Ontogeny and Dietary Modulation of 3-Hydroxy-3-Methylglutaryl-CoA Reductase Activities in Neonatal Pigs

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ABSTRACT: The development of hepatic and ileal 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase was studied in three types of young pigs (crossbred, obese, and lean pigs). Pigs were fed one of two diets: a high-fat (coconut oil), high-cholesterol (1.0%; designated HC) diet or a high-fat, no-cholesterol (designated NC) diet from postpartum d 3 to d 13, 25, and 42 (crossbred only). There were four pigs per age per diet group (except for obese pigs). Liver and ileal mucosal microsomal reductase activities were determined by the conversion of [14C]HMG-CoA to mevalonic acid followed by lactonization of the product. The samples were analyzed by thin layer chromatography and liquid scintillation spectrometry. Hepatic reductase activity (1 unit of specific activity = 1 pmol·min⁻¹·mg protein⁻¹) was <20 units on d 3 in all groups. By d 13, the activity was 40 to 46 units in all groups of pigs fed HC and approximately 50 to 80 units in pigs fed NC. Reductase activity then decreased at d 25 to 18 to 40 units in pigs fed NC and to <14 units in pigs fed HC. The d 42 reductase values (crossbred only) were approximately 14 units for pigs fed both HC and NC diets. Intestinal reductase activity was not affected (P > .1) by either age or diet. The data suggest that dietary cholesterol suppressed hepatic reductase activity in young pigs (d 13 and 25) from divergent genetic backgrounds. The data also suggest that the stage of development is a dominant factor in regulating porcine hepatic HMG-CoA reductase activity, which was considerably increased at d 13, even in pigs fed HC diets. The relatively modest increase in plasma cholesterol, even in pigs fed cholesterol during the suckling period, provides evidence that both dietary and endogenously synthesized cholesterol are probably used predominantly for tissue building in very young pigs (d 13).

Key Words: Cholesterol, Swine, 3-Hydroxy-3-methylglutaryl-coenzyme A Reductase, Liver, Neonatal Pigs, Dietary Cholesterol

Introduction

Suckling mammalian neonates, including pigs, have a relatively high cholesterol intake. Artificial milk formulas usually contain a very low cholesterol concentration and a relatively high polyunsaturated fatty acid concentration (Friedman and Goldberg, 1975; Carlson et al. 1982). The relatively high concentration of cholesterol in milk suggests the possibility of a dietary requirement for cholesterol in neonatal mammals. Cholesterol as a component of membranes, as a precursor of hormones, and as a metabolite is essential for growth of all tissues. Addition of dietary cholesterol to subsets of a population of pigs genetically selected for low serum cholesterol caused an increase in growth rate and brain cholesterol concentration (Schoknecht et al., 1994; Abbott et al., 1995), implying a dietary cholesterol requirement, at least in this particular population of pigs. The liver enzyme 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase is prob-
ably the rate-limiting enzyme in cholesterol synthesis (Hunter and Rodwell, 1980). Reductase activity is increased in pigs after weaning or dietary transition from the relatively high-cholesterol sow’s milk to a no-cholesterol, grain-based diet (Kwekkeboom et al., 1990). Reductase is suppressed in neonatal pigs fed sow’s milk compared with pigs fed a low-cholesterol formula (Jones et al., 1990). The purpose of our experiments was to ascertain the effect of age from d 3 to 42, as well as the effect of dietary cholesterol compared with no cholesterol, on plasma lipids and the activity of HMG-CoA reductase in liver and small intestine of young pigs. To obviate the discrepancy between diet composition of milk and formula (Jones et al., 1990), we fed all pigs the same modified sow milk replacer diet, with or without added cholesterol.

### Methods

**Experiment 1.** Twenty-eight crossbred male and female pigs were obtained from a local producer (DeKalb crossbred pigs predominantly composed of Large White, Landrace, Duroc, and Hampshire breeds), weaned at 3 d postpartum (2 to 4 d), and transported to the Children’s Nutrition Research Center. Four pigs, each from a different litter, were killed by captive bolt gun and exsanguination immediately upon arrival to obtain baseline data. Twenty-four pigs were individually housed in a controlled environment (28 ± 1°C with supplemental heat provided by heat boards or heat lamps) in 76 x 76 cm stainless steel cages. Pairs of littermate pigs were fed either a milk protein-based diet (25% protein, 0.5% fiber, 50% lactose) containing 11% coconut oil (either a milk protein-based diet (25% protein, 0.5% fiber, 50% lactose) containing 11% coconut oil (Hunter and Rodwell, 1980). Propylene glycol improves pelleting properties in cases requiring a pelleted diet; Soweena Pig Krave Extra is a non-nutritive sweetener to enhance diet palatability; BHA and propionic acid provide antioxidant and mold inhibition, respectively; neomycin sulfate minimizes diarrhea.

<table>
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<th>Ingredient</th>
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<td>Whey protein concentrate</td>
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<td>Neomycin sulfate</td>
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<td>Cholesterol (USP grade)</td>
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*Modified Soweena (coconut and corn oil replacing tallow) from Merrick Foods (Union Center, WI). Each dry diet contained 13.65 j of ME per kilogram. Propylene glycol improves pelleting properties in cases requiring a pelleted diet; Soweena Pig Krave Extra is a non-nutritive sweetener to enhance diet palatability; BHA and propionic acid provide antioxidant and mold inhibition, respectively; neomycin sulfate minimizes diarrhea.

**Experiment 2.** A second experiment with similar design used genetically obese and lean pigs obtained from Prairie View A&M University (Prairie View, TX). These pigs were derived from the selection experiments of Hetzer and Harvey (1967) via the U.S. Meat Animal Research Center (Clay Center, NE). Purebred Duroc and purebred Yorkshire pigs were selected for thick (=obese) or thin (=lean) backfat at 80 kg body wt; the selection was for 18 and 14 generations for Duroc and Yorkshire, respectively. At the Nebraska site, obese Duroc pigs were mated to obese Yorkshire pigs to obtain the current obese pigs (50% Duroc, 50% Yorkshire), with similar type matings to obtain the current lean pigs.

In this second experiment, the ages studied were d 3, 13, and 25 (d 42 was not included). The number of water until the end of the experiment. Excessive feed wastage precluded measurement of feed intake. Littermate pairs fed either the HC or NC diet were killed by captive bolt gun followed by exsanguination at 0800 to 0900 on d 13 (12 to 14 d), 25 (24 to 26 d), or 42 (41 to 43 d) after birth; thus, they were fed the experimental diets for 10, 22, or 39 d, respectively. All procedures were approved by the Institutional Animal Care and Use Committee, Baylor College of Medicine.
pigs for each age-diet group was four, except for some obese pig groups because of breeding difficulty in the herd. (There were two d3 and two d13 obese pigs fed the NC diet and three d13 obese pigs fed the HC diet). The diets were the same as those used in Exp. 1 (Table 1) and were fed from d3 to 25.

Blood and Tissue Analyses (Experiments 1 and 2). Blood was collected from the cut jugular vein into heparinized tubes for the determination of plasma total cholesterol, HDL cholesterol, and triglyceride concentrations (Ciba Corning 550 Express Chemistry Analyzer, Ciba Corning Diagnostic, OH). A 0.5-mm section of ileum was removed from each pig beginning at 0.3 to 0.6 m proximal to the ileocecal junction in d3 and d13 pigs and 1.0 to 1.3 m proximal to the ileocecal junction in d25 and d42 pigs. A 5- to 10-g section of the left liver lobe adjacent to that containing the gall bladder was excised; the ileal and liver sections were placed in ice-cold .9% NaCl. The tissues were transported immediately to the laboratory (<10 min) to measure HMG-CoA reductase (EC 1.1.1.34) activity. Preliminary experiments indicated a dramatic decrease in both hepatic and ileal HMG-CoA reductase activities when either the tissue or the microsomes were frozen in liquid N2, stored at −70°C, and thawed rapidly before enzyme assay, in agreement with the observation of Kwakkeboom et al. (1990). Thus, all assays used fresh tissue.

The activity of HMG-CoA reductase was measured using a modified method of Brown et al. (1979). Tissues were kept on ice, all solutions were in ice baths, and all centrifugations were at 3°C. The peritoneum and large veins were removed from the liver, which then was passed through a tissue press with a screen mesh of 1 mm. One gram of pressed liver or 1 g of the ileal mucosa (ileal section opened lengthwise and mucosa scraped with a glass slide) was homogenized using a motor-driven Potter-Elvehjem homogenizer (7 strokes at 55 rpm) with 9 mL of buffer (10 mM Tris-HCl with pH adjusted to 7.4 at 22°C, 250 mM sucrose, 2 mM dithiothreitol, and 1 mM EDTA). An inactive form of reductase is assayed in rat liver in the presence of fluoride to inhibit a phosphatase necessary for activation of the enzyme (Brown et al., 1979). Inhibition is reversed by subsequent incubation with exogenous alkaline phosphatase. In pigs, homogenization of liver or ileal mucosa with 50 mM NaF inhibited reductase, but exogenous phosphatase did not reverse the inhibition in liver (data not presented) and only partially reversed the inhibition in ileal mucosa (data not presented). Although an inactive form of the porcine hepatic or ileal reductase could be assayed in the presence of fluoride, the tissue had to be processed extremely rapidly to avoid a highly variable degree of activation. Consequently, we did not report inactive reductase.

The homogenate was centrifuged at 8,5000 g for 20 min, and the infranate (below the floating fat cake) was centrifuged at 100,000 g for 60 min. The resulting pellet (microsomes) was resuspended in the homogenization buffer with a Potter-Elvehjem homogenizer (pellet from 1 g of liver in 3.5 mL of buffer). Microsomal protein was determined on trichloroacetic acid precipitates using the Peterson modification (1977) of the Lowry method (1951).

Reductase activity was determined by measuring the conversion of [14C]-3-hydroxy-3-methylglutaryl-CoA to [14C]-mevalonate in an assay mixture containing approximately 600 μg and 300 μg of microsomal protein for the liver and ileum, respectively. Preliminary experiments demonstrated that the enzyme activity for both liver and ileum was within the linear range for amount of microsomal protein and for time. The liver microsomes were preincubated at 37°C for 60 min; then the assay medium was added, and the reaction was allowed to proceed for 15 min. Preliminary experiments indicated that the pig liver enzyme (data not reported), like the rat liver enzyme (Brown et al., 1979), was activated by preincubation under these conditions. The ileal microsomes were incubated for 30 min without a preincubation period because preliminary experiments indicated the gut microsomes were not activated by preincubation. The incubation medium contained 90 μL microsomes plus 47.6 mM KH2PO4, 9.5 mM glucose-6-phosphate, 1.2 mM NADP, 4.8 mM dithiothreitol, and 2.38 mM EDTA with pH adjusted to 7.4 at 22°C with KOH, 16.8 μM HMG-CoA (H-6132, Sigma Chemical, St. Louis, MO), 1 unit glucose-6-phosphate dehydrogenase (G 7878, Type XII from Torula yeast; Sigma Chemical), and 0.1 μCi of [DL-3 (glutaryl-3-14C)]HMG-CoA (NEC-642; Du Pont NEN Products, Boston, MA) in a total volume of 210 μL. Preliminary experiments indicated that addition of greater amounts of glucose-6-phosphate, the dehydrogenase, or HMG-CoA did not increase the reaction velocity. The reaction was terminated and the product, mevalonate, was lactonized by addition of 25 μL of 6 N HCl and incubation at 37°C for 30 min. Mevalonolactone was isolated by thin layer chromatography on silica gel G plates (#31511 with a 250-μm thick layer and a preabsorbent area; Analtech, Newark, DE) using a solvent system of toluene-acetone (50:50 vol/vol). The mevalonolactone area of the plate (Rf 0.4 to 0.7) was scraped and the radioactivity was counted using liquid scintillation spectrometry. The HMG-CoA reductase specific activity was expressed as units (1 unit = 1 pmol mevalonate formed·min−1·mg microsomal protein−1).

Statistical Analyses (Experiments 1 and 2). Experiment 1 was treated as a split plot design in which litters were randomly assigned to age group and litter mates were randomly assigned to diet. There are two experimental units. Litter (L) is the larger experimental unit or whole plot, which provides the error term for age (A), and pig (P) is the smaller experimental unit or split plot, which provides the error term for diet (D) and the age × diet interaction.
Results

There were no differences at d 3 in body weight of pigs assigned to NC or HC diets; there were no differences in body weight between pigs fed the two experimental diets on d 13, 25, or 42 (Figure 1). Obese pigs were smaller than lean pigs at all ages, as previously observed (Mersmann, 1991). Liver weight increased with age as expected; there were no significant diet or diet × age effects (Figure 2).

Suckling pigs at d 3 seemed to have elevated plasma total cholesterol (not statistically tested; Figure 3), presumably because their diet (sow milk) was relatively high in fat and cholesterol. Plasma total cholesterol concentration seemed to be lower at d 13.
than at d 3 and increased with age and with dietary cholesterol in all groups; plasma cholesterol was especially elevated at d 42 in crossbred pigs fed cholesterol. Obese pigs tended to have higher plasma cholesterol than lean pigs (P < .1), especially at d 25 (gene × age, P < .05). Plasma HDL cholesterol concentration followed the same pattern as total cholesterol concentration (Figure 4). The HDL cholesterol age × diet effect was less in the crossbred pigs (P = .11) than that for total cholesterol; this effect was not present in the obese and lean pigs. The obese pigs had a greater HDL cholesterol response to dietary cholesterol than the lean pigs.

Pigs fed HC compared with NC had similar plasma triglycerides at d 13, but by d 25 the HC diet tended to cause an increase in plasma triglycerides (Figure 5). This diet × age interaction was stronger in the crossbred pigs (P < .05) than in the lean and obese pigs (P < .1). Plasma triglyceride was lower in crossbred pigs fed cholesterol than in those fed diets with no cholesterol at d 42.

Liver HMG-CoA reductase activity (Figure 6) was significantly affected by age in all groups. Reductase activity seemed to increase at least threefold between d 3 and 13 in the group fed NC and by at least onefold in the group fed HC (not statistically tested). In the crossbred pigs, after d 13, reductase activity declined to low levels in both dietary groups; reductase activity
also declined in the obese and lean pigs. Dietary cholesterol did not significantly reduce reductase activity in the crossbred pigs, although activity was numerically lower at d 13 and 25 in pigs fed HC than in pigs fed NC. In the obese and lean pigs, dietary cholesterol caused a decrease in reductase activity, but this was nominal at d 13 in the lean pigs (gene x age, P < .1). Ileal HMG-CoA reductase activity (pmol mevalonate formed·min⁻¹·mg microsomal protein⁻¹) was not affected by age or diet; the diet means were 41.1 for NC and 28.8 for HC (SD = 16.7) and 46.7 for d 13, 27.8 for d 25, and 30.5 for d 42 (SD = 17.9). Ileal reductase activity seemed to be lower at d 3 (10.1 with SD = 2.8) compared with other ages. Expression of either the liver or ileal HMG-CoA reductase activity on the basis of units per gram of tissue, or of the liver HMG-CoA reductase activity on the basis of total activity per liver, did not alter the patterns compared with expression on the basis of activity per milligram of microsomal protein (data not reported).

Discussion

In general terms, all three genetic groups of pigs responded to the experimental diets in the same manner. There were several cases where a variable reached P < .05 for age or diet in one experiment but not in the other; in most of these cases the numerical direction was the same. There were scattered cases of age x diet and age x gene interactions that approached significance at P < .1.

Plasma cholesterol concentration is low at birth in mammals, including pigs (Carroll and Hamilton, 1973; Mersmann et al., 1979; Jones et al., 1990). Cholesterol concentration increases in suckling neonates as a result of the high-fat, relatively high-cholesterol (approximately 200 mg/100 g milk) milk diet. Our suckling pigs had high plasma cholesterol concentrations at d 3 that seemed lower after 10 d of feeding the 11% coconut oil diet and increased progressively at older ages. Addition of dietary cholesterol resulted in higher plasma total cholesterol concentrations at older ages than at d 13. The pattern for plasma total cholesterol in young pigs was similar to that observed by Jones et al. (1990). However, the high-cholesterol sow milk or low cholesterol formula diets fed by Jones et al. were confounded by more unsaturated fatty acids in the low-cholesterol formula diet than in the high-cholesterol sow milk diet. Although we did not study it, the type of dietary fat may influence plasma cholesterol concentrations; saturated fats may elevate plasma cholesterol concentration more than polyunsaturated fats (Grundy and Denke, 1990; Gurr, 1992). We fed a high-fat (coconut oil) diet to all pigs with or without supplemental cholesterol; thus, the changes we observed in plasma cholesterol are attributable only to the absence or presence of dietary cholesterol. Our results with lean and obese pigs agree with those of Patterson et al. (1992) and Hackman et al. (1996), who observed a steady increase in plasma cholesterol throughout the period to 8 wk of age in both genotypes.

Porcine hepatic HMG-CoA reductase activity had a distinct developmental pattern, with a large apparent increase in activity between d 3 and 13; the activity increased threefold in the NC-fed group and onefold in the HC-fed group. Enzyme activities decreased by d 25 and remained relatively constant at d 42 in both dietary groups. The increased enzyme activity at d 13 and the subsequent decrease in both dietary groups at d 25 and d 42 seem to be attributable to age.

A variety of results have been reported with respect to effects of diet composition on HMG-CoA reductase activity. The degree of saturation of the dietary fat and its quantity may be significantly involved in modulating hepatic reductase activity. Studies with several species have shown suppression of HMG-CoA reductase activity with high-fat, high-cholesterol diets; in some studies the reduction in enzyme activity was less in animals fed diets high in saturated fatty acids than in animals fed diets high in unsaturated fatty acids (Field et al., 1987; Garg and Sabine, 1988; Spady and Dietschy, 1988; Kuan and Dupont, 1989; Jackson et al., 1990; Fernandez and McNamara, 1991).

In our experiments (Figure 6), hepatic reductase activity was numerically less (statistically significant in obese and lean but not in crossbred pigs) in d 13 and 25 pigs fed cholesterol (HC group) than in pigs fed no cholesterol (NC group); mean reductase activity was approximately 50 to 80 units at d 13 in pigs fed no cholesterol and 40 to 46 units in pigs fed cholesterol. At d 25, mean reductase was 18 to 40 units in pigs fed no cholesterol and 7 to 13 units in pigs fed cholesterol. Others have observed suppression of hepatic reductase activity by dietary cholesterol in young pigs. Hackman et al. (1996) reported a lower liver reductase activity at 8 wk of age in genetically lean and obese pigs fed .5% cholesterol than in those fed no cholesterol. Jones et al. (1990) reported approximately 30 units of hepatic reductase in d 15 pigs fed sow’s milk (relatively high cholesterol) and 68 units reductase in pigs fed a low-cholesterol formula, whereas at d 25, reductase was 30 units in both groups (no effect of dietary cholesterol). Rioux and Innis (1993) fed two groups of 1-d-old pigs the same diet with high or low cholesterol; after 17 d of feeding, hepatic reductase was 30 units and 95 units in the two groups, respectively. In a previous study from our laboratory (Schoknecht et al., 1994) using pigs genetically selected for elevated and reduced plasma cholesterol concentration and fed a high-fat diet (coconut oil) with or without 0.2% cholesterol, hepatic reductase was 7 and 25 units, respectively. In somewhat older pigs (10 to 12 wk), dietary cholesterol
suppressed hepatic reductase (Rogers et al., 1981). Thus, our results indicate an ontogenic elevation of hepatic reductase at d 13, regardless of diet, and an apparent suppression of reductase by dietary cholesterol, at least at the younger ages, as indicated by others.

In agreement with Jones et al. (1990), we found no effect of diet or age on porcine ileal reductase activity; Schoknecht et al. (1994) also did not observe an effect of dietary cholesterol on ileal reductase, whereas Rogers et al. (1981) indicated dietary cholesterol suppressed ileal reductase in older pigs. The role of dietary cholesterol in regulation of gut HMG-CoA reductase is unclear in that some studies show suppression of activity in animals fed cholesterol, and others do not (Rogers et al., 1981; Field et al., 1987; Field et al., 1990).

The current results indicate that in three different genetic groups of pigs, dietary cholesterol suppressed hepatic cholesterol synthesis (as indicated by HMG-CoA reductase activity) during the neonatal period. This was also observed by Hackman et al. (1996) in genetically lean and obese pigs, in pigs selected for high or low plasma cholesterol (Schoknecht et al., 1994), and in obese pigs in vivo, using deuterium to detect synthesis (Wong et al., 1991). The interesting observation is that young pigs increased reductase activity at d 13 in spite of the very high dietary cholesterol concentration. Speculatively, the demand for cholesterol must be so great in these early stages of growth that endogenous synthesis was increased in the presence of 1% dietary cholesterol. One might also speculate that most of the dietary and synthesized cholesterol was used for membrane biosynthesis at this early stage of rapid growth, because plasma cholesterol was only minimally increased by dietary cholesterol. By d 25, there was less biosynthetic activity, and plasma cholesterol increased with dietary supplementation, perhaps as a result of lesser growth rates by various tissues.

Obese pigs apparently have a different ability to metabolize or use cholesterol than lean pigs (and perhaps crossbred pigs, although they were not studied in the same experiment). Plasma cholesterol was increased to a greater extent at d 25 in obese than lean pigs, as observed previously (Patterson et al., 1992; Hackman et al., 1996). Because obese pigs grow more slowly than lean pigs and have less muscle and visceral mass, they may require less cholesterol for tissue membrane and brain growth, so that more cholesterol enters the plasma pool.

Finally, dietary cholesterol may be a requirement for optimal growth in at least some young pigs (Schoknecht et al., 1994; Abbott et al., 1995). There may be impaired behavioral development when diets do not contain cholesterol, but growth rates are also accelerated by exogenous cholesterol (Schoknecht et al., 1994). The exact age for this apparent dietary requirement is not known (cholesterol was added to the diet between d 2 and 35 postpartum). We do know that pigs allowed to suckle for 4 wk do not seem to have a requirement for dietary cholesterol for optimal growth thereafter (Harris et al., 1993). These observations suggest that early weaning schemes or artificial rearing of neonatal pigs may require supplemental dietary cholesterol for optimal growth and development in some, but not necessarily all, genetic lines.

Implications

The results indicate that dietary cholesterol in suckling pigs increases plasma cholesterol modestly and depresses cholesterol synthesis, as indicated by reduced liver HMG-CoA reductase activity. However, the activity of this enzyme is increased from birth to 2 wk of age, followed by a decline by d 25 in divergent genetic lines of pigs, even in the presence of dietary cholesterol. This persistent cholesterol synthesis and the relatively small increase in plasma cholesterol in response to dietary cholesterol in the 1- to 25-d-old pig imply that the large demand for cholesterol to satisfy requirements for tissue growth and obligatory cholesterol accretion requires appreciable endogenously produced cholesterol and, in some genetic lines, dietary cholesterol to allow normal growth and development.

Literature Cited


