Down-regulation of peroxisome proliferator-activated receptor-γ gene expression by sphingomyelins

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Received 26 February 2001; accepted 2 March 2001

First published online 13 March 2001

Edited by Shozo Yamamoto

Abstract We recently demonstrated that the sphingomyelin (SM) content of adipocyte membranes was negatively correlated with the expression of peroxisome proliferator-activated receptor-γ (PPARγ) in the subcutaneous adipose tissue of obese women with variable degrees of insulin resistance. We have now investigated whether SM really does have an impact on the expression of PPARγ in 3T3-F442A adipocytes. Adding SM to the culture medium for 24 h caused a significant increase in SM content of adipocyte membranes and an acyl chain length-dependent decrease in the levels of PPARγ mRNA and protein. The longer the acyl chain of the fatty acid of SM, the greater was the decrease in PPARγ. These data suggest that the nature of the fatty acid is important in the regulation of PPARγ by the SM pathway.

Key words: Sphingomyelin; Hydrocarbon chain; Cholesterol; PPARγ; Gene expression

1. Introduction

Sphingomyelin (SM) is a phosphosphingolipid that has long been known to play a part in the structure and dynamic properties of cell membranes [1]. Recent studies have revealed the critical role of SM in signal transduction [2,3]. SM and its metabolites, ceramide and sphingosine, are important intracellular messengers and cell regulatory molecules that mediate at least part of the action of extracellular agents such as tumor necrosis factor-α (TNF-α), γ-interferon, interleukin-1β and 1α,25-dihydroxyvitamin D_3 [4,5].

There is also evidence that the SM pathway plays a role in insulin signal transduction by modulating gene expression in insulin-sensitive cells. TNF-α inhibits insulin signaling by stimulating sphingomyelinase activity and generating ceramide [6,7]. Ceramide mediates the down-regulation of glucose transporter (Glut4) mRNA [8]. TNF-α also down-regulates the production of the peroxisome proliferator-activated receptor-γ (PPARγ) [9], a transcription factor that is abundant in adipose tissue and may also play a role in the sensitivity of cells to insulin [10]. Our study on obese women with varying degrees of insulin resistance showed that the concentrations of PPARγ mRNA are negatively correlated with fasting plasma insulin, a marker of insulin resistance, and with the membrane SM contents of the subcutaneous adipose tissue [11]. The most insulin-resistant women had higher SM contents in their adipocyte plasma membranes, and also lower concentrations of PPARγ mRNA.

This link between PPARγ expression and SM led us to investigate the regulation of PPARγ by SM. This study was done to determine whether the in vitro uptake of exogenous SMs into cultured adipocytes could regulate PPARγ expression. We show that PPARγ gene expression and protein concentration undergo SM-dependent down-regulation in 3T3-F442A adipocytes.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), SMs (palmitic, stearic and nervonic, and lignoceric) and insulin were purchased from Sigma (Saint Quentin Fallavier, France). Fetal bovine serum was obtained from Eurobio (Les Ulis, France) and donor bovine serum from Life Technologies (Cergy Pontoise, France). The primers for PPARγ and β-actin were obtained from Pharmacia Biotech (Orsay, France). The anti-PPARγ$_1$/γ$_2$ polyclonal antibody raised against both PPARγ$_1$ and PPARγ$_2$ was from Interchim (Montluçon, France). The specific polyclonal antibody directed against PPARγ$_2$ has been previously described [12]. The sheep anti-rabbit immunoglobulin G polyclonal antibody conjugated with peroxidase and the chemiluminescent substrate used in ECL-Western analysis were provided by Boehringer Mannheim Biochemica (Mannheim, Germany). Molecular mass markers for proteins were obtained from Pharmacia (Orsay, France). Polyvinylidene difluoride membranes for Western blotting were purchased from NEN (Paris, France). The protein assay kit was from Bio-Rad (Ivry-sur-Seine, France).

2.2. Cell culture

3T3-F442A cells [13] were cultured in DMEM containing 25 mM glucose and gentamicin (0.1 g/l), supplemented with 10% (v/v) donor bovine serum, and were grown to confluence at 37°C in an atmosphere of air/CO_2 (93:7, v/v) in 75 cm^2 cell culture flasks. The medium was changed every 2 days. Two days after confluence, cells were fed with differentiation medium (DMEM containing 50 nM insulin and supplemented with 10% fetal bovine serum). The medium was changed every 2 days. Before experiments, culture medium was removed and replaced by DMEM without insulin for 2 days. Cells were used 9–10 days after the induction of differentiation when more than 90% of the cells had an adipocyte-like phenotype.

2.3. Addition of SMs

SM-PA (from chicken eggs) with mainly palmitic acid, SM-SNA (from bovine brain) with mainly stearic and nervonic acids and SM-

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0014-5793/01/$20.00
PII: S0014-5793(01)02278-5

LA (from bovine erythrocytes) containing mainly lignoceric acid were used. SMs in absolute ethanol at 37°C were added to DMEM (SM final concentration: 15 μM; ethanol to medium ratio: 1/200 (v/v)) and kept at 37°C for 10 min. Standard medium was then removed from the culture flasks and replaced by medium with SM. Control cells received equivalent amounts of ethanol.

2.4. Semi-quantitative RT-PCR

The culture medium was removed 2 or 24 h after SM or ethanol treatment, and the cells were washed with cold phosphate-buffered saline (PBS) (136 mM NaCl, 10 mM Na_2HPO_4, 2 mM KCl, 1.46 mM KH_2PO_4 and 5.5 mM D-glucose, pH 7.4).

Total cellular RNA was prepared with the Trizol reagent (Gibco, Cergy Pontoise, France) following the manufacturer's instructions. After electrophoretic verification of their integrity, mRNAs were reverse-transcribed (0.5 μg) with the First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Orsay, France) in 15 μl final volume. 2 μl of the amplicons so obtained was used for duplex PCR coamplifying β-actin and PPARγ. Compatible primer sequences were chosen from the GenBank Database Document Reader. The sense and antisense primers were respectively: 5′-AAA CAT ATC ACC CCC CTG CA-3′ and 5′-GCA GCA GGT TGT CTT GGA TG-3′ for PPARγ; 5′-CCG TGA AAA GAT GAC CCA GA-3′ and 5′-GCA CGA TTT CCC TCT CAG CT-3′ for β-actin to amplify the following genomic region from bp 267 to bp 550 (β-actin) and from bp 1277 to bp 1727 (PPARγ). The sense primers were 5′ end-labeled with Cy-5. Duplex PCRs were carried out in 20 μl final volume including 0.25 μl of Taq polymerase (1.25 U), 2 μl of PCR mix, 0.5 μl of a mixture of the four deoxynucleotide triphosphates (200 μM each), 2 μl of each primer pair (20 pM each) and 2 μl of the RT reaction (50–70 ng). The cDNA amplification program included 28 cycles of denaturation at 92°C for 1 min, primer annealing at 70°C for 1 min and extension at 72°C for 1 min, followed by a final 10 min extension step at 72°C. 10 μl of each RT-PCR reaction was analyzed by non-denaturing gel electrophoresis on an ALFexpress sequencing machine (Pharmacia Biotech, Orsay, France) fitted with short plates. The results were analyzed on-line with the Allele Links software, which allows quantification of amplicons.

2.5. Western blot analysis

The treated and control cells were harvested and disrupted in cold 10 mM Tris–HCl, pH 7.4, containing 1% (w/v) Triton X-100 at 4°C and centrifuged ($17\,000\times g$) for 20 min. The resulting supernatants were used for protein determination and Western blot analysis. Protein content was measured by the method of Lowry et al. [14], using bovine serum albumin as a standard. The total PPARγ (PPARγ$_1$ plus PPARγ$_2$) and PPARγ$_2$ proteins were quantified using the ECL-Western blot procedure. Proteins (25 μg) were resolved by SDS–PAGE, then transferred to a polyvinylidene difluoride membrane [15]. PPARγ$_1$/γ$_2$ and PPARγ$_2$ were detected with polyclonal antibodies diluted 1/1000 and visualized using an anti-immunoglobulin G polyclonal antibody conjugated to peroxidase exposed to a chemiluminescent substrate. The commercial anti-PPARγ$_1$/γ$_2$ was directed against the amino acid sequence MMGEDKIKFKHITPL common to both PPARγ$_1$ and PPARγ$_2$ (amino acids 256–270 of human PPARγ$_1$, 284–298 of human PPARγ$_2$). We used the fact that PPARγ$_2$ differs from PPARγ$_1$ in having an additional specific N-terminal amino acid region, to map the sequence of the hapten used to produce the anti-human PPARγ$_2$ described in [11] in that region. It was $_2$GETLGDSPIDPESDS$_{16}$ of human PPARγ$_2$ [16,17]. The polyclonal antibodies cross-reacted with the corresponding rat PPARγ proteins. The intensities of the bands were quantified by densitometry with a computerized image processing system (Gel Doc 2000, Bio-Rad).

2.6. Membrane cholesterol and phospholipid composition

The culture medium of 3T3-F442A adipocytes in 100 mm dishes was supplemented or not with SMs (15 μM). After 24 h, the medium was removed and cells washed twice with cold PBS. Cells were then processed for the isolation of total membranes. The cells were pipetted off the dish 5 min after incubation with 3 ml of homogenization buffer (250 mM sucrose, 1 mM EDTA, 10 mM Tris–HCl, pH 7.4), centrifuged at $200\times g$ for 10 min and resuspended in 6 ml of ice-cold homogenization buffer. The cell suspension was homogenized with 15 strokes in a Potter–Kontes homogenizer with a Teflon pestle. Cell disruption was monitored under the light microscope. Total membranes were pelleted at $250\,000\times g$ for 90 min [18]. The pellet was washed twice with 1 mM EDTA, 10 mM Tris–HCl, pH 7.4, and finally resuspended in 1 ml of 1 mM EDTA, 1 mM Tris–HCl, 10 mM NaCl, pH 7.4. A 50 μl aliquot was used for the determination of protein content according to the method of Lowry et al. [14]. Total membranes were kept at −80°C.

2.7. Membrane lipid determination

Lipids were extracted from cell total membranes with methanol and chloroform (11:7, v/v) according to the method of Rose and Oklander [19]. The amount of membranes used for optimal extraction was 500 μg protein. The organic phase was evaporated to dryness under a stream of nitrogen at room temperature. The lipid residue was redissolved in chloroform, and its components were separated by HPLC (Gold HPLC System, Beckman, Palo Alto, CA, USA) [20]. 20 μg of lysophosphatidylcholine (lyso-PC) per 100 μg proteins was added as internal standard before the extraction. Phospholipids were detected with an evaporative light scattering detector (SEDERE, Orléans, France). Quantification was based on the comparison of integrated peak area with curves prepared from standard phospholipid solutions. Membrane cholesterol was quantified by the method of Zlatkis and Zak [21].

2.8. Statistical analysis

The results are presented as means ± S.E.M. and have been analyzed for statistical significance using Student's *t*-test (Statgraphics software, Manugistics, Rockville, MD, USA). The level of significance was set at $P < 0.05$.

3. Results

3.1. Effect of SMs on PPARγ mRNA levels

We determined whether SMs had an effect on PPARγ production in the adipocytes by supplementing the culture medium of 3T3-F442A with SM for 2 h or 24 h. Fig. 1A shows the results of three experiments in which SM was added for 2 h. The ratio of relative fluorescence units of PPARγ to β-actin expressed as a percentage of control values showed no difference between cells treated or untreated with SM-PA. Treatment with SM-SNA or SM-LA caused a 6–8% reduction of the ratio PPARγ/β-actin mRNA. However, these decreases were not significant.

Table 1
Changes in membrane phospholipid contents of adipocytes following 24 h incubation with SMs

Phospholipid	Untreated cells	SM		
		SM-PA	SM-SNA	SM-LA
SM	4.7 ± 0.2[a]	5.6 ± 0.2*	5.4 ± 0.3*	6.6 ± 0.5*
PS	6.6 ± 0.2	6.4 ± 0.4	7.5 ± 0.1	5.8 ± 0.3*
PI	12.9 ± 0.3	13.0 ± 0.4	15.1 ± 1.6	11.0 ± 0.5*
PE	32.7 ± 1.9	31.9 ± 1.4	31.0 ± 1.3	30.5 ± 1.6
PC	43.1 ± 1.2	43.2 ± 2.6	41.1 ± 0.9	46.1 ± 1.3

*$P < 0.05$: SM-treated cells versus untreated cells.
[a]Data are reported as relative percentage of total phospholipids. Values are the mean ± S.E.M. ($n = 3$). PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

By contrast, adding SM to the culture medium for 24 h significantly decreased the PPARγ/β-actin mRNA levels (Fig. 1B). The reductions were 8% ($P<0.05$), 13% ($P<0.01$) and 33% ($P<0.001$) for cells treated with SM-PA, SM-SNA and SM-LA respectively. The decreases in PPARγ mRNA were not due to some toxic effect of SM. There was no loss of adhesion of 3T3-F442A adipocytes under these conditions. Cell viability determined by trypan blue was greater than 98% in all cases.

3.2. Effect of SMs on PPARγ protein synthesis

We used Western blotting to determine whether the fall in PPARγ mRNA after incubation of adipocytes with SM for 24 h resulted in a fall in PPARγ protein. There was a significant decrease in total PPARγ (PPARγ$_1$ plus PPARγ$_2$) protein in 3T3-F442A adipocytes incubated for 24 h with SM (Fig. 2A). In rank order the decreases were SP-PA < SM-SNA < SM-LA. As percentages they were −15% ($P<0.05$), −18% ($P<0.05$) and −22% ($P<0.05$), respectively.

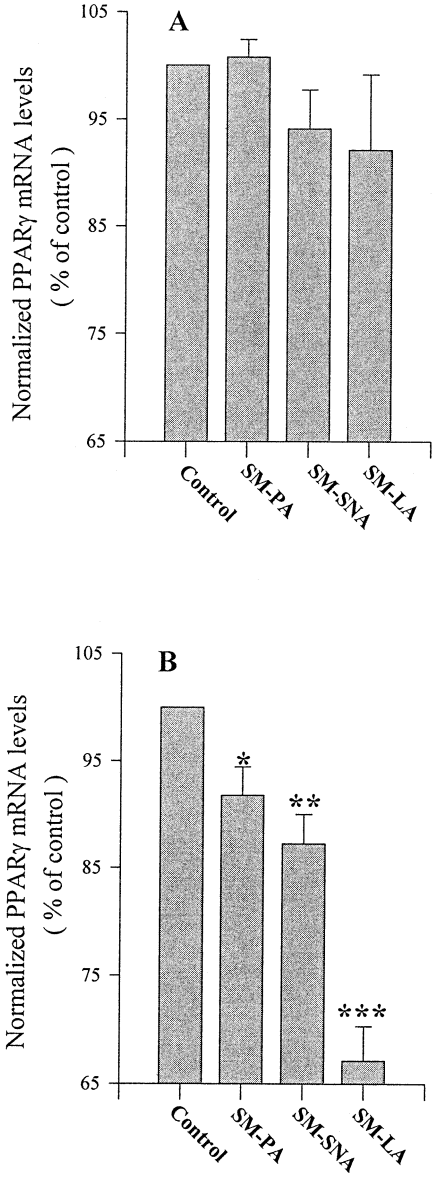

Fig. 1. The effect of SMs on PPARγ mRNA levels in 3T3-F442A adipocytes. Cells were incubated for 2 h (A) or 24 h (B) with or without 15 μM SM. SMs were dissolved in ethanol. Control cells were incubated with the same volume of ethanol. mRNA PPARγ was measured by fluorescent RT-PCR. The effects of three SMs with different acyl chains were determined. Each histogram represents the average ± S.E.M. of three experiments. $*P<0.05$, $**P<0.01$, $***P<0.001$: SM-treated cells versus cells without SM.

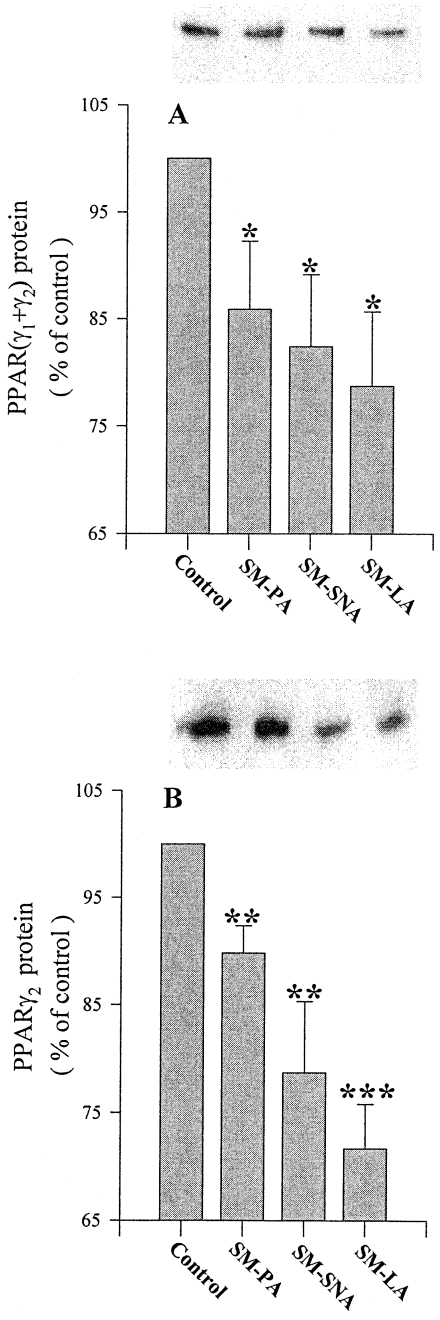

Fig. 2. The effect of SMs on PPARγ protein in 3T3-F442A adipocytes. Cells were incubated for 24 h with or without 15 μM SM. SMs were dissolved in ethanol. Control cells were incubated with the same ratio of ethanol. PPARγ protein was determined using an antibody that recognizes the two isoforms of PPARγ (A) or an antibody that recognizes the γ$_2$ isoform of PPARγ (B). The effects of three SMs having different acyl chains were determined. Each histogram represents the average ± S.E.M. of three experiments. $*P<0.05$, $**P<0.01$, $***P<0.001$: SM-treated cells versus cells without SM.

A similar result was obtained for the PPARγ$_2$ protein (Fig. 2B). SM-PA, SM-SNA and SM-LA significantly decreased the concentration of this PPAR subtype compared to controls. The effect of SM-LA was much more pronounced than that of SM-PA or of SM-SNA: −29% ($P < 0.001$), as against −11% ($P < 0.01$) and −22% ($P < 0.01$), respectively.

3.3. Effect of SMs on phospholipid profile and cholesterol content of adipocyte membranes

To verify whether the changes in PPARγ mRNA and protein levels were related to a significant uptake of SM in cell membranes, we determined the phospholipid composition and cholesterol of the total membranes of adipocytes treated with SM for 24 h. The major phospholipids were 629.5 ± 20.1 μg/mg protein in SM-untreated cells. The SM content was 29.4 ± 1.5 μg/mg protein and represented 4.7 ± 0.2% of the phospholipids. Cholesterol was 68.4 ± 4.4 μg/mg protein.

There was a significant ($P < 0.05$) increase in SM levels: 19%, 15% and 40% for cells treated with SM-PA, SM-SNA and SM-LA, respectively (Table 1). No significant change occurred in the other phospholipid classes, except for PS and PI in SM-LA-treated cells (Table 1). Membrane ceramide and sphingosine contents were very weak and there was no significant change in SM-treated cells (data not shown). The increase in SM content of total membranes was associated with an increase in cholesterol levels: 19% (81.7 ± 7.0 μg/mg protein, not significant), 21% (83.0 ± 4.4 μg/mg protein, $P < 0.05$) and 20% (82.7 ± 3.8 μg/mg protein, $P < 0.01$) for cells treated with SM-PA, SM-SNA and SM-LA, respectively.

4. Discussion

We have shown that exogenous SMs in the culture medium of 3T3-F442A adipocytes for 24 h significantly reduce the concentrations of PPARγ mRNA and protein. TNF-α has also been found to reduce the levels of PPARγ mRNA in 3T3-L1 adipocytes [9,22]. Intracellular signaling molecules such as H_2O_2, lyso-PC, and phorbol 12-myristate 13-acetate also decrease the expression of PPARγ in rat mature adipocytes [23]. Phorbol ester stimulates SM synthesis in HL-60 and NIH 3T3 cells [24,25]. TNF-α, H_2O_2 and lyso-PC directly or indirectly affect the SM pathway in a variety of cells by activating sphingomyelinase [26–30]. The major products of SM hydrolysis are phosphocholine and ceramide, with the latter being further converted to sphingosine and fatty acids by ceramidase. However, no direct link between the SM metabolites and PPARγ has been reported. Previous studies carried out in the absence of an agent activating sphingomyelinase have shown that loading SM into human fibroblasts and lymphoid cell lines is followed by an intracellular catabolism of exogenous SM with the formation of ceramide [31]. In our hands, only very small amounts of ceramide and sphingosine were detected in 3T3-F442A adipocytes and the uptake of SM into cells caused no significant increments in SM metabolites.

Our data indicate that the regulation of PPARγ expression depends on the length of the acyl chain of SM. The longer the acyl chain, the greater the fall in PPARγ gene expression and protein levels. Our results are in agreement with published data suggesting that fatty acids with 24 carbons inhibit expression of such PPARs. In fact the activation of PPARα, determined by the chloramphenicol acetyltransferase activity in HeLa cells, was below the basal levels found with the very long chain (24:1) nervonic acid [32]. In the present study, the membrane SM contents of adipocytes treated with the three SMs are different. Although the differences are not significant, the higher membrane SM content in SM-LA-treated adipocytes could explain the higher inhibition of PPARγ gene expression and protein levels. It also seems possible that the large chain asymmetry of SM-LA could play a role in the mechanisms whereby SM blocks the synthesis of PPARγ.

It is noteworthy that the increased membrane SM contents were related with increased cholesterol contents. These data are in agreement with previous reports showing that loading cells with SM causes an increased cholesterol synthesis [33,34] and positive correlations between SM and cholesterol levels in biological membranes [35]. The increased cholesterol content could also have an indirect role in PPARγ expression. SREBP-1 is a member of the family of transcription factors designated ADD1/SREBP (adipocyte determination differentiation-dependent factors/sterol regulatory element binding protein), which are proteins bound to the membranes of the endoplasmic reticulum. Proteolytic cleavage releases active NH_2-terminal fragments from cell membranes [36]. Upon entering the nucleus, these active domains activate the transcription of several genes involved in lipid metabolism and PPARγ [37,38]. Now, cholesterol inhibits the proteolytic cleavage of ADD1/SREBP1 [36] and an increased cholesterol synthesis in 3T3-F442A could lead to an inhibition of PPARγ expression and protein levels.

Other mechanisms may be involved. They may include a reduced transcription and/or an increased turnover of the receptor mRNA levels. A mechanism involving auto-regulation of the receptor may also play a part, as suggested in a recent report on the interaction of troglitazone with 3T3-L1 adipocytes [39]. A regulation pathway involving p38 mitogen-activated protein kinase is also possible [40]. The transcription of the PPARγ gene is impaired by p38 inhibitors [41].

In conclusion, these in vitro findings support our previous in vivo report that the membrane SM content is inversely correlated with the levels of PPARγ mRNA in the adipose tissue of obese women [11]. The fatty acid content of SM, particularly the very long chain fatty acids, could be important. The biosynthesis of these fatty acids can be modified in response to a diet [42]. They are also increased in pathological states. These very long chain fatty acids accumulate in the cell membranes of alcoholic patients [43]. Lastly, the levels of the PPAR mRNA are decreased in the livers of ethanol-fed rats [44]. The role of these very long chain fatty acids in the regulation of nuclear receptors therefore deserves further investigation.

Acknowledgements: This work was partly supported by the Fondation pour la Recherche Médicale. N.Al-M. was the recipient of a predoctoral fellowship from the Région Lorraine. We thank Dr. Owen Parkes for help in correcting the English text.

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