Lauric acid inhibits the maturation of vesicular stomatitis virus

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In the presence of lauric acid (C12), the production of infectious vesicular stomatitis virus (VSV) was inhibited in a dose-dependent manner. The inhibitory effect was reversible: after removal of C12 the antiviral effect disappeared. In addition, the chain length of the monocarboxylic acids proved to be crucial, as those with shorter or longer chains were less effective or had no antiviral activity. Concomitant with the C12-induced inhibition was the stimulation of triacylglycerol synthesis, increasing the amount up to ninefold. Analysis of the antiviral mechanism of C12 revealed that the correct assembly of the viral components was disturbed, but viral RNA and protein synthesis remained unimpaired.

Introduction

Monocarboxylic acids with 11 to 14 C atoms stimulate the de novo synthesis of triacylglycerols, thus increasing the amount of triacylglycerols in the cell (Hornung et al., 1992). The chain length of the monocarboxylic acids has proved to be crucial, as those with shorter or longer chains are less effective or have no effect. Generally, newly synthesized triacylglycerols are stored as lipid droplets in the cytoplasm (Schneeberger et al., 1971; Spector et al., 1981), where they act as temporary lipid and energy sources. A small quantity of these triacylglycerols, however, becomes part of the plasma membrane. A recently published new model of plasma membrane structure proposes that neutral lipids are intercalated with the bilayer lipids (Mountford et al., 1982; Mountford & Wright, 1988). Proton magnetic resonance spectroscopy identified these neutral lipids as predominantly triacylglycerols. In cells with a high degree of proliferation and an increased capacity for mobility, such as stimulated immune cells and malignant cells, the triacylglycerols make up about 6% of the lipid content of the plasma membrane (Mountford et al., 1984; May et al., 1986; Mountford & Wright, 1988; Sze & Jardetzky, 1990; Freitas et al., 1990; Dingley et al., 1992; Mackinnon et al., 1992). This supports the suggestion that the accumulation of triacylglycerols might be correlated with increases in membrane fluidity and permeability (Mountford et al., 1982; Mountford & Wright, 1988).

The present study focused on the altered maturation of an enveloped virus at the plasma membrane as a result of such triacylglycerol-induced membrane alterations. The prototype rhabdovirus vesicular stomatitis virus (VSV) was chosen. VSV is an enveloped, negative-stranded RNA virus with an 11 kb genome that encodes five functional proteins (Banerjee, 1987a). Two proteins, L and NS, together make up the viral RNA polymerase (Banerjee, 1987b). The other three proteins are structural components: the membrane-embedded glycoprotein (G protein), the nucleocapsid protein (N protein), which complexes with the viral RNA to form a nucleocapsid, and the matrix protein (M protein). The M protein is synthesized on free polyribosomes and is initially a soluble cytoplasmic protein that binds to cellular membranes during the budding process. It promotes budding by interacting with the membrane-embedded viral G protein and the viral nucleocapsid, which condenses into a tightly coiled structure (Ono et al., 1987). Subsequently, the host plasma membrane encloses the nucleocapsid forming the viral envelope, and the virus is pinched off. The present study has shown that the budding process is drastically disturbed after C12-induced enhancement of the triacylglycerol level in the host cell. The budding of enveloped viruses is dependent...
that elevated triacylglycerol levels induce membrane alterations. The present study thus shows that medium chain length monocarboxylic acids both stimulate the synthesis of triacylglycerols and suppress the maturation of enveloped viruses, such as VSV, at the plasma membrane.

**Methods**

**Cells and viruses.** Rita cells (African green monkey kidney cells; Italodiagnostics Products) were used for experiments with VSV (Indiana strain, ATCC) and herpes simplex virus type 1 (HSV-1, Angelotti strain). CV-1 cells (African green monkey kidney cells) were used for experiments with simian virus 40 (SV40; Rh 911 strain). NIH3T3 cells (non-transformed mouse fibroblasts, ATCC) and C12C cells (Moloney murine leukaemia virus (MoMuLV)-transformed NIH3T3 cells; obtained from Professor W. Ostertag, Heinrich Pette Institute, Hamburg, Germany) were used for experiments with MoMuLV.

**Cell culture.** Cells were seeded in Eagle’s basal medium with 2.2 g/l NaHCO₃, 5% fetal calf serum (FCS), 1% streptomycin and 1% penicillin at an initial density of 1.3 × 10⁷ cells/cm² Petri dish. Incubation took place in a 5% CO₂/95% air atmosphere at 37 °C. The cells were grown to confluence and subsequently treated with monocarboxylic acids (octanoic acid, decanoic acid, lauric acid, palmitic acid and stearic acid; Sigma). A 1% stock solution of each monocarboxylic acid in 80% acetone and 20% H₂O was used.

**Toxicity assay.** For determination of cell density, quadruplicate monolayer cultures were fixed with 3% formaldehyde and stained with 1% crystal violet, after which the amount of bound dye was evaluated as described elsewhere (Sauer et al., 1984). Prior to fixation and staining, the dead cells were washed off and removed with the cell supernatant, and thus only the density of the adherent cells was quantified.

**Cell proliferation assay.** The mitotic activity of cells was determined by the incorporation of radiolabelled thymidine into the DNA. Quadruplicate monolayer cultures were grown to subconfluence and subsequently incubated with 3 µCi/ml [³H]thymidine (Amersham Buchler; specific activity 25 Ci/mmol) simultaneously with the C12 treatment. After 24 h the cell monolayers were washed twice with PBS and the cells were lysed in lysis buffer (PBS, 0.5% SDS, 10 mM-EDTA). The DNA was then precipitated by adding TCA to a final concentration of 5%. The precipitated DNA was collected on Whatman glass fibre filters, washed twice with 5% TCA and once with 70% ethanol, and the [³H]thymidine incorporation into the DNA was determined using a liquid scintillation counter.

**Viral infectivity assay.** Cells were infected at an m.o.i. of 0.001 to 0.1 p.f.u./cell, as indicated in the figure legends. After 1 h adsorption at 37 °C in a 5% CO₂/95% air atmosphere, the inoculum was removed and medium containing 5% FCS and monocarboxylic acids was added to the cells. The viral progeny was harvested at the time points indicated, and the virus yield was determined by plaque assay in Limbro plates. The XC plaque assay for MoMuLV was carried out as described elsewhere (Music & Sauer, 1992).

**Quantification of triacylglycerols.** Treated cells were washed twice with cold PBS, scraped from the dishes and collected by centrifugation. Total lipids were extracted by the method of Bligh & Dyer (1959). After extraction the lipids were dried in vacuo, resuspended in 10 µl isopropanol and mixed with an enzymatic assay kit (GPO-Trinder, Sigma) for quantitative determination of total triacylglycerols.
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cell numbers and the incorporation of [3H]thymidine into
cell DNA (Fig. 1b). Up to 750 μg/ml C12 was tolerated
by uninfected cells, whereas concentrations as low as
40 μg/ml of C12 reduced the titre of the virus progeny by
98%. However, changes in cell morphology could be
detected by light microscopy at C12 concentrations
higher than 100 μg/ml (data not shown). Therefore a
concentration between 30 and 70 μg/ml C12 was chosen
for further experiments (unless otherwise stated) as an
appropriate inhibitory concentration of virus maturation
that did not lead to recognizable morphological alter-
ations of the treated cells.

The antiviral potential of monocarboxylic acids was
observed exclusively with the medium chain length
monocarboxylic acid. In contrast, treatment of infected
Rita cells with equimolar concentrations of mono-
carboxylic acids with shorter (C6, C8) or longer (C16,
C18) chain lengths was much less effective (Fig. 2). Thus,
the chain length of the monocarboxylic acid is of crucial
importance for inhibition of the release of infectious
virus.

The inhibitory effect was reversible. When C12 was
removed from the tissue culture medium after a 16 h
treatment the virus titre in the supernatant of C12-
treated cells increased to the same level as in untreated
cells within 28 h (Fig. 3). This indicates that the
inhibitory effect of C12 is dependent on its continued
presence.
had been treated with C12 was examined (Fig. 4). In addition, the virus yield in the supernatant was determined. C12 treatment simultaneously enhanced the level of triacylglycerols by up to ninefold and inhibited the release of infectious virus particles. The increase in triacylglycerol level in the cells was found to correlate with the degree of reduction of infectious virus in the medium.

Whether the C12-induced enhancement of the triacylglycerol level in the host cells affected the infectivity of VSV, the infectibility of the host cell, or the production of virus progeny was considered (Table 1). When Rita cells were pretreated with C12 for 16 h and subsequently infected with VSV, the yield of infectious virus, determined after another 24 h incubation in C12-free medium, was only slightly reduced in comparison with the untreated control (factor of inhibition 2.86). The adsorption and penetration of VSV into the host cell were both seemingly undisturbed by the initial presence of C12. In contrast, after simultaneous C12 treatment of Rita cells for 24 h and infection with VSV, the titre of the released virus was reduced by 1000-fold, indicating a C12-induced disturbance of virus production. Pretreatment of VSV with 120 µg/ml C12 prior to infection had no significant effect on the virus yield, which indicates that the infectivity of the virus particles was not irreversibly affected by C12.

To provide circumstantial evidence for inhibition by C12 of VSV maturation at the plasma membrane, several virus species that use different pathways of maturation were examined (Table 2). HSV-1, for example, matures by budding at the nuclear membrane rather than at the plasma membrane. The yield of infectious HSV-1 particles was only slightly reduced after treatment with medium.

It has already been reported that medium chain length monocarboxylic acids are ‘consumed’ by cells, on the one hand stimulating triacylglycerol synthesis and, on the other, being incorporated into the newly synthesized triacylglycerols (Hornung et al., 1992). Therefore, the triacylglycerol content of VSV-infected Rita cells that

Table 2. Effect of C12 on the replication of various taxonomically unrelated viruses

<table>
<thead>
<tr>
<th>Virus species</th>
<th>Virus titre in the absence of C12</th>
<th>Virus titre in the presence of C12</th>
<th>Factor of inhibition of virus release</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSV*</td>
<td>6.60 x 10⁸</td>
<td>4.40 x 10⁵</td>
<td>1500</td>
</tr>
<tr>
<td>MoMuLV†</td>
<td>2.83 x 10⁵</td>
<td>2.80 x 10⁵</td>
<td>101</td>
</tr>
<tr>
<td>SV40§</td>
<td>2.79 x 10⁵</td>
<td>1.85 x 10⁴</td>
<td>15.1</td>
</tr>
<tr>
<td>HSV-1</td>
<td></td>
<td></td>
<td>7.93 x 10⁵</td>
</tr>
</tbody>
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* The VSV, HSV-1 and SV40 yields in the cell supernatants were determined by plaque assay. The MoMuLV yield was determined by XC plaque assay in NIH3T3 cells, as described elsewhere (Music & Sauer, 1992).
† Rita cells were infected with VSV (m.o.i. 0.01) and treated with 60 µg/ml C12 for 24 h.
‡ MoMuLV-transformed NIH3T3 cells (C12C) were treated with 60 µg/ml C12 for 48 h.
§ CV1 cells were infected with SV40 (m.o.i. 0.01) and treated with 60 µg/ml C12 for 5 days.
|| Rita cells were infected with HSV-1 (m.o.i. 0.01) and treated with 60 µg/ml C12 for 48 h.

Table 1. Effect of C12 pretreatment of host cells or virus particles on the replication of VSV

<table>
<thead>
<tr>
<th>Pretreatment of virus particles with (A) or without C12 (B), followed by infection of host cells*</th>
<th>Virus titre (B) in absence of C12</th>
<th>Virus titre (A) in presence of C12</th>
<th>Factor of inhibition of virus release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment of host cells with (A) or without C12 (B), followed by infection with VSV†</td>
<td>5.90 x 10⁸</td>
<td>5.14 x 10⁶</td>
<td>1.15</td>
</tr>
<tr>
<td>Treatment of host cells with (A) or without C12 (B); simultaneous infection with VSV‡</td>
<td>1.06 x 10⁸</td>
<td>3.70 x 10⁸</td>
<td>2.86</td>
</tr>
<tr>
<td></td>
<td>7.23 x 10⁷</td>
<td>6.65 x 10⁶</td>
<td>1087.2</td>
</tr>
</tbody>
</table>

* The virus yield in the supernatant of Rita cells infected for 24 h with VSV (m.o.i. 0.01) was determined by plaque assay. C12 (120 µg/ml) was added to the virus pool to be used for infection for 3 h prior to infection.
† The virus yield in the supernatant of Rita cells infected for 24 h with VSV (m.o.i. 0.01) was determined by plaque assay. Prior to infection the cells were treated with 60 µg/ml C12 for 18 h.
‡ The virus yield in the supernatant of Rita cells infected for 24 h with VSV (m.o.i. 0.01) and treated with 60 µg/ml C12 was determined by plaque assay.

Fig. 4. Correlation of the C12-induced inhibition of VSV replication and the C12-induced stimulation of triacylglycerol synthesis. Rita cells were infected with VSV (m.o.i. 0.01) and incubated in the presence or absence of 60 µg/ml C12. At the time points indicated the virus yield in the supernatant was examined by plaque assay and the degree of inhibition was determined by dividing the virus yield in the supernatant of untreated cells by the yield of treated cells (■). Simultaneously, the triacylglycerol content of the cells was determined and the degree of stimulation was calculated (▲).
Fig. 5. Influence of C12 on viral RNA synthesis. Cells were infected with VSV (m.o.i. 0.1) in the absence (lanes 1, 3) or presence of 30 µg/ml (lane 4), 50 µg/ml (lanes 2, 5) or 70 µg/ml (lane 6) C12. At 8 h after infection, total RNA was separated in a 1.4% agarose/2.2 M-formaldehyde gel, transferred to nylon membranes (Biodyne B) and hybridized with clone 6 32P-labelled DNA, which contained sequences for the L region of VSV RNA. RNA from mock-infected cells was run in lanes 1 and 2. Positions of rRNA species are given on the left.

60 µg/ml C12 (factor of inhibition 12.2). Similar results were obtained with the non-enveloped virus SV40, which leaves the host cells by exocytosis (factor of inhibition 15.1). The production of the retrovirus MoMuLV, however, which buds at the plasma membrane, was inhibited by two orders of magnitude. Taken together, these data support the conclusion that the medium chain length monocarboxylic acid inhibits the release of enveloped viruses at the host plasma membrane.

The different magnitudes of virus inhibition induced by C12 on the two plasma membrane-maturing viruses, VSV and MoMuLV, may be explained by differences in the two host cell systems. VSV-infected Rita cells and MoMuLV-infected NIH3T3 cells reacted differently upon treatment with C12. It was found that the triacylglycerol level was enhanced by C12 in Rita cells by a factor of 8.8, whereas in NIH3T3 cells it was stimulated by a factor of only 5.7 (data not shown). The C12-induced triacylglycerol stimulation correlates directly with the inhibition of virus maturation, as can be seen in Fig. 4.

The mechanism of virus inhibition was investigated in more detail. First the influence of C12 on the synthesis of viral RNA was examined by Northern blot analysis (Fig. 5). Total RNA was prepared from treated and untreated infected Rita cells and, after subsequent electrophoresis and transfer to nylon filters, hybridization was carried out using a 32P-labelled VSV cDNA probe encoding the L region of VSV RNA. One major band representing the viral L mRNA was detected in untreated and C12-treated cells in equal amounts, indicating that C12 had no effect on the viral RNA synthesis. Plaque assays of the medium from the cultures from which the RNA had been prepared showed that the virus yield was inhibited by a factor of 88.2 (lane 6) after C12 treatment.

The effect of C12 on viral protein synthesis was examined by autoradiography of 35S-labelled proteins...
from treated and untreated infected cells. The synthesis of all five viral proteins (L, G, NS, N and M) remained unimpaired regardless of the C12 concentration (Fig. 6a). In contrast, a reduction of the viral proteins in the cell supernatant was observed (Fig. 6b), and plaque assays carried out with the supernatant from treated cultures revealed a drastic reduction in the titre of released viral progeny (up to a factor of inhibition of 43.2).

The lack of an inhibitory effect of C12 on the synthesis of the viral components was indicative of a disturbance in the maturation of the viral progeny. Therefore, the distribution of the viral proteins in the host cell was examined by cell fractionation followed by SDS–PAGE. Despite treatment of infected Rita cells with increasing amounts of C12 the incorporation of [35S]methionine into the viral proteins of the cytoplasma was undisturbed (Fig. 7a). In the plasma membrane, however, a decrease in the two membrane-associated viral proteins, G and M, was observed (Fig. 7b).

The reduced incorporation of M protein into the plasma membrane after treatment with C12 was further verified with the aid of Western blot analysis (Fig. 8). Indeed, there was a reduced amount of M protein in the plasma membrane (Fig. 8c) whereas, in contrast, almost no decrease of M protein in the cytoplasma was detectable (Fig. 8b). In the cell supernatant a reduction of the M protein after incubation with C12 was also observed (Fig. 8a). By measuring the intensity of the band representing the M protein (Fig. 8a), inhibition was shown to be 1.46-fold in the presence of 40 μg/ml C12 (lane 3), rising to 13.88-fold in the presence of 60 μg/ml C12 (lane 6). This corresponds to the results obtained by plaque assay which indicated that the release of virus into the medium was inhibited 1.64-fold in the presence of 40 μg/ml C12, and 15.63-fold in the presence of 60 μg/ml C12. Thus, the reduced incorporation of the viral M protein into the host plasma membranes correlated directly with the inhibition of release of infectious virus particles.

Discussion

In this report we have shown that in the presence of lauric acid (C12) the replication of vesicular stomatitis virus was inhibited by several orders of magnitude (Fig. 1). Simultaneously, the triacylglycerol level in the host cell rose up to ninefold, as reported previously (Fig. 4; Hornung et al., 1992). The virus inhibition and the triacylglycerol stimulation were highly dependent on the chain length of the monocarboxylic acid used: those with shorter or longer chains were less effective or had no effect at all (Fig. 2). In addition, both events occurred in a dose-dependent manner (Fig. 1) and both were reversible (Fig. 3). This correlation between the two events led to the conclusion that the C12-induced enhancement of the triacylglycerol level was involved in the inhibition of VSV maturation.

After incubation of cells with a surplus of exogenous fatty acids the newly synthesized triacylglycerols accumulate as cytoplasmic droplets (Mackenzie et al., 1966; Schneeberger et al., 1971; Spector et al., 1981;
Gavino et al., 1981; Denning et al., 1983). Small quantities of triacylglycerols are incorporated into the plasma membrane, where they might influence cell permeability and membrane fluidity (Mountford et al., 1982; Mountford & Wright, 1988). This, in turn, may disturb the reproduction of enveloped viruses which bud from the plasma membrane of infected cells and acquire an envelope which includes lipids derived from the host cell. Therefore the manipulation of the lipid composition or the fluidity of the plasma membrane, or both, may provide an antiviral approach. It has already been reported that the infection of chickens with Newcastle disease virus is suppressed by administration of butylated hydroxytoluene (BHT), a membrane-fluidizing drug that interferes with the budding process (Brugh, 1977). BHT is also active against HSV, cytomegalovirus and Semliki Forest virus, all viruses that have a lipid envelope (Snipes et al., 1975; Kim et al., 1978). Poliovirus, however, which does not possess a lipid envelope, was not inactivated by BHT (Snipes et al., 1975). Other membrane-fluidizing drugs with antiviral potential are amphoterin C methylster (Schaffner et al., 1986) and the liposome mixture AL721, consisting of 70% triacylglycerol, 20% phosphatidylcholine and 10% phosphatidyl ethanolamine (Lyte & Schmitzky, 1985; Sarin et al., 1985). Both drugs reduce the infection of lymphocytes by human immunodeficiency virus type 1 (HIV-1), the aetiological agent of AIDS. They influence a variety of enveloped RNA and DNA viruses by complexing membrane cholesterol, an event that causes changes in cell permeability and function and results in a significant resistance to virus replication (Schaffner et al., 1986; Stevens et al., 1975).

Here we deal with the antiviral activity of an agent that interferes with plasma membrane triacylglycerol. Although little is known about the effects of triacylglycerols in membranes, studies have already shown that the triacylglycerols have the effect of disordering the molecular organization of membrane cholesterol ester and probably altering the lipid composition of the plasma membrane (Deckelbaum et al., 1977; Tall et al., 1978). Furthermore, the triacylglycerols apparently influence membrane fluidity and permeability, parameters which increase with elevated lipid/protein ratio (Alonio et al., 1988). Membrane permeability to certain chemical agents, including anti-cancer drugs and anaesthetics, is impaired by triacylglycerols. For example, human leukaemia lymphoblasts resistant to the vinca alkaloid vinblastine could be resensitized to the drug by reducing the plasma membrane triacylglycerol content (Holmes et al., 1989).

Mellert et al. (1988) reported that medium chain length fatty acids in combination with tricyclodecan-9-yloxanthenogenate (D609) inhibit the release of infectious HIV-1 from chronically infected lymphoma cells, apparently by disturbing the assembly of viral components. The application of D609 or fatty acid alone, however, failed to show any antiviral effect. This can be explained by the fact that in chronically infected lymphoma cells incubated for 2 to 5 days with only small amounts of fatty acids (10 µg/ml), the fatty acids were quickly consumed and used for the synthesis of triacylglycerols, which have a short half-life and are rapidly hydrolysed. When sufficient amounts of fatty acid were applied, however, the antiviral effect on retroviruses (MoMuLV) (Table 2) and other enveloped viruses that mature at the plasma membrane was shown to persist.

Mellert et al. (1988) were able to inhibit the replication of HIV-1 by offering fatty acid in combination with D609 which prevents the hydrolysis of triacylglycerols (Hornung et al., 1992). These results support the proposition that elevated plasma membrane triacylglycerol leads to inhibition of virus assembly.

Treatment of VSV-infected cells with C12 neither disturbed viral RNA synthesis (Fig. 5) nor had any influence on viral protein synthesis (Fig. 6a). The correct assembly of these viral components at the host plasma membrane, however, seemed to be impaired by C12. Thus a reduction in membrane-embedded viral G protein and M protein was observed in the plasma membrane fraction (Fig. 7). The latter protein is known to be associated with the cytoplasmic surface of the plasma membrane.
membrane bilayer (Pepinsky & Vogt, 1979), where it initiates the process of virus budding by interacting with the nucleocapsid and the G protein located in the plasma membrane (Dubovi & Wagner, 1977; Pepinsky & Vogt, 1979; Zakowski & Wagner, 1980; Mancarella & Lenard, 1981). It appears, therefore, that the decreased insertion of G and M proteins into the plasma membrane, possibly as a result of the incorporation of triacylglycerols, consequently leads to an inhibition of virus release.

Thus, with the aid of a physiological substance, a medium chain length fatty acid, it is shown here that theoretically it might be possible to intervene in the maturation process of an enveloped virus. Further research is needed to determine whether this observation can be used for the development of new antiviral therapeutic strategies.

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