figure 6, except that the level of [^{3}H]-choline incorporated into lipids was assessed. *Greater than control with $P < 0.01$. **Less than in tissues treated in HBSS or KRB.

unstirred fluid layers than single cells; this can lead to an overestimation of the K_m value.

The results of the hormone treatment experiments were quite clear. I is the only hormone that stimulates choline uptake into the explants. I expressed its effect by itself and in the presence of H and/or PRL. In contrast, PRL stimulated the incorporation of choline incorporation into lipids, but only when the tissues were concurrently treated with I and H; it is assumed that most of the choline in the lipid fraction was present in phospholipids. The stimulation of choline uptake specifically by I, but not by PRL, in the mammary gland is different from the regulation of a number of other milk constituents. The uptake of many other substances—including glucose, iodide, amino acids, nucleosides, and phosphate (8–12)—are all regulated by PRL when it is present with I and H. The lactogenic hormones thus appear to have specific actions on the accumulation of selected nutrients in milk.

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