Dietary linoleic acid increases and palmitic acid decreases hepatic LDL receptor protein and mRNA abundance in young pigs

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Abstract The present study was conducted to determine the effects of dietary fatty acids on hepatic LDL receptor (LDLr) protein abundance and mRNA levels. Sixty pigs were randomized into 10 groups and fed corn–soybean meal diets containing three cholesterol levels (0.25%, 0.5%, and 1.0%, w/w) with no added fat, or fats rich (30% of calories) in palmitic acid or linoleic acid. A control group was fed the base diet with no added fat. After 30 days, plasma LDL-cholesterol (LDL-C) levels increased as the dietary cholesterol increased ($P < 0.05$); however, there was no significant effect of either fatty acid. Dietary fatty acids, however, had distinctly different effects on hepatic LDLr protein (analyzed by ELISA) and mRNA (analyzed by Northern blot) abundance. When pigs consumed diets containing 0.25% cholesterol, linoleic acid increased hepatic LDLr protein 40% whereas palmitic acid reduced it 40% ($P < 0.05$). These changes in LDLr protein abundance were accompanied by parallel changes in hepatic LDLr mRNA; linoleic acid increased LDLr mRNA 2-fold ($P < 0.01$), whereas palmitic acid decreased it 60% ($P < 0.01$). The differential effects of fatty acids on LDLr expression were only observed at 0.25% cholesterol, suggesting that higher intakes of cholesterol have a dominant and repressive effect on regulation of LDLr expression. Cholesterol intake increased hepatic total cholesterol levels ($P < 0.01$) while dietary fatty acids had no effect on hepatic sterols. In summary, our results indicate that dietary linoleic acid and palmitic acid have markedly different effects on hepatic LDLr protein abundance that are mediated by differential effects on LDLr mRNA levels. Further studies are needed to fully elucidate the molecular mechanisms by which fatty acids regulate LDLr mRNA and protein levels.—**Mustad, V. A., J. L. Ellsworth, A. D. Cooper, P. M. Kris-Etherton, and T. D. Etherton.** Dietary linoleic acid increases and palmitic acid decreases hepatic LDL receptor protein and mRNA abundance in young pigs. *J. Lipid Res.* 1996. **37:** 2310–2323.

Supplementary key words LDL receptor • mRNA levels • linoleic acid • palmitic acid • pigs

There is evidence to suggest that dietary fatty acids affect LDL-cholesterol (LDL-C) levels via several different mechanisms including effects on whole animal sterol balance and LDL production or turnover (1); however, modulation of LDL receptor (LDLr)-mediated uptake of LDL is thought to be the primary pathway regulating LDL-C levels. Studies in humans (2) and numerous animal models (3–16) have shown that changes in plasma LDL-C as a result of dietary fatty acid modification are associated with changes in its fractional catabolic rate (FCR). Alterations in the FCR are presumably mediated via changes in the receptor-dependent uptake of the LDL particle by the liver. Consequently, there is considerable interest in studying fatty acid regulation of the LDLr.

Dietary modification of hepatocyte membrane fluidity may be one means by which diets high in polyunsaturated fatty acids (PUFA) affect LDLr activity differently than diets enriched in saturated fatty acids (SFA). Support for this comes from in vitro studies (17, 18) and studies with rats (19) that have shown significant alterations in LDL binding to the LDLr as a result of changes in membrane fluidity. It also has been proposed that dietary fatty acids can directly influence the number of receptors available for uptake of the circulating LDL particle by specifically affecting the synthesis of the

Abbreviations: LDL-C, low density lipoprotein cholesterol; VLDL-C, very low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; LDLr, LDL receptor; SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids.

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LDLr. In vitro binding studies have demonstrated that alterations in LDL uptake that are associated with the dietary fatty acid composition can be attributed to changes in the B_{max}, an indication of maximal receptor number (5–8, 20). This is only, however, an indirect measure of LDLr number and it is possible that other changes (e.g., changes in membrane fluidity affecting cell recycling of receptors) could result in an increased expression of membrane receptors.

A number of groups have measured the effects of dietary fatty acids on the expression of the LDLr gene in different animal models (21–28). The results of these studies, however, have been inconsistent. For example, two studies with either African green or Cebus monkeys found a greater effect of dietary cholesterol than dietary fatty acids on LDLr mRNA levels (22, 25). A study with hamsters found that palmitic acid increased LDLr mRNA compared to other SFA (23) while a more recent study with hamsters (28) found the opposite. A recent study with HepG2 cells also found equivocal results (29). Furthermore, the majority of studies have made relative comparisons (i.e., PUFA vs. SFA) and did not include a neutral control (i.e., comparison to carbohydrate); thus, the independent effects of fatty acids on LDLr mRNA levels remain unclear.

In order to gain a better understanding of how diet regulates the LDLr, we conducted the present study to quantify the effects of dietary SFA (palmitic acid) and PUFA (linoleic acid) on hepatic LDLr protein abundance and to determine whether this is associated with a parallel change in LDLr mRNA levels. We also analyzed the relationship between these effects and changes in hepatic lipid content to further define the mechanisms by which fatty acids alter liver cholesterol homeostasis. We used pigs in this study because they have been regarded as one of the best models for human lipid metabolism, their lipoprotein profile is similar to that of humans and a number of studies have shown that their lipid metabolism and lipoprotein response to diet also are similar (30). Moreover, because the habitual diet of the pig is so low in fat (i.e., less than 6% of energy) and cholesterol, we can gain a perspective about the independent effects of dietary fat on LDL-C levels and LDLr mRNA levels.

METHODS

Animals and study design

Duroc (n = 30) and Yorkshire (n = 30) gilts, 26 ± 5 kg initial weight (~3 months of age) were used in this study. The animals were randomized (balanced by breed) into 10 treatment groups with six pigs per dietary treatment. The experimental diets were fed for 30 days. Animals were killed by exsanguination at The Pennsylvania State University Meats Laboratory. Blood was collected for lipoprotein separation and cholesterol analysis. Liver samples were removed rapidly (within 20–30 min of death) and stored in liquid N_2 (for membrane isolation and lipid analysis) or immediately homogenized in a guanidinium-thiocyanate buffer (31) for subsequent RNA extraction. The homogenate was stored at −70°C until total RNA was extracted (within 3 months). Procedures for animal care and use were approved by The Pennsylvania State University's Institutional Animal Care and Use Committee.

Diets

Pigs were fed a corn–soybean meal based diet that was formulated to meet the NRC requirements for swine (32) to avoid nutrient dilution associated with increasing the energy density of the diet (**Table 1**). Animals were fed diets containing fat at 30% of energy, rich in either SFA (palmitic acid, provided by palm oil) or PUFA (linoleic acid, provided by sunflower oil) (Premier Edible Oils, Portland, OR) and were formulated to contain cholesterol at either 0, 0.25%, 0.5% or 1.0% (wt%; 0, 0.60 mg/kcal, 1.2 mg/kcal, or 2.4 mg/kcal, respectively). The fatty acid composition and the cho-

TABLE 1. Composition of the experimental diets

	Control Diet	Test Fat Diet
Ingredient, %		
Base mix[a]	3.0	3.6
Corn, ground	72	44.4
Soybean meal	25	34
Test fat	3	16[b]
Cholesterol, crystalline	[c]	[c]
Cholic acid	[d]	[d]
Total	100	100
Nutrient composition[e]		
ME, % from fat	6	30
Lysine, g/Mcal ME	2.75	2.75
Base mix, g/Mcal ME	9.2	9.1

[a]Contains vitamin and trace mineral premix (Walnut Grove, Ames, IA), and antibiotic-premix (Tylan-10, ELANCO Animal Health Division, Eli Lily, Indianapolis, IN).

[b]Test fats include palm oil and sunflower oil (Premier Edible Oils, Portland, OR).

[c]Crystalline cholesterol (Sigma Chemical Co., St. Louis); added at 0, 0.25%, 0.5%, and 1% (wt%).

[d]Cholic acid (Sigma Chemical Co., St. Louis); added at 10% level of that of cholesterol.

[e]ME, metabolizable energy; Mcal, 1000 kcal. Nutrient information based on NRC calculated values (31).

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TABLE 2. Fatty acid and cholesterol composition of experimental diets

	Palm Oil	Sunflower Oil
Fatty acids (% of calories)[a]		
C14:0	0.3	—
C16:0	14	1.5
C18:0	1.2	1.2
C18:1n-9	10	6.0
C18:2n-6	3.0	20
Other fatty acids	2.0	1.3
Cholesterol (wt%)		
0.0	0[b]	0[b]
0.25%	0.22 ± 0.09[b]	0.21 ± 0.09[b]
0.5%	0.40 ± 0.12[b]	0.42 ± 0.09[b]
1.0%	0.89 ± 0.14[b]	0.91 ± 0.14[b]

[a]Fatty acids analysis based on GC analysis of oils provided by Premier Edible Oils (Portland, OR).

[b]Assayed cholesterol content of the experimental diets.

lesterol content (which was 20% lower than the target levels) of the diets are shown in **Table 2.** A pilot study we conducted with Yorkshire gilts demonstrated that high SFA diets alone or those containing less than 0.12% cholesterol did not elicit a hypercholesterolemic response; however, pigs were responsive when higher levels of cholesterol (i.e., 1.5%) were consumed. Thus, in the present study, we examined the effects of dietary SFA or PUFA at three levels of dietary cholesterol that represented a range between those tested in the pilot study. Crystalline cholesterol (Sigma Chemical Co., St. Louis, MO) was completely dissolved in warmed fat and then mixed with the base diet (Table 1). A group of animals consumed the base diet supplemented only with cholesterol (which was dissolved in warmed ethanol then evaporated under N_2 after it was added to the base diet) and no added fat. To enhance the absorption of the cholesterol in this diet, cholic acid (Sigma Chemical Co., St. Louis, MO) was added at 10% of the level of crystalline cholesterol. This modest addition was chosen to prevent any additional effect on hepatic sterol metabolism reported when higher levels are fed (33). A control group was fed a base diet (6% kcal from fat) with no added test fat or cholesterol (Table 1). All pigs were fed twice daily at 08:00 and 19:00 to minimize feed wastage and to control feed intake. (This feeding regimen did not affect growth rate or plasma cholesterol response in our preliminary studies.) Pigs were weighed weekly to monitor growth rate.

Lipoprotein isolation

Blood was collected from the jugular vein into centrifuge tubes containing EDTA (1 mg/ml of blood). Plasma was immediately isolated by low speed centrifugation at 1500 *g* for 20 min and lipoproteins were isolated by ultracentrifugation using procedures established for isolating lipoproteins from pigs (34). Briefly, 3 ml of plasma was adjusted to density (d) of 1.21 g/ml with NaBr and sucrose. This was overlaid sequentially with 3.0 ml each of solutions having d 1.09 g/ml, d 1.063 g/ml, and d 1.006 g/ml. Lipoprotein fractions were obtained by ultracentrifugation at 17°C at 250,000 *g* for 24 h in a Beckman L2-65B centrifuge using a Beckman SW40 Ti swinging bucket rotor. The bands corresponding to VLDL-(VLDL-C), LDL-C and HDL-(HDL-C) cholesterol were aspirated with a syringe. The purity of the lipoprotein fractions was confirmed using SDS-PAGE (not shown).

Membrane isolation

Hepatic membranes were prepared from liver homogenates by centrifugation at 10,000 *g* for 30 min at 4°C and further centrifugation of the supernatant at 100,000 *g* for 60 min at 4°C (35). Liver membrane preparations were resuspended in ice-cold 250 mM Tris-maleate (pH 7.4), 2 mM $CaCl_2$ and 1 mM phenylmethylsulfonyl fluoride (PMSF), solubilized with 30 mM CHAPS (3-[3-cholamidopropyl)-dimethylamminio]-1-propane-sulfonate) and centrifuged at 100,000 *g* for 60 min (36). Samples of the supernatant were stored at −80°C. Protein assays were performed using the Pierce BCA microprotein assay kit (Rockford, IL).

RNA extraction

Total RNA was extracted from guanidium-thiocyanate using acid phenol-chloroform according to the method described by Chomczynski and Sacchi (31). The RNA was quantified by absorbance A280/260 (the ratio was greater than 1.75). The integrity of the 28S and 18S ribosomal RNAs was verified by agarose/formaldehyde gel electrophoresis followed by staining with ethidium bromide (see below).

Biochemical analyses

Plasma cholesterol and triglycerides were quantified using enzymatic methods (Boehringer Mannheim, Indianapolis, IN). Liver total and microsomal lipids were extracted by a modification of the method of Folch, Lees, and Sloane Stanley (37). Briefly, 1 gram of liver (wet weight) was homogenized in 5 ml of chloroform–methanol) (C–M) 2:1 (v/v) to which 0.01% butylated hydroxytoluene (Sigma Chemicals, St. Louis, MO) was added as an antioxidant. Fifteen ml of C–M was added to the homogenate and the mixture was allowed to sit (with occasional vortexing) for at least 8 h. The mixture was filtered through Whatman filter paper (# 1) and the filtrate was washed 2 times each with 3.7 ml of 0.05% $CaCl_2$ and 9.7 ml of 0.025% $CaCl_2$ in C–M–H_2O 4:92:4 (v/v/v). After each wash, the samples were

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centrifuged at 500 *g* for 2 min and the upper aqueous layer was removed. After the last phase separation, the chloroform layer was removed and evaporated under N_2; the lipid extract was dissolved in approximately 5 ml of C–M 2:1 and stored at −20°C under N_2. Total lipid content was determined gravimetrically. Tissue cholesterol and triglyceride levels were determined enzymatically using reagents purchased from Boehringer Mannheim (Indianapolis, IN). Cholesteryl esters were determined as the difference between total and nonesterified (free) cholesterol using the method of Sale et al. (38).

Determination of LDL-receptor protein

LDLr protein in solubilized hepatic membrane proteins was determined using the ELISA method previously described by May et al. (36). The polyclonal rabbit anti-rat LDLr IgG antibody used in this assay has been characterized in a number of previous studies and does not recognize the LDLr-related protein (36) nor the oxidized LDL receptor (39). The antibody was validated for use with the pig LDLr first by confirming its specific binding by Western blotting. Briefly, 100 µg of solubilized membranes was loaded onto a 6.5% polyacrylamide gel. The samples were electrophoresed at 30 mA for 18 h at 4°C and the proteins were transferred to a nitrocellulose membrane. After transfer, the membrane was blocked with 2% nonfat dried milk in Tris-buffered saline (pH 7.4) containing 0.05% Tween-20. After blocking, membranes were incubated with the anti-LDLr IgG (1:5000) and secondary antibody (biotinylated-GAR; 1:3000; streptavidin-HPR conjugate; 1:3000) in sequence, according to the ECL-Western blotting protocol (Amersham Life Sciences, Boston, MA). The adherent biotinylated-IgG was detected using reagents provided in the kit. After detection, the membranes were exposed to X-ray film (Kodak X-OMAT AR) with two intensifying screens for 15 sec.

The ELISA assay conditions were optimized using 2 mg of solubilized membrane protein per well. Membrane proteins were incubated overnight at 37°C in 96-well plates (Immulon-2, Dynatech, Chantilly, VA). After blocking with bovine serum albumin [BSA; 3.0% in phosphate-buffered saline (PBS)], the anti-LDLr IgG was added in excess at a concentration of 50 µg/ml. The amount of adherent antibody was determined using a second alkaline phosphatase-conjugated IgG (Zymed Laboratories, San Francisco, CA) and the color reaction was quantified in a microelisa reader by absorbance at a dual wavelength of A405/490. Nonspecific binding was determined in parallel samples using nonimmune rabbit IgG (Zymed Laboratories, San Francisco, CA) and was generally less than 20% of specific binding. The nonspecific binding was subtracted from the total binding to determine specific binding and the absorbance units of the unknown samples were compared to that obtained from the standard curve. To construct a standard curve, plates were coated with excess goat anti-rabbit IgG (1 µg/ml in PBS) and anti-LDLr IgG was added to triplicate wells (0.078, 0.156, 0.312, 0.625, 1.25, 2.5, and 5.0 ng/100 µl in PBS containing 0.3% BSA and 1 mM $CaCl_2$). After incubation and washing, alkaline phosphatase was quantified colorimetrically and a standard curve was constructed (**Fig. 1**). Typically, the R^2 was always greater than 0.95. The absorbance of unknown samples was expressed as ng of LDLr antibody bound.

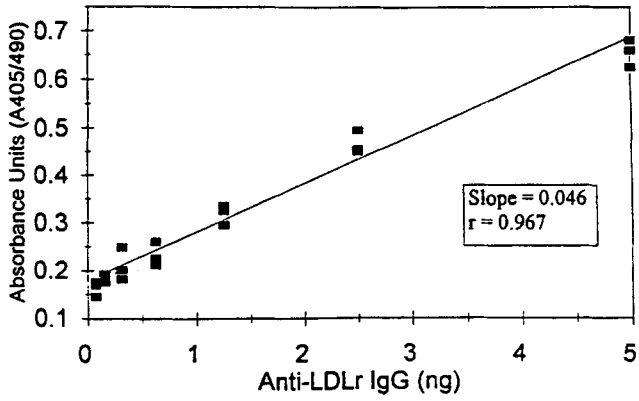

Fig. 1. A typical standard curve obtained using the ELISA assay for LDL receptor (LDLr) protein. ELISA plates were coated with excess goat anti-rabbit IgG (1 µg/ml in phosphate-buffered saline) and anti-LDLr IgG was added to triplicate wells (0.78, 0.156, 0.312, 0.625, 1.25, 2.5, 5 ng protein/100 µl phosphate-buffered saline). The amount of adherent anti-LDLr IgG was determined using a second IgG conjugated to alkaline-phosphatase, and the color reaction was quantified in a microelisa reader using a dual wavelength absorbance of A405/490.

Northern blot analysis of LDL-receptor mRNA

Because changes in LDLr protein may be the result of changes in mRNA levels, LDLr mRNA abundance was determined by Northern blot analysis using a 1.2 kb *BamHI-EcoRI* fragment of the human LDLr cDNA. The cDNA insert was excised from the plasmid [pBluescript SK+ (Stratagene, La Jolla, CA)] and labeled with ^{32}P dCTP (DuPont-NEN, Boston, MA) using a random oligonucleotide priming kit (Ready-to-Go, Promega, Madison, WI). We confirmed that the human LDLr cDNA would bind specifically to the pig LDLr mRNA by comparing the size of the transcript to that obtained from a human LDLr RNA transcript (RNA extracted from HepG2 cells; ATCC, Rockville, MD) (data not shown). Twenty µg of total RNA was subjected to electrophoresis on a 1.0% agarose gel containing 0.66 M formaldehyde in MOPS buffer (50 mM MOPS, pH 7.0, 0.22 M formaldehyde) according to the procedures of Sambrook,

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Fritsch, and Maniatis (40). Gels were rinsed twice in 1 M ammonium acetate for 15 min each and RNA was transferred overnight onto a GeneScreen membrane (DuPont-NEN, Boston, MA) by capillary action. LDLr mRNA was identified by hybridization with a ^{32}P-labeled LDL-receptor cDNA (added at 1×10^6 cpm/ml). Hybridization with the labeled cDNA was carried out for 16–24 h at 42°C. After hybridization, membranes were washed sequentially for 2×5 min in 2× SSC (250 mM NaCl, 30 mM Na citrate) at room temperature; 2×30 min in 2× SSC; 1% SDS at 65°C; and 2×30 min in 0.1× SSC at room temperature. After washing, the membranes were exposed to Kodak X-OMAT AR film with two intensifying screens at −70°C for up to 4 weeks. The hybridization signals for LDLr transcripts were adjusted for loading errors after hybridization with an 18S ribosomal cDNA (data not shown).

Statistics

Three-way ANOVA was used to determine interactions between dietary fat type, dietary cholesterol, and breed, as well as their independent effects. When this analyis indicated significant F values ($P < 0.05$), Tukey's test was used to determine significant differences among treatment means. Finally, regression analysis was performed and Pearson's correlation coefficients were determined to evaluate the relationship between LDLr mRNA and protein abundance and hepatic lipid levels.

RESULTS

The experimental diets were well accepted and all food was consumed within 1 h. Because the diets (provided isocalorically) were based on calculated energy requirements, as expected, no significant differences were found in the rate of weight gain (average rate of gain = 0.9 ± 0.3 kg/day). This rate of growth is within the range observed in animals fed ad libitum (32).

Plasma cholesterol levels

Table 3 shows the plasma lipids for pigs in each of the diet groups at the end of the 30-day experimental period. Plasma total cholesterol and LDL-C increased as the intake of cholesterol increased from 0 to 1% (wt%). As is apparent from Table 3, most of the increase in the plasma total cholesterol was due to a rise in LDL-C. There was no cholesterolemic effect of either test fat at any level of cholesterol intake. ANOVA revealed a significant breed effect ($P < 0.01$) for total and LDL-C response to dietary cholesterol. There were no differences in total or lipoprotein-cholesterol levels between the two breeds on the low fat/cholesterol-free control diet or the diets with 0.25% cholesterol; however, the magnitude of the responses differed between the two breeds consuming 0.5% and 1.0% cholesterol. The greatest increase in total and LDL-C levels in response to dietary cholesterol was observed in the Duroc pigs

TABLE 3. Plasma lipid values (mg/dl) of pigs fed experimental diets

	TC	LDL-C	VLDL-C	HDL-C	Triglycerides
Control group	84 ± 10^a	43 ± 9^a	4 ± 1	24 ± 4	26 ± 5
Experimental group					
0.25% cholesterol					
No added fat	94 ± 15^a	49 ± 7^{ab}	5 ± 2	28 ± 8	38 ± 20
SFA	97 ± 5^a	48 ± 7^{ab}	5 ± 2	29 ± 8	34 ± 19
PUFA	86 ± 9^a	46 ± 8^{ab}	3 ± 2	26 ± 4	24 ± 14
0.5% cholesterol					
No added fat	101 ± 14^{ab}	55 ± 10^{ab}	6 ± 5	28 ± 5	43 ± 33
SFA	93 ± 11^{ab}	48 ± 7^{ab}	5 ± 2	31 ± 4	37 ± 21
PUFA	101 ± 14^{ab}	57 ± 11^{ab}	5 ± 2	27 ± 4	35 ± 16
1.0% cholesterol					
No added fat	112 ± 20^b	60 ± 15^b	14 ± 6	25 ± 6	63 ± 43
SFA	112 ± 21^b	60 ± 13^b	4 ± 1	34 ± 6	28 ± 18
PUFA	104 ± 12^b	61 ± 13^b	4 ± 1	27 ± 4	34 ± 14
Statistics, main effects					
Cholesterol	$P < 0.05$	$P < 0.01$	NS	NS	NS
Fat type	NS	NS	NS	NS	NS
Breed	$P < 0.01$	$P < 0.001$	NS	NS	NS
Interactions	NS	NS	NS	NS	NS

Values are means ± SD (n = 6 animals per dietary treatment). Main effects in the ANOVA model were differences due to amount of dietary cholesterol as determined by three-way ANOVA; differences due to dietary fat type (no added fat, SFA, PUFA); differences due to breed (Duroc versus Yorkshire); differences due to interactive effects among dietary fat type × cholesterol × breed. Abbreviations: TC, total cholesterol; LDL-C, LDL cholesterol; VLDL-C, VLDL cholesterol; HDL-C, HDL cholesterol; NS, not significant.

^{a,b,c} Values within a column with different superscripts are significantly different ($P < 0.05$).

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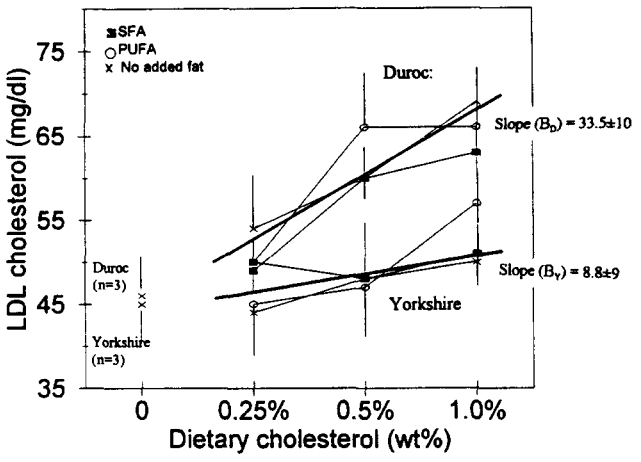

Fig. 2. LDL cholesterol response (mg/dl) to increasing levels of dietary cholesterol with or without added fat from sunflower oil (PUFA) or palm oil (SFA) at 16% (wt%) in Duroc and Yorkshire pigs. Values are means ± SD (n = 3 pigs per dietary treatment). Comparison of the slopes of the regression lines (thick lines) showed that the Duroc breed was more responsive [slope (β_D) = 33.5 ± 10] to increasing dietary cholesterol compared to Yorkshire pigs [slope (β_Y) = 8.8 ± 9].

(**Fig. 2**). Comparison of regression coefficients (β) of the response curves revealed that Duroc pigs were significantly more responsive ($P < 0.01$) to increasing intakes of dietary cholesterol ($\beta_D = 33.5 \pm 10$) compared to Yorkshire pigs ($\beta_Y = 8.8 \pm 9$).

Hepatic lipid content

Table 4 shows the effect of diet on the liver total lipids and lipid fractions. Increasing cholesterol intake was associated with a 6-fold increase in hepatic cholesterol, from 4 ± 1 mg/g to 15 ± 2 mg/g liver wet weight in pigs consuming 0 and 1% cholesterol (no added fat) diets ($P < 0.01$). All of the increase in hepatic cholesterol levels in response to the high cholesterol diets was in the cholesteryl ester fraction; there were no significant effects of cholesterol intake on hepatic total or microsomal free cholesterol levels. There was a significant ($P < 0.05$) increase in triglycerides as a result of consumption of fat but there was no differential effect of fat type. In fact, the type of fat had no significant effect on the levels of hepatic lipids. Lastly, no detectable differences were observed in hepatic lipids between the two breeds.

LDL-receptor protein abundance

Specific binding of the antibody to LDLr protein was confirmed by Western blotting (**Fig. 3**). The antibody bound to one band corresponding to a protein of approximately 126 kDa. Marked changes were observed in the level of hepatic LDLr protein in response to the diet. LDLr protein abundance decreased in a dose-dependent manner with increasing cholesterol intake ($P < 0.05$; **Fig. 4**). As cholesterol intake increased from 0 to 0.25%, there was no significant effect on LDLr protein abundance; however, at 0.5% and 1% cholesterol, LDLr protein was reduced by 20% and 60%, respectively, compared to either control pigs or those consuming 0.25% cholesterol ($P < 0.05$). Furthermore, there was a distinct fatty acid effect; hepatic LDLr protein levels were approximately 40% higher in pigs consuming PUFA and approximately 40% lower in those fed SFA compared to control pigs consuming no added fat ($P < 0.05$; Figs. 3 and 4). It is important to note that these differential effects of the dietary fatty acid saturation were only distinguishable at the lowest intake of cholesterol and were not observed at higher intakes of dietary cholesterol. No detectable differences were observed in hepatic LDLr protein abundance between the two breeds.

LDL-receptor mRNA

Dietary fatty acids had a profound effect on LDLr mRNA (Figs. 5 and 6). As shown in **Fig. 5,** the human LDLr cDNA hybridized to a single pig mRNA transcript of approximately 5 kb [while this is slightly smaller than the human receptor (~5.3 kb), this likely reflects the smaller size of the pig receptor]. As expected, increasing dietary cholesterol intake reduced hepatic LDLr mRNA abundance (**Fig. 6**). LDLr mRNA levels were reduced by 37% when dietary cholesterol intake increased from 0.25% to 0.5%, and were barely detectable in samples from animals consuming 1.0% cholesterol. Consistent with the observed effects of dietary fatty acids on LDLr levels, consumption of PUFA or SFA had striking effects on LDLr mRNA levels at different levels of cholesterol intake. At 0.25% cholesterol, PUFA increased mRNA levels by 2-fold ($P < 0.01$), whereas SFA decreased them by ~60% ($P < 0.01$). In contrast, PUFA or SFA had no significant effect on mRNA levels at higher cholesterol intakes. No detectable differences were observed in hepatic LDLr mRNA abundance between the two breeds.

Correlation between hepatic LDL-receptor protein abundance, LDL-receptor mRNA abundance, and other lipid parameters

The relationship between hepatic LDLr mRNA and LDLr protein is shown in **Fig. 7A.** As expected, hepatic LDLr abundance was significantly correlated with hepatic LDLr mRNA ($r = 0.747$; $P = 0.05$). There was no significant correlation, however, between plasma LDL-C levels and LDLr protein or mRNA levels (Fig. 7B, 7C). Correlations between hepatic lipid levels and LDLr protein and mRNA abundance are shown in **Fig. 8A–F.** Hepatic cholesteryl ester content was significantly and nega-

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TABLE 4. Hepatic lipid levels (mg/g) in pigs fed PUFA or SFA and 0.25, 0.5 and 1.0% cholesterol

	Total Lipid	Total Cholesterol	Free Cholesterol	Cholesteryl Ester	Triglycerides	Microsomal Cholesterol
						μg/mg protein
Control group	12 ± 2[a]	4 ± 1[a]	2 ± 1	1 ± 1[a]	9 ± 1[a]	10 ± 1
Experimental group						
0.25% cholesterol						
No added fat	19 ± 3[b]	4 ± 1[b]	2 ± 2	3 ± 1[b]	12 ± 1.9[a]	11 ± 1
SFA	39 ± 6[c]	6 ± 1[b]	2 ± 1	3 ± 1[b]	24 ± 3.5[b]	9 ± 1
PUFA	39 ± 6[c]	5 ± 1[b]	2 ± 1	3 ± 1[b]	30 ± 4.7[b]	10 ± 1
0.5% cholesterol						
No added fat	25 ± 4[b]	9 ± 2[c]	2 ± 1	7 ± 1[c]	14 ± 3[a]	9 ± 1
SFA	48 ± 9[d]	10 ± 2[c]	2 ± 1	9 ± 1[c]	35 ± 6[c]	12 ± 1
PUFA	47 ± 8[d]	11 ± 2[c]	2 ± 1	9 ± 1[c]	34 ± 6[c]	11 ± 1
1.0% cholesterol						
No added fat	34 ± 6[c]	15 ± 2[d]	3 ± 1	12 ± 3[d]	16 ± 3[b]	11 ± 1
SFA	68 ± 11[e]	17 ± 3[d]	3 ± 1	14 ± 3[d]	40 ± 6[d]	11 ± 1
PUFA	72 ± 11[e]	22 ± 3[e]	3 ± 1	19 ± 3[d]	48 ± 8[e]	12 ± 2
Statistics, main effects						
Cholesterol	$P < 0.01$	$P < 0.01$	NS	$P < 0.001$	$P < 0.05$	NS
Fat type	NS	NS	NS	NS	NS	NS
Breed	NS	NS	NS	NS	NS	NS
Interactions	NS	NS	NS	NS	NS	NS

Values are means ± SD (n = 6 animals per dietary treatment). Main effects were differences due to amount of dietary cholesterol (0, 0.25, 0.5, 1.0 wt%) as determined by three-way ANOVA; differences due to fat type (no added fat, SFA, PUFA) determined by three-way ANOVA; differences due to breed (Duroc versus Yorkshire); and differences due to interactive effects among dietary fat × cholesterol × breed, NS, not significant.
[a,b,c,d,e]Values within a column with different superscripts are significantly different ($P < 0.05$).

tively correlated with both LDLr mRNA (Fig. 8A, $r = -0.651$; $P < 0.05$) and LDLr protein abundance (Fig. 8B, $r = -0.682$; $P < 0.05$). Hepatic cholesteryl ester concentrations were best fit with a curved line using a quadratic equation. No relationship was observed between hepatic free cholesterol or triglycerides and LDLr protein abundance and mRNA levels (Fig. 8C–F).

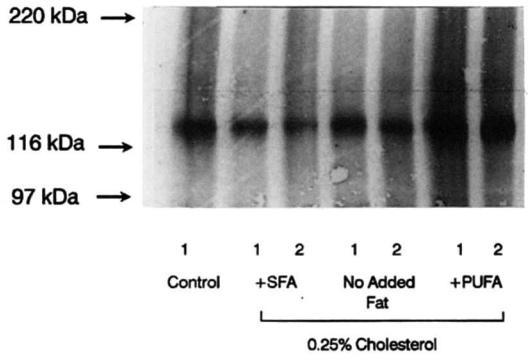

Fig. 3. Western blot of hepatic LDLr protein from a control pig or pigs fed 0.25% cholesterol (wt%) with no added fat, palm oil (SFA), or sunflower oil (PUFA) at 16% (wt%). Solubilized hepatic membrane proteins (100 mg) were randomly selected from one control pig and two pigs from each group fed 0.25% cholesterol. Proteins were separated on SDS-PAGE, transferred to nitrocellulose, and LDL receptor protein abundance was analyzed using a polyclonal antibody to the rat LDLr as described in the Methods section. The autoradiograph was developed after 15 sec exposure at room temperature.

DISCUSSION

The results of the present study provide new insight into the mechanisms by which fatty acids affect the LDLr. When pigs consumed a diet containing 0.25% cholesterol, SFA (palmitic acid) markedly decreased whereas PUFA (linoleic acid) increased LDLr protein

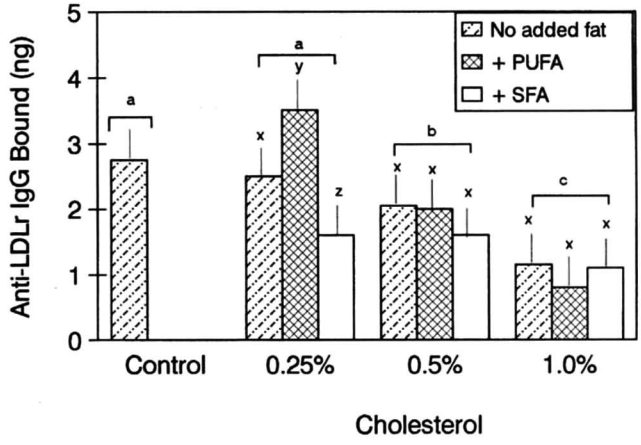

Fig. 4. Effect of dietary treatments on porcine hepatic LDLr protein abundance (ng anti-LDLr IgG bound). Values are means ± SD (n = 6 pigs per dietary treatment group). Bars with different superscripts (a, b, c) indicate significant ($P < 0.05$) effects of level of dietary cholesterol. Bars with different superscripts (x, y, z) within a cholesterol level are significantly different ($P < 0.05$).

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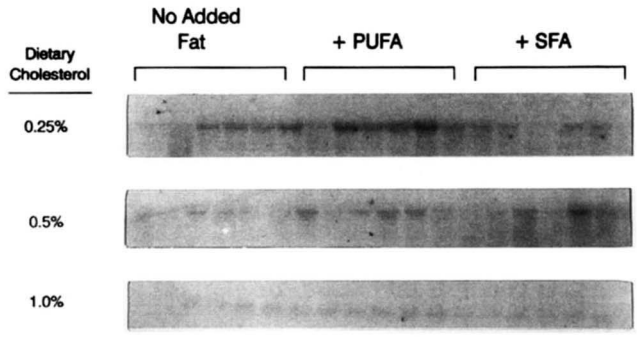

Fig. 5. Northern blot analyses of hepatic LDLr mRNA. Total RNA (20 μg) was isolated from liver of pigs (n = 6 per treatment) fed 0.25, 0.5, or 1% cholesterol (wt%), with or without sunflower oil (PUFA) or palm oil (SFA) at 16% (wt%). Total RNA was separated on agarose gels, transferred to membranes, and probed for LDL receptor mRNA using a ^{32}P-labeled human LDLr cDNA as described in the Methods section. The autoradiographs were developed after 4 weeks exposure at −70°C.

relative to animals consuming cholesterol only or to controls maintained on a low fat/cholesterol-free diet. Furthermore, we have shown that these distinct effects of dietary fatty acids were accompanied by parallel changes in LDLr mRNA levels. The data from the present study support those from other studies in nonhuman primates (21, 24, 25). In these studies, LDLr mRNA levels were reduced between 30–50% in animals consuming SFA from coconut oil (predominately lauric acid and myristic acid) or lard (~24% palmitic acid), and including a wide range of dietary cholesterol, when compared to animals consuming unsaturated fatty acids. Collectively, these data provide strong evidence

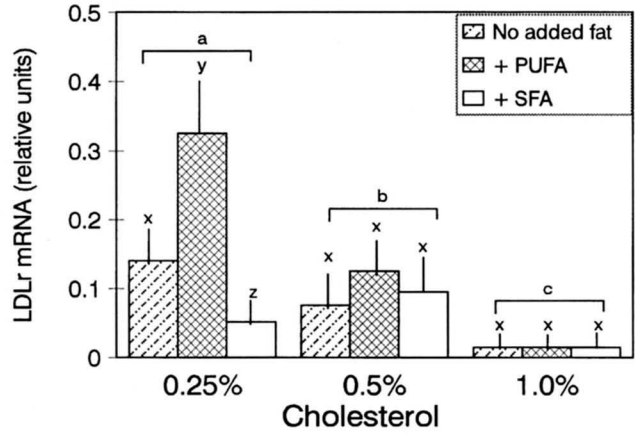

Fig. 6. Effects of dietary treatments on porcine hepatic LDLr mRNA (relative units), by Northern blot analyses. Values are mean ± SD (n = 6 pigs per dietary treatment group). Bars with different superscripts (a, b, c) indicate significant ($P < 0.05$) effects of level of dietary cholesterol. Bars with different superscripts (x, y, z) within a level of cholesterol are significantly different ($P < 0.01$).

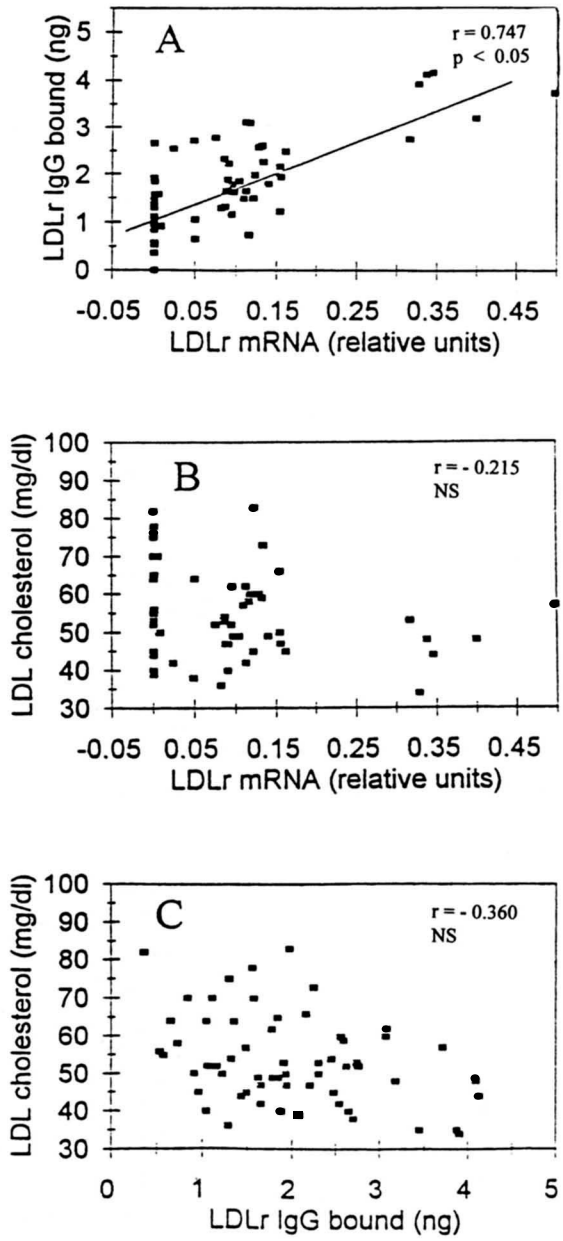

Fig. 7. Correlation scatter plots of the relative abundance of hepatic LDLr mRNA (relative units) and LDLr protein (ng LDLr IgG bound) and plasma LDL-cholesterol levels (mg/dl). Data are from individual animals (n = 60). The Pearson's correlation coefficients (r values) and level of significance are noted for each plot. Plot A displays the correlation between LDLr mRNA and LDLr protein abundance. Plot B displays the correlation between LDLr mRNA abundance and plasma LDL-cholesterol levels. Plot C displays the correlation between LDLr protein abundance and plasma LDL-cholesterol levels.

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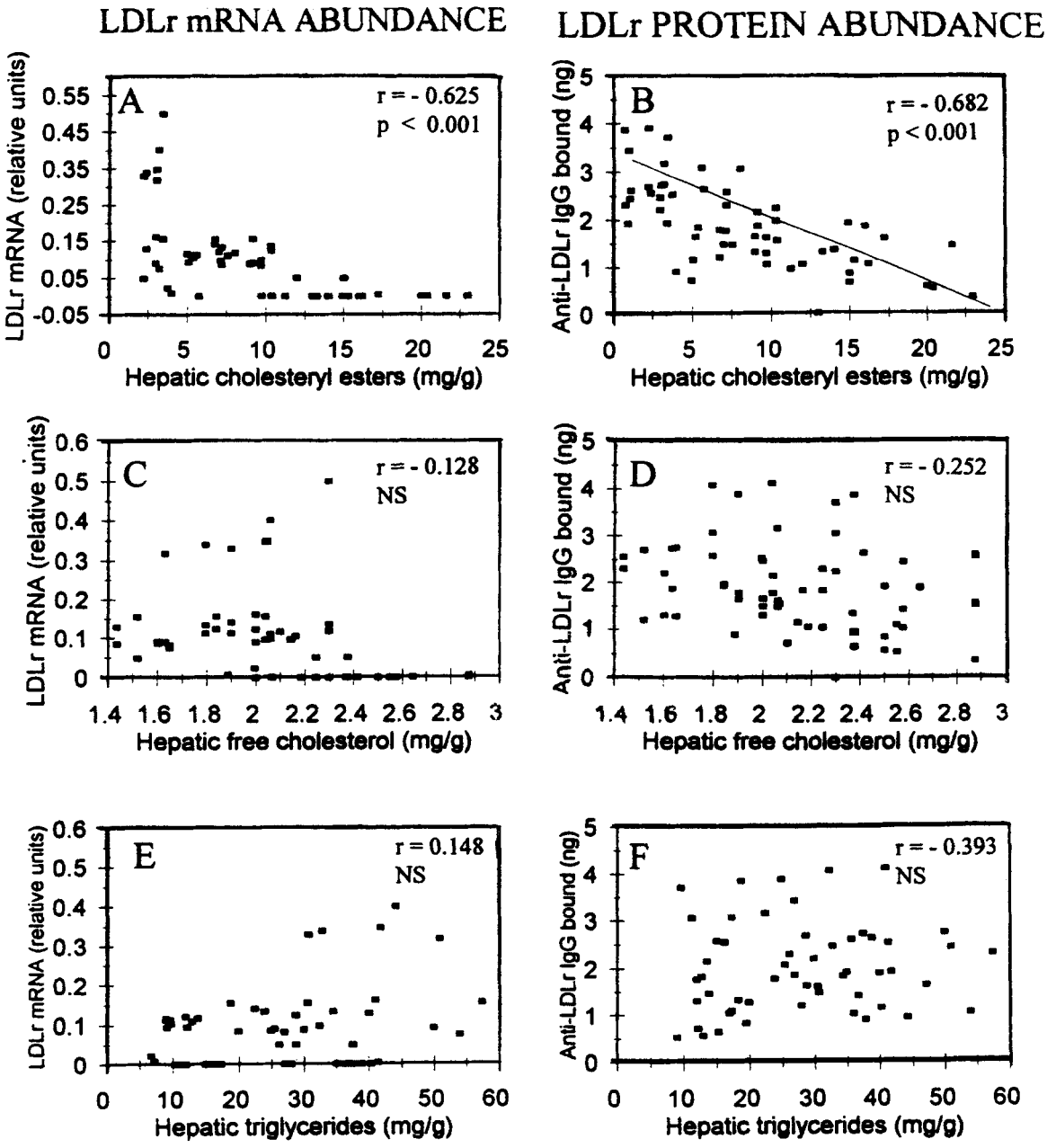

Fig. 8. Correlation scatter plots of hepatic lipid levels and hepatic LDLr mRNA and protein levels. Data are from individual animals (n = 60). The Pearson's correlation coefficients (r values) and level of significance are noted for each plot. For ease of comparison, the plots are grouped in columns by LDLr mRNA or LDLr protein abundance, with the three different hepatic lipid measures shown side by side for comparison. Plots A and B display the correlation between total hepatic cholesteryl esters, and hepatic LDLr mRNA and protein abundance, respectively. Plots C and D display the correlation between hepatic free cholesterol and hepatic LDLr mRNA and protein abundance, respectively. Plots E and F display the correlation between hepatic triglycerides and hepatic LDLr mRNA and protein abundance, respectively.

that dietary fatty acids differentially regulate LDLr mRNA levels.

Of particular interest in the present study is the finding that LDLr protein and mRNA abundance were higher when PUFA was added to diets containing cholesterol (0.25%) compared to animals consuming low fat/cholesterol-free diets which theoretically should exhibit maximal LDLr expression. These results are consistent with those reported by Horton et al. (26) in hamsters consuming PUFA with no added cholesterol. Of importance is that the study by Horton, Cuthbert, and Spady (26) as well as the present study in pigs observed these effects compared to a carbohydrate control (which is the standard comparison to which fatty acids

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are compared in human dietary studies) rather than relative to other fatty acids (e.g., medium-chain triglycerides such as caprylic or capric) which may also have independent cholesterolemic effects (41). Thus, taken together, these data provide strong evidence for an independent and positive effect of PUFA on the regulation of LDLr expression.

A unique aspect of this study is that these differential effects of fatty acids on LDLr expression were only observed when pigs consumed the lowest level of dietary cholesterol. This was surprising given that, in humans, the effect of dietary cholesterol on LDL-cholesterol levels has been shown to be quantitatively less important than chain length and the degree of fatty acid saturation (42, 43). Furthermore, studies in hamsters (4, 11), guinea pigs (7, 8), and monkeys (9) have shown that PUFA attenuate while SFA exacerbate the suppressive effects of dietary cholesterol on LDLr activity. Nevertheless, at the lowest intakes of cholesterol (0.25%) used in this study, the changes in LDLr expression observed in these pigs fed PUFA or SFA are consistent with these studies and indicate that fatty acids have potent effects on LDLr expression. It is also important to note that an interaction between fatty acid saturation and cholesterol was reported in a recent study using guinea pigs (44) in which the effect of fatty acids on LDL cholesterol FCR was not observed at high intakes of cholesterol. Collectively, these data suggest that at high intakes, cholesterol has a dominant and repressive effect on LDLr mRNA levels that cannot be attenuated by fatty acids.

In light of the striking changes in levels of both LDLr protein and mRNA observed in the present study, it was surprising that neither the consumption of SFA, PUFA, nor cholesterol resulted in a more significant hypercholesterolemic response. This is particularly so because our rationale for using these pigs as a model was a result of their being regarded by some as one of the best models for human lipid and lipoprotein metabolism (30). Other studies using pigs (similar in age and gender) have found significant and predictable cholesterolemic responses to changes in dietary lipids (45–48), although, as in a human population, marked variability in these responses exists (49, 50). It also is important to note that studies with the baboon have found no apparent relationship between changes in LDLr mRNA and plasma LDL-C levels (21, 24), suggesting that some species have a very efficient mechanism(s) to compensate for a large change in fat and cholesterol intake (51).

Whether this lack of effect observed (and the differential response observed between the two breeds) is related to genetic variation in one or multiple pathways of cholesterol regulation (52–54) is unknown. In addition, variants in the apoB-100 allele have been shown to contribute to differences in response to a dietary cholesterol challenge in pigs (55); however, these are also associated with marked hypercholesterolemia in animals consuming a low fat/cholesterol-free diet which was not observed in the present study. While we studied only the effects of dietary fatty acids on LDLr regulation, the plasma LDL-C levels can be affected by changes in both LDL uptake as well as production. For example, the addition of dietary lipid to low fat diets has been shown to increase LDL production rate in the hamster (4, 10–13), although this has not been observed in Cebus (9) or cynomolgous (14) monkeys. Accordingly, if basal cholesterol production was low in the pigs used in this study, and cholesterol production was not significantly stimulated by dietary lipid, or, if these pigs had an efficient mechanism to compensate for the increased dietary cholesterol and lipid, then conceivably even large changes in LDLr number would have only small effects on the circulating LDL-C levels. In fact, Spady, Woollett, and Dietschy (56) propose that if production rates were low the plasma LDL-C levels would not increase until more than 50% of the receptor activity is lost. Although we did not measure LDL production in these animals, the fact that the pigs consuming 0.25% cholesterol did not have elevated hepatic sterol levels suggests that they have a very efficient mechanism to compensate for the increased dietary cholesterol and fat, and that this, in addition to a low cholesterol production rate, may prevent the increase in plasma LDL levels regardless of effects on hepatic LDLr levels. Thus, our data clearly demonstrate that the liver LDLr are of little significance in controlling the plasma LDL-C levels in pigs.

Collectively, our data and those from other animal models described above suggest that other mechanism(s) or pathway(s) independent of the LDLr function to maintain the plasma cholesterol levels in these diverse animal models. It is important to note that, in humans, we have found the opposite to be true; specifically, increasing dietary SFA decreases the level of LDLr in circulating mononuclear cells and this is associated with an increase in plasma LDL-C (57). Moreover, in this latter study, we found that the percentage increase in LDLr protein abundance in response to changes in dietary SFA was significantly and negatively associated with the percentage decrease in LDL-C levels ($r = -0.655$; $P < 0.001$). It is important to note, however, that in human populations, non-responders to large changes in dietary fat and cholesterol also have been identified under controlled clinical settings (58, 59). The metabolic bases underlying these responses are unknown, and it is apparent that our understanding of the interrelationships between genetic background and re-

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sponses to dietary cholesterol (and fatty acids) is incomplete. It is likely that pigs could be an appropriate model to study these mechanisms.

Our understanding of the molecular mechanisms that explain these effects of fatty acids on LDLr mRNA levels is incomplete. Most of our knowledge pertains to the control of transcription of the LDLr gene by the intracellular sterol content of the cell (and the putative sterol regulatory pool) (60). Progressively increasing the cholesterol content of the diet increases this sterol regulatory pool and induces a number of predictable events: 1) an increase in hepatic cholesteryl esters; *2*) a suppression of cholesterol synthesis; *3*) an increase in LDL-C production; and *4*) a decrease in LDLr synthesis. These events collectively contribute to the elevation of plasma LDL-C. Other responses, including increased secretion of VLDL, may also contribute to the net response of the plasma cholesterol levels as well and may explain some of the differences in response. While we did not measure all of these parameters that contribute to cholesterol homeostasis, our observations with respect to changes in LDLr mRNA levels and lipid accumulation in response to dietary cholesterol are consistent with the events described above (i.e., we observed a dose-dependent increase in liver cholesteryl esters, a reduction in LDLr mRNA, and an increase in the plasma LDL-C). In addition, our results are consistent with those recently reported in guinea pigs demonstrating that cholesteryl ester concentrations were logarithmically related to hepatic LDLr B_{max} (44). In the present study, hepatic cholesteryl ester levels above 14 mg/g were associated with no further decrease in receptor expression. In comparison, this level was 4 mg/g in guinea pigs (44) while in the hamster (4, 11–13, 25, 61) and in Cebus monkeys (9), there appears to be a linear relationship between LDLr activity and cholesteryl ester levels up to 30 mg/g. In the present study, we observed no relationship between LDLr mRNA levels and the level of hepatic total or microsomal free cholesterol. Although the prevailing theory is that free cholesterol (or an oxysterol) regulates LDLr gene expression, others also did not find significant changes in free cholesterol levels as affected by dietary lipid (9, 25). This occurs presumably because the liver prevents the accumulation of free cholesterol by several mechanisms described above; however, it is possible that some separate, unidentified regulatory pool of cholesterol is affected.

A key question that emerges from these observations is: What are the molecular mechanisms by which fatty acids affect the expression of the LDLr gene? Our results show that the influence of dietary fatty acids is dependent on the level of dietary cholesterol intake; clearly, in pigs, the latter has a dominant influence. Thus, it is likely that fatty acids interact with cholesterol to regulate mRNA levels. In vitro studies with different cell types (62) and studies with hamsters (56, 61) have shown that fatty acids can affect the storage of cholesteryl esters and influence intracellular sterol distribution. Activation of the ACAT enzyme (which controls the esterification of intracellular cholesterol) by oleic acid has been hypothesized to cause a shift in sterol distribution away from the regulatory pool of cholesterol toward the non-active cholesteryl esters (61). SFA have been shown to suppress this enzyme which may result in a greater proportion of cholesterol remaining in the regulatory pool. An increase in hepatic cholesteryl esters has been shown to be negatively correlated with LDL-receptor activity in hamsters (12, 61). However, our data with pigs and those from other animal models (9) are not consistent with this hypothesis. In the present study, hepatic cholesteryl ester concentrations in pigs increased as cholesterol consumption increased, and there were no differences in hepatic cholesterol attributed to the dietary fatty acid composition. This is likely because oleic acid, the preferred substrate for ACAT, was similar in the experimental diets. Another interaction between dietary fatty acids with the sterol regulation of LDLr mRNA is that fatty acid modification of membranes could lead to an increased/decreased responsiveness to intracellular sterols. For example, fatty acids might alter membrane properties of the nuclear membrane or endoplasmic reticulum and thereby interfere with (SFA) or enhance (PUFA) the release of membrane-bound SREBP that confers sterol regulation of the LDLr gene (63–65).

An interesting observation in the present study is that animals that consumed PUFA had higher LDLr protein and mRNA abundance relative to control animals (which theoretically should exhibit maximal LDLr expression). These results support a theory for a positive regulatory effect of dietary fatty acids that may be independent of cholesterol; however, the mechanisms by which fatty acids regulate LDLr message levels are obscure. It is possible that fatty acids affect post-transcriptional processing and/or mRNA stability (66) or, perhaps, transcription of the LDLr gene. Nuclear run-on assays with nuclei prepared from livers of animals fed dietary PUFA have shown effects on the transcription rate of a number of genes including fatty acid synthase (FAS) and S14 mRNA (67), stearoyl Co A desaturase (68), pancreatic lipase (69), pyruvate kinase (70), and lipid binding proteins (67, 71). (It is of interest that in these studies, MUFA and SFA have no apparent influence on the expression of these particular genes.) Although a putative fatty acid response element has been localized in the promoter region of FAS and S14 genes

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(72), the specific sequence necessary to confer fatty acid regulation of gene transcription has not been fully delineated. Alternatively, it also is conceivable that fatty acids could act as mediators of transcriptional regulation via covalent modification (e.g., myristoylation or palmitoylation) (73) of key *trans*-acting nuclear proteins. Another consideration is that the effects of fatty acids could be mediated by binding to peroxisome proliferator activated receptors (PPAR). Recently, dietary PUFA, and the synthetic arachidonic acid analog ETYA, have been shown to be very potent activators of PPARs (74–76). It remains to be resolved, however, whether the differential effects of SFA and PUFA on expression of the LDLr gene involve PPAR and subsequent changes in transcription of the LDLr gene. The examples above present several plausible mechanisms by which fatty acids may control gene expression. Whether these have any relevance to the regulation of the transcription of the LDLr gene remains to be resolved. Studies are ongoing in our laboratory to learn more about the molecular mechanisms for the disparate effects that fatty acids have on LDLr mRNA levels; it may only be possible to discern their true independent/interactive effects using an in vitro cell culture system in which the independent effects of fatty acids and sterols can be studied.

In summary, we have shown that dietary fatty acids have very potent and distinctly different effects on LDLr protein abundance which are the result of changes in LDLr mRNA levels. Furthermore, our results demonstrate that PUFA, specifically linoleic acid, exerts a strong and independent positive effect on LDLr expression. Further studies are needed to fully elucidate the molecular mechanisms by which fatty acids affect LDLr mRNA abundance.

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