Modulation of protein kinase C by endogenous sphingosine: inhibition of phorbol dibutyrate binding in Niemann–Pick C fibroblasts

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The abnormal and variable increase in levels of free sphingoid bases recently described in fibroblasts from Niemann–Pick C patients allowed us to investigate the modulation of protein kinase C *in vivo* by endogenous sphingosine. The specific binding of [20-^{3}H]phorbol 12,13-dibutyrate to the regulatory domain of membrane-bound protein kinase C was significantly decreased in fibroblasts from patients compared with controls. A pronounced difference between the two groups ($P < 0.0001$) was demonstrated in low-density lipoprotein-supplemented medium, i.e. under conditions known to disclose abnormal mobilization of unesterified cholesterol in Niemann–Pick C fibroblasts. Furthermore the degree of impairment of [^{3}H]phorbol 12,13-dibutyrate binding was highly correlated ($r = 0.95$) with the sphingosine levels measured in fibroblasts from those patients. Scatchard analysis of the binding data indicated that Niemann–Pick C and control fibroblasts contained almost the same number of binding sites per cell. A 8–34-fold increase in K_d was measured in Niemann–Pick C fibroblasts with at least a 5-fold increase in sphingosine levels. Removal, by cell fractionation, of membrane-bound protein kinase C from the bulk of sphingosine induced a normalization of K_d values. The overall results suggest that protein kinase C inhibition is directly related to sphingosine accumulation.

INTRODUCTION

Sphingosine has gained attention as a bioactive compound since it was shown to induce a potent and reversible inhibition of protein kinase C (PKC) after addition to cell cultures [1,2] and to affect other signal transduction pathways (reviewed in [3]). Furthermore the *N*-acyl (i.e. ceramide) and 1-phosphate derivatives of sphingosine are highly bioactive and are emerging as intracellular mediators. Amounts of free sphingoid bases in tissues and cultured cells are generally low and constant [4,5]. We have previously described a 20–40-fold elevation of sphingosine and sphinganine levels in tissues from patients affected by the genetic disease Niemann–Pick C (NPC) [6] and its Balb/c murine model [7]. A significant increase in free sphingoid bases also occurred in NPC fibroblasts, disproportionate to the minimal degree of lipid storage, and varying between cell lines from near normal up to a 10-fold increase. Furthermore addition of 10 μM sphinganine to normal fibroblast cultures has been shown to induce a biochemical NPC phenotype [8] characterized by abnormalities in cellular trafficking of unesterified cholesterol associated with its lysosomal accumulation (reviewed in [9]). These two observations suggest that sphingoid bases might participate in the physiopathology of the disease because PKC has been shown to be involved in the activation of cholesterol transport [10,11]. Moreover, NPC provides the first tool for studying the modulation of PKC activity by endogenous sphingosine. Because sphingosine is postulated to inhibit PKC by interaction at the regulatory domain with the phorbol ester binding sites or with other lipidic cofactors [2], the binding of [20-^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDB) to membrane-bound PKC was studied in living fibroblasts from NPC patients in relation to cholesterol overload and sphingosine levels.

MATERIALS AND METHODS

Chemical and biological reagents

[^{3}H]PDB (20.0–20.7 Ci/mmol) was obtained from New England Nuclear (Les Ulis, France). Unlabelled PDB, sphingosine, sphinganine, fumonisin B1 and other chemical reagents were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Human lipoprotein-deficient serum (LPDS) and low-density lipoproteins (LDLs) were prepared in the laboratory from fresh human plasma [12].

Cell culture conditions

Skin fibroblasts from 12 NPC patients belonging to the major group of complementation and showing severe alterations in intracellular LDL-cholesterol processing [9] were used in this study. Ten control cell lines were obtained from healthy volunteers and children with other lysosomal storage diseases (Krabbe and Sandhoff diseases). Cells grown by standard procedures [13] were seeded either in 6-well culture plates (Nunc) (40000 cells per well) for [^{3}H]PDB binding studies or in 75 cm^2 flasks for sphingosine analysis and for fractionation procedures. In most experiments they were grown either for 3 days in a cholesterol-deprived medium [McCoy's 5A medium supplemented with 10 % (v/v) human LPDS] followed by 24 h in an LDL-supplemented medium (10 % LPDS/50 μg/ml LDL) or 4 days in an LPDS medium.

Intoxication with sphinganine

Fibroblasts were preincubated for 20 min at 37 °C with increasing concentrations of sphinganine for dose–effect studies. Sphing-

Abbreviations used: LDL, low-density lipoprotein; LPDS, lipoprotein-deficient serum; NPC, Niemann–Pick C disease; [^{3}H]PDB, [20-^{3}H]phorbol 12,13-dibutyrate; PKC, protein kinase C.

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anine addition was maintained in the medium during the binding procedure.

Intoxication with fumonisin

Control fibroblasts grown under standard conditions were pre-incubated for 48 h with 20 μM fumonisin B1 before binding studies or quantification of sphingoid bases.

Binding of [^{3}H]PDB to living fibroblasts

Kinetics of [^{3}H]PDB binding

Non-confluent fibroblasts, conditioned as above, from NPC patients and controls were incubated at 37 °C for increasing durations with 1 ml of the same fresh medium containing 10 nM [^{3}H]PDB. The reaction was terminated by two washes of 5 min each at 4 °C with ice-cold 150 mM NaCl/50 mM Tris buffer (pH 7.4) containing 2 mg/ml BSA, followed by one wash in the same buffer without BSA. Cells were then dissolved at 25 °C by incubation for at least 20 min in 1 ml of 0.5 M NaOH. One 0.5 ml aliquot was counted to determine total [^{3}H]PDB binding and 0.1 ml was used to determine the content of cellular protein by the Lowry procedure [14]. Non-specific binding was determined in the presence of 10 μM unlabelled PDB and subtracted from the total counts to yield the specific binding. Binding assays were performed in triplicate and results were expressed as pmol/mg protein of [^{3}H]PDB bound (means ± S.E.M.).

Influence of LDL-cholesterol on [^{3}H]PDB binding

Fibroblasts were conditioned by the procedure described for cell culture conditions and then incubated at 37 °C in the same fresh medium with 10 nM [^{3}H]PDB for 30 min.

Scatchard analyses

Fibroblasts were incubated at 4 °C for 30 min with increasing concentrations of [^{3}H]PDB (5–200 nM). Scatchard plots were obtained by use of the computer program EBDA, which calculated the K_d coefficient and B_{max} and allowed the determination of the number of sites per cell.

Binding of [^{3}H]PDB to membrane fractions

Preparation of membrane fractions

Cells from two 75 cm^2 flasks were collected by scraping, pooled and pelleted by low-speed centrifugation. They were then disrupted in a Potter–Elvehjem homogenizer in 1 ml of ice-cold buffer containing 0.25 M sucrose, 50 mM Tris/HCl, pH 7.4, and 10 μg/ml leupeptin. The homogenate was centrifuged at 10000 ***g*** for 15 min at 4 °C in a Beckman TL100 ultracentrifuge. The pellet was discarded and the supernatant centrifuged at 100000 ***g*** for 75 min. The 100000 ***g*** pellet was resuspended in 0.2 ml of 50 mM Tris/HCl buffer and stored at −80 °C until needed for the binding assay.

Binding assay

The soluble phorbol ester receptor assay was done as described by Driedger and Blumberg [15], with the after modifications: the sample was reduced to 0.3–0.4 μg of the membrane-bound protein preparation, the specific radioactivity of [^{3}H]PDB used was 20-fold higher, 50 mM Tris/HCl buffer was supplemented with 0.5% BSA, and incubations were performed at 37 °C instead of 39 °C. Scatchard experiments proceeded with increasing concentrations of [^{3}H]PDB (7.25 to 200 nM) and the analysis of results by the Scatchard method was done as described above.

Content of free sphingoid bases in fibroblasts

Quantification of sphingosine and sphinganine was performed on a total lipid extract by HPLC [6]. Results are expressed as pmol/mg of protein, measured by the Hartree method [16] with serum albumin as the standard.

RESULTS

General characteristics of [^{3}H]PDB binding to living skin fibroblasts

The whole-cell phorbol receptor assay was adapted to fibroblast cultures from that described by Hannun et al. [1] for suspended cells; special conditions of washing and cell collection were defined. Figure 1 shows the kinetics of [^{3}H]PDB binding obtained for NPC and control fibroblasts as a function of culture conditions and temperature. The two conditions of culture classically used to discriminate NPC fibroblasts from normal ones

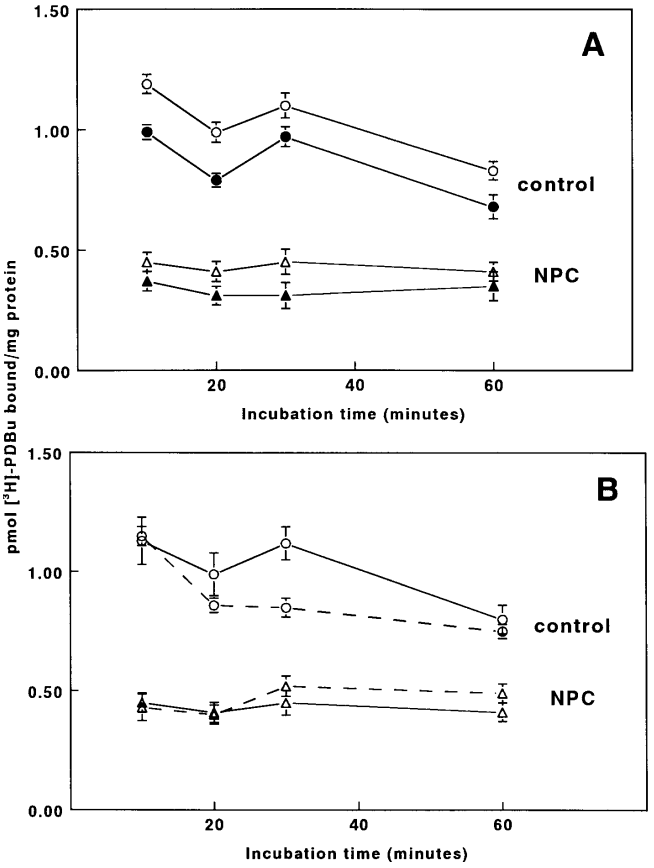

Figure 1 Effect of incubation time on [^{3}H]PDB binding to living fibroblasts as a function of conditions of culture (A) and temperature (B)

(**A**) Fibroblasts from controls (○,●) or NPC patients (△,▲) were grown for either 4 days in a cholesterol-deprived medium (LPDS medium) (○,△) or 3 days in LPDS medium followed by 24 h of LDL (50 μg/ml) addition (●,▲) (see the Materials and methods section). [^{3}H]PDB (10 nM) was added in the same fresh medium and incubation was performed at 37 °C for various periods. (**B**) Fibroblasts from controls (○) or NPC patients (△) grown for 4 days in LPDS medium were incubated as above, either at 37 °C (solid lines) or 4 °C (broken lines). Results are expressed as pmol of [^{3}H]PDB bound/mg of protein (means ± S.E.M.; $n = 3$), including a correction for non-specific binding.

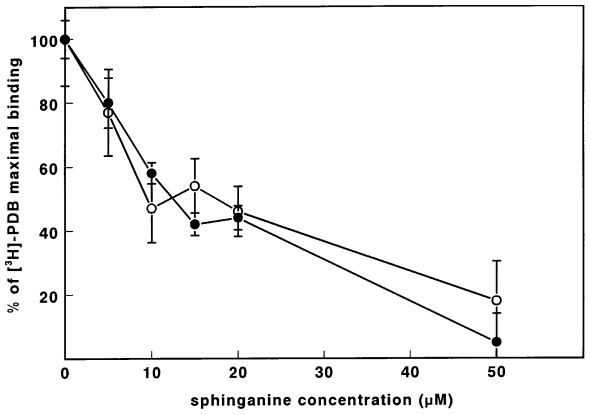

Figure 2 Sphinganine inhibition of [^{3}H]PDB binding to control fibroblasts

Sphinganine was added at the indicated concentration to normal fibroblasts grown for either 4 days in a cholesterol-deprived medium (○) or 3 days in a cholesterol-deprived medium followed by 24 h of LDL (●). After incubation for 20 min at 37 °C with sphinganine, 10 nM [^{3}H]PDB was added to cells for 30 min and binding (including correction for non-specific binding) was determined as described in the Materials and methods section. Results are expressed as percentages of [^{3}H]PDB bound in untreated cells (means ± S.E.M.; $n = 3$).

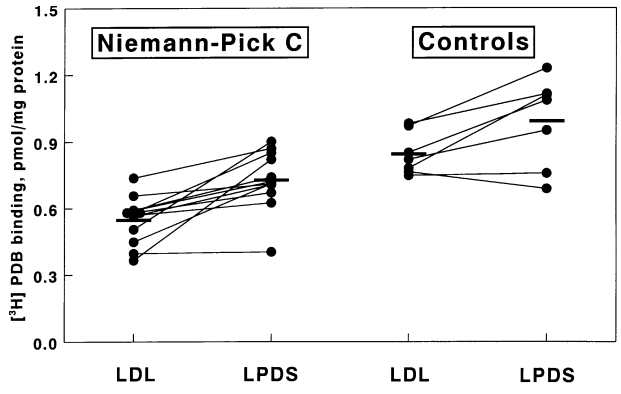

Figure 3 [^{3}H]PDB binding to living fibroblasts from NPC patients and control subjects

Fibroblasts from 12 NPC patients and seven control subjects were grown for either 3 days in LPDS medium followed by 24 h of LDL (50 μg/ml) (LDL) or 4 days in a cholesterol-deprived medium (LPDS) and then subjected to [^{3}H]PDB binding studies. The first conditions are those classically used to display cholesterol sequestration in NPC cells; under the second conditions cholesterol processing seemed normal in NPC cells. Results of specific binding are expressed as pmol/mg of protein of [^{3}H]PDB bound. The horizontal lines represent the means.

were studied as shown in Figure 1(A). Cells were grown for 3 days in a lipoprotein-deficient medium to express the LDL receptors and were thereafter either challenged in an LDL-supplemented medium for 24 h (classical conditions for displaying cholesterol sequestration in NPC fibroblasts) (LDL conditions in Figure 1) or maintained for 24 h in a cholesterol-deprived medium, run as negative control (LPDS in Figure 1). The time course of specific binding was rapid, with maximal binding being achieved within 10 min. A downward drift in the amount of [^{3}H]PDB bound was generally observed at 20 min and after 30 min the specific radioactivity started to decrease with increasing incubation time. Cell culture conditions did not affect the kinetics of [^{3}H]PDB binding in either NPC or control fibroblasts (Figure 1A); neither did incubation temperature (37 or 4 °C) (Figure 1B). For further studies, optimal binding conditions were established as 30 min incubation time and 37 °C temperature (or 4 °C for Scatchard analyses). Experiments were validated only when non-specific binding accounted for less than 30 % of the total bound [^{3}H]PDB and less than 1 % of the total radioactivity added, as recommended previously [17].

Specificity of binding was achieved by showing that 20 min of preincubation of fibroblasts with an increasing concentration of sphinganine (less toxic for cells than sphingosine) induced a dose-dependent reduction of [^{3}H]PDB binding (Figure 2), observed in either cholesterol-deprived or cholesterol-supplemented medium. A 50 % inhibition was obtained with 10 μM of sphinganine.

[^{3}H]PDB binding to living NPC fibroblasts

The results of the binding studies obtained from 12 NPC cell lines and seven healthy controls are presented in Figure 3: individual results obtained under the LDL or LPDS conditions of culture are connected by lines. Binding of [^{3}H]PDB was decreased in fibroblasts from NPC patients compared with control subjects, especially when cholesterol overload was present. In fibroblasts from NPC patients in which abnormalities of cholesterol processing have been induced under LDL-supplemented conditions of culture, the mean specific binding of [^{3}H]PDB (mean ± S.D. 0.55 ± 0.11 pmol/mg of protein, $n = 12$) was significantly lower ($P < 0.0001$, unilateral t test) than in controls (0.84 ± 0.10, $n = 7$). This difference was less evident ($P < 0.005$) under conditions of cholesterol deprivation because the mean specific binding was 0.73 ± 0.13 pmol/mg of protein in NPC fibroblasts and 0.99 ± 0.20 in controls. Despite a wide range of variation for NPC patients under the LDL or LPDS condition of culture, a significant decrease in [^{3}H]PDB binding ($P < 0.001$) was observed when the results were analysed by a paired t test. The difference was less significant ($P < 0.05$, paired t test) when cholesterol-deprived cells from control subjects were challenged in an LDL-supplemented medium, indicating an elective effect of LDL on NPC fibroblast cultures. Mean binding results in three pathological controls (two Krabbe, one Sandhoff disease) were not statistically different from those in normal controls for either the LDL (0.79 ± 0.17) or LPDS (1.08 ± 0.17) culture conditions.

Correlation of [^{3}H]PDB binding with sphingosine levels in NPC fibroblasts

To investigate the relationship between decreased [^{3}H]PDB binding and sphingosine levels in NPC fibroblasts, quantification of sphingosine by HPLC was run in parallel with binding studies. Individual results obtained in eight NPC cell lines are presented in Figure 4. For each of the two parameters, areas delimited by broken lines define the range of values obtained in four control cell lines. The mean sphingosine levels were 460 ± 235 pmol/mg of protein ($n = 8$) in NPC fibroblasts and 80 ± 20 pmol/mg of protein ($n = 4$) in controls. As shown in the inset to Figure 4, the graded impairment of [^{3}H]PDB binding to fibroblasts from the eight NPC patients studied was highly correlated ($r = 0.95$) with their sphingosine levels. This observation strongly suggests a direct relation between inhibition of [^{3}H]PDB binding and sphingosine accumulation, and thus a potential modulation of PKC activity by endogenous sphingosine.

Scatchard analyses

The previous results imply that sphingosine interacts with the surface-bound PKC, probably by interfering with its regulatory domain. To test this hypothesis, Scatchard analyses were made on data from binding studies, performed at 4 °C on fibroblasts from five of the previous NPC patients (2, 3, 5, 6 and 8), three healthy controls and two pathological controls (Krabbe disease),

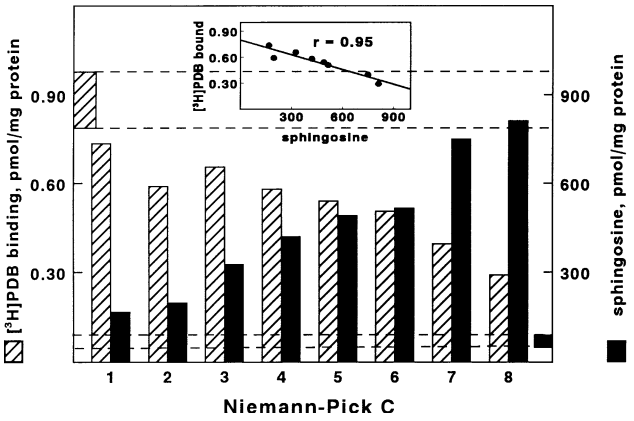

Figure 4 Correlation between [^{3}H]PDB binding and sphingosine levels in NPC fibroblasts

Fibroblasts from eight NPC patients were cultured for 3 days in LPDS medium and then for 1 day in LDL-enriched medium; they were then analysed for [^{3}H]PDB binding (hatched columns) and sphingosine concentration (filled columns). The correlation between sphingosine levels and [^{3}H]PDB binding is illustrated in the inset. The normal range of data obtained from four control cell lines for [^{3}H]PDB binding and sphingosine level (means $\pm$ S.D.) are represented by broken lines. All NPC cell lines expressed massive lysosomal sequestration of endocytosed LDL-cholesterol (classical biochemical phenotype) and were selected to cover the varying clinical phenotypes (patients 1 and 7, late infantile form; patients 2–6, juvenile form; patient 8, severe infantile form).

Table 1 K_d and sites per cell for [^{3}H]PDB binding to NPC and control fibroblasts at 4 °C

NPC patients 2, 3, 5, 6 and 8 were the same as for Figure 4. The experimental procedure was as described in the Materials and methods section. Scatchard analyses of results were performed with the EBDA software.

	Sphingosine (pmol/mg of protein)	K_d (nM)	$10^{-6} \times$ Sites per cell
NPC patients			
2	195	9.8	1.64
3	325	7.7	1.25
5	490	154.0	1.76
6	520	159.0	1.56
8	810	610.0	2.40
Normal controls	120	6.4	1.10
	65	21.7	2.22
	–	28.0	1.9
Pathological controls	120	6.5	0.97
	142	27.6	1.25

in the presence of increasing concentrations of [^{3}H]PDB. K_d values and numbers of sites per cell are given in Table 1. The analysis was linear for all cell lines tested, indicative of there being only one population of receptors. Fibroblasts from NPC patients or controls contained almost the same number of binding sites for [^{3}H]PDB. No significant difference in the K_d coefficient was observed for patients 2 and 3, who displayed moderate alteration in both sphingosine levels and [^{3}H]PDB binding. In contrast, in patients 5, 6 and 8 with sphingosine levels more than 5-fold the control value, 8.5-fold, 9-fold and 34-fold increases in K_d values respectively were observed.

A pharmacological modulation of intracellular sphingoid bases content was undertaken to strengthen our findings. Control fibroblasts were incubated for 48 h with 20 μM fumonisin B1, an inhibitor of ceramide synthase [18], to induce the accumulation of sphingoid bases. A marked increase in sphinganine levels (590 pmol/mg of protein, 24-fold increase) was obtained without a concomitant sphingosine increase. A 40 % decrease in [^{3}H]PDB binding was observed but without a significant increase in K_d value (28.6 compared with 25.0 in non-treated cells).

Table 2 K_d values for [^{3}H]PDB binding to plasma membrane (100000 *g* pellet) or to intact fibroblasts

	K_d (nM)		Total cellular sphingosine (pmol/mg of protein)
	Plasma membrane fraction	Intact cells	
NPC 5	144	154	490
NPC 8	113	610	810
Control	98	25	95

Because the addition of 10 μM sphinganine to normal cells can induce an NPC phenotype [8] we investigated whether this would also occur after fumonisin B1 treatment. There was no evidence of vesicular accumulation of unesterified cholesterol after 24 h of LDL uptake, whereas a marked decrease in cholesteryl ester formation after 4.5 h of LDL addition was observed.

Reversibility of inhibition of [^{3}H]PDB binding in NPC fibroblasts

To corroborate our results and to evaluate the reversibility of the inhibition of [^{3}H]PDB binding in NPC fibroblasts, the removal of membrane-bound PKC from the bulk of sphingosine was attempted. Low-speed centrifugation (10000 ***g***) was first applied to fibroblasts of NPC patients 5 and 8 with high sphingosine levels and K_d values, as well as those of one healthy control. The pellet obtained from NPC fibroblasts contained 81 % of total cellular sphingosine; the supernatant was subjected to a 100000 ***g*** centrifugation to obtain a plasma membrane fraction (6 % sphingosine) and a cytosolic fraction (13 % sphingosine). [^{3}H]PDB binding studies were performed on the membrane-bound fraction (100000 ***g*** pellet) of NPC and control fibroblasts by the method of Driedger and Blumberg [15]; K_d values were compared with those previously obtained for intact cells (Table 2). The results indicate that removing sphingosine from the PKC environment induces a normalization of K_d values and a reversal of [^{3}H]PDB binding inhibition.

DISCUSSION

In the present report we provide the first evidence that endogenous sphingosine, significantly increased in fibroblasts from a pathological genetic model, NPC, can interfere with the PKC signalling pathway by an inhibitory mechanism. Using [^{3}H]PDB as a ligand of membrane-bound PKC, we have demonstrated a linear relation between the inhibition of [^{3}H]PDB binding to NPC fibroblasts and the elevation of sphingosine levels, modulated by the presence of cholesterol overload. Reversal of inhibition was obtained after removal of membrane-bound PKC from the bulk of sphingosine by cell fractionation. [^{3}H]PDB binding studies in living fibroblasts were preferred to measurement of PKC catalytic activity in cell homogenates, as reported for mucolipidosis 4 and Zellweger syndrome [19,20] because some alterations of cholesterol metabolism observed in intact NPC fibroblasts are corrected in sonicated homogenates [21]. Furthermore sphingosine is supposed to inhibit PKC in a lipidic membrane environment by chemical interaction with phosphatidylserine or by competitive inhibition at the regulatory domain of PKC with activator substrates, such as diacylglycerol or phorbol esters [2].

Kinetics of [^{3}H]PDB binding to fibroblasts and dose–response

inhibition by sphinganine were in good agreement with experiments performed on other cellular models [1,22]. The K_d values calculated from Scatchard analysis in control fibroblasts were very close to those in the literature, published data falling between 1.3 nM for mink lung cells [23] and 60 nM for lymphocytes [17]. Our values for the number of sites per cell were significantly higher than those reported for platelets (0.29×10^5) or monocytes (1.55×10^6) [17] but were consistent with the greater size of fibroblasts.

The major difference between NPC and control [^{3}H]PDB binding results was observed after 24 h of cholesterol overload, suggesting that unesterified cholesterol might contribute to decreasing the binding of PKC to the membrane. Previous studies with immunoblotting or [^{3}H]PDB binding procedures [24,25] have shown that LDL induces a rapid but transient cytosol-to-membrane translocation of PKC in human vascular smooth-muscle cells or skin fibroblasts. However, these findings concerned the early response to LDL uptake, whereas the present study reports the effects of a long-term stimulation. Decreased binding of PKC to the membrane observed in NPC fibroblasts in relation to unesterified cholesterol overload might reflect a down-regulation process counteracting further putative LDL-mediated PKC activation. The catalytic activity of PKC has been shown to be decreased by 70 % in mucolipidosis 4 and to a lesser extent in Niemann–Pick A cells [19] or, as recently published, to be stimulated in fibroblasts from Zellweger patients [20]. We did not find any significant variation in either [^{3}H]PDB binding or sphingosine levels in fibroblast cultures from patients with Krabbe and Sandhoff diseases.

Our study corroborates the hypothesis first raised by Hannun and Bell [26] and recently revived by Boneh [27], who speculated that a modulation of PKC activity, subsequent to the accumulation of naturally occurring PKC modulators, might occur in sphingolipidoses and participate in the physiopathology of the diseases through the derangement of various cellular functions. An alteration of signal transduction in NPC fibroblasts is in good agreement with the data of Yamamoto et al. [28], who found that the early response of NPC fibroblasts to LDL uptake by changes in intracellular Ca^{2+} is significantly decreased compared with control cells.

There was no evidence for a direct relation between the variable decrease in [^{3}H]PDB binding (or increase in sphingosine levels) and the biochemical or clinical phenotypes of NPC patients. The selected cell lines showed a uniform severe block in intracellular sterol trafficking and were obtained from patients spanning the entire spectrum of clinical phenotypes (Figure 4). Sphingosine accumulation might be related to several perturbations of the sphingolipid metabolism (C. Rodriguez-Lafrasse, J. Ledrinova and M. T. Vanier, unpublished work). The pharmacological modulation of intracellular sphinganine content in control cells by fumonisin B1 was also associated with a 40 % decrease in [^{3}H]PDB binding. We have observed that fumonisin B1 strongly inhibits cholesterol esterification but cannot induce a lysosomal sequestration of LDL-cholesterol, at variance with the exogenous addition of 10 μM sphinganine [10], suggesting that the drug blocks LDL uptake or transport to the lysosome. Therefore the absence of K_d modification might be explained by differences in cholesterol homeostasis of the cells.

Our Scatchard analyses, combined with the previous interpretation of experiments *in vitro* [2], lead us to propose the following mechanism for [^{3}H]PDB-binding inhibition in NPC fibroblasts. At low concentration, considering the absence of K_d modification, endogenous sphingosine could act either by means of chemical interaction with lipidic cofactors, possibly with the negative charge of phosphatidylserine, or by inhibition of the release of PKC activators such as Ca^{2+}. At high concentrations of endogenous sphingosine (beyond a 5-fold increase), the significant K_d increase suggests a competitive inhibition. Both mechanisms would result in an increased threshold of PKC activation.

This study first documents the inhibition of [^{3}H]PDB binding by endogenous sphingosine and gives a potentially relevant insight into NPC, because PKC has been widely involved in the activation of cholesterol transport by sterol carrier protein 2 [10] and intermediate filaments [29] and of HDL-mediated cholesterol efflux [11].

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