A high-cholesterol diet increases the association between caveolae and insulin receptors in rat liver

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Abstract Caveolin-1, a component of caveolae, regulates signaling pathway compartmentalization by interacting with tyrosine (Tyr) kinase receptors and their substrates. Perturbations in caveolae lipid composition have been shown in vitro to displace proteins from lipid microdomains, thereby altering their functionality and subsequent downstream signaling. The role of caveolin-1 in insulin receptor (IR) signaling has been widely investigated in vitro mainly in 3T3-L1 adipocyte cells. However, in vivo experiments investigating this connection in liver tissue have not been carried out. The objective of the present study was to investigate the effects of a high-cholesterol diet on caveolin-1 expression and IR localization and activity in the rat liver. Compared with a standard diet, rats fed with diet rich in cholesterol significantly altered liver caveolae by increasing both caveolin-1 (66%, $P < 0.05$) and caveolin-2 (55%, $P < 0.05$) expression while caveolin-1 mRNA levels were reduced. Concomitantly, a 25% increase in localization of the caveolae-resident signaling protein IR was observed. The distribution of caveolar and noncaveolar phosphorylated IR was unaffected but insulin-induced IR activation was significantly enhanced following consumption of the high-cholesterol diet (120%, $P < 0.001$). However, the downstream molecules IRS-1 and Akt have shown impaired activity in cholesterol-fed rats suggesting insulin resistance condition. Insulin stimulation failed to induce Tyr phosphorylation of caveolin-1 in cholesterol-fed rats. These findings suggest a mechanism by which a high-cholesterol diet altered caveolin-1 expression in vivo accompanied by altered IR localization and activity.—Hahn-Obercyger, M., L. Graeve, and Z. Madar. **A high-cholesterol diet increases the association between caveolae and insulin receptors in rat liver. *J. Lipid Res.* 2009.** 50: **98–107.**

Supplementary key words caveolin • insulin signaling • lipid rafts

Caveolae are flask-shaped vesicular invaginations in the plasma membrane and are considered to be a subset of lipid rafts (1, 2). They are enriched in cholesterol and sphingolipids (3) and insoluble in nonionic detergents, which can facilitate isolation of caveolae as detergent-resistant membrane microdomains. On the basis of their lipid composition, lipid rafts and caveolae may "trap" proteins that reveal higher affinity to membrane microdomains than to the surrounding plasma membrane (4, 5).

The importance of lipid composition for the function of membrane proteins has been demonstrated for a number of relevant proteins involved in signal transduction (6, 7) and membrane trafficking (8). Cholesterol is an essential structural component of lipid rafts and caveolae (9, 10). The function of caveolae is dependent on a sufficient level of cholesterol in the plasma membrane and caveolae (11). Fielding et al. (12, 13) have recently reported that in human fibroblasts, the cellular free cholesterol level regulates the transcription of the gene encoding caveolin, the structural protein of caveolae. Caveolin-1 forms high molecular weight homo-oligomers or hetero-oligomers with caveolin-2 (14, 15). Caveolin-1 and caveolin-2 often colocalize in the same membrane compartment; in the absence of caveolin-1, caveolin-2 remains exclusively in the Golgi apparatus (16). The transport of caveolin-2 to the plasma membrane appears to require caveolin-1 (17). Caveolin-1 and -2 may participate in the formation of caveolae (14, 18).

Lipid rafts and caveolae have been shown to support signaling by functioning as platforms for recruitment and organization of signal transduction molecules and to suppress signaling by sequestering signaling proteins (19, 20). A number of studies, predominately in adipocytes, suggest that intact caveolae are necessary for insulin signaling. In 3T3-L1 adipocytes, insulin receptor (IR) was reported to be concentrated in caveolae (21) and to interact with caveolin-1 to modulate insulin signaling (22, 23). It has also been reported that insulin induced tyrosine (Tyr) phosphorylation of caveolin (24). Disrupting the lipid structure of caveolae by depleting cholesterol content with

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Abbreviations: IR, insulin receptor; TBST, tris-buffered saline with 0.1% Tween; TC, total cholesterol; TG, triglycerides; Tyr, tyrosine.

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methyl-β-cyclodextrin attenuated IR signaling (25). Rafts/caveolae have also been implicated in insulin-stimulated glucose transport in 3T3-L1 adipocytes by a mechanism independent of phosphatidylinositol 3-kinase (26, 27). Caveolin-1 knockout mice have been created that are viable despite a complete ablation of caveolae (28). The mice suffer from impaired nitric-oxide signaling, vascular dysfunction, hyperproliferation of endothelial cells, abnormalities in lipid homeostasis, insulin resistance, and decreased IR expression in adipose tissue (29, 30). These observations suggest that caveolae in adipocytes contribute to the strength and specificity of insulin signaling. However, the role of caveolae in insulin signaling in liver tissue has received little attention. This may be due to the fact that the liver contains a lower level of caveolin-1. Nevertheless, it has been shown that caveolin-1 is located in liver parenchymal cells, with negligible levels detected in endothelial cells (31, 32). The discovery that the IR is compartmentalized into caveolae (33, 34) introduced a new framework for the understanding of the molecular mechanisms whereby exposure to a high-cholesterol diet may alter insulin signaling. Therefore, the aim of this study was to determine whether a high-cholesterol diet modulates the hepatic caveolar lipid environment and IR microdomain localization and function in vivo. We observed that a high-cholesterol diet significantly upregulated caveolin-1 content in caveolae in rat liver. In addition, alterations in caveolar IR localization associated with modulation of IR function were observed. Taken together, these findings indicate that a high-cholesterol diet can alter the caveolae resident proteins and thereby influence insulin signaling in the liver.

METHODS

Materials

OptiPrep and TriReagent were purchased from Sigma (Rehovot, Israel). SYBR-green PCR master mix was purchased from ABgene (Epsom, UK). Protein A-Agarose and polyclonal antibodies against insulin Rβ and caveolin-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), antibodies against phosphor-IGF-I receptor β (Tyr1131)/IR β (Tyr1146), phospho-Akt (Ser473) were obtained from Cell signaling (Denvers, MA), and antibody against IRS-1 was purchased from Upstate (Upstate Biotechnology, Lake Placid, NY) . Monoclonal antibody against phosphotyrosine (Clone PY20) was obtained from Exalpha Biologicals (Watertown, MA). Monoclonal antibody against phospho-caveolin-1 (P-Tyr-14) was purchased from BD transduction laboratories (BD Pharmingen, San Jose, CA). A HRP-conjugated secondary antibody against rabbit was purchased from Pierce (Rockford, IL) and against mouse was obtained from Jackson ImmunoResearch (West Grove, PA). All chemicals were obtained from Sigma (Rehovot, Israel).

Experimental design

All experimental procedures using rats were approved by the Institutional Animal Care Committee of the Hebrew University of Jerusalem. Twenty-four Sprague-Dawley rats (Harlan, Jerusalem, Israel) were housed in a controlled environment (22–24°C and 12 h light-12 h dark) with free access to tap water and foods. Diets were randomly ascribed to two groups of rats (n = 12) fed: *1*) standard diet, and *2*) a high-cholesterol diet: 1% (w/w) cholesterol. Body weights were recorded weekly while both groups of rats were maintained on the diets for 21 days and fasted over night before liver removal. The rats were anesthetized with ketamine-xylazine (100 mg/10 mg per kilogram, ip) and then half animals in each group received insulin (10 U, ip) while the remainder received a saline injection. After 30 min, when glucose levels were reduced to approximately 40 mg/dl in animals receiving insulin (measured in blood samples from tail tips) liver tissue along with blood samples were collected and stored at −80°C until use.

Intravenous insulin tolerance test

Cholesterol and control-fed rats (fasted overnight) were subjected to an i.v insulin tolerance test (ITT; 1 U/kg body weight of regular insulin was injected into the portal vein) and tail blood samples were collected at 0 (basal) 4, 8, 12, and 16 min after injection for blood-glucose measurements. The rate constant for plasma glucose disappearance (K_{itt}) was calculated using the formula 0.693/(t1/2). The plasma glucose t1/2 was calculated from the slope of the least squares analysis of the plasma glucose concentrations during the linear phase of decay (35).

Metabolic measurements

Plasma glucose concentrations were measured by the glucose oxidase method (36). Plasma NEFA levels were determined using an acyl-CoA oxidase based colorimetric kit (WAKO NEFA-C; WAKO Pure Chemical Industries, Osaka, Japan). Plasma insulin was determined using a human insulin ELISA kit (LINCO Research, Inc., Saint Charles, MO) according to supplier's instructions. Plasma concentrations of total cholesterol (TC), LDL, HDL, and triglycerides (TGs) were determined using commercial kits.

Hepatic glycogen and cholesterol

Hepatic glycogen and cholesterol was extracted from nonfasting rats. Briefly, liver tissue samples were boiled with 33% KOH and pelleted with ethanol as previously described for glucose concentration measurement (37). Hepatic cholesterol levels were determined enzymatically using a commercial kit.

Tissue plasma membrane isolation

Plasma membrane isolation was performed as previously described (38). Briefly, 0.4 g liver tissue was homogenized in 4 ml sucrose solution containing 0.25 M sucrose, 10 mM $NaHCO_3$, pH 7.4, 5 mM NaN_3, 100 μM PMSF, 10 μg/ml leupeptin, 10 μg/ml aportinin. The homogenates were centrifuged at 1,000 *g* at 4°C for 10 min. After centrifugation, the supernatant was removed, the pellet was resuspended in 4 ml sucrose solution and centrifuged once again. The combined supernatants were centrifuged at 9,000 *g* for 15 min, and the resulting supernatant was then centrifuged at 105,000 *g* for 110 min at 4°C. The pellet (plasma membranes) was resuspended in 1 ml sucrose solution and stored at −80°C until caveolin-1 and caveolin-2 protein levels were determined. Protein concentration was determined using the Bradford method (39). Bovine serum albumin served as a standard.

Western blot analysis

Aliquots of protein (100 μg) were subjected to SDS-PAGE and transferred to blotting nitrocellulose membrane. The membrane was incubated with 5% nonfat dry milk dissolved in tris-buffered saline with 0.1% Tween (milk-TBST) for 1 h at room temperature to block the nonspecific sites on the membrane. Blots were

probed with primary antibodies against caveolin-1, caveolin-2, IR and p- caveolin-1 in milk-TBST and against p-IR in 5% bovine serum albumin-TBST, overnight at 4°C. The membrane was then washed in TBST for 10 min, four times, followed by incubation with suitable secondary antibody conjugated with horseradish peroxidase for 1 h at room temperature. The membrane was washed for 10 min in TBST four times; visualization of hybridization was carried out using chemiluminescence's reagent. Optical density of each band was analyzed by Gel Pro program (Media Cybernatics, Silver Spring, MD) and normalized against β-actin.

Immunoprecipitation and Western blot analysis

Liver samples were homogenized (1:10) in buffer lysis (50 mM HEPES [pH 7.4], 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM EDTA, 2 mM Na_3 VO_4, 10 mM $Na_4P_2O_2$, 10 mM NaF, 1% NP-40, 10% glycerol, 0.2 mM phenylmethylsulfonyl fluoride, 20 μg of aprotinin per ml, 10 μg of leupeptin per ml) and insoluble protein was removed by centrifugation at 30,000 *g* for 10 min. The protein content was determined by the Bradford method (39). For immunoprecipitation, 5,000 μg of cellular protein was incubated with 0.2 μg of IR-β or IRS-1 antibodies for 2 h at 4°C. Twenty microliters of protein A agarose were added to each sample and incubated for 1 h. Immunocomplexes were collected and washed with lysis buffer three times and resuspended in 60 μl sample buffer × 2. The samples were boiled for 3 min and the beads were removed by centrifugation. Proteins were separated by 7.5% SDS-PAGE and transferred to a nitrocellulose membrane. The blots were blocked with 5% milk solution in TBST buffer (10 mM Tris [pH 7.5], 150 mM NaCl), incubated with αIR-β, αIRS-1 or anti-phosphotyrosine antibodies in TBST containing 5% milk, and then incubated with secondary antibodies conjugated to horseradish peroxidase. The immunoreactive bands were visualized by enhanced chemiluminescence.

Detergent-free lipid raft preparation

Lipid raft isolation was performed as previously described (40). Briefly, all procedures were carried out on ice. Liver tissue (0.2 g) was homogenized in 2ml base buffer (20 mM Tris-HCl, pH 7.8, 250 mM sucrose) to which had been added 1 mM $CaCl_2$ and 1 mM $MgCl_2$ and protease inhibitors at final concentrations of 0.2 mM aminoethyl-benzene sulfonyl fluoride, 1 μg/ml aprotinin, 10 μM bestatin, 3 μM E-64, 10 μg/ml leupeptin, 2 μM pepstatin, and 50 μg/ml calpain inhibitor I. Homogenates were centrifuged at 1,000 *g* for 10 min. The resulting postnuclear supernatant was collected and transferred to a separate tube. The pellet was again lysed by the addition of 0.83 ml base buffer plus divalent cations and protease inhibitors. After centrifugation at 1,000 *g* for 10 min, the second postnuclear supernatant was combined with the first. An equal volume (0.83 ml) of base buffer containing 50% Opti-Prep was added to the combined postnuclear supernatants (equal amount of protein was taken for each sample) and placed in the bottom of a 5 ml centrifuge tube. A 3.34 ml gradient of 0% to 20% OptiPrep in base buffer was poured on top of the homogenate, which was now 25% in OptiPrep. Gradients were centrifuged for 90 min at 52,000 *g* using a TLS-55 rotor in a Beckman ultracentrifuge. Six fractions of 0.83 ml each were collected from the top of the tube. Based on the distribution of the caveolae marker protein caveolin-1 in these fractions we pooled fractions 1–3 and 4–6 and marked these fractions as caveolae and noncaveolae, respectively. A small proportion of caveolin-1 was present in fraction 4, which probably represented nonplasma membrane intracellular species. These pools were then analyzed by immunoblotting with antibodies against IR, pIR, caveolin-1 and p-caveolin-1.

Quantitative RT-PCR

RNA was extracted from liver using TriReagent (Sigma, Rehovot, Israel). Total RNA was DNase I treated using RQ1 DNase (Promega, Madison, WI) for 2 h at 37°C, as previously described. Two micrograms of DNase I-treated RNA were reverse transcribed using MMLV RT (Promega) and random hexamers; 1/20 of the reaction was then subjected to quantitative real-time PCR using the ReadyMix, Sybr Green Master kit (Abgene, Epsom, UK) and the ABI Prism 7300 Sequence Detection system. The following primers were used: IR: 5′ AACCTGTGAGGATGAGTGTCAGAGT 3′ and 5′ CCTTGCTCTTCATCAGTTTCCA 3′; IRS-1: 5′ CCTACTGCTATGGGCTCCG 3′ and 5′ AGGATCTGCAGATGGCCCTC 3′; Caveolin-1: 5′ GACTTTGAAGATGTGATTGC 3′ and 5′ AGATGGAATAGACACGGCTG 3′; Caveolin-2: 5′ GACGTACAGCTCTTCATGGAC 3′ and 5′ GGCTGACAGAAGAGAAGCATC 3′; samples were tested alongside the normalizing gene glyceraldehyde-3-phosphate dehydrogenase (GapdH) 5′ CTGGAAGATGGTGATGGGTT 3′,5′ ATGATTCTACCCACGGCAAG 3′). Samples were analyzed by the $2^{-[delta][delta]Ct}$ method.

Statistical analysis

The significance of the differences between means was determined by Student's *t*-test when a single comparison was performed. When multiple comparisons were carried out, the significance was tested using 2-way ANOVA. A posthoc test (Tukey-Kramer) was performed when the interaction between treatments was significant. Differences were considered significant at $P < 0.05$. JMP version 3.1.6 (SAS Institute) was used for all analyses.

RESULTS

Body weight

Rats consuming the high-cholesterol diet had significantly greater weight gain ($P < 0.05$) when compared with rats receiving the control diet (**Table 1**).

Biochemical analysis and intravenous insulin tolerance test

As shown in Table 1, the plasma levels of TC, HDL and fasting glucose were not statistically different in rats during

TABLE 1. Biochemical measurements taken on the last day of the feeding experiment in male Sprague Dawley rats fed either HC or standard diet

	Standard diet	HC diet	*P*
Final weight, g	297.5 ± 3.9 (21)	310.4 ± 3.4 (21)	0.017
Fasting glucose, mmol/L	7.9 ± 0.28 (6)	7.6 ± 0.53 (6)	0.64
Kitt,%/min	18.7 ± 2.6 (3)	19.4 ± 4.4 (3)	0.89
Fasting insulin, μIU/ml	10.8 ± 4.2 (4)	36.0 ± 16.5 (4)	0.085
Glycogen, μg/g liver tissue	30.1 ± 4.6 (6)	36.7 ± 2.6 (6)	0.24
Cholesterol, mg/g liver tissue	6.6 ± 0.4 (6)	50.5 ± 3.2 (6)	<0.0001
TC, mg/dl	86 ± 7.04 (4)	123 ± 27.5 (4)	0.12
LDL, mg/dl	33 ± 3.02 (4)	76.7 ± 9.7 (4)	0.004
HDL, mg/dl	59.7 ± 4.2 (4)	48.4 ± 5.3 (4)	0.14
Serum triglycerides, mg/dl	63 ± 5.6 (4)	34.25 ± 2.4 (4)	0.003
Serum NEFA, mEq/L	0.40 ± 0.036 (6)	0.28 ± 0.017 (6)	0.001

HC, high cholesterol; TC, total cholesterol. Values are means ± SE. The number of animals in each subgroup is indicated in parentheses.

3 weeks of a high-cholesterol feeding. The insulin levels of cholesterol-fed rats were slightly higher, but not statistically significant. Plasma triglycerides (TGs) and NEFA were dramatically decreased in rats given a high-cholesterol diet for 3 weeks, while LDL was markedly elevated suggests hypercholesterolemia condition. To determine whether cholesterol-fed rats have a defect in insulin responsiveness, an intravenous insulin tolerance test was performed. Despite the increased insulin levels, after 3 weeks of high-cholesterol feeding, blood glucose levels (*Kitt*) were not reduced after insulin injection compared with controls. In contrast to plasma cholesterol, hepatic cholesterol content was significantly elevated in the cholesterol-fed rats. As for hepatic glycogen content, no differences were observed between control and cholesterol-fed rats.

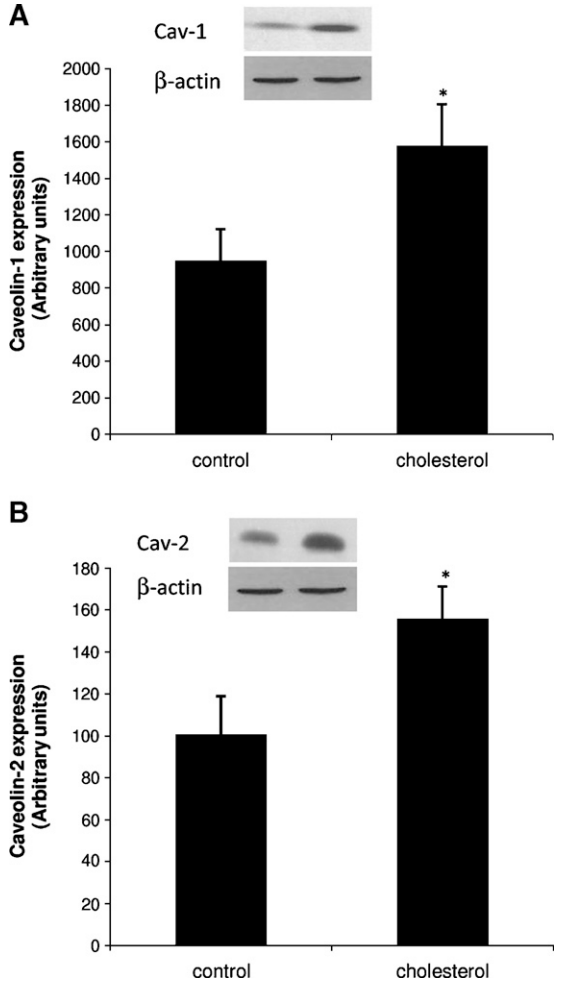

Fig. 1. The effect of a high-cholesterol diet on the expression of caveolin-1 and caveolin-2 protein levels in rat liver. Plasma membrane fractions were isolated from rat liver and 100 μg of protein was subjected to SDS-PAGE (15%) and analyzed by immunoblotting with anti-caveolin-1 and β-actin (A) or anti-caveolin-2 and β-actin (B). Band intensity was determined by the Gel-Pro program, and the results were expressed in arbitrary units. The data are representative of two independent experiments for caveolin-1 and one experiment for caveolin-2. Values represent mean ± SE (n = 6). * $P < 0.05$ for a high-cholesterol diet vs. control.

Caveolin-1 and caveolin-2 protein expression in isolated plasma membrane fractions in response to a high-cholesterol diet

Caveolin-1 is the most abundant protein associated with caveolae located in the plasma membrane; thus, it is commonly used as a marker of caveolae. Caveolin-1 and caveolae play important roles in the activity of IR and cholesterol transport. Stimuli, which increase or decrease caveolin-1 levels, regulate the number of caveolae and thus the IR activity. The effect of a high-cholesterol diet on caveolin-1 and caveolin-2 protein expression was therefore examined in plasma membranes of liver tissue. In the present study, plasma membrane was isolated from controls and cholesterol-fed rats. As shown in **Fig. 1**, caveolin-1 and caveolin-2 protein levels were upregulated by a high-cholesterol diet. These results clearly demonstrate that a high-cholesterol diet alters the liver caveolae microenvironment by localizing the major structural protein, caveolin-1.

Regulation of in vivo caveolin-1, caveolin-2, IRS-1, and IR mRNA expression by a high-cholesterol diet

The increase in caveolin-1 and caveolin-2 protein levels may result from either enhanced transcription of mRNA or a reduced rate of degradation. Therefore, the impact of a high-cholesterol diet on caveolin-1 and caveolin-2 mRNA along with IRS-1 and IR mRNA was measured. The corresponding mRNAs were quantified using RT-PCR and were expressed as the ratio over the expression of gapdh mRNA, as described in Methods. **Fig. 2** shows that IRS-1, IR, and caveolin-2 mRNA expression did not alter after a high-cholesterol diet. However, caveolin-1 was significantly reduced in cholesterol-fed rats compared with control rats ($P < 0.05$).

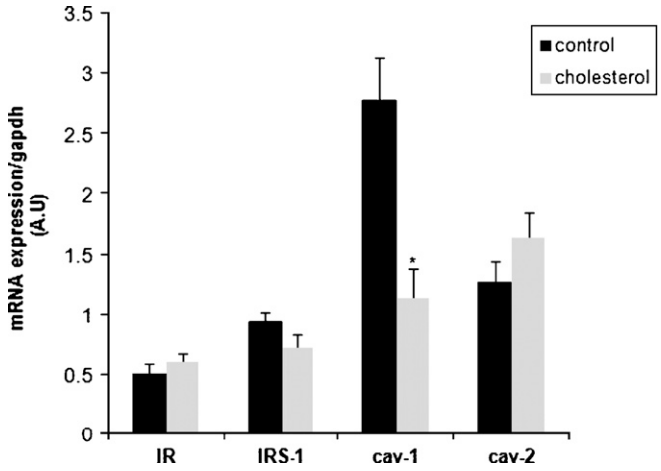

Fig. 2. Caveolin-1, caveolin-2, IRS-1, and IR mRNA in liver of controls and cholesterol-fed rats. Total RNA was extracted from liver tissue and mRNA expression levels of caveolin-1, caveolin-2, IRS-1, and IR were determined by quantitative real-time RT-PCR. The mRNA levels were normalized for each target gene against GAPDH as an internal control. The results (n = 6) were expressed in arbitrary units. Data represent mean ± SE. *$P < 0.05$ for a high-cholesterol diet vs. control.

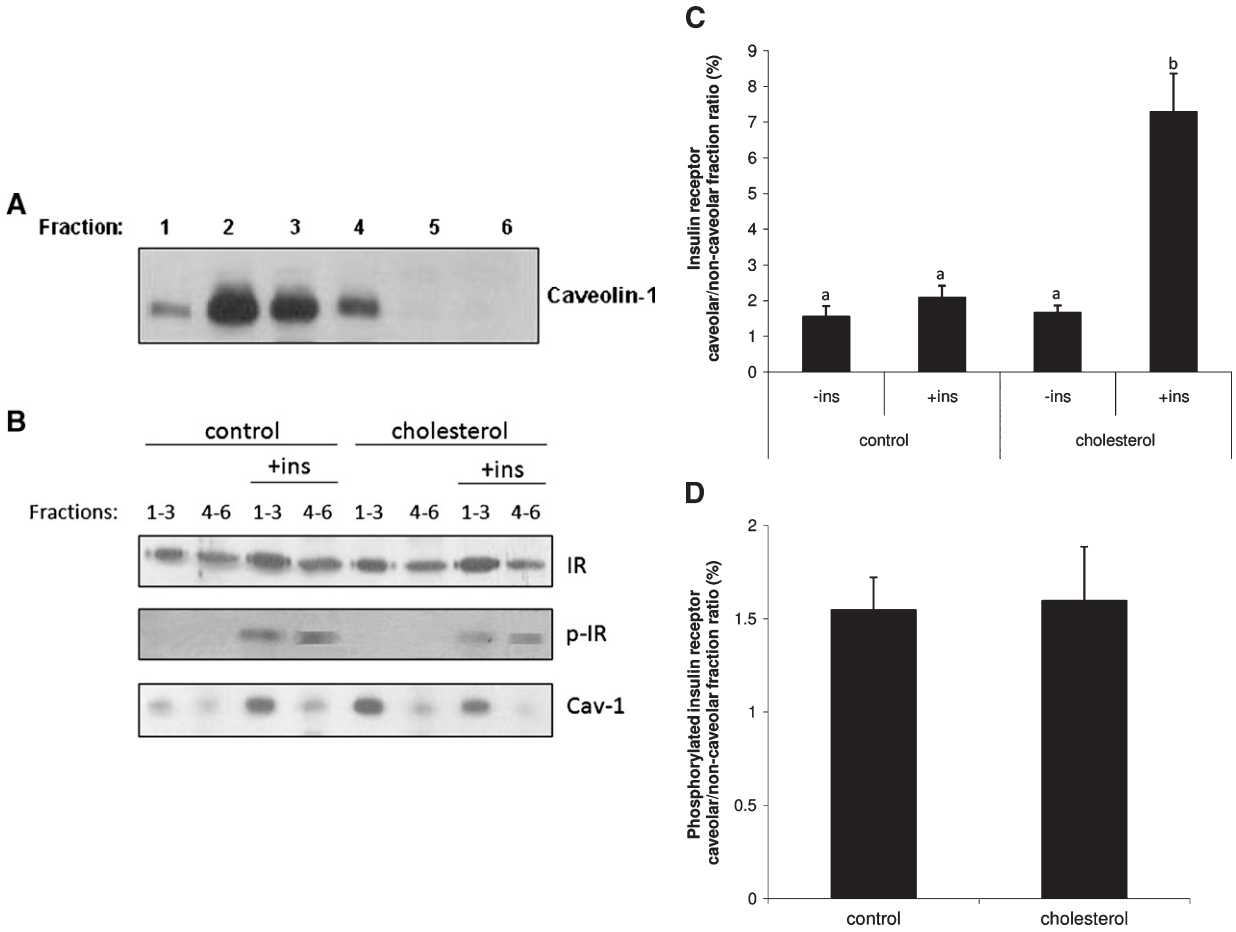

Fig. 3. Insulin receptor (IR) localization in liver caveolae of cholesterol-fed rats. Caveolar fractions were prepared from liver tissue using a 5/25% discontinuous OptiPrep gradient. Fractions 1–3 and 4–6 were pooled and termed caveolar (Cav) and noncaveolar (non-Cav), respectively (A). Aliquots of the fractions were analyzed by immunoblotting using antibodies against IR, phosphorylated IR or caveolin-1 (B). Caveolin-1 staining was used as a raft marker. Ratio of raft versus nonraft associated IR (C) and p-IR (D) in liver tissue of hypercholesteroliemic rats compared with control and rats treated with insulin prior to the liver removal. Distribution of IR (E) and p-IR (F) in caveolar and noncaveolar fractions. The results (n = 3) were expressed as percents of total protein. Data represent mean ± SE. [ab] Different subscripts represent significant differences among groups at $P < 0.05$.

Subcellular localization of IR in cholesterol-fed rats

Insulin signaling is highly sensitive to perturbations of cholesterol-rich microdomains by cholesterol depletion or caveolin-1 knockout, as it requires access to lipid rafts/caveolae for proper activation (29, 41). Therefore, the impact of a high-cholesterol diet on IR localization in rat liver lipid rafts/caveolae was examined. In cholesterol-fed rats that received insulin, the levels of IR in the caveolae fraction was increased compared with controls. (**Fig. 3B**, upper panel). Caveolin-1 protein was observed exclusively in the caveolae fraction (Fig. 3B, lower panel). Quantification of the scanned immunoblots revealed a 7-fold increase in the ratio of caveolea versus noncaveolae associated IR in cholesterol-fed rats compared with controls (Fig. 3C). The induction of IR in caveolae in cholesterol-fed rats was not due to an effect on protein expression, as total cellular levels of IR (**Fig. 4B**) were not affected by diet. Despite the higher receptor levels in the caveoale fraction, the amount of activated IR in caveolae did not differ between control and cholesterol-fed rats (Fig. 3D, F).

Regulation of IR phosphorylation and downstream signaling by high-cholesterol diet

To further elucidate the biological significance of alterations in signaling protein localization, the activation status (Tyr phosphorylation) of IR and downstream molecules, such as IRS-1 and Akt/PKB, in response to a high-cholesterol diet was determined using immunoprecipitation of supernatants from liver homogenates with αIR or αIRS-1 antibodies. After SDS-PAGE and electrotransfer, nitrocellulose membranes were incubated with αPY20. As shown in Fig. 4A, the level of Tyr phosphorylation of IR β-subunit was induced significantly in cholesterol-fed rats compared with controls. Total cellular of IR-β was unaffected by diet (Fig. 4B). We have seen a significant decrease by 50% in insulin-stimulated IRS-1 Tyr phosphorylation in cholesterol-

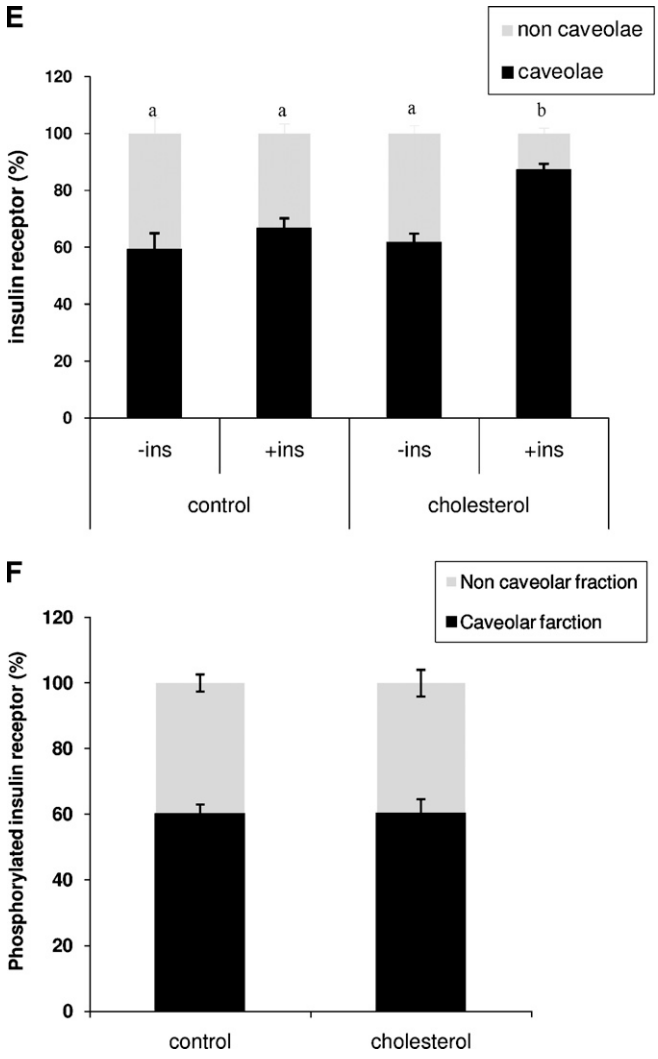

Fig. 3.—*Continued.*

fed rats when compared with controls (Fig. 4C). However, basal IRS-1 levels were essentially similar both treatment groups (Fig. 4D). In cholesterol-fed rats, insulin-induced Akt phosphorylation tended to reduce but with no statistically significance (Fig. 4E). These results indicate that a high-cholesterol diet led to insulin resistance despite the distinct effect of a high-cholesterol diet on IR localization.

Tyrosine phosphorylated caveolin-1 in noncaveolar fractions

Based on the presence of IR in caveolae and on the inducible effect of caveolin-1 on its activity, the extent of caveolin-1 phosphorylation (Tyr 14) induced by insulin stimulation in control and cholesterol-fed rats was investigated. In this study *p*-caveolin-1 at Tyr 14 was only detected in noncaveolar fractions. Control rats treated with insulin significantly increased *p*-caveolin-1 expression in noncaveolar fractions (**Fig. 5**). The *p*-caveolin-1 levels in liver of cholesterol-fed rats were similar with or without insulin treatment meaning that a high-cholesterol diet prevented the induction of cavolin-1 phosphorylation by insulin stimulation. These results suggest that IR activation might result from an increased association of IR with caveolin-1.

DISCUSSION

Cholesterol is an essential component of lipid rafts/caveolae and contributes to the formation of these microdomains. The connection between caveolae integrity and insulin signaling is well documented. To date, in vivo experiments investigating the effect of a high-cholesterol diet on caveolae protein composition and insulin signaling have not been carried out. In this study, for the first time, in vivo modulation of caveolin-1 expression along with IR localization and activity has been demonstrated in the liver of rats fed a high-cholesterol diet.

Animals fed on a high-cholesterol diet for 3 weeks have shown similar glucose disappearance constants (*Kitt*) compared with control animals (Table 1), indicating that cholesterol had no effect on insulin sensitivity. An increase in insulin level was observed and accompanied in a decrease as expected with in NEFA levels. Significant reduction in circulating TG was found in the cholesterol-fed rats, as compared with controls, which may be associated with an inadequate synthesis of lipoproteins contained high levels of cholesterol and low levels of TG. Serum LDL cholesterol have shown elevated levels in cholesterol-fed rats while TC was similar for both groups ($P > 0.05$).

In the current study, the presence of caveolin-1 and caveolin-2 in isolated plasma membranes from rat livers has been confirmed. Our data are consistent with previously reported presence of caveolin in caveolae at the surface of hepatocytes (32). In addition, several studies have shown the distinct presence of caveolin-1 in hepatocytes with negligible amounts in rat liver endothelial cells (31, 32, 42). Fig. 3 shows how a high-cholesterol diet can dramatically upregulate both caveolin-1 and caveolin-2 expression in the liver. In vitro assays have shown that caveolin-1 interacts with cholesterol (43) and has a role in delivering it to cell surface caveolae (44). Therefore, the induction of caveolin-1 expression by a high-cholesterol diet may be explained, at least in part, by a protective mechanism against cellular cholesterol accumulation in the rat liver. Caveolin-1 expression directly correlates with caveolae number (11, 45). Therefore, the results also suggest that a high-cholesterol diet might affect the number of caveolae present on the cell surface through regulation of caveolin-1. The upregulation of caveolin-1 in caveolae is consistent with previous reports describing cholesterol dependency of caveolin-1. Cholesterol loading by LDL exposure to human endothelial cells has been shown to increase caveolin-1 in caveolae (46). More relevant to our observations, Lin et al. (47) demonstrated that in rabbits fed a high-cholesterol diet for 5 weeks, the expression of caveolin-1 was induced. In contrast to the effect of a high-cholesterol diet on caveolin-1 protein levels, we detected a significant decline in caveolin-1 mRNA levels (Fig. 2). Indeed these results are in agreement with a recent study demonstrating that supplementation of exogenous

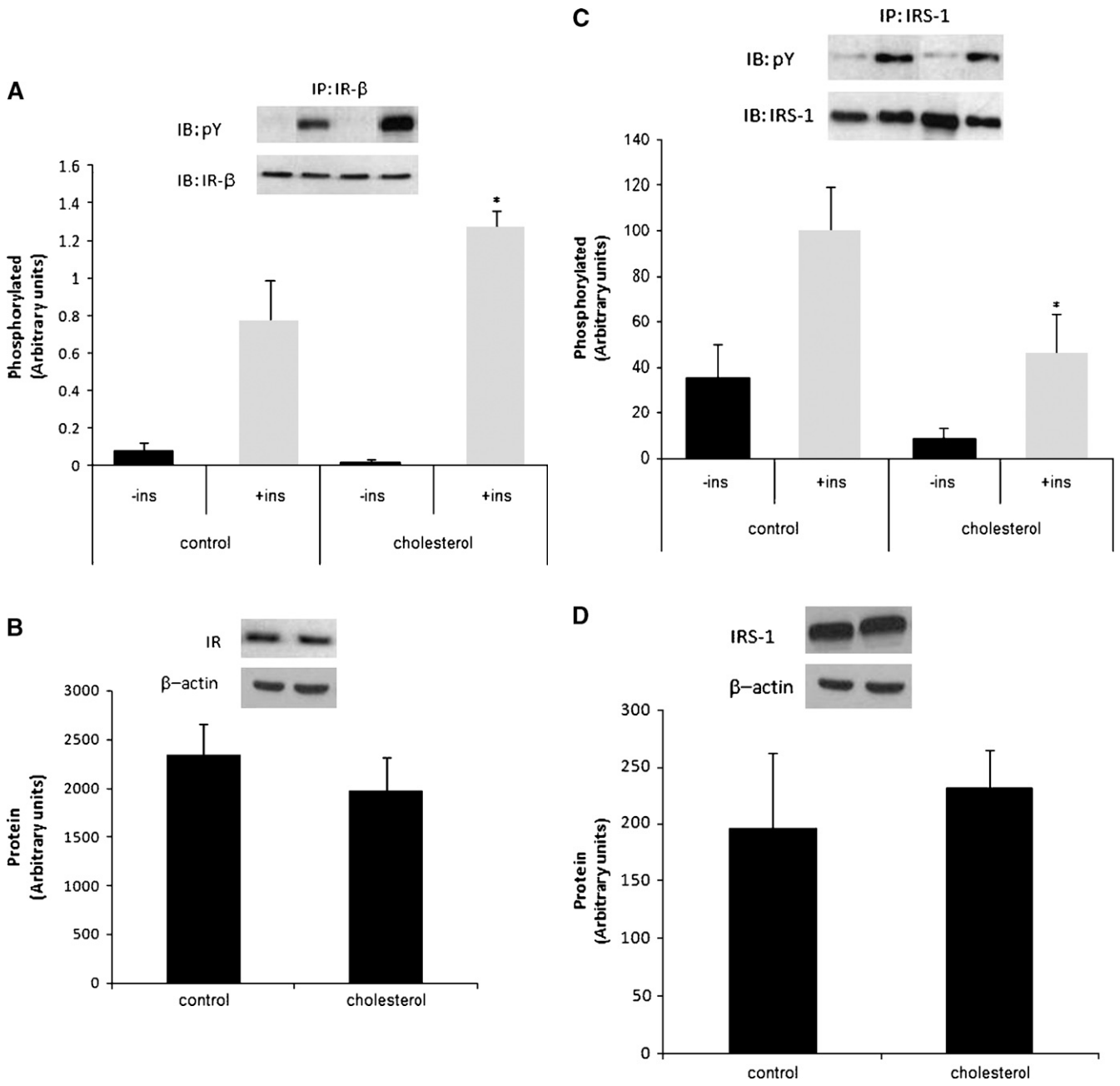

Fig. 4. Insulin signaling in liver of cholesterol-fed rats. Male Sprague-Dawley rats subjected to the control or high-cholesterol diet for 3 wk received an ip (dose of) saline (−) or 10U insulin (+) and were killed after 30 min. Liver lysates were immunoprecipitated with αIR-β and immunoblotted with αPY or αIR-β (A). Liver lysates were also immunoprecipitated with αIRS-1 antibody and immunoblotted with αPY or αIRS-1 (C). Whole-liver lysates were immunoblotted with αIR-β (B), αIRS-1 (D), or anti-phospho-Akt (E) antibodies. The result of scanning densitometry (n = 6) were presented in arbitrary units. Data represent mean ± SE. * $P < 0.05$ insulin control vs. insulin cholesterol.

LDL to endothelial cells reduced caveolin-1 mRNA levels (48). Moreover, exposure of human endothelial cells to LDL resulted in caveolin-1 protein migrated from the cytoplasm to the cell membrane (46). Taken together with the observations of the present study, it is possible that caveolin-1 trafficking regulated by a high-cholesterol diet is not due to increase de novo protein synthesis but rather to movement of pre-existing caveolin-1 protein from the cytoplasm to the membrane caveolae microdomains. This assumption can be supported also by the different cellular fractions that have been used for caveolin-1 protein and mRNA levels determinations (isolated plasma membranes and total liver homogenate, respectively). Caveolin-1 in total liver homogenate was undetected by available methodologies, and therefore it was impossible to determine whether the caveolin-1 upregulation was mediated by alterations in caveolin-1 trafficking, recycling, or by protein expression changes, as demonstrated by others (46, 49).

In vitro studies have shown that caveolin-1 can function as a positive regulator of IR activation (22, 23). IR interacts

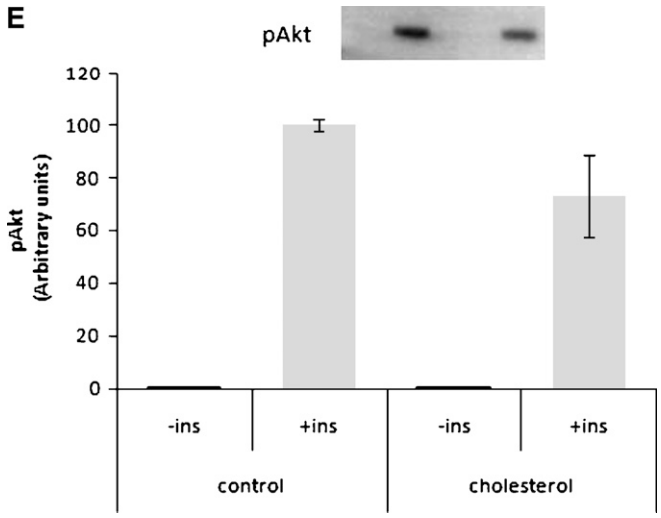

Fig. 4.—*Continued.*

with caveolin and binds to the scaffolding region of specific subtypes of caveolin (caveolin-3, and -1, but not caveolin-2). The IR/caveolin binding is direct and serves to increase the activity of the IR kinase. Thus, caveolin appears to target the IR kinase to a specific subcellular location (caveolae) and stimulate the IR kinase activity toward substrates in caveolae (22). The modification of caveolae environment by a high-cholesterol diet (Fig. 1) coincided with localization of caveolae resident IR (Fig. 3). Conversely, in a study by Oh et al. (50) in L6 skeletal muscle cells, it was reported that down-regulation of caveolin-1 levels did not change the distribution of IRβ in caveolae. These diverse findings might be due to the fact that regulation of insulin signaling by caveolin-1 is carried out in a tissue-specific manner.

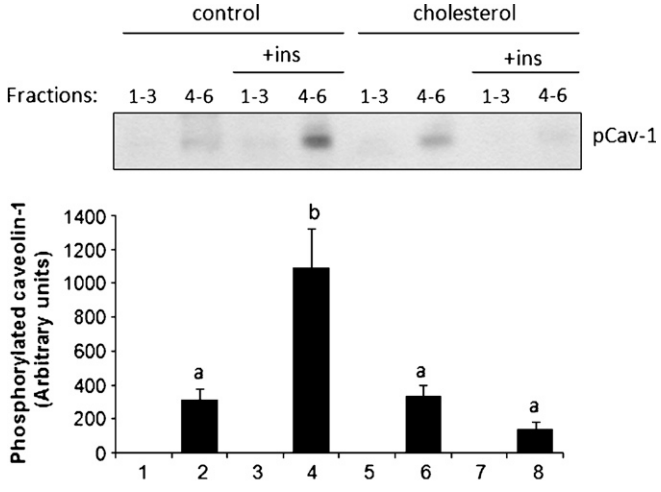

Fig. 5. The effect of a highcholesterol diet on insulin-induced tyrosine (Tyr) phosphorylation of caveolin-1 in rat liver. Caveolar fractions were prepared from liver tissue using a 5/25% discontinuous OptiPrep gradient. Fractions 1–3 and 4–6 were pooled and termed caveolar (Cav) and noncaveolar (non-Cav), respectively. Aliquots of the fractions were analyzed by immunoblotting using antibody against phsphorylated caveolin-1 (A). Disturibution of p- caveolin-1 in noncaveolar fractions in control and hypercholesterolaemic rats. Data represent mean ± SE (n = 3). [ab] Different subscripts represent significant differences among groups at $P < 0.05$.

The alterations in protein localization, observed in the present study, translated to modulation of protein function. Insulin-induced phosphorylation of IR was markedly increased by a high-cholesterol diet as determined by immunopercipitation of the IR in liver homogenate (Fig. 4A). However, IR mRNA and protein levels were unaffected by the treatment, supporting a specific alteration of kinase activity by a high-cholesterol diet (Figs. 2 and 4B, respectively).

Caveolin-1 up-regulation, observed in the present study (Fig. 1), may explain the increased phosphorylation of IR by a high-cholesterol diet. If the amount of caveolin-1 in the plasma membrane is increased, a certain number of IRs are associated with the caveolae, where it is required for efficient insulin signaling. Despite the enhanced activity of the IR, the distribution of Tyr phosphorylated IR between caveolar and noncaveolar fractions was similar following a high-cholesterol diet compared with controls (Fig. 3B). This may explain the impaired signaling downstream to the receptor (phosphorylated IRS-1 and phospho-Akt). Attenuated insulin signaling was not followed by altered glycogen content in the liver (Table 1) as expected possibly due to insufficient time (3 weeks) of the feeding experiment to decrease hepatic glycogen concentration.

Caveolin-1 can be phosphorylated on Tyr residues at the N terminus (51, 52). Some effectors like growth factors (epidermal growth factor, platelet-derived growth factor), insulin, and IGF-I as well as osmotic shock or oxidative stress can lead to Tyr phosphorylation (24, 53, 54). Moreover, IR catalyzes the Tyr phosphorylation of caveolin-1 supporting a role of caveolin-1 phosphorylation in insulin signaling (55). In the present study, a high-cholesterol diet prevented the induction of caveolin-1 phosphorylation obsereved in control rats after insulin stimulation (Fig. 5). It should be noted that in caveolar-fractions isolated from control and cholesterol-fed rats, no Tyr phosphorylated caveolin-1 could be detected. These results are similar to those reported recently in v-Src expressing cells, caveolin-1 phosphorylation is associated with flattening and aggregation of caveolae (56). Termination of certain signaling cascades associated with rafts is a consequence of caveolae internalization. Caveolin-1 phosphorylation could thus be involved in the internalization of caveolae in response to stimuli. This provides a possible explanation for the observed augmentation of IR phosphorylation following a high-cholesterol diet (Fig. 3) where internalization, or down-regulation mechanisms, are inhibited. Receptors remain at the cell surface for a longer period of time, resulting in its hyperphosphorylation.

Caveolin-1 plays an integral part in maintaining normal lipid and glucose homeostasis. Caveolin-1 can regulate hepatic lipid metabolism by several ways: transit fatty acids across the plasma membrane or by the transporter CD36/FAT, which localizes in caveolae and storing triacylglycerol in lipid droplets and modulation of triacylglycerol catabolism.

The disruption of caveolae by depleting membrane cholesterol or by inhibiting caveolin expression or function reduces the uptake of fatty acids into cells (57). Conversely, overexpression of caveolin-1 increases the cholesterol content of the plasma membrane and fatty acid uptake into cultured cells (58). It has been shown that caveolin-1 null hepatocytes reduced triacylglycerol accumulation after partial hepatectomy (59). Down-regulation of caveolin-1 production in C57BL/6 mice have shown increased glucose levels and impaired glucose tolerance and insulin sensitivity. In contrast, overproduction of caveolin-1 in JYC mice (model for type 2 diabetes without obesity) improved glucose tolerance and insulin sensitivity in skeletal muscle (60). Taken together, it can be speculated from our data that reduction in serum NEFA and triglycerides levels in cholesterol-fed rats might be a result of increased caveolin-1 protein levels. Also, caveolin-1 induction possibly prevented or delayed hyperglycemia and reduction in insulin sensitivity as expected during high-cholesterol feeding.

In summary, it has been demonstrated, for the first time, that a high-cholesterol diet can markedly alter protein composition of liver caveolae microdomains. These current findings indicate a possible mechanism by which a high-cholesterol diet can modulate insulin signaling.

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