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# Increased Expression of Caveolin-1 in Heterozygous Niemann-Pick Type II Human Fibroblasts

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**Human Niemann-Pick type II fibroblasts, which encompass the panethnic type C (NPC) and Nova Scotia Acadian type D (NPD) variants, exhibit altered expression of caveolin-1 protein when examined by immunoblotting using an anti-caveolin-1 monoclonal antibody. Unexpectedly, caveolin-1 in heterozygous fibroblasts was significantly elevated as much as 10-fold compared to caveolin-1 in normal and homozygous affected fibroblasts. Homozygous NPC fibroblasts expressed caveolin-1 levels similar to those in normal fibroblasts, while the expression of caveolin-1 in homozygous NPD fibroblasts was slightly elevated. Northern analysis indicates that normal fibroblasts and NPC heterozygous fibroblasts have similar amounts of caveolin-1 mRNA, while NPC homozygous fibroblasts have significantly less caveolin-1 mRNA. In contrast, heterozygous and homozygous NPD fibroblasts exhibit increased levels of caveolin-1 mRNA. These novel findings suggest that caveolin-1 containing subcellular structures are involved in the pathophysiology of Niemann-Pick type II disease. Furthermore, altered caveolin-1 protein expression may serve as a useful marker for the diagnosis of carriers of NPC or NPD.** 

Niemann-Pick type C (NPC) and Niemann-Pick type D (NPD), collectively referred to as Niemann-Pick type II (NPII) disease, are autosomal recessive lipid storage disorders (1). NPC is a panethnic form of NPII, that is clinically heterogenous and characterized by an impaired ability of cells to metabolize low density lipoprotein (LDL) derived cholesterol. NPD is a clinically more homogenous form of NPII, originating in the Acadian population of southwestern Nova Scotia in Canada (2,3), and is likely an allelic varient of NPC (4). Though the defective gene responsible for NPII is currently unknown, studies have demonstrated that heterozygous and homozygous fibroblasts incubated with LDL accumulate unesterified cholesterol in lysosomes and the Golgi apparatus (5-8). In addition, it has been shown that there is impaired esterification of plasma membrane cholesterol following treatment of NPC and NPD fibroblasts with sphingomyelinase, indicating that the transport of cholesterol from the plasma membrane to the endoplasmic reticulum is deficient (9). The block in cholesterol transport prevents the proper maintenance of intracellular cholesterol homeostasis by : 1) preventing inhibition of cholesterol biosynthesis, a reaction catalyzed by $\beta$-hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase; 2) impaired down-regulation of LDL receptors; and 3) activation of acyl-CoA : cholesterol acyltransferase (ACAT), an enzyme responsible for storing excess unesterified cholesterol as relatively inert cholesteryl ester lipid droplets (10).

Recently, interest has developed concerning the function of caveolin, an avid cholesterol binding protein of 22-25 kDa, and a coat protein for specialized plasma membrane invaginations called caveolae (11). Three different tissue specific forms of caveolin (caveolin-1, caveolin-2 and caveolin-3) characterize the caveolin gene family (12-14). Caveolin-1, the best characterized, is capable of forming oligomers of up to 400 kDa. Its importance in forming and maintaining an invaginated structure representative of caveolae was shown by inducing expression of caveolin-1 in lymphoblasts, a cell-type which does not normally express caveolin-1 (15). Treatment of human fibroblasts with cholesterol oxidase has detailed a constitutive caveolin-1 cycling pathway, whereby caveolin-1 migrates by a microtubule-dependent pathway from plasma membrane caveolae to the Golgi complex by way of the endoplasmic reticulum (ER) (16, 17). Subsequent return of caveolin-1 to

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the plasma membrane from the Golgi complex is microtubule independent. Presumably, this novel bidirectional caveolin-1 pathway may have a crucial role in transporting unesterified cholesterol to the plasma membrane. These findings are supported by others indicating a role of caveolin-1 containing vesicles in the transport of unesterified cholesterol (18, 19), and the involvement of plasma membrane caveolae in mediating the desorption of unesterified cholesterol into the media (20).

Besides its structural functions, caveolin-1 may also function in signal transduction processes. It contains conserved serine and tyrosine residues near its N-terminus that constitute phosphorylation consensus sequences (21). Indeed, activators of protein kinase C (PKC) such as phorbol myristate acetate (PMA) and diacylglycerol have been found to modulate the morphology and function of caveolae (22). Caveolin-1 has also been shown to associate with the $\alpha$ subunit of specific heterotrimeric GTP (G) binding proteins, inhibiting GTPase activity analogous to effects of the GDP-dissociation inhibitor (GDI) protein (23). Studies have also shown that the cytosolic domain of caveolin-1 interacts with inactive conformations of Src family tyrosine kinases and H-Ras (24, 25). Preferring the inactive conformation of these cytoplasmic signaling molecules indicates that caveolin-1 may function as a common membrane-anchored scaffolding protein (25).

Using the murine model for Niemann-Pick type C (26), we have previously shown that liver endothelial cells from heterozygous mice have significantly elevated levels of caveolin-1 as assessed by immunoblotting and *in situ* analysis (27). In the present study, we have extended our investigation to humans and have measured the relative expression of fibroblast caveolin-1 and caveolin-1 mRNA from both heterozygous and homozygous individuals with NPC and NPD. In accordance with our previous findings in the NPC murine model, we found that the expression of caveolin-1 in both NPC and NPD heterozygous fibroblasts was significantly elevated compared to normal fibroblasts; NPC and NPD homozygous fibroblasts had similar or slightly elevated levels of caveolin-1 compared to normal fibroblasts. Unexpectedly, we found disparity between the levels of caveolin-1 mRNA and caveolin-1. Levels were markedly dissimilar between NPC and NPD fibroblasts, with heterozygous and homozygous NPD fibroblasts displaying elevated levels of caveolin-1 mRNA compared to normal and NPC fibroblasts.

## MATERIALS AND METHODS

*Reagents.* Phenylmethylsulfonylfluoride (PMSF), pepstatin, leupeptin and aprotinin were purchased from Sigma Chemical Company (St. Louis, MO). Bicinchoninic acid (BCA) protein assay reagent and Supersignal Substrate Solution were purchased from Pierce Chemical Company (Rockford, IL). Mouse anti-caveolin-1 IgG and mouse anti-annexin II IgG were purchased from Transduction Laboratories (Lexington, KY). Peroxidase-conjugated anti-mouse IgG was purchased from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD). Chloroform, isopropanol and ethanol were all of analytical reagent grade and purchased from Mallinckrodt Chemical Company (Paris, KY). Dulbecco's Modified Eagles's Medium (DMEM), fetal bovine serum and Trizol Reagent were purchased from Gibco BRL (Gaithersburg, MD). $^{32}$P-dCTP (3000 Ci/mmole) was purchased from NEN Life Science Products (Boston, MA).

*Cells.* Normal human skin fibroblast cell lines were obtained from the American Type Culture Collection (Rockville, MD). Human NPC and NPD (heterozygous and homozygous) fibroblasts were obtained from the Mutant Cell Repository at the Coriell Institute for Medial Research (Camden, NJ) and from established cell lines derived from heterozygous and homozygous individuals. Cells were maintained in plastic tissue culture flasks in DMEM supplemented with L-glutamine and 10% fetal bovine serum at 37°C in a humidified incubator equilibrated with 5% $CO_2$ and 95% air. Cells were plated in 25 $cm^2$ flasks at approximately $5 \times 10^5$ cells and grown to confluence (3-6 days) prior to use.

*Preparation of protein.* After reaching confluence, cells were rinsed three times with ice-cold phosphate buffered saline (0.010 M $Na_2HPO_4$ adjusted to pH 7.4, 0.150 M NaCl). Two ml of solubilization buffer (0.010 M Tris adjusted to pH 8.0, 0.150 M NaCl, 10 mM SDS containing 10 $\mu$M each of PMSF, pepstatin, leupeptin and aprotinin) was used to solubilize the cell monolayer for 5 minutes at room temperature. Solubilized protein was collected and concentrated using Centricon 10 microconcentrators (Amicon Inc., Beverly, MA) at 4°C. Protein concentration from each sample was then determined.

*Western blot analysis and quantitation.* Equal amounts of protein, 25 $\mu$g, were separated using 10% sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) under reducing conditions and transferred to 0.45 micron nitrocellulose membranes (BioRad). Nonspecific sites were blocked with blocking buffer (0.010 M sodium phosphate pH 7.4, 0.150 M NaCl, 0.05% Tween 20 and 4.0% non-fat dry milk) for 2 h at room temperature. Blots were then incubated with mouse anti-caveolin-1 IgG (1:500 dilution) or mouse anti-annexin-II IgG (1:1000 dilution) overnight at 4° C, followed by rinsing three times each for 10 min. Next, blots were incubated with peroxidase-conjugated goat anti-mouse IgG (1:5000 dilution) for 1 h at room temperature. Finally, blots were rinsed three times for 10 min each followed by immediate incubation in Supersignal Substrate Solution for 5 min to initiate enhanced chemiluminescence (ECL). Autoradiography was performed using BioMax MR Scientific Imaging Film (Kodak). After multiple exposures, caveolin-1 protein levels were quantitated using a Model GS-700 Imaging Densitometer (BioRad). Each band was quantitated using an equal area and background counts were subtracted and reported as caveolin-1/$\mu$g protein.

*RNA isolation and Northern blot analysis.* Total RNA from confluent fibroblasts was isolated using Trizol Reagent (28). RNA purity and concentration were determined spectrophotometrically using the absorbance ratio of 260/280 nm and 260 nm, respectively. Total RNA ($\approx$10 $\mu$g) from each sample was separated on formaldehyde-agarose (0.8%) gels and transferred to a Hybond-N+ nylon membrane (Amersham). Immobilized RNA was probed with randomly-primed $^{32}$P-labeled canine caveolin-1 cDNA (kindly provided by Michael P. Lisanti) and human $\gamma$-actin cDNA (control) using standard procedures (29). Blocking of nonspecific sites, hybridization and washes were performed by preparing dilutions from a 20$\times$ stock solution of SSPE (0.2 M $Na_2HPO_4$ adjusted to pH 7.7, 3.6 M NaCl and 0.02 M EDTA). After a 1 h prehybridization in hybridization buffer (5$\times$ SSPE, 5$\times$ Denhardt's solution and 0.5% (w/v) SDS), the radiolabeled cDNA probe was added for 14 h at 65°C. Blots were washed sequentially with 2$\times$ SSPE/0.1% (w/v) SDS (10 min at room temperature), 1$\times$ SSPE/0.1% (w/v) SDS (15 min at 65°C) and 0.1$\times$ SSPE/0.1% (w/v) SDS (10 min at 65°C), followed by autoradiography. Quantitation of caveolin-1 and $\gamma$-actin mRNA levels were performed using imaging densitometry after autoradiography. Caveolin-1 mRNA levels were

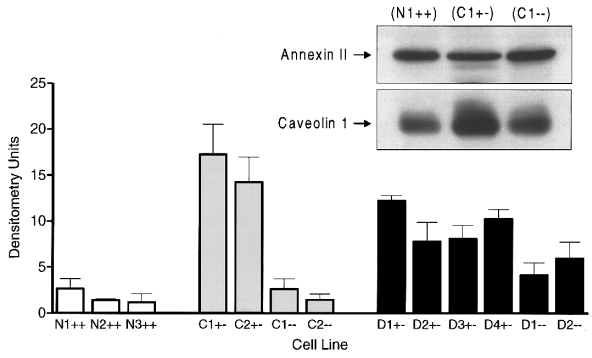


**FIG. 1.** Increased expression of caveolin-1 in heterozygous NPC and NPD fibroblasts. Human fibroblasts were grown to confluence in DMEM containing 10% FCS. Protein (25 $\mu$g) was separated using SDS-PAGE (10%) and transferred to a nitrocellulose membrane. Blots were probed with mouse anti-caveolin-1 IgG and mouse anti-annexin II (control) as described in Methods. A total homogenate, prepared from a representative normal, NPC heterozygous and NPC homozygous cell lines, was probed with mouse anti-caveolin-1 IgG (1/500 dilution) and mouse anti-annexin II IgG (1/1000 dilution), followed by visualization using peroxidase-conjugated goat anti-mouse IgG to perform ECL (Insert). Quantitative analysis of caveolin-1 protein expression from NPC and NPD heterozygous and homozygous fibroblasts was determined by scanning the film using a BioRad Model GS-700 Imaging Densitometer at high (21 $\mu$m) resolution. Background was subtracted from each respective sample. Data for each cell line is represented as the mean ± S.D. of four samples analyzed in parallel. Normal (N), NPC (C), NPD (D) and the accompanying numerical designations represent specific cell lines within a given genotype denoted as homozygote normal (++), heterozygous (+−) and homozygous affected (−−).

calculated in relation to measured $\gamma$-actin mRNA levels to compensate for inexact loading of total RNA.

*Statistical analysis.* All quantitative data are represented as the mean ± standard deviation (S.D.) for each cell line derived from four separate flasks. Data from similar genotypes have been grouped and expressed as the mean ± S.D. where indicated. Significant differences between groups of data were determined using a two-tailed students t-test assuming equal variance.

## RESULTS

### *Caveolin-1 and Annexin II Protein Expression in NPC Fibroblasts*

Shown in Fig. 1 (insert) is a representative autoradiogram depicting the relative amounts of caveolin-1 and annexin-II in total homogenates from normal fibroblasts (control), heterozygous NPC fibroblasts and homozygous NPC fibroblasts. Both proteins, caveolin-1 and annexin II, have traditionally been used as marker proteins for caveolae. Annexin II expression was included and monitored as an internal control. As is evident, heterozygous fibroblasts have upregulated caveolin-1 protein expression, but the homozygous affected fibroblasts do not have an altered expression of caveolin-1 protein. Under the described experimental conditions, no other immunoreactive proteins were detected.

The relative quantitation of caveolin-1 from three different homozygous normal human fibroblast cell lines (controls), two NPC and four NPD heterozygous fibroblast cell lines, and a homozygous fibroblast cell line each from a NPC and NPD individual is also shown in Fig. 1. Each of the NPC and NPD heterozygous fibroblasts expressed significantly elevated levels of caveolin-1 compared to normal fibroblasts. The average expression of caveolin-1 from the two NPC heterozygotes is elevated 9.3-fold ($P = 0.0016$) compared to the expression of caveolin-1 measured in the three normal fibroblasts. In comparison, the average expression of caveolin-1 from the four NPD heterozygotes was elevated 5.6-fold ($P = 0.0017$) compared to the expression of caveolin-1 measured in the three normal fibroblasts. Comparing NPC and NPD heterozygotes, the expression of caveolin-1 in the two heterozygous NPC fibroblasts is elevated 1.6-fold ($P = 0.0270$) compared to the expression of caveolin-1 in the four heterozygote NPD fibroblasts. These data indicate that the expression of caveolin-1 measured from NPD heterozygous fibroblasts is intermediate between the expression of caveolin-1 measured from NPC heterozygous fibroblasts and that measured in homozygous normal fibroblasts. In contrast, there was no detected significant elevation (1.2-fold, $P = 0.6921$) in the two NPC homozygotes when compared to the three normal fibroblasts. The two NPD homozygous fibroblasts expressed significantly elevated levels of caveolin-1 (3.0-fold, $P = 0.0335$) when compared to the three normal fibroblasts.

### *Northern Blot Analysis*

An autoradiogram of a Northern Blot depicting the levels of caveolin-1 and $\gamma$-actin (control) mRNA from representative cell lines is shown in Fig. 2. Quantitational analysis of caveolin-1 and $\gamma$-actin (control) mRNA levels was performed using imaging densitometry. Caveolin-1 mRNA levels were normalized to $\gamma$-actin mRNA levels (caveolin-1 mRNA/$\gamma$-actin mRNA) to compensate for loading effects (Fig. 2). As shown, NPC heterozygous fibroblasts had slightly decreased levels of caveolin-1 mRNA compared to normal fibroblasts, and this decrease was more evident in the NPC homozygous fibroblast cell line analyzed. In contrast, both NPD heterozygous and homozygous fibroblasts had increased amounts of caveolin-1 mRNA compared to normal fibroblasts. Noticably, the levels of caveolin-1 mRNA detected in both the NPC and NPD homozygous fibroblasts was somewhat less than that detected in the corresponding heterozygous fibroblasts.

## DISCUSSION AND CONCLUSION

Caveolin-1 is a membrane-bound phosphoprotein that avidly binds unesterified cholesterol (10). Caveolin-1 associates with plasma membrane caveolae and

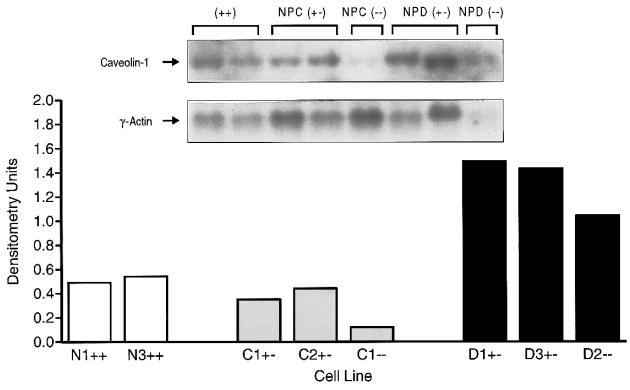


**FIG. 2.** Northern Blot analysis of caveolin-1 mRNA levels from representative NPC and NPD heterozygous and homozygous fibroblasts. Total RNA from each of the indicated fibroblast cell lines (10 $\mu$g) was separated using 0.8% a formaldehyde-agarose gel, then transferred to a Hybond N+ membrane. The membrane was probed sequentially with $^{32}$P-labeled canine caveolin-1 and $^{32}$P-labeled $\gamma$-actin cDNA (control utilized for normalizing the amount of RNA). An approximate 2.5 kb transcript for caveolin-1 mRNA and 1.5 kb transcript for $\gamma$-actin mRNA were detected in all cell lines. Molecular sizes were derived from ethidium bromide-stained marker RNA. The insert shows an autoradiogram exhibiting caveolin-1 and $\gamma$-actin mRNA levels in the indicated fibroblasts. The main figure quantitatively displays caveolin-1 mRNA levels after precisely correcting for total RNA loading using $\gamma$-actin for normalization. Normal (N), NPC (C), NPD (D) and the accompanying numerical designations represent specific cell lines within a given genotype denoted as homozygote normal (++), heterozygous (+−) and homozygous affected (−−).

intracellular vesicles that may facilitate cholesterol transport and have a role in promoting intracellular cholesterol homeostasis (18-20). For this reason, we investigated the expression of caveolin-1 in heterozygous and homozygous fibroblasts from both NPC and NPD individuals. Similar to our results using liver homogenates from heterozygous mice with NPC (26), both NPC and NPD heterozygous fibroblasts express significantly elevated levels of caveolin-1 compared to normal fibroblasts. Comparing the expression of caveolin-1 between the two different NPC heterozygous cell lines and the four different NPD heterozygous cell lines, it is evident that the NPD caveolin-1 expression is intermediate between NPC and homozygous normal fibroblasts. This difference in caveolin-1 protein expression is intriguing considering that NPD heterozygous and homozygous fibroblasts have an intermediate deficiency in cholesterol transport compared to NPC fibroblasts (3). With respect to the homozygous fibroblast cell lines, the expression of caveolin-1 in homozygous NPC fibroblasts was similar to levels measured in normal fibroblasts, while homozygous NPD fibroblasts had slight, but significantly elevated levels of caveolin-1.

To investigate the regulatory basis of altered caveolin-1 protein expression, we measured levels of caveolin-1 mRNA using Northern analysis. Our results demonstrate that caveolin-1 mRNA levels in NPC heterozygous fibroblasts were slightly decreased compared to normal fibroblasts. To account for the increased levels of caveolin-1 measured by immunoblotting, either translation of caveolin-1 mRNA is increased or the degradation of caveolin-1 protein is decreased. In the one NPC homozygous fibroblast cell line analyzed, the level of caveolin-1 mRNA was decreased compared to normal and heterozygous fibroblasts. Again, to compensate for apparently normal levels of caveolin-1 protein, similar translational or protein degradation regulatory mechanisms must be active. Surprisingly, NPD heterozygous and homozygous fibroblasts had elevated levels of caveolin-1 mRNA, compared to caveolin-1 mRNA levels measured in normal and NPC fibroblasts. This would indicate that transcriptional regulation of the caveolin-1 gene may account for increased levels of caveolin-1 protein only in the NPD variant. Currently, we are unable to explain the differences in caveolin-1 mRNA levels between NPC and NPD cell lines, and further investigation into the transcriptional, translational and protein degradation mechanisms of caveolin-1 must be explored. Biochemical investigations have identified other dissimilarities between NPC and NPD fibroblasts (30). For example, though defective downregulation of HMG-CoA reductase activity by LDL is similar between NPD fibroblasts and NPC fibroblasts, LDL receptor downregulation is less affected in NPD fibroblasts.

The pattern of caveolin-1 protein expression shown in this study is similar to the pattern of caveolin-1 protein expression we measured in total liver homogenates from the murine NPC model. The reason for altered caveolin-1 protein and caveolin-1 mRNA levels in NPC and NPD is unknown. It is possible that the over production of caveolin-1 in heterozygous fibroblasts is a response to one defective gene that facilitates the transport of unesterified cholesterol to those sterol regulatory pools required for maintaining intracellular cholesterol homeostasis. This would prevent accumulation of excess unesterified cholesterol in heterozygous individuals. However, if this were true, we would expect homozygous fibroblasts to produce caveolin-1 above that detected in the heterozygous fibroblasts. The disparity of caveolin-1 mRNA and protein levels between NPC and NPD fibroblasts demonstrate the yet undefined complex regulatory mechanisms that are responsible for altered caveolin-1 expression as a result of this disorder.

Currently, our laboratories are combining efforts to map and clone the defective gene responsible for Niemann-Pick type II disease. We do not propose that caveolin-1 is the responsible gene, since lymphocytes derived from NPC and NPD homozygotes exhibit the classical NPC and NPD phenotype (31), even though lymphocytes do not normally express caveolin-1 (15). We do hypothesize though, that caveolin-1 may have an important role in compensating for the accumulation of

excess unesterified cholesterol in heterozygous cells, and may therefore be classified as a putative modifying gene. Finally, we believe that caveolin-1 protein expression may serve as a sensitive and convenient means to detect heterozygous individuals.

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