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The *Npc1* mutation causes an altered expression of caveolin-1, annexin II and protein kinases and phosphorylation of caveolin-1 and annexin II in murine livers

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Abstract

We have previously demonstrated (1) an increased expression of caveolin-1 in murine heterozygous and homozygous Niemann-Pick type C (NPC) livers, and (2) an increased concentration of unesterified cholesterol in a detergent insoluble caveolae-enriched fraction from homozygous livers. To define further the relationship between caveolin-1 function and the cholesterol trafficking defect in NPC, we examined the expression and distribution of additional caveolar and signal transduction proteins. The expression of annexin II was significantly increased in homozygous liver homogenates and the Triton X-100 insoluble floating fraction (TIFF). Phosphoamino acid analysis of caveolin-1 and annexin II from the homozygous TIFF demonstrated an increase in serine and tyrosine phosphorylation, respectively. To determine the basis for increased phosphorylation of these proteins, the expression and distribution of several protein kinases was examined. The expression of PKCα, PKCζ and pp60-src (protein kinases) were significantly increased in both heterozygous and homozygous liver homogenates, while PKCδ was increased only in homozygous livers. Of the protein kinases analyzed, only CK IIα was significantly enriched in the heterozygous TIFF. Finally, the concentration of diacylglycerol in the homozygous TIFF was significantly increased and this elevation may modulate PKC distribution and function. These results provide additional evidence for involvement of a caveolin-1 containing cellular fraction in the pathophysiology of NPC and also suggest that the *Npc1* gene product may directly or indirectly, regulate the expression and distribution of signaling molecules.

Keywords: Niemann-Pick type C; Caveolin-1; Annexin II; Protein kinase C; Protein kinase A; Casein kinase IIα; pp60-src

1. Introduction

Niemann-Pick type C (NPC) disease is an autosomal recessive lipid storage disorder characterized by hepatosplenomegaly and neurological deterioration [1]. Elevated intracellular cholesterol is the major biochemical finding in NPC. NPC fibroblasts grown in media with low density lipoprotein (LDL) accumulate unesterified cholesterol within lysosomes and the *trans*-Golgi cisternae [2,3]. The inability of unesterified cholesterol to traffic to specific intracellular compartments is responsible for the dysregulation of cholesterol metabolism seen in this disorder [4,5]. Recently, the NPC genes from both human (*NPC1*) and mouse (*Npc1*) were cloned, and the gene product determined to be an integral membrane protein with

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regions homologous to the Hedgehog signaling molecule PATCHED and to the sterol-sensing domain associated with 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase [6,7]. Although the subcellular location and function of the NPC1 protein is unknown, specifically its role in intracellular cholesterol movement, definition of its function is under intense investigation.

Our previous studies demonstrated an altered expression of caveolin-1 in livers from heterozygous and homozygous mice with NPC [8]. Caveolin-1 is a cholesterol-binding protein that serves a structural role in the formation of plasma membrane caveolae which are membrane domains that participate in potocytosis, transcytosis and signal transduction [9–11]. Recently, caveolae have been shown to participate in cholesterol trafficking [12]. Incubation of cultured fibroblasts with cholesterol oxidase results in caveolin-1 cycling from the plasma membrane to the endoplasmic reticulum and back to the plasma membrane through the Golgi [13,14]. Other studies show that caveolin-1 facilitates the transport of unesterified cholesterol to plasma membrane caveolae where it then becomes available for efflux, a mechanism presumably important for maintaining intracellular cholesterol homeostasis [12,13,15]. Our demonstration that caveolin-1 is altered in NPC suggests that a caveolin-1 containing cellular component is involved in the NPC cholesterol trafficking defect.

Like caveolin-1, annexin II has been found to be associated with caveolae and intracellular vesicles [16–18]. Structurally, annexin II contains a highly conserved C-terminal domain responsible for facilitating Ca^{2+}-dependent association with negatively charged phospholipids [19]. Recent studies show that the N-terminal domain participates in cholesterol-dependent binding within cholesterol-rich membrane domains including caveolae [20]. Annexin II may, therefore, be important for caveolar structure and function.

It appears that phosphorylation is important in regulating the function of both caveolin-1 and annexin II. Both caveolin-1 and annexin II contain protein kinase C (PKC) phosphorylation consensus sequences, although only annexin II serves as a direct substrate for PKC [21,22]. Caveolin-1 is, instead, serine phosphorylated by casein kinase IIα (CK IIα), which is, in turn, activated by PKC [23]. Tyrosine phosphorylation of both proteins can be mediated by pp60-src [24,25]. Both CK IIα and pp60-src are found in caveolae and are thus poised to modulate caveolae structure and function possibly through phosphorylation of caveolin-1 and annexin II [11,26].

Results obtained in the present study indicate that the expression of annexin II in homozygous liver homogenates is increased and that caveolin-1 and annexin II associated with the homozygous Triton X-100 insoluble floating fraction (TIFF) are increased in serine and tyrosine phosphorylation, respectively. To help determine the underlying mechanism responsible for caveolin-1 and annexin II phosphorylation, we examined and found alterations in expression and distribution of specific protein kinases (PKCα, PKCδ, PKCε and PKCξ, PKA-cs, pp60-src and CK IIα). The alteration found in the expression of these kinases may help to define further the basis of the defective intracellular cholesterol trafficking in NPC.

The additional findings of alterations in annexin II and kinase expression suggest that a caveolin-1 containing cell component is involved in the pathophysiology of NPC, and that alterations in intracellular signaling mechanisms are an important pathophysiological consequence of NPC.

2. Methods

2.1. Animals

Two breeding pairs of BALB/c heterozygous Niemann-Pick type C (NPC) mice were the generous gift of Dr. Peter Pentchev from the National Institute of Health. These mice were bred to produce affected offspring and additional heterozygotes. Mice were identified as homozygous affected when symptoms of NPC developed and as heterozygous by the production of affected offspring. Homozygous normal BALB/c mice were purchased and bred separately from the NPC colony. Animals were killed using cervical dislocation, and the livers were removed and stored frozen at −70°C. Males and females were equally represented. Homozygous normal mice were killed between the ages of 1.5 and 12 months. Homozygous affected mice were killed between the time of diagnosis (≈1.5 months) and death (≈2.5

months). Heterozygous mice were killed between the ages of 3 and 12 months. Animals were maintained at the University of Arizona Animal Care facility on mouse chow containing 6% fat and water ad libitum.

2.2. Reagents

Cholesterol, phosphatidylcholine (dioleoyl), lysophosphatidylcholine (oleoyl), phosphatidylethanolamine (dioleoyl), phosphatidylserine (dioleoyl), 1,2-dioleoylglycerol, sphingomyelin (oleoyl), pepstatin, leupeptin, aprotinin and bovine albumin (fraction V) were purchased from Sigma (St. Louis, MO). Glacial acetic acid, hexane, isopropanol and chloroform were purchased from Fisher Scientific (Pittsburgh, PA). Diethyl ether was obtained from Aldrich (Milwaukee, WI). Bicinchoninic acid (BCA) protein assay reagent and SuperSignal Substrate for Western blotting were purchased from Pierce Chemical (Rockford, IL). Cholesterol oxidase was purchased from Calbiochem (La Jolla, CA) and horseradish peroxidase from Boehringer Mannheim (Indianapolis, IN). Affi-Gel Protein A Gel agarose was purchased from Bio-Rad Laboratories (Hercules, CA). Mouse anti-caveolin-1, mouse anti-annexin II, mouse anti-PKC (α, δ, ε and ζ isotypes), and casein kinase IIα (CK IIα) were purchased from Transduction Laboratories (Lexington, KY). Mouse anti-pp60-src was purchased from Oncogene Research Products (Cambridge, MA). Mouse anti-phosphoserine and anti-phosphotyrosine were purchased from Sigma (St. Louis, MO). Peroxidase-conjugated anti-mouse IgG was purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD).

2.3. Isolation of the TIFF

The TIFF from mouse livers was isolated as previously described [27]. Briefly, mouse liver was minced using a razor blade, followed by homogenization in 10 ml of ice-cold buffer A (0.025 M MES, pH 6.5, 1.0 mM EDTA, 10 μM each of PMSF, pepstatin, leupeptin and aprotinin) using a motor-driven Dounce homogenizer. The homogenate was centrifuged at $500\times g$ for 10 min to remove nuclei and cell debris. The resulting post-nuclear supernatant was centrifuged at $100\,000\times g$ for 1 h to obtain a pellet representing the total membrane preparation. This pellet was solubilized in 2 ml of ice-cold buffer A containing 150 mM NaCl and 1% Triton X-100 (buffer B) using needle aspiration. A sucrose step gradient was generated using 1.5 ml 42% sucrose, 3.0 ml 25% sucrose and 3.5 ml 15% sucrose in buffer B containing 0.5% Triton X-100 at 4°C, followed by layering 2.0 ml of the detergent solubilized membranes on top of the sucrose step gradient and centrifuged at $100\,000\times g$ for 2 h at 4°C. The TIFF migrated to the 15–25% sucrose interface and the opalescent interface was collected directly using needle aspiration. Collected fractions were concentrated using Centricon-10 concentrators (Amicon, Beverly, MA) in preparation for immunoblot analysis, immunoprecipitation and lipid analysis.

2.4. Immunoprecipitation of caveolin-1 and annexin II

TIFF-derived protein (500 μg) from normal, heterozygous and homozygous mouse livers was solubilized using 1.0 ml of buffer C (0.010 M Tris, pH 7.4, 0.150 M NaCl, 1.0 mM EDTA, 0.5% Triton X-100, 30 mM CHAPS, 10 mM NaF and protease inhibitors) for 1 h at 4°C followed by centrifugation at $100\,000\times g$ for 1 h. Supernatants were precleared using 50 μl of 10% Protein A-agarose for 2 h at 4°C followed by overnight nutation at 4°C with either mouse anti-caveolin-1 or anti-annexin II (5.0 μg). Protein A-agarose (50 μl) was added and allowed to nutate for an additional 2 h at 4°C to initiate immunoprecipitation. The agarose beads were then rinsed 4 times with 1.0 ml of buffer C. Immunoprecipitated protein was solubilized from the agarose beads using SDS-PAGE sample buffer under reducing conditions and boiled for 5 min before electrophoresis.

2.5. Immunoblot analysis

Samples were separated using 10% SDS-PAGE under reducing conditions and transferred to a nitrocellulose membrane. In most instances, immunoblot buffer D (0.010 M sodium phosphate, pH 7.4, 0.150 M NaCl, 0.05% Tween 20 and 4% non-fat dry milk) was used for blocking non-specific sites for 2 h at room temperature. When probing for the presence of phosphotyrosine, immunoblot buffer E (0.010 M sodium phosphate pH 7.4, 0.150 M NaCl, 0.05%

Tween 20 and 2% bovine albumin) was used for 3 h at room temperature. Immunoblots were routinely performed by incubating with antibody overnight at 4°C. Blots were rinsed 3× for 10 min, then incubated with peroxidase-conjugated goat anti-mouse IgG (1:5000 dilution in the appropriate blocking buffer) for 1 h at room temperature. After rinsing 3× for 10 min, the immunoblots were incubated in SuperSignal Substrate solution for 5 min and enhanced chemiluminescence (ECL) was detected using Kodak BioMax MR Scientific Imaging Film. Signals were quantitated using a Bio-Rad Model GS-700 Imaging Densitometer.

2.6. Phosphoamino acid analysis of caveolin-1 and annexin II

To measure the phosphorylation of caveolin-1 and annexin II, these proteins were first immunoprecipitated using anti-caveolin-1 and anti-annexin II followed by immunoblot analysis. To detect phosphorylated amino acids, the blots were first incubated with an antibody directed against a particular phosphorylated amino acid (mouse anti-phosphoserine or mouse anti-phosphotyrosine) followed by quantitation using ECL and imaging densitometry. The primary and secondary antibodies were then removed from the immunoblot using stripping buffer (65 mM Tris, pH 6.8, 1.5% SDS and 150 mM 2-mercaptoethanol for 30 min at room temperature) before reblocking. To assure adequate removal of antibodies, immunoblots were screened by reblocking and then incubating with secondary antibody followed by ECL. To normalize for the degree of serine and tyrosine phosphorylation, immunoblots were then incubated with antibodies against caveolin-1 or annexin II. The degree of protein phosphorylation was reported as a ratio of specific serine and tyrosine phosphorylation to the amount of protein.

2.7. Cholesterol determination

The isolated TIFF was extracted with hexane:isopropanol (3:2) for 1 h, followed by centrifugation. The organic solvent was removed, dried, and spotted onto a thin-layer chromatography plate (Redi-Plate, 250 μm Silica Gel G, Fisher Scientific). Neutral lipids were separated using hexane:diethyl ether:glacial acetic acid (100:30:1.1). Unesterified cholesterol and cholesteryl ester were localized by comparison with standards after staining with iodine vapors. After destaining, unesterified cholesterol was collected into glass culture tubes and extracted with 3 ml hexane and 1 ml water, with the hexane phase being separated into a separate glass tube. Cholesterol concentrations were then determined using the cholesterol oxidase method [28]. Briefly, to each tube was added 0.4 ml of assay reagent (0.1 ml cholesterol oxidase (8 U/ml), 0.1 ml peroxidase (3000 U/ml), 1.0 ml of hydroxyphenylacetic acid (1.5 mg/ml) and 8.8 ml 0.05 M sodium phosphate pH 7.0) followed by incubation for 20 min at room temperature. The reaction was terminated by adding 0.8 ml of 0.5 N NaOH. A Perkin-Elmer Model LS-40 Fluorescence Spectrometer (excitation 325 nm and emission 415 nm) was used for measuring fluorescence. The unknown cholesterol concentrations were determined using cholesterol standards and linear regression analysis.

2.8. Phospholipid determination

Phospholipids, which do not migrate in the solvent system utilized for separation of sterols, were collected from the origin and extracted using Folch reagent (chloroform:methanol, 2:1). The extracted lipids were spotted onto a heat activated (100°C for 30 min) 250 μm Silica Gel H thin-layer chromatography plate (Analtech), and the phospholipids (phosphatidylcholine, lysophosphatidylcholine, sphingomyelin, phosphatidylserine, phosphatidylethanolamine) were separated using chloroform:methanol:glacial acetic acid:water (100:60:16:8) as the solvent system. This solvent system does not allow adequate separation of phosphatidic acid and cardiolipin from the other phospholipids, since both migrate together at the solvent front. Therefore, these two lipids were collected and extracted from the resin using chloroform and reapplied to a 250 μm Silica Gel G plate and separated using petroleum ether:diethylether:zglacial acetic acid (90:10:5). After staining with iodine vapors, the resin containing the lipids was scraped into a separate tube and analyzed for phosphorus by adding 150 μl of concentrated perchloric acid (60%) and incubated for 1 h at 170°C [29]. After incubation, 1.7 ml of water, 0.4 ml acid molybdate

solution (1.25% w/v ammonium molybdate in 2.5 M sulfuric acid) and 0.1 ml Fiske Subbarow reducer solution (1 g/6.3 ml of water) were added to each sample and reincubated at 95°C for 15 min. Samples were read at a wavelength of 820 nm and the concentration of phosphorus from each sample was determined by extrapolation using a phosphorus standard (Sigma Diagnostics) analyzed in parallel.

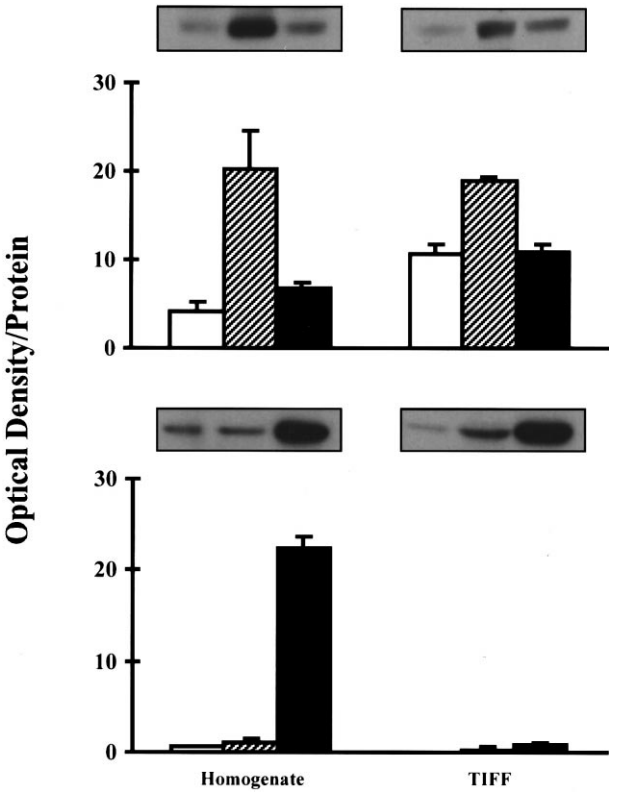

Fig. 1. Altered expression of caveolin-1 (top) and annexin II (bottom) in heterozygous and homozygous liver homogenates and the relative amount associated with the corresponding TIFF. Equal amounts of liver homogenate protein and TIFF derived protein from four sets of mice were separated in parallel using 10% SDS-PAGE and transferred to nitrocellulose for immunoblot analysis. Primary antibodies, mouse anti-caveolin-1 IgG and mouse anti-annexin II IgG, were detected using peroxidase-conjugated goat anti-mouse IgG developed with ECL. A representative immunoblot showing the relative amount of caveolin-1 and annexin II in the liver homogenate and the isolated TIFF is provided for normal, heterozygous and homozygous animals, respectively. Quantitation was performed using an imaging densitometer, where the background optical density was subtracted from each sample. Shown is the mean ± S.D. of the optical density normalized to protein for the three genotypes. White bars represent homozygous normal (+/+), diagonally hatched bars represent heterozygous (+/−) and black bars represent homozygous affected (−/−).

2.9. Diacylglycerol determination

The concentration of diacylglycerol was determined using the Triglyceride/Glycerol Assay Kit (Sigma Diagnostics). Briefly, diacyglycerol was extracted from the resin using hexane and hydrolyzed by incubating with 0.5 ml of 0.1 N KOH at 80°C for 1 h. Upon cooling to room temperature, 1 ml of 0.15 M $MgSO_4$ was added to form a precipitate that was removed by centrifugation to obtain a supernatant fraction containing the resultant glycerol. To 0.2 ml of this supernatant was added 1.0 ml of assay solution (5 min at room temperature) and read at 340 nm. To each of the respective samples was added 4 μl of glycerokinase responsible for oxidizing NADH to NAD, followed by reincubation at room temperature for 15 min before reading absorbance again at 340 nm. The difference between the latter and former absorbances were used to calculate the absolute amount of glycerol derived from the hydrolysis of diacylglycerol.

2.10. Statistical analysis

All quantitative data is represented as the mean ± S.D. of four separate mouse livers isolated from each of the three different genotypes (homozygous normal, heterozygous and homozygous affected). Each of the isolated TIFF samples was derived from a corresponding liver homogenate. Significant differences between groups of data were determined using the two-tailed Student's *t*-test assuming equal variance.

3. Results

3.1. Expression of caveolin-1 and annexin II in liver homogenates and the TIFF

The altered expression of caveolin-1 and annexin II in heterozygous and homozygous liver homogenates is shown in Fig. 1. Caveolin-1 expression is elevated in both heterozygous (4.9 fold, $P = 0.0004$) and homozygous livers (1.6 fold, $P = 0.0153$). In con-

trast, the expression of annexin II was increased only in homozygous livers (23 fold, $P = 4.97 \times 10^{-8}$) compared to the expression of annexin II in normal and heterozygous livers. The amount of caveolin-1 in the heterozygous TIFF was increased (1.8 fold, $P = 5.5 \times 10^{-5}$) compared to the amount of caveolin-1 associated with the normal TIFF; the amounts of caveolin-1 in the normal and homozygous TIFFs were similar. The amount of annexin II associated with the homozygous TIFF was significantly increased (4.3 fold, $P = 0.0014$) compared to the amount of annexin II associated with the normal and heterozygous TIFF.

3.2. Phosphoamino acid analysis of caveolin-1 and annexin II

Using antibodies generated against phosphoserine and phosphotyrosine, we determined quantitatively the degree of caveolin-1 and annexin II phosphorylation after selective immunoprecipitation from the TIFF. To accurately determine the amount of phosphoserine and phosphotyrosine associated with caveolin-1 and annexin II, immunoblots were initially probed with antibodies against a respective phosphoamino acid, stripped of both primary and secondary antibodies, and reprobed with antibody against the protein of interest to normalize for protein. This procedure assured that alterations in amino acid phosphorylation were not the result of differences in the amount of protein detected during immunoblot analysis. The degree of serine phosphorylation in caveolin-1 immunoprecipitated from the homozygous TIFF was significantly increased compared to caveolin-1 from normal (1.84 fold, $P = 0.0003$) and heterozygous TIFFs (1.96 fold, $P = 0.0403$) (Fig. 2). No significant difference in caveolin-1 tyrosine phosphorylation was detected. The degree of annexin II tyrosine phosphorylation was increased in the homozygous TIFF compared to annexin II from the normal TIFF (2.41 fold, $P = 0.0155$). Although annexin II from the homozygous TIFF was increased in tyrosine phosphorylation compared to annexin II from the heterozygous TIFF (1.97 fold, $P = 0.0600$), the measured increase was not statistically significant. There was no significant difference in the degree of annexin II serine phosphorylation between the three genotypes.

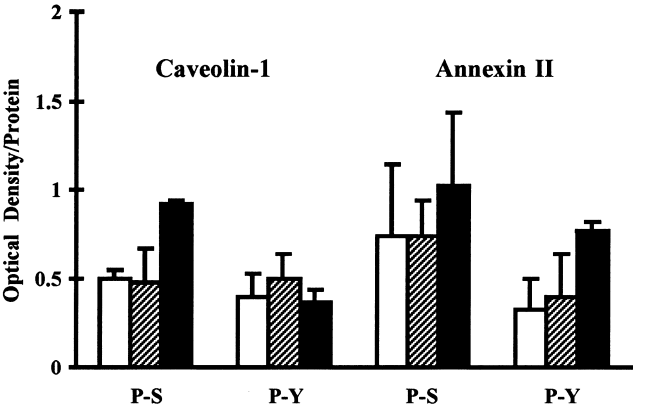

Fig. 2. Phosphoamino acid analysis of caveolin-1 and annexin II associated with the TIFF derived from normal, heterozygous and homozygous livers. Caveolin-1 and annexin II were immunoprecipitated from four sets of TIFFs and then separated using 10% SDS-PAGE and transferred to nitrocellulose for immunoblot analysis. Primary antibodies, mouse anti-phosphoserine IgG and mouse anti-phosphotyrosine IgG, were detected using peroxidase-conjugated goat anti-mouse IgG developed with ECL. To normalize for the amount of immunoprecipitated protein, blots were stripped of primary and secondary antibodies and reprobed for either caveolin-1 or annexin II. Shown is the mean ± S.D. of phosphoamino acid measured after normalizing for the amount of caveolin-1 or annexin II. White bars represent homozygous normal (+/+), diagonally hatched bars represent heterozygous (+/−) and black bars represent homozygous affected (−/−).

3.3. Expression of protein kinases in liver homogenates

The expression of several protein kinases (PKCα, PKCδ, PKCε, PKCζ, PKA-cs, CK IIα and pp60-src) was measured in liver homogenates prepared from normal, heterozygous and homozygous mice (Fig. 3). The expression of PKCα in heterozygous and homozygous liver homogenates was significantly increased compared to normal liver homogenates (3.7 fold, $P = 0.0130$ and 3.5 fold, $P = 0.0004$, respectively). The expression of PKCδ from homozygous livers was increased approximately 35 fold compared to normal ($P = 0.0002$) and heterozygous ($P = 0.0006$) livers. The minor higher molecular weight bands, present in both the PKCα and PKKζ immunoblots, are believed to represent phosphorylated isoforms. In both instances, these minor bands were quantitated along with the major protein kinase band. The expression of PKCε was decreased in heterozygous and homozygous livers (1.4 fold, $P = 0.0320$ and 2.2 fold,

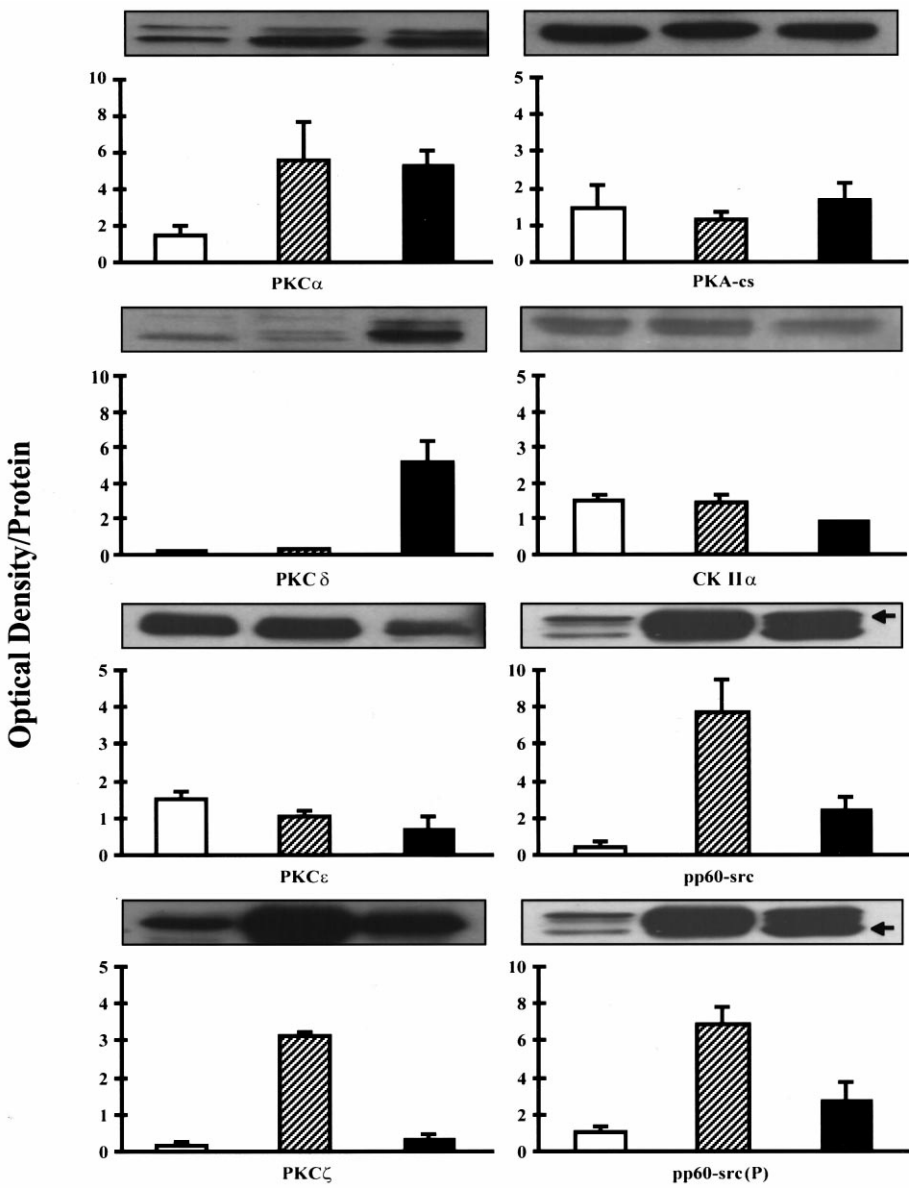

Fig. 3. Relative amounts of select protein kinases expressed in normal, heterozygous and homozygous liver homogenates. Equal amounts of liver homogenate protein from four sets of mice were separated in parallel using 10% SDS-PAGE and transferred to nitrocellulose for immunoblot analysis. Primary antibodies against select protein kinases were detected using peroxidase-conjugated goat anti-mouse IgG developed with ECL. A representative immunoblot showing the relative amount of each protein kinase in the liver homogenate is provided for normal, heterozygous and homozygous animals, respectively. Quantitation was performed using an imaging densitometer, where the background optical density was subtracted from each sample. Shown is the mean ± S.D. of the optical density normalized to protein from the three genotypes. White bars represent homozygous normal (+/+), diagonally hatched bars represent heterozygous (+/−) and black bars represent homozygous affected (−/−). Arrowheads in the pp60-src panels represent the phosphorylated (higher band) and unphosphorylated (lower band) forms.

$P = 0.0126$, respectively) compared to normal. The expression of PKCζ was increased in heterozygous livers (20 fold, $P = 1.72 \times 10^{-7}$) compared to normal liver. The expression of PKA-cs was not significantly different ($P \geq 0.0500$) in heterozygous and homozygous liver homogenates compared to normal liver homogenates. The expression of CK IIα in homozygous liver homogenates was decreased (1.7 fold,

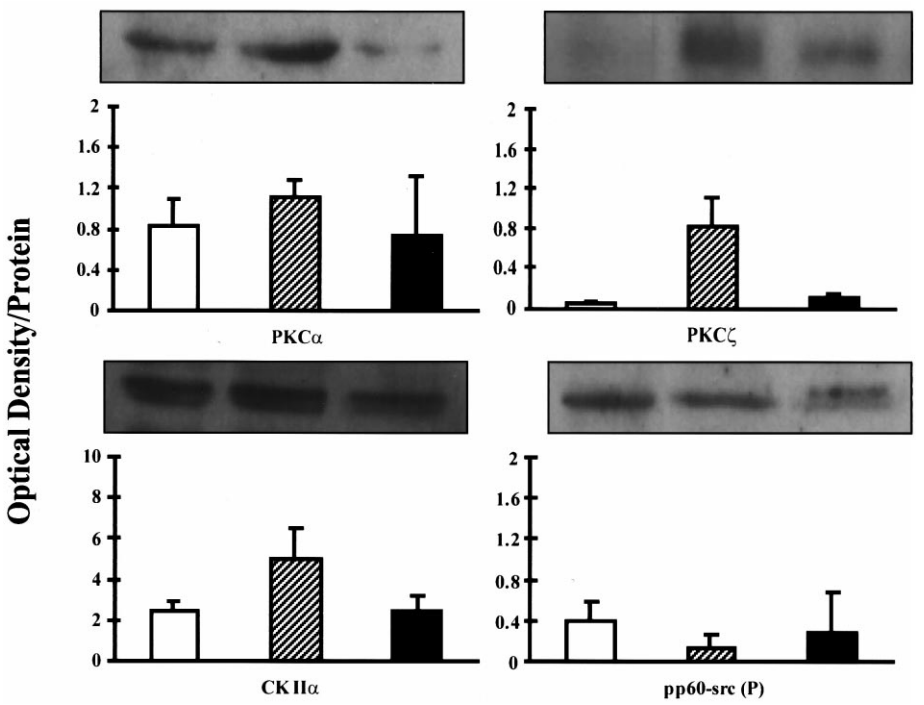

Fig. 4. Relative amounts of select protein kinases associated with the TIFF isolated from normal, heterozygous and homozygous livers. Equal amounts of the TIFF derived protein from four sets of mice were separated in parallel using 10% SDS-PAGE and transferred to nitrocellulose for immunoblot analysis. Primary antibodies against select protein kinases were detected using peroxidase-conjugated goat anti-mouse IgG developed with ECL. A representative immunoblot showing the relative amount of select protein kinases in the isolated TIFF is provided for normal, heterozygous and homozygous animals, respectively. Quantitation was performed using an imaging densitometer, where the background optical density was subtracted from each sample. Shown is the mean ± S.D. of the optical density normalized to protein for the three genotypes. White bars represent homozygous normal (+/+), diagonally hatched bars represent heterozygous (+/−) and black bars represent homozygous affected (−/−).

$P = 0.0013$) compared to normal homogenates. pp60-src was resolved into two distinct bands during immunoblot analysis: a lower molecular weight band believed not to be phosphorylated and a higher molecular weight band representing the phosphorylated and active pp60-src [30]. The lower molecular weight form of pp60-src was increased 16 fold ($P = 0.0002$) in heterozygous livers and 5.2 fold ($P = 0.0012$) in homozygous livers compared to normal liver homogenates, while the higher molecular weight form of pp60-src was increased 6.5 fold ($P = 0.0003$) in heterozygous livers and 2.6 fold ($P = 0.0130$) in homozygous liver homogenates compared to normal liver homogenates.

3.4. Relative amount of protein kinases associated with the TIFF

Only four of the eight protein kinases examined were found in the TIFF (Fig. 4). There was no significant difference among the three genotypes in the amount of PKCα measured in the TIFFs. For PKCζ, an increased amount was found associated with the heterozygous TIFF (15 fold, $P = 0.0041$) and the homozygous TIFF (2.0 fold, $P = 0.0155$) compared to the normal TIFF. CK IIα was increased (1.99 fold, $P = 0.0224$) only in the heterozygous TIFF compared to the normal TIFF. Finally, only the lower molecular weight form of pp60-src was significantly represented in the TIFF. No significant difference ($P \geq 0.05$) in the relative amount of pp60-src was measured when comparing the TIFFs isolated from normal, heterozygous and homozygous livers.

3.5. Amount of total protein and relative enrichment of specific proteins in the TIFF

The amount of total protein recovered in the normal, heterozygous, and homozygous TIFFs was, re-

Table 1
Analysis for enrichment of specific proteins in the TIFF fraction vs. total livers homogenates

Mouse genotype	Homogenate protein (mg)	TIFF protein (mg)	Caveolin-1	Annexin II	PKCα	PKCζ	CK IIα	pp60-src
+/+	53.24 ± 7.16	1.28 ± 0.14	2.97 ± 0.28	0.11 ± 0.08	0.56 ± 0.17	0.35 ± 0.14	1.67 ± 0.35	0.83 ± 0.41
+/−	69.29 ± 33.39	1.41 ± 0.61	0.93 ± 0.03	0.16 ± 0.04	0.20 ± 0.03	0.26 ± 0.10	3.42 ± 1.06	0.02 ± 0.02
−/−	36.80 ± 2.57	0.34 ± 0.05	1.62 ± 0.14	0.03 ± 0.01	0.14 ± 0.11	0.34 ± 0.08	2.73 ± 1.14	0.12 ± 0.14

Liver homogenates and the corresponding TIFF from each liver were prepared as described in Section 2. Protein derived from each of the homogenates and the corresponding TIFFs were used for the determination of protein and the relative amount of select proteins associated with the TIFF. Data is represented as the mean ± S.D. of four different homogenates and the corresponding TIFF.

spectively, 2.4, 2.0% and 0.9% that found in the total homogenates (Table 1). Caveolin-1 was enriched in both the normal and homozygous TIFFs but not in the heterozygous TIFF. This indicates that the increased expression of caveolin-1 measured in the heterozygous homogenate does not result in an increased distribution of caveolin-1 to the TIFF. However, caveolin-1 levels in the heterozygous TIFF are still greater than levels in the normal or homozygous TIFF (Fig. 1). Annexin II is not enriched within any of the TIFFs suggesting it was solubilized with Triton X-100 detergent during TIFF isolation. Of the four protein kinases found in the TIFF (PKCα, PKCζ, CK IIα, and pp60-src), only CK IIα was enriched compared to total liver homogenates.

3.6. Concentration of select lipids in the TIFF

The masses of select lipids in the TIFF derived from normal, heterozygous and homozygous liver homogenates are shown in Table 2. The concentration of common glycerolipids (phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine) and lysophosphatidylcholine were similar among the three genotypes. Unesterified cholesterol and sphingomyelin, which have previously been shown to be elevated in liver homogenates of mice affected with NPC, were increased approximately 15 fold ($P = 0.0012$) and 3 fold ($P = 0.0012$), respectively, in the homozygous TIFF compared to the normal TIFF. The concentration of diacylglycerol was increased 6.6 fold ($P = 0.0016$) in the TIFF from homozygous livers compared to the TIFF from normal livers. None of the lipids analyzed in the TIFF derived from heterozygous livers were significantly increased in concentration compared to the TIFF derived from normal livers. Additional lipids (monoacylglycerol, triacylglycerol, phosphatidic acid and cardiolipin) were below the limits of mass detection.

Table 2
Quantitation of specific lipids associated with the isolated TIFF derived from livers of normal, heterozygous and homozygous mice

Lipid	Normals	Heterozygous	Homozygous
Cholesterol	7.71 ± 0.26	6.45 ± 2.33	1.06 ± 34.3*
Phosphatidylcholine	2.90 ± 0.78	2.94 ± 1.45	3.43 ± 0.82
Phosphatidylserine	1.22 ± 0.13	1.17 ± 0.42	1.31 ± 0.06
Phosphatidylethanolamine	1.04 ± 0.14	0.92 ± 0.57	1.15 ± 0.24
Lysophosphatidylcholine	0.86 ± 0.06	0.85 ± 0.28	0.99 ± 0.16
Sphingomyelin	1.20 ± 0.15	0.96 ± 0.25	3.61 ± 0.83*
Diacylglycerol	1.10 ± 0.45	1.25 ± 0.24	6.68 ± 1.82*

Detailed methods utilized to isolate the TIFFs from the liver homogenates with subsequent determination of lipid mass are described in Section 2. The concentration of each lipid is expressed as μg/mg protein. Data is represented as the mean ± S.D. of TIFFs isolated from four different liver homogenates within a specified genotype. Statistically significant differences, $P < 0.050$, in the concentration of specified lipids derived from TIFFs isolated from normal and homozygous mice are indicated (*). Monoacylglycerol, triacylglycerol, phosphatidic acid, cardiolipin and fatty acids were not detected within any of the TIFF samples.

4. Discussion

Heterozygous and homozygous mice with NPC have an increased expression of caveolin-1 in liver homogenates [8]. These results can now, perhaps, be partially explained by recent studies indicating that LDL-derived cholesterol upregulates the expression of caveolin-1 in normal cells [31–33]. The conclusion from these latter experiments was that caveolin-1, in association with plasma membrane caveolae, is responsible for maintaining cholesterol levels by promoting the efflux of excess intracellular unesterified cholesterol. This relationship between LDL-cholesterol and caveolin-1 levels may explain the increase in caveolin-1 seen in NPC. Since the transport of LDL-derived cholesterol is impaired in NPC and cells accumulate LDL-derived cholesterol, this excess intracellular cholesterol may result in an increase in caveolin-1 expression. However, the elevation of caveolin-1 in heterozygous NPC liver homogenates and fibroblasts is greater than that seen in the homozygous genotype, even though accumulation of cholesterol is not detected in the heterozygous state. It has been shown, however, that the heterozygous genotype is not without its effects on cholesterol trafficking, and that some cholesterol accumulation does occur [34,35]. Heterozygous cells may be 'sensing' this cholesterol accumulation. Since there is one functional *Npc1* gene in heterozygous cells, this may in some way result in a greater expression of caveolin-1 than that seen in homozygous cells to 'compensate' for the deficient amount of NPC1 protein. To discern further the mechanisms of this cellular response, we investigated the role of potential regulatory proteins associated with caveolae and caveolin-1 containing vesicles that may also serve to modulate intracellular cholesterol trafficking pathways.

Our present study demonstrates an increased expression of annexin II in homozygous NPC livers. Work performed by other investigators have suggested that an increased expression of annexin II in hepatocytes may result from hepatocyte proliferation secondary to liver damage (a state that may indeed exist in NPC), but these studies have also demonstrated an upregulation of annexin I [36]. We were unable to detect annexin I within any of the liver homogenates, suggesting that the altered expression of annexin II may, like caveolin-1, be in response to the NPC cholesterol trafficking defect. Although annexin II has been found associated with caveolae, our results indicate that annexin II was not enriched within the isolated TIFF. A possible explanation for this finding is that annexin II is capable of being solubilized, unlike caveolin-1, with Triton X-100 during isolation of the TIFF [37]. Other studies indicate that the majority of cellular annexin II is associated with endosomal membranes, where it participates in the reorganization of vesicular structures by facilitating the fusion of lipid bilayers. The nature of these endosomal membranes may have different detergent insolubility characteristics than caveolae [38].

Recent studies demonstrate that annexin II participates in cholesterol metabolism [20]. Traditionally, annexins were classified only as calcium and phospholipid-binding proteins [39,40], but a novel mechanism has been described demonstrating that a significant portion of total cellular annexin II is tightly associated with membranes in a Ca^{2+}-independent manner [20]. It was concluded that a cholesterol-dependent binding mechanism mediated by the N-terminal domain of annexin II maybe specific for membrane domains rich in cholesterol, such as caveolae. Although the cholesterol-dependent binding mechanism is uncharacterized, annexin II may interact directly with cholesterol and participate in the transport of cholesterol between subcellular compartments. Because of the lack of information concerning the exact function of annexin II, we are limited in our conclusions regarding its role in NPC beyond that of its participation in endosomal membrane and vesicular transport processes including, perhaps, those involving the NPC1 protein [41–43].

The functions of caveolin-1 and annexin II are believed to be regulated in part by serine and tyrosine phosphorylation [25,44]. Serine phosphorylation of caveolin-1 has been shown to flatten caveolae and prevent folate uptake by potocytosis [45], while tyrosine phosphorylation of caveolin-1 may facilitate the docking of specific signaling molecules [46]. Likewise, the phosphorylation of serine and tyrosine residues on annexin II is believed to influence aspects of membrane binding, vesicle aggregation and vesicle internalization [47–49]. Comparison of the phosphorylation of caveolin-1 in the normal, heterozygous, and homozygous TIFF reveals increased phosphorylation

on serine residues in the homozygous TIFF, but not on tyrosine residues, suggesting the involvement of a serine specific kinase. The primary structure of caveolin-1 contains two serine residues, both residing in a phosphorylation consensus sequence and flanked by a regulatory scaffolding domain [21,23]. One of these residues, Ser-37, represents a PKC consensus site, while the other, located at Ser-88, is believed to be phosphorylated by CK IIα. Since studies indicate that caveolin-1 is not a direct substrate for PKC, the increased serine phosphorylation that was detected on caveolin-1 is most likely catalyzed by CK IIα at Ser-88 [23]. It has been demonstrated that activation of PKC modulates both the morphology and function of caveolae, and that phosphorylation of CK IIα by PKC may represent a phosphorylation cascade that facilitates this event [11,23,26]. Indeed, CK IIα is a substrate for PKCα and both protein kinases are associated with caveolae [23,50]. An additional explanation for the activation of CK IIα and subsequent serine phosphorylation of caveolin-1 is somewhat more non-specific. Sphingosine and sphinganine, which are potent activators of CK IIα [51], have been shown to be elevated in the livers of mice with NPC and NPC fibroblasts grown in culture [52–54]. Whatever the mechanism, serine phosphorylation of caveolin-1 may impute specific properties that negatively regulate the normal transport of unesterified cholesterol.

With respect to the phosphorylation of annexin II, our results indicate that tyrosine phosphorylation of annexin II in the homozygous TIFF was significantly increased compared to annexin II in the normal and heterozygous TIFFs, while the level of serine phosphorylation remained unchanged. Annexin II is known to be a substrate for specific protein tyrosine kinases, most notably (1) pp60-src, (2) the insulin receptor kinase, and (3) the PDGF receptor kinase, all of which phosphorylate Tyr-23 located near the N-terminal domain [25,55,56]. Although studies suggest that insulin-induced tyrosine phosphorylation of annexin II may regulate the internalization and sorting mechanism for this receptor, the precise role of annexin II tyrosine phosphorylation remains to be determined.

Enhanced phosphorylation of both caveolin-1 and annexin II associated with the homozygous TIFF prompted us to investigate the expression and distribution of select serine and tyrosine protein kinases. As our results demonstrate, a number of these protein kinases were altered to some degree within heterozygous and homozygous livers which may provide an explanation for the increased phosphorylation of caveolin-1 and annexin II. Compared to normal homogenates, the amounts of PKCα and pp60-src were increased in both heterozygous and homozygous liver homogenates, while the amount of PKCζ and PKCδ were selectively increased in heterozygous or homozygous liver homogenates, respectively. A possible explanation for the increased expression of these protein kinases could be related to recent studies that have established a link between caveolin-1 and the inhibition of specific protein kinases [57,58]. It is believed that the caveolin-1 scaffolding domain interacts and inhibits the enzymatic activity of particular protein kinases (PKCα, PKCζ and pp60-src, but not PKCε). Therefore, it is possible that the increased expression of caveolin-1 we previously measured in heterozygous and homozygous livers has a role in negatively regulating protein kinase activity, and that the increased protein expression of these protein kinases is an attempt to compensate for this inhibitory effect on catalytic activity.

Since the homozygous TIFF contained an increased concentration of diacylglycerol, it is possible that certain PKC isotypes associated with the TIFF could be modulated by this lipid. Investigators working with monkey kidney epithelial cells have found that an increased concentration of PMA displaces PKCα from caveolae [26]. This would at least provide an explanation for the decreased amount of PKCα associated with the homozygous TIFF, but would not provide an explanation for the decreased amount of PKCα associated with the heterozygous TIFF, unless it was displaced by a resident protein kinase, such as PKCζ upon activation within the heterozygous TIFF. Since PKCζ is activated independent of calcium and diacylglycerol, the induced translocation of this particular PKC isotype to the TIFF is intriguing and may yield important regulatory information regarding the pathophysiology of NPC. Importantly, other inborn errors of metabolism, such as mucolipidosis type 4 and Zellweger syndrome, have demonstrated major perturbations in PKC-mediated signal transduction which are be-

lieved to contribute to pathogenesis of these disorders [59,60].

Of the protein kinases examined, the expression and distribution of CK IIα and pp60-src are of primary importance in delineating the mechanism responsible for serine phosphorylation of caveolin-1 and tyrosine phosphorylation of annexin-II, respectively. The relative expression of CK IIα was decreased in homozygous homogenates, but the amount of CK IIα associated with the homozygous TIFF was similar to the amount associated with the normal TIFF. Based solely on these results, we are unable to provide a plausible explanation accounting for the increased serine phosphorylation of caveolin-1 in the homozygous TIFF, besides the possibility that sphingosine and sphinganine are activating CK IIα as previously described. With respect to pp60-src, examination of the immunoblots revealed increased amounts of two forms of pp60-src due to phosphorylation: a tight doublet representing an inactive (lower molecular weight) form and an active (higher molecular weight) form of pp60-src [61]. By measuring both proteins independently and comparing the relative expression among the three genotypes, we determined that the inactive and active forms of pp60-src were expressed highest in heterozygous livers, while homozygous livers expressed intermediate levels. Distribution of pp60-src to the TIFF indicated that only the lower molecular weight form was present and in similar amounts, consistent with studies demonstrating that caveolae, and caveolin-1 in particular, may represent a scaffolding structure that holds the inactive form of pp60-src [62].

Our previous results have indicated that unesterified cholesterol was enriched within the TIFF derived from homozygous livers. To complete the analysis of lipids associated with the TIFF, we determined the mass of additional glycerolipids and sphingolipids in an attempt to further characterize the role of this subcellular compartment in NPC. In doing so, we found that the concentration of major phospholipids (phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, lysophosphatidylcholine) were similar among the three genotypes analyzed, although sphingomyelin was significantly increased in the TIFF derived from homozygous livers. This result would be consistent with an increased concentration of unesterified cholesterol in the homozygous TIFF, since these two lipids form a tight complex [63]. As described above, our results indicate that diacylglycerol is elevated in the TIFF derived from homozygous livers, which may in part contribute to the altered distribution and activity of specific PKC isotypes.

The function of the *Npc1* gene product is currently uncharacterized, but the primary amino acid sequence indicates a sterol-sensing domain and other important regulatory domains that will no doubt have an important role in modulating cholesterol homeostasis. With this study, we describe differential alterations in the expression, phosphorylation and localization of caveolin-1, annexin II and protein kinases that may have an important role in diverse signal transducing pathways. Alteration in these proteins may be involved in modulating and controlling intracellular cholesterol trafficking, specifically the distribution of cholesterol to various cellular compartments. How these proteins are regulated by the NPC1 protein is an important first step in defining key processes in maintaining cholesterol homeostasis and the molecular basis for NPC disease.

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