PERGAMON

Journal of Steroid Biochemistry and Molecular Biology 70 (1999) 123–131

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Progesterone metabolism in human fibroblasts is independent of P-glycoprotein levels and Niemann–Pick type C disease

Jie Zhang[a], Ling-Jie Ming[b], Jan Sjövall[b], Harold W. Cook[a], Neale D. Ridgway[a], David M. Byers[a,*]

[a] *Atlantic Research Centre, Departments of Pediatrics and Biochemistry, Dalhousie University, Halifax, NS, Canada*
[b] *Department of Medical Biochemistry and Biophysics, Karolinska Institute, Stockholm, Sweden*

Received 27 November 1998; accepted 10 March 1999

Abstract

Progesterone inhibits intracellular transport of lysosomal cholesterol in cultured cells, and thus at least in part mimics the biochemical phenotype of Niemann–Pick type C disease (NPC) in human fibroblasts. The goal of this study was to determine whether metabolism of progesterone to other steroids is affected by the NPC mutation or by P-glycoprotein (a known progesterone target). We found that human fibroblasts metabolize progesterone in three steps: rapid conversion to 5α-pregnane-3,20-dione, which is then reduced to 5α-pregnane-3β(α)-ol-20-one with subsequent 6α-hydroxylation. The pattern and rates of progesterone metabolism were not significantly different in a variety of fibroblasts from normal individuals, NPC patients, and obligate heterozygotes. Inhibition of steroid 5α-reductase with finasteride completely blocked metabolism of progesterone but had no effect on inhibition of LDL-stimulated cholesterol esterification ($IC_{50} = 10$ μM). Progesterone also partially inhibited 25-hydroxycholesterol-induced cholesterol esterification, with similar dose-dependence in normal and NPC fibroblasts. P-glycoprotein levels varied significantly among the various fibroblasts tested, but no correlation with NPC phenotype or rate of progesterone metabolism was noted, and P-glycoprotein inhibitors did not affect conversion of progesterone to products. These results indicate that metabolism of progesterone in human fibroblasts is largely independent of its ability to interfere with cholesterol traffic and P-glycoprotein function. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Niemann–Pick type C disease; Progesterone; Cholesterol esterification; 25-Hydroxycholesterol; P-glycoprotein; Human fibroblasts

1. Introduction

It is becoming clear that the functions of progesterone go beyond reproduction. Progesterone is produced not only in the ovaries, but also in significant amounts by the adrenal glands and in the brain [1]. Progesterone interacts with intracellular receptors to directly regulate gene expression. However, some of its effects are extragenomic and involve interactions with cellular membranes and membrane proteins. It has been demonstrated that some progesterone metabolites are potent regulators in the central nervous system by rapidly increasing the affinity of the γ-aminobutyric acid receptors for this inhibitory neurotransmitter [2,3]. The enzymes required for formation of these biologically active metabolites, such as steroid 5α-reductase and 3α/β-hydroxysteroid dehydrogenase, have a wide tissue and organ distribution including the brain [1,4], suggesting that progesterone metabolites may have functions in many cell types.

Progesterone is also known to influence intracellular cholesterol homeostasis [5,6]. First described as an inhibitor of ACAT in cell-free extracts [7,8], progester-

Abbreviations: ACAT: acyl–CoA:cholesterol acyltransferase; FBS: fetal bovine serum; Finasteride: *N*-(1,1-dimethylethyl)-3-oxo-(5α17β)-4-azaadrost-1-ene-17-carboxamide; LDL: low density lipoprotein; LPD: lipoprotein-deficient; MEM: minimal essential medium; NPC: Niemann–Pick disease type C; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; U18666A: 3-β-[(2–diethyl–amino)ethoxy] androst-5-en-17-one.

* Corresponding author. Tel.: +1-902-494-7084; fax: +1-902-494-1394.

E-mail address: david.byers@dal.ca (D.M. Byers)

0960-0760/99/$ - see front matter © 1999 Elsevier Science Ltd. All rights reserved.
PII: S0960-0760(99)00107-7

one has more recently been shown to impair transport of cholesterol from lysosomes [9,10] and from the plasma membrane to the endoplasmic reticulum [11]. Progesterone inhibits cholesterol biosynthesis resulting in accumulation of various precursors at the plasma membrane [12] and inhibition of cholesterol esterification [9,13,14]. The effects of progesterone and other steroids appear to correlate with their ability to inhibit the activity of P-glycoprotein, the multidrug transporter which also plays a role in traffic and disposition of endogenous lipids [13–15].

The ability of progesterone to block egress of lysosomal cholesterol and to impair regulatory responses to LDL-derived cholesterol is reminiscent of the biochemical phenotype of Niemann–Pick type C (NPC) disease [9], a fatal neurodegenerative disorder [16]. Other steroids and hydrophobic amines such as U18666A (3-β-[(2–diethyl–amino)ethoxy] androst-5-en-17-one) are also capable of causing similar cholesterol regulatory defects, but the mechanisms have not been elucidated and indeed may be distinct for different compounds [17]. One possibility is that agents such as progesterone and U18666A may interact directly with and inhibit the NPC gene product, instead of or in addition to any effects on other cellular targets such as P-glycoprotein. The NPC gene has recently been identified and is predicted to encode a 142-kDa membrane protein (NPC1) with a putative sterol sensing domain that is likely involved in directing cholesterol traffic through intracellular membranes [18].

The present study was undertaken to clarify the relationship between metabolism of progesterone and cholesterol regulation in cultured human fibroblasts. The objective was to determine whether progesterone is converted to other products in these cells, and to examine whether proteins (P-glycoprotein, NPC1) that might interact with progesterone can affect this metabolism. Conversely, rapid metabolism of progesterone to products of greater or lesser potency might influence its ability to interfere with cholesterol trafficking and regulation. Our results indicate that progesterone is metabolized in three steps initiated by 5α-reduction, but that neither P-glycoprotein nor a functional NPC gene product are required for this process.

2. Materials and methods

2.1. Materials

[1,2–(N)^{3}H]Progesterone (47.5 Ci/mmol) and [9,10(N)–^{3}H]oleic acid (10 Ci/mmol) were obtained from NEN Products (Canada). Progesterone, 5α-pregnane-3,20-dione and 25-hydroxycholesterol were purchased from Sigma (USA). Oleic acid was from Serdary Research Laboratories (London, Ont., Canada). Finasteride was donated by Merck Laboratories (Rahway, NJ, USA). Octadecylsilane (ODS)-bonded silica (Chromosep C_{18} Sep) was from Chromatographic Specialities (Brockville, Ont., Canada). Triethylaminohydroxypropyl Sephadex LH-20 (TEAP-LH-20) was synthesized as described previously [19]. All other reagents and solvents were of the highest quality available from commercial suppliers.

2.2. Cell culture

Fibroblasts were obtained from skin biopsies of unaffected individuals (abbreviated as NN), local patients diagnosed with Niemann–Pick disease type C (CC) or obligate heterozygotes (NC). The established NPC cell line GM3123 (CC-1) was obtained from the Human Genetic Mutant Cell Repository (Camden, NJ, USA). Cell lines CC-3, CC-4 and NC-3 were from the Nova Scotia Acadian Niemann–Pick type D kindred, which has recently been shown to contain a point mutation in the NPC1 gene [20]. Cells were maintained at 37°C in an atmosphere of 95% air/5% CO_2 and were used between the 7th and 13th passage. In most experiments, cells were seeded in minimal essential medium (MEM) from Gibco (USA) containing 10 vol% FBS or 5 vol% LPD-FBS at densities of 150,000 per 35 mm dish or 500,000 cells per 60 mm dish. Following a culture period of 3 days, this medium was replaced with 2 ml of fresh medium for varying periods of time (0–48 h).

2.3. Analysis of steroid metabolites

Cells were incubated with radiolabeled progesterone dissolved in ethanol or ethanol alone as indicated in specific experiments (final ethanol concentration was $<0.5\%$). Labeled medium was removed and a portion was directly applied to Fisher Redi-Plate G TLC plates and developed for 30 min in methylene chloride/acetone (80:20, v/v). Labeled products were quantified using a Bioscan System 200 imaging scanner, based on the percentage of total radioactivity (determined by scintillation counting) associated with each peak. Some radioactive material at the origin was routinely observed when labeled medium was spotted directly onto TLC plates. However, this unidentified material did not appear to be a metabolic product as it was also seen with [^{3}H]progesterone mixed with fresh medium, but not in lipid extracts of labeled medium. Cellular steroid metabolites were extracted with 2 ml of hexane/isopropanol (3:2, v/v) [21] and analyzed by TLC as described above.

Metabolism of unlabeled progesterone was assessed by GLC and GLC–mass spectrometry. The incubation medium was applied to an ODS-bonded silica car-

tridge followed by a wash with 3 ml of water. Cells were extracted with 5 ml of ethanol and this was heated for 5 min at 60°C. After centrifugation, the supernatant was diluted to 30% ethanol with water and applied to ODS-bonded silica as above. Steroids were eluted from the cartridge with 5 ml methanol and one half of the sample was applied to a column (6×0.4 cm) of TEAP-LH-20 in the HCO_3^- form packed in 95% aqueous methanol, followed by elution with 5 ml of methanol. The eluate was dried under nitrogen and converted into trimethylsilyl ethers prior to analysis. The other half of the sample was resolved by TLC as described above. Selected regions of the plate (indicated by co-migration with radioactive metabolites) were scraped, extracted with ethyl acetate and analyzed by GLC and GLC–mass spectrometry.

GLC was performed using a Hewlett Packard 5890 Series II gas chromatograph equipped with a fused silica capillary column (30 m × 0.32 mm) coated with a 0.25 μM layer of cross-linked methyl silicone (Supelco, Bellefonte, PA). Samples were injected in the split mode; oven temperature at injection was 60°C and was increased to 290°C at 10°C/min. Retention indices were calculated by comparison of retention times with those of the C-26 to C-30 *n*-hydrocarbons analyzed under the same conditions. GLC-mass spectrometry was performed as described previously [22].

2.4. Cholesterol esterification assay

Cholesterol esterification was measured as incorporation of [^{3}H]oleic acid into cholesteryl-[^{3}H]oleate as described by Ref. [23] with slight modification. After incubation as described for specific experiments, fibroblasts were pulse-labeled 1 h with 2 ml of MEM containing [^{3}H]oleic acid (final concentration 0.2 mM, 3.6 mCi per dish), prepared by suspension in bovine serum albumin at an oleate/albumin molar ratio of 5:1 [24]. Cells were harvested by washing twice with 2 ml of phosphate-buffered saline and lipids were extracted directly from culture dishes with hexane/isopropanol (3:2, v/v). Lipids were separated by TLC in petroleum ether/diethyl ether/acetic acid (85:15:1, v/v) and identified by comparison to known standards. Incorporation of [^{3}H]oleic acid into cholesterol ester and other neutral lipids was determined from the percentage of label associated with the corresponding peak (measured using a Bioscan System 200 imaging scanner) and the amount of radioactivity in the lipid extract.

2.5. Western blot analysis of P-glycoprotein

Cells were washed twice with ice-cold phosphate-buffered saline, scraped and harvested by centrifugation (5 min) in an Eppendorf tube. One hundred μl of SDS sample buffer (1.25% SDS, 60 mM Tris–HCl, pH 6.8, 12% glycerol, Bromphenol Blue) was added directly to the cell pellet and incubated 10 min at 4°C. Total protein was measured using the micro-bicinchoninic acid assay (Pierce Assay kit). β-Mercaptoethanol (2 vol% final) was added and samples containing equivalent protein were separated by SDS-PAGE (8% running gel). Proteins were transferred to 0.45 μ nitrocellulose membranes using a BioRad Mini-Protean II apparatus and membranes were blocked overnight at 4°C with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20. P-glycoprotein was detected by incubation 4 h at room temperature with monoclonal antibody C219 (Centocor, PA) at 3 μg/ml, followed by goat anti-mouse IgG conjugated to horseradish peroxidase (BioRad) at 1:20,000 dilution (1 h). Western blots were developed using the ECL kit (Amersham) and immunoreactive bands were quantified by densitometric scanning using an Apple OneScanner with NIH Image v. 1.49 software.

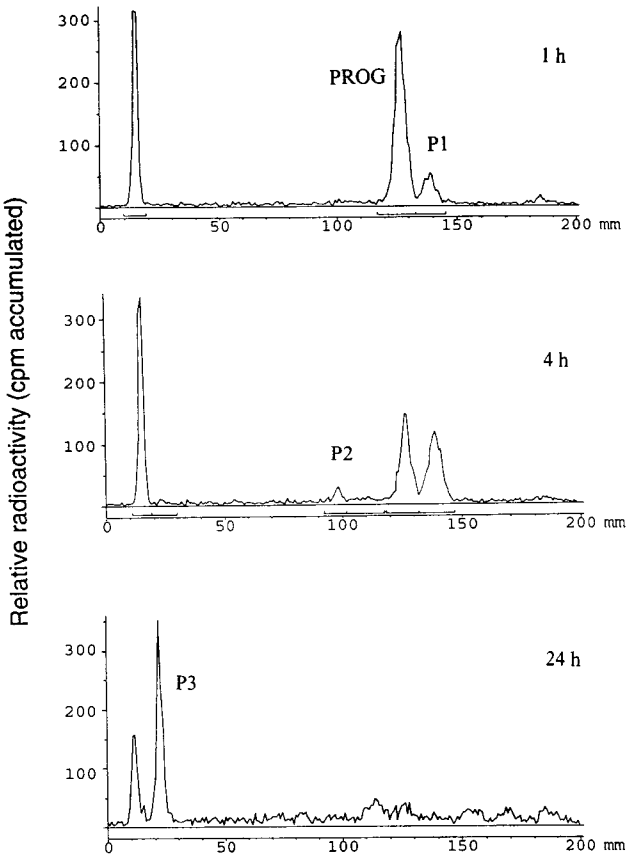

Fig. 1. TLC analysis of [^{3}H]progesterone metabolism in human fibroblasts. Normal fibroblasts (cell line NN-1, ~150 μg of protein) were incubated with 40 nM [^{3}H]progesterone (2 μCi) in 1 ml of MEM plus 10% FBS. Ten μl of medium was removed at 1, 4 and 24 h, separated by TLC, and radioactivity as a function of migration distance (in mm) was imaged as described in the text.

Table 1
Identification of progesterone metabolites[a]

TLC peak	Retention index	Major ions	Compound
P1	2652	316, 198, 231	5α-pregnane-3,20-dione
P2	2622	390, 375, 300, 215	5α-pregnane-3α-ol-20-one
P2	2705	390, 375, 300, 215	5α-pregnane-3β-ol-20-one
P3	2778	478, 463, 388, 298	5α-pregnane-3α,6α-diol-20-one
P3	2841	478, 463, 388, 298	5α-pregnane-3β,6α-diol-20-one

[a] Fibroblasts (NN-1) were incubated with 7.5 μM progesterone for 12 or 38 h (five 60 mm dishes for each time point, ~2.4 mg protein). Lipids were extracted, separated by TLC, and processed for GLC (retention index) and GLC-mass spectrometry (major ions) as described in the text. Samples from the earlier time point were used to identify P1 and P2, while the 38 h sample was used to identify P3.

3. Results

3.1. Metabolism of progesterone in human fibroblasts

To determine whether progesterone is stable or is converted to other products in human fibroblasts, cells (NN-1) were incubated with [^{3}H]progesterone (40 nM) for varying periods of time between 1 and 48 h. Analysis of medium radioactivity by TLC at three time points is shown in Fig. 1. TLC resolved at least three metabolites (P1-P3) which appeared to be formed in the sequential order P1 → P2 → P3. At all time points, approx. 5% of total radioactivity was associated with lipid extracts of [^{3}H]progesterone-labeled cells and a similar TLC profile to that of the medium was observed (not shown), indicating that progesterone and its metabolites rapidly equilibrate between media and cells. No conversion was noted when cells were killed by treatment with 95% ethanol for 2 h prior to incubation with [^{3}H]progesterone, confirming that progesterone metabolism in cultured human fibroblasts is enzyme catalyzed. The pattern and rate of progesterone metabolism was not significantly different when serum was omitted from the incubation medium or when cell density was varied from subconfluent to confluent.

Progesterone metabolites were scraped from TLC plates and identified by comparison of GLC retention indices and mass spectra with those of authentic compounds [25] (Table 1). Metabolite P1 was identified as 5α-pregnane-3,20-dione, while P2 consists of two isomers, 5α-pregnane-3β-ol-20-one and 5α-pregnane-3α-ol-20-one. The two compounds comprising P3 are 5α-pregnane-3β,6α-diol-20-one and 5α-pregnane-3α,6α-

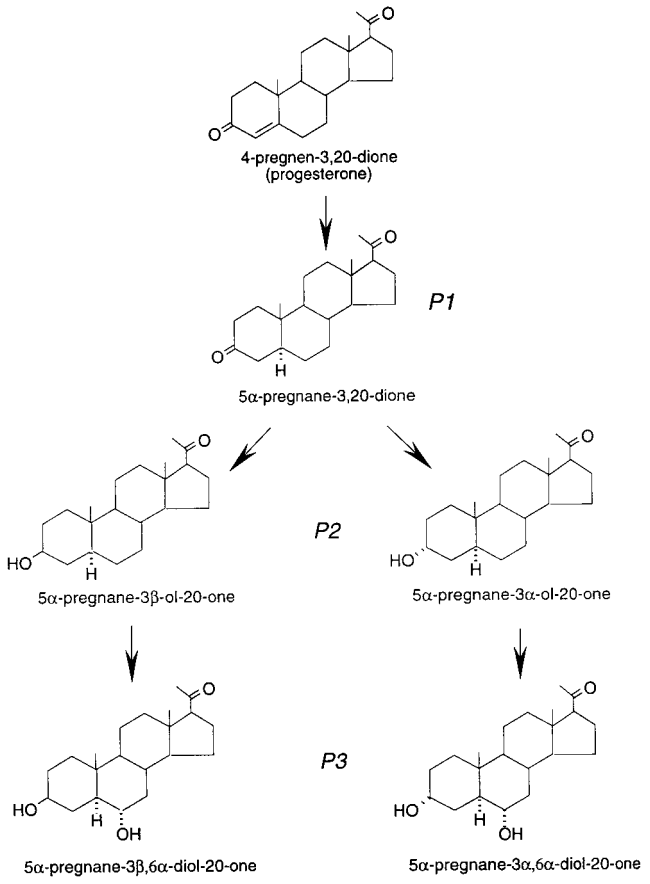

Fig. 2. Pathway of progesterone metabolism in human fibroblasts.

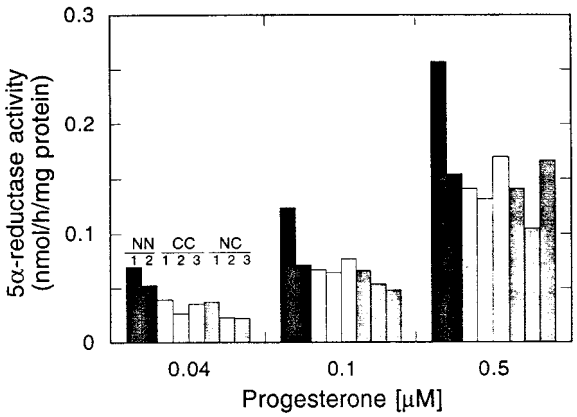

Fig. 3. 5α-Reduction of progesterone in several normal and NPC fibroblast cell lines. Cells were grown for 72 h in MEM + 10% FBS, then incubated with the indicated concentrations of [^{3}H]progesterone in fresh medium for 3 h. The formation of labeled 5α-pregnane-3,20-dione was determined from the amount of radioactivity associated with P1 following TLC separation as described in the text. The mean of duplicate dishes which differed by < 10% are shown.

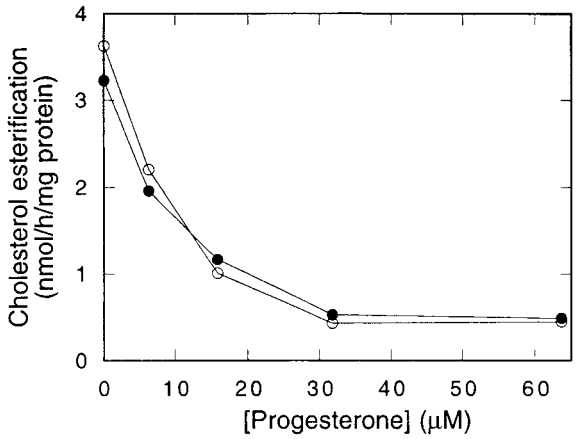

Fig. 4. Finasteride does not affect inhibition of LDL-stimulated cholesterol esterification by progesterone. Normal fibroblasts (NN-1) were cultured in MEM + 5% LPD-FBS for 72 h and then switched to MEM + 10% FBS with (●) or without (○) 1.0 μM finasteride and the indicated concentrations of progesterone for 18 h. Cholesterol esterification was determined by measuring the incorporation of [^{3}H]oleic acid into cholesteryl-[^{3}H]oleate during a 1 h pulse as described in the text. The average of duplicate values from one of three similar experiments are shown.

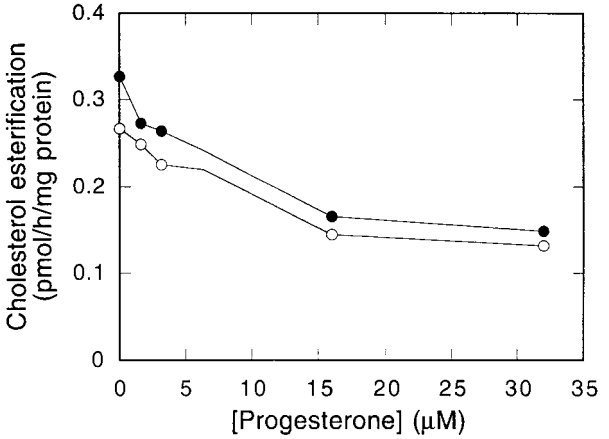

Fig. 5. Effect of progesterone on 25-hydroxycholesterol-stimulated cholesterol esterification in normal and NPC fibroblasts. Normal (NN-2, ●) and NPC (CC-1, ○) fibroblasts were cultured in MEM + 5% LPD-FBS for 72 h and then switched to the same medium containing 25-hydroxycholesterol (1.0 μg/ml) and progesterone at the indicated concentrations for 6 h. Cholesterol esterification was determined by measuring the incorporation of [^{3}H]oleic acid into cholesteryl [^{3}H]oleate during a 1 h pulse as described in the text. The mean of duplicate dishes which differed by < 10% are shown.

diol-20-one. The suggested pathway of progesterone metabolism in fibroblasts is shown in Fig. 2. That progesterone is exclusively metabolized by this pathway was confirmed using finasteride (1.0 μM), an inhibitor of steroid 5α-reductase [4], that completely blocked conversion of [^{3}H]progesterone to other labeled TLC products during a 24 h incubation (data not shown). Moreover, only P2 and P3 were detected by GLC when fibroblasts were incubated for 24 h with unlabeled 5α-pregnane-3,20-dione (P1); 75% of the products in each case consisted of the 3β isomer. Finally, incubation with 5α-pregnane-3β-ol-20-one (P2) under the same conditions produced only the product 5α-pregnane-3β,6α-ol-20-one (P3).

No difference in the pathway of progesterone metabolism between normal and NPC fibroblasts was observed when labeled or unlabeled products were analyzed by TLC or GLC, respectively. 5α-Pregnane-3,20-dione was the only metabolite detected following short incubation periods (< 4 h) with [^{3}H]progesterone. Figure 3 compares 5α-reductase activity among several normal, mutant and carrier cell lines under these conditions. 5α-Reductase activity increased with progesterone concentration between 0.04 and 0.5 μM. No correlation in activity with the NPC phenotype was observed, but some variation among individual cell lines was noted: the normal fibroblast NN-1 consistently exhibited higher 5α-reductase activity than other normal or mutant fibroblasts. Similar cell-specific differences were observed when total progesterone metabolites were measured over longer incubation time (up to 36 h; data not shown).

3.2. Relationship between progesterone metabolism and inhibition of cholesterol esterification in normal and NPC fibroblasts

The discovery that progesterone is rapidly metabolized in fibroblasts raises the question of whether this metabolism attenuates or enhances its ability to interfere with cholesterol trafficking, i.e. by depletion of progesterone or by its conversion to a more potent steroid derivative. Consistent with previous data [9], progesterone effectively inhibited LDL-stimulated cholesterol esterification in fibroblasts, with an IC_{50} of 10 μM during an 18 h incubation. Completely blocking progesterone metabolism with finasteride (1.0 μM) had no significant effect on the dose response of this inhibition (Fig. 4). Moreover, no difference was observed between the potency of progesterone and its product 5α-pregnane-3,20-dione (P1) in inhibition of LDL-stimulated cholesterol esterification (data not shown). These results indicate that progesterone and at least its first metabolic product are of similar potency in impairing intracellular transport and esterification of LDL-derived cholesterol.

Several studies have reported that 25-hydroxycholesterol-stimulated esterification of endogenous cholesterol is unaffected by the NPC mutation [21,24,26,27] and the dose response of cholesterol esterification to this agent is similar in normal and NPC fibroblasts [26]. It is not known whether 25-hydroxycholesterol-

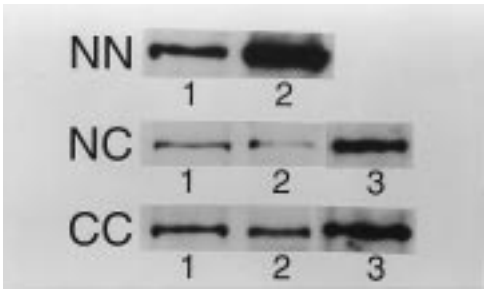

Fig. 6. Western blot analysis of P-glycoprotein levels in normal and NPC fibroblasts. Cells were grown 72 h in MEM + 10% FBS, harvested, and proteins (20 μg/lane) separated by SDS-PAGE as described in the text. The 170 kDa P-glycoprotein band (PGP) was detected by immunoblotting with monoclonal antibody C219 and visualized by ECL detection. The results are representative of two different experiments, with duplicate dishes of each cell line measured in each.

stimulated cholesterol esterification is similarly resistant to progesterone in human fibroblasts, although one study reported that progesterone (5 μM) significantly blocks this activity in mouse peritoneal macrophages [28]. As shown in Fig. 5, progesterone inhibited 25-hydroxycholesterol-induced cholesterol esterification to a similar extent in normal and NPC fibroblasts, although inhibition was incomplete (~50%) by comparison to that of LDL-stimulated esterification. This difference is likely not due to the shorter incubation time (6 h) for 25-hydroxycholesterol stimulation, as progesterone effectively blocks LDL-stimulated cholesterol esterification over similar periods [9]. It should be noted that a low sub-optimal [26] concentration of 25-hydroxycholesterol (1 μg/ml) was used in this experiment in order to reveal any cell-specific differences in the inhibitory effect of progesterone. At higher concentrations of 25-hydroxycholesterol (5–10 μg/ml), higher concentrations of progesterone were required for inhibition of 25-hydroxycholesterol-stimulated cholesterol esterification (not shown). These results suggest that a component of the stimulatory effect of 25-hydroxycholesterol on cholesterol esterification is resistant to inhibition by progesterone, and that the progesterone-sensitive component does not require a functional NPC1 gene product.

3.3. P-glycoprotein levels do not correlate with rate of progesterone metabolism in fibroblasts

Previous studies have indicated that progesterone inhibits cholesterol synthesis and esterification primarily through interaction with P-glycoprotein [13–15,29]. Indeed, progesterone can be photoaffinity cross-linked to this plasma membrane protein [30]. To determine whether the capacity of different normal and NPC fibroblast cell lines to metabolize progesterone might be correlated with P-glycoprotein levels, the latter was measured by Western blot analysis using a specific monoclonal antibody. As shown in Fig. 6, P-glycoprotein was present in all normal and NPC cells tested, although levels varied considerably among individual cell lines. This variability did not parallel differences in rates of progesterone metabolism: for example, P-glycoprotein levels were consistently higher in a different normal cell line (NN-2) than that showing the greatest rate of progesterone metabolism (NN-1, Fig. 3). In other experiments (not shown), progesterone metabolism was not affected by vinblastine (2 μM), a non-steroidal inhibitor of P-glycoprotein activity. Thus, there does not appear to be any relationship between rates of progesterone metabolism and the level of P-glycoprotein, and the latter does not appear to be required for progesterone metabolism in human fibroblasts.

4. Discussion

The present investigation has shown that progesterone is metabolized in human fibroblasts by three sequential steps to form 5a-pregnane-3,20-dione, 5α-pregnane-3β(α)-ol-20-one, and 5α-pregnane-3β(α),6α-diol-20-one. A similar pathway has also been reported in other cell types, including $T47D_{co}$ breast cancer cells and neurons [4,31,32]. The reason for the wide distribution of steroid 5α-reductase, the first enzyme in the pathway, is not clear. The main function of this enzyme is believed to be conversion of testosterone into dihydrotestosterone, and disturbances in this reaction have been associated with a number of human disorders [4]. However, 5α-reductase has a wide substrate specificity and distribution including human fibroblasts [33], and progesterone seems to be a more efficient substrate than testosterone [34]. The biosynthesis of progesterone and 5α-reduced progesterone metabolites have also been demonstrated in both the central and peripheral nervous systems, where they are collectively termed neurosteroids, and regulate a variety of neuronal and glial functions [1,35]. In peripheral tissues, the final step in inactivation of 5α-reduced bioactive metabolites of progesterone is selective introduction of a hydroxy group to the 6α-position [36].

Compounds that affect intracellular cholesterol transport and regulation generally fall into two categories: steroids and hydrophobic amines [6]. Of these compounds, progesterone [9,11,13,15] and U18666A [37] most closely mimic the biochemical phenotype of NPC: (i) inhibition of cholesterol transport both from lysosomes to plasma membrane and from plasma membrane to endoplasmic reticulum and (ii) delayed cholesterol regulatory responses such as LDL receptor and HMG–CoA reductase downregulation and ACAT stimulation. At the outset of this investigation, we hy-

pothesized that progesterone might interact directly or indirectly with the NPC gene product, and that this might be reflected in altered metabolism of or response to progesterone in NPC fibroblasts. Our experiments have shown that this is not so. The pattern and rates of progesterone metabolism are not significantly different between normal and NPC cells. Thus, a functional NPC gene product, recently identified as a membrane protein with putative sterol sensing capability (NPC1) [18], does not appear to be required for transport or metabolism of progesterone.

Other studies have shown that relatively low (<10 μM) concentrations of progesterone inhibit cholesterol transport or esterification in a variety of cell types [7,11,13,14,28,29]. Given our observation that progesterone added at micromolar levels can be quickly converted to other steroids, it is reasonable to ask how much progesterone remained and what effect its metabolism might have had in earlier investigations. The present results would suggest that metabolism of progesterone is unlikely to have been a significant factor in those studies. Finasteride completely blocked progesterone metabolism in fibroblasts yet had no effect on inhibition of LDL-stimulated cholesterol esterification, even at low progesterone concentrations. Moreover, the first metabolic product (5α-pregnane-3,20-dione) inhibited cholesterol esterification with similar potency. Although inhibitory properties of the other metabolites were not investigated, the results of a previous structure-function analysis of steroids on cholesterol regulation suggest that all metabolic products of progesterone would be likely to retain their inhibitory ability as they all possess a 20-oxo group [28].

While both the NPC mutation [21,24,26,27] and U18666A [37] impair transport and regulation of LDL-derived cholesterol, they have relatively little effect on regulation by the non-lipoprotein modulator 25-hydroxycholesterol. In contrast, progesterone did partially inhibit 25-hydroxycholesterol-stimulated cholesterol esterification. This observation is consistent with previous studies using mouse peritoneal macrophages [28] and FU5AH hepatoma cells [14]. This suggests that progesterone inhibits cholesterol trafficking and activation of ACAT by different mechanism(s) than either U18666A or the NPC mutation (see below), and is consistent with our observation that inhibition of 25-hydroxycholesterol-induced cholesterol esterification by progesterone occurred with similar dose dependence in normal and NPC fibroblasts. Presumably, 25-hydroxycholesterol acts downstream of NPC1 in regulation of cholesterol metabolism, possibly by interaction with ER- or Golgi-specific proteins such as oxysterol binding protein [38].

Recent evidence suggests that inhibition of cholesterol transport and esterification by progesterone and other steroids occurs through interaction with the multidrug resistant P-glycoprotein [13,14,39] and there is a strong correlation between the hydrophobicity of specific steroids and their ability to inhibit both cholesterol esterification and P-glycoprotein-catalyzed drug efflux [13,15]. We have found that the level of P-glycoprotein varies considerably among individual human fibroblast lines, but is not correlated with either the NPC phenotype or the capacity of cells to metabolize progesterone. A recent report also indicated that P-glycoprotein activity is not affected by U18666A [17]. Together with the finding that another inhibitor of P-glycoprotein activity, vinblastine, did not alter progesterone metabolism in fibroblasts, this would suggest that P-glycoprotein is not required (or at least not limiting) for progesterone metabolism and that its levels are not affected by the NPC mutation.

In summary, neither the known progesterone target P-glycoprotein (at the plasma membrane) nor NPC1 (location unknown, but possibly endosomal/lysosomal [18]) appears to affect the ability of fibroblasts to transport and deliver progesterone to intracellular membranes for further metabolism by steroid 5α-reductase [33]. Moreover, our results are consistent with earlier work showing that progesterone and U18666A act at distinct intracellular sites to block cholesterol transport and esterification. As discussed above, P-glycoprotein likely represents the major site of progesterone action, while U18666A may target NPC1 and/or other unidentified proteins involved in sterol trafficking [17]. These possibilities can be directly tested as soon as molecular tools for analysis of NPC1 become available.

Acknowledgements

The authors thank Heather Keith for excellent technical support and Robert Zwicker and Gladys Keddy for cell culture assistance. This work was supported by a Medical Research Council of Canada Program Grant (PG-11476) and a research fellowship to J.Z. from the IWK Grace Health Centre.

References

[1] E.E. Baulieu, M. Schumacher, H. Koenig, I. Jung-Testas, Y. Akwa, Progesterone as a neurosteroid: actions within the nervous system, Cell. Mol. Neurobiol. 16 (1996) 143–154.

[2] M.D. Majewska, N.L. Harrison, R.D. Schwartz, J.L. Barker, S.M. Paul, Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor, Science 232 (1986) 1004–1007.

[3] R.H. Purdy, A.L. Morrow, J.R. Blinn, S.M. Paul, Synthesis, metabolism, and pharmacological activity of 3 alpha-hydroxy steroids which potentiate GABA-receptor-mediated chloride

ion uptake in rat cerebral cortical synaptoneurosomes, J. Med. Chem. 33 (1990) 1572–1581.
[4] V.A. Randall, Role of 5α-reductase in health and disease, Bailliere's Clinical Endocrinology and Metabolism 8 (1994) 405–431.
[5] Y. Lange, T.L. Steck, The role of intracellular cholesterol transport in cholesterol homeostasis, Trends Cell Biol. 6 (1996) 205–208.
[6] L. Liscum, K.W. Underwood, Intracellular cholesterol transport and compartmentation, J. Biol. Chem. 270 (1995) 15443–15446.
[7] J.L. Goldstein, J.R. Faust, J.H. Dygos, R.J. Chorvat, M.S. Brown, Inhibition of cholesterol ester formation in human fibroblasts by an analogue of 7-ketocholesterol and by progesterone, Proc. Natl. Acad. Sci. USA 75 (1978) 1877–1881.
[8] S. Synouri-Vrettakou, K.A. Mitropoulos, On the mechanism of the modulation in vitro of acyl–CoA:cholesterol acyltransferase by progesterone, Biochem. J. 215 (1983) 191–199.
[9] J.D. Butler, J. Blanchette-Mackie, E. Goldin, R.R. O'Neill, G. Carstea, C.F. Roff, M.C. Patterson, S. Patel, M.E. Comly, A. Cooney, M.T. Vanier, R.O. Brady, P.G. Pentchev, Progesterone blocks cholesterol translocation from lysosomes, J. Biol. Chem. 267 (1992) 23797–23805.
[10] T. Mazzone, M. Krishna, Y. Lange, Progesterone blocks intracellular translocation of free cholesterol derived from cholesteryl ester in macrophages, J. Lipid Res. 36 (1995) 544–551.
[11] Y. Lange, Cholesterol movement from plasma membrane to rough endoplasmic reticulum. Inhibition by progesterone, J. Biol. Chem. 269 (1994) 3411–3414.
[12] J.E. Metherall, K. Waugh, H.J. Li, Progesterone inhibits cholesterol biosynthesis in cultured cells: accumulation of cholesterol precursors, J. Biol. Chem. 271 (1996) 2627–2633.
[13] P. Debry, E.A. Nash, D.W. Neklason, J.E. Metherall, Role of multidrug resistance P-glycoproteins in cholesterol esterification, J. Biol. Chem. 272 (1997) 1026–1031.
[14] Y. Lange, T.L. Steck, Cholesterol homeostasis: modulation by amphiphiles, J. Biol. Chem. 269 (1994) 29371–29374.
[15] J.E. Metherall, H.J. Li, K. Waugh, Role of multidrug resistance P-glycoproteins in cholesterol biosynthesis, J. Biol. Chem. 271 (1996) 2634–2640.
[16] P.G. Pentchev, M.T. Vanier, K. Suzuki, M.C. Patterson, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), Niemann–Pick Disease Type C: a Cellular Cholesterol Lipidosis. Metabolic Basis of Inherited Disease, McGraw-Hill, New York, 1995, pp. 2625–2639.
[17] K.W. Underwood, B. Andemariam, G.L. McWilliams, L. Liscum, Quantitative analysis of hydrophobic amine inhibition of intracellular cholesterol transport, J. Lipid Res. 37 (1996) 1556–1568.
[18] E.D. Carstea, J.A. Morris, K.G. Coleman, S.K. Loftus, D. Zhang, C. Cummings, J. Gu, M.A. Rosenfeld, W.J. Pavan, D.B. Krizman, J. Nagle, M.H. Polymeropoulos, S.L. Sturley, Y.A. Ioannou, M.E. Higgins, M. Comly, A. Cooney, A. Brown, C.R. Kaneski, E.J. Blanchette-Mackie, N.K. Dwyer, E.B. Neufeld, T.Y. Chang, L. Liscum, Niemann–Pick C1 disease gene: homology to mediators of cholesterol homeostasis, Science 277 (1997) 228–231.
[19] M. Axelson, B.J. Sahlberg, J. Sjövall, Analysis of profiles of conjugated steroids in urine by ion-exchange separation and gas chromatography–mass spectrometry, J. Chromatogr. Biomed. Appl. 224 (1981) 355–370.
[20] W.L. Greer, D.C. Riddell, T.L. Gillan, G.S. Girouard, S.M. Sparrow, D.M. Byers, M.J. Dobson, P.E. Neumann, The Nova Scotia (Type D) form of Niemann–Pick disease is caused by a G3097 to T transversion in NPC1, Am. J. Human Genet. 63 (1998) 52–54.
[21] L. Liscum, J.R. Faust, Low density lipoprotein (LDL)-mediated suppression of cholesterol synthesis and LDL uptake is defective in Niemann–Pick type C fibroblasts, J. Biol. Chem. 262 (1987) 17002–17008.
[22] J. Zhang, O. Larsson, J. Sjövall, 7α-hydroxylation of 25-hydroxycholesterol and 27-hydroxycholesterol in human fibroblasts, Biochim. Biophys. Acta 1256 (1995) 353–359.
[23] J.L. Goldstein, S.K. Basu, M.S. Brown, Receptor-mediated endocytosis of low-density lipoprotein in cultured cells, Methods Enzymol. 98 (1983) 241–260.
[24] H.S. Sidhu, S.A.R. Rastogi, D.M. Byers, H.W. Cook, F.B.St.C. Palmer, M.W. Spence, Cultured fibroblasts from patients with Niemann–Pick disease type C and type D exhibit distinct defects in cholesterol esterification, Biochim. Biophys. Acta 1124 (1992) 29–35.
[25] L.-J. Meng, J. Sjövall, Method for combined analysis of profiles of conjugated progesterone metabolites and bile acids in serum and urine of pregnant women, J. Chromatogr. B 688 (1997) 11–26.
[26] M.R. Natowicz, J.M. Stoler, E.M. Prence, L. Liscum, Marked heterogeneity in Niemann–Pick disease, type C: clinical and ultrastructural findings, Clin. Pediatr. (Phila.) 34 (1995) 190–197.
[27] P.G. Pentchev, M.E. Coml, H.S. Kruth, S. Patel, M. Proestel, H. Weintroub, The cholesterol storage disorder of the mutant BALB/c mouse: a primary genetic lesion closely linked to defective esterification of exogenously derived cholesterol and its relationship to human type C Niemann–Pick disease, J. Biol. Chem. 261 (1986) 2772–2777.
[28] K. Aikawa, T. Furuchi, Y. Fujimoto, H. Arai, K. Inoue, Structure-specific inhibition of lysosomal cholesterol transport in macrophages by various steroids, Biochim. Biophys. Acta Lipids Lipid Metab. 1213 (1994) 127–134.
[29] F.J. Field, E. Born, H. Chen, S. Murthy, S.N. Mathur, Esterification of plasma membrane cholesterol and triacylglycerol-rich lipoprotein secretion in CaCo-2 cells: possible role of P-glycoprotein, J. Lipid Res. 36 (1995) 1533–1543.
[30] X. Qian, W.T. Beck, Progesterone photoaffinity labels P-glycoprotein in multidrug-resistant human leukemic lymphoblasts, J. Biol. Chem. 265 (1990) 3–18756.
[31] P.V. Fennessey, A.W. Pike, C. Gonzalez-Aller, K.B. Horwitz, Progesterone metabolism in $T47D_{co}$ human breast cancer cells-I: 5α-pregnan-3β,6α-diol-20-one is the secreted product, J. Steroid Biochem. 25 (1986) 641–648.
[32] K.B. Horwitz, A.W. Pike, C. Gonzalez-Aller, P.V. Fennessey, Progesterone metabolism in T47Dco human breast cancer cells. II. Intracellular metabolic path of progesterone and synthetic progestins, J. Steroid Biochem. 25 (1986) 911–916.
[33] R.J. Moore, J.D. Wilson, Steroid 5α-reductase in cultured human fibroblasts, J. Biol. Chem. 251 (1976) 5895–5900.
[34] D.W. Fredericksen, J.D. Wilson, Partial characterization of the nuclear reduced nicotinamide adenine dinucleotide phosphate: D4-3-ketosteroid 5α-oxidoreductase of rat prostate, J. Biol. Chem. 246 (1971) 2584–2593.
[35] S.H. Mellon, Neurosteroids: biochemistry, modes of action and clinical relevance, J. Clin. Endocrin. Metab. 78 (1994) 1003–1008.
[36] S. Chantilis, R. Dombroski, C.H.L. Shackleton, M.L. Casey, P.C. MacDonald, Metabolism of 5α-pregnane-3,20-dione in women and men: 3β- and 3α-,6α-dihydroxy-5α-pregnan-20-ones are major urinary metabolites, J. Clin. Endocrinol. Metab. 81 (1996) 3644–3649.
[37] L. Liscum, J.R. Faust, The intracellular transport of low density lipoprotein-derived cholesterol is inhibited in CHO cells cultured with 3-β-[2-(diethylamino)ethoxy]androst-5-en-17-one, J. Biol. Chem. 264 (1989) 11796–11806.
[38] N.D. Ridgway, P.A. Dawson, Y.K. Ho, M.S. Brown, J.L. Goldstein, Translocation of oxysterol binding protein to Golgi

apparatus triggered by ligand binding, J. Cell Biol. 116 (1992) 307–319.
[39] C.P.H. Yang, S.G. DePinho, L.M. Greenberger, R.J. Arceci, S.B. Horwitz, Progesterone interacts with P-glycoprotein in multidrug-resistant cells and in the endometrium of gravid uterus, J. Biol. Chem. 264 (1989) 782–788.