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The human fatty acid synthase: A new therapeutic target for coxsackievirus B3-induced diseases?

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Abstract

Coxsackievirus is linked to a large variety of severe human and animal diseases such as myocarditis. The interplay between host factors and virus components is crucial for the fate of the infected cells. However, host proteins which may play a role in coxsackievirus-induced diseases are ill-defined. Two-dimensional gel electrophoresis of protein extracts obtained from coxsackievirus B3 (CVB3)-infected and uninfected HeLa or HepG2 cells combined with spot analysis revealed several proteins which are exclusively up-regulated in infected cells. One of these proteins was identified as the fatty acid synthase (FAS). By using cerulenin and C75, two known inhibitors of FAS we were able to significantly block CVB3 replication. FAS appears to be directly involved in CVB3-caused pathology and is therefore suitable as a therapeutic target in CVB3-induced diseases.

Keywords: Coxsackievirus; Fatty acid synthase (FAS); Cerulenin; c75; TOFA

1. Introduction

Picornaviruses are small positive-stranded RNA viruses. They are linked to a variety of human and animal diseases. Coxsackievirus B3 (CVB3) is an important pathogenic member of the picornavirus family, which can cause severe complications predominantly in heart function. Clinically, CVB3 infections are known to be associated with different forms of myocarditis (Reyes and Lerner, 1985; Woodruff, 1980). It can cause cardiac arrhythmias and acute heart failure, and chronic forms of this disease may lead to dilated cardiomyopathy (DCM). This is characterized by an enlargement and reduced contractibility mainly of the left heart ventricle. Consequently, DCM leads to a progressive heart insufficiency and a significantly increased death rate (Gillum, 1986; Sugrue et al., 1992), which can be cured by heart transplantation only. CVB3 and other enteroviruses cause up to half of all cases of acute myocarditis and 25% of DCM (Bowles et al., 1986; Frisk et al., 1984). CVB3 also replicates in the pancreas, which results in a destruction of the exocrine tissue, whereas the Langerhans islets remain intact (Henke et al., 2001; Mena et al., 1999). CVB3 infections are often accompanied by programmed cell death. Apoptotic processes were found in myocardial tissue of patients with DCM (Olivetti et al., 1997). It was shown that apoptotic cell death occurs inside and outside of inflamed areas in the myocardial tissue of CVB3-infected mice (Gebhard et al., 1998; Henke et al., 1995; Huber et al., 1996). Despite accumulating data, no promising compounds for the treatment of CVB3-induced heart diseases have been forthcoming. CVB3 infections in children and infants have been treated with pleconaril with some efficacy in reducing mortality and improving recovery, albeit the treatment is often on a compassionate use basis (Barnard, 2006).

The pathogenesis of coxsackievirus infections has been studied intensively in different murine models, demonstrating that the outcome of the disease is determined by complex variables, such as the immune status of the host (Henke et al., 1995; Huber et al., 1999; Leipner et al., 1999). The interaction between virus

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components and host factors is an important determinant for the course and severity of a virus infection. So far only a few host proteins have been identified which influence the response after infection with CVB3, including p56lck and ERK1/2 (Liu et al., 2000; Opavsky et al., 2002). We previously described a direct interaction between the viral capsid protein VP2 and the proapoptotic protein Siva, which is overexpressed upon a CVB3 infection in mice (Henke et al., 2000, 2001; Martin et al., 2004). Siva is involved in the CD27/CD70-transduced apoptotic pathway (Prasad et al., 1997).

By using proteomics, we have identified several host factors which are up- or down-regulated in CVB3-infected human cells (Rassmann et al., 2006). Functional studies revealed that one of these proteins, the fatty acid synthase (FAS), might be a potent therapeutic target for CVB3-induced diseases. Relevant hints were obtained by blocking FAS activity through the inhibitors cerulenin and/or C75. FAS is a lipogenic enzyme that catalyzes the condensation of acetyl-CoA and malonyl-CoA to generate long-chain fatty acids (Wakil, 1989). When CVB3-infected human cells were treated with cerulenin or C75, which have both been described as inhibitors of FAS (Funabashi et al., 1989; Kuhajda et al., 2000), virus replication decreased significantly, thus indicating the involvement of FAS in the pathogenicity of CVB3.

2. Materials and methods

2.1. Viruses and cells

Viruses were propagated in HeLa (for 6 h) and HepG2 (for 12 h) cells at 37 °C and 5% CO_2, using DMEM for HeLa and RPMI-1640 for HepG2 cells supplemented with 10% fetal bovine serum. The onset of the cytopathic effect was inspected visually at the indicated time points. Typically, 90–95% confluent cell monolayers were infected with CVB3 at a multiplicity of infection (MOI) of 5. The cell lines were obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany) and cultivated according to the manufacturer's instructions. Media and serum were purchased from Biochrom (Berlin, Germany). Control cells were mock-infected.

2.2. Compound treatment of CVB3-infected cells

Cerulenin and C75 were used according to Pizer et al. (2000), i.e. at a final concentration of 45 μM (not toxic for HeLa cells), whereas TOFA (5-(tetradecyloxy)-2-furoic acid) was applied at 5 μM. Compounds were purchased from Cayman Chemicals (Ann Arbor, MI) and dissolved in DMSO (stock solutions: cerulenin, 9 mM; C75, 9 mM; TOFA, 5 mM). The dissolved compounds were added to the cells simultaneously with the viruses. Due to the high decay rate of cerulenin in water, the treatment was repeated after 1.5 h of incubation. CVB3-infected control cells were treated with DMSO only. Additional controls were non-infected cells in the presence of cerulenin, C75, TOFA or DMSO, respectively.

2.3. Sample preparation for proteome analysis

The cells were incubated with lysis buffer (8 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT). After sonification (10× 5 s pulses; 60 W) the lysate was separated by acetone/TCA precipitation (Görg et al., 1997) followed by incubation in rehydration buffer (8 M urea, 2 M thiourea, 4% CHAPS, 12 μl/ml DeStreak™, 2% Pharmalyte™). Insoluble elements were removed by centrifugation at 75.000 × *g* for 15 min. The protein concentration was determined by Bradford analysis (Bradford, 1976) with bovine serum albumin as reference. DeStreak™ and Pharmalyte™ were obtained from Amersham Pharmacia Biotech.

2.4. Sample preparation for RNA analysis and $CCID_{50}$ analysis

RNA was prepared using the Qiagen RNeasy Mini Kit according to the manufacturer's instructions. The virus titer was determined according to Reed and Muench (1938).

2.5. 2D electrophoresis

Isoelectric focussing (IEF) was performed by the Multiphor II system (Amersham Pharmacia Biotech, Freiburg, Germany) according to the manufacturer's instructions, i.e. in-gel rehydration (Rabilloud et al., 1994; Sanchez et al., 1997) of 500 μg protein using 24 cm long IPG (immobilised pH gradient) strips (pH 3–10; Amersham Pharmacia Biotech).

The IPG strips were transferred onto a 10% SDS-polyacrylamide gel. Electrophoresis was carried out in an Ettan DALT*twelfe*™-System (Amersham Pharmacia Biotech) according to Laemmli (1970). Protein spots were visualised in the gels by colloidal Coomassie Brilliant Blue G-250 staining (Doherty et al., 1998).

Spots were picked manually by the GelPal Excision System (Genetix GmbH, Munich-Dornach, Germany). After destaining, washing and drying the gel pieces were reswollen in 5 μl protease solution, containing 20 ng trypsin per ml of protease buffer (10 mM Tris–HCl pH 8.5). After 10 min of incubation, 5 μl of protease buffer was added. Tryptic digestion was performed for 16 h at 37 °C. Trypsin (modified sequencing grade) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Protein identification was performed by matrix assisted laser desorption/ionisation-mass spectrometry (MS/MS) in combination with SWISS-PROT-database searching.

2.6. Reverse transcription PCR

Reverse transcription of 5 μg total RNA was performed with SUPERSCRIPT™ II-RT/M-MLV RT (Invitrogen GmbH, Karlsruhe, Germany) according to the manufacturer's instructions. cDNA of VP1 (viral capsid protein 1) and cDNA of β-actin (host gene as control) were amplified using the Qiagen Taq-Polymerase Kit in combination with the SpeedCycler (Rapid PCR System, Analytik Jena, Jena, Germany), using the following primer pairs:

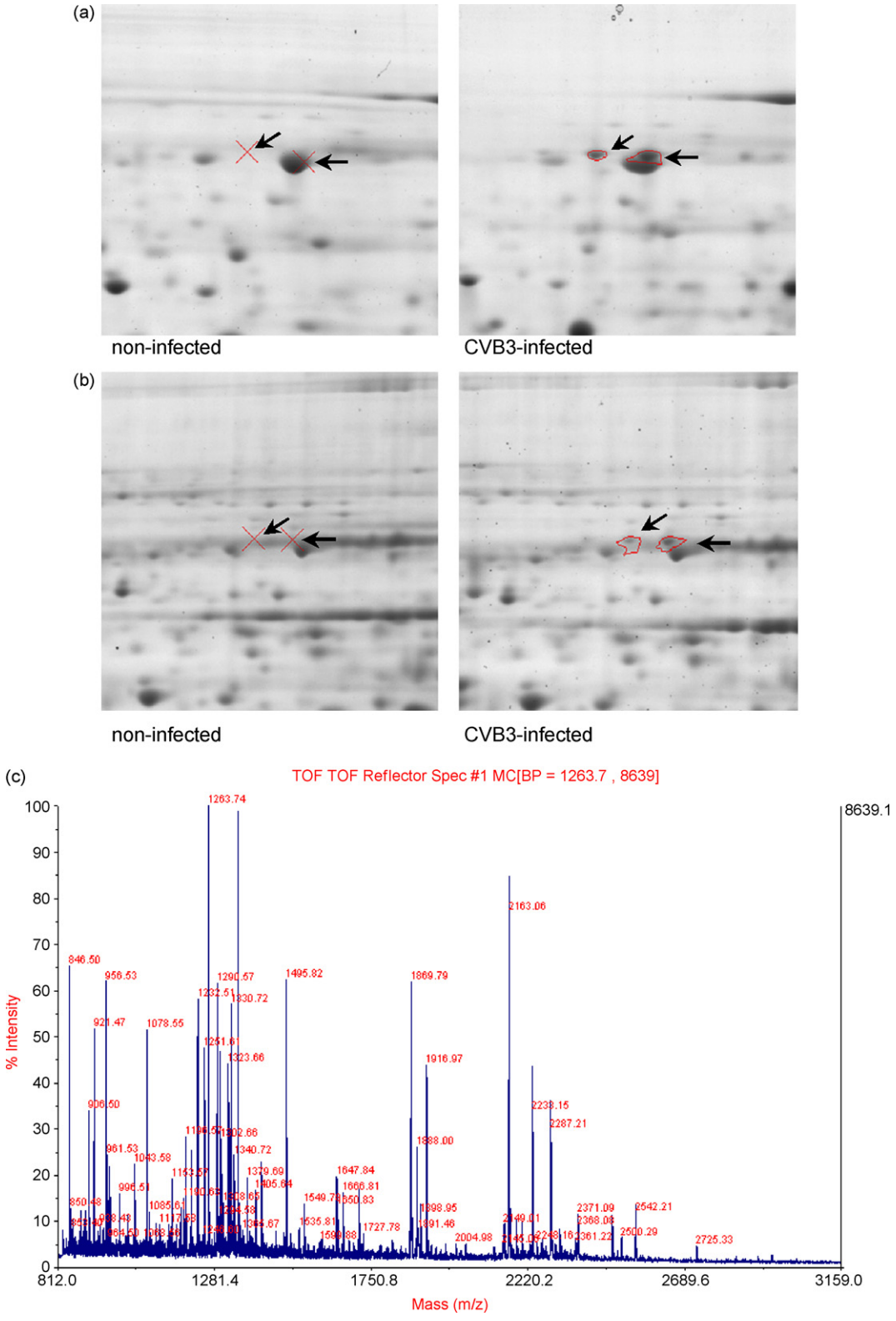

Fig. 1. Sections of 2D gels showing upregulation of FAS in CVB3-infected HeLa (a) or HepG2 (b) cells. The arrows show the localisation of FAS. Crosses indicate the region lacking the framed protein spot of the corresponding gel. (c) An example of mass spectra of a tryptic digested spot of FAS is shown. (d) Complete amino acid sequence of human FAS. The underlined peptide sequences were found in the mass spectrometry sequence analysis.

Fig. 1. (*Continued*).

- β-actin-5′ (5′-ATCTGGCACCACACCTTCTACAATGAG-CTGCG-3′);
- β-actin-3′ (5′-CGTCATACTCCTGCTTGCTGATCCACAT-CTGC-3′);
- VP1-5′ (5′-GGCCCAGTGGAAGACGCG-3′);
- VP1-3′ (5′-AAATGCGCCCGTATTTGTCATTG-3′).

Analysis of the amplification products was performed by DNA agarose gel electrophoresis according to Sambrook et al. (1989).

2.7. Real time PCR

Quantification of cDNA samples was performed using the QuantiTect™SYBR Green PCR Kit (Qiagen Hilden, Germany) in combination with the LightCycler system (Roche, Idaho Technologies) according to the Manufacturer's instructions. Initially, HotStar Taq DNA Polymerase was activated by a 95 °C heating step for 15 min. Subsequently, a total of 32 cycles was performed, consisting of denaturation at 94 °C for 15 s (20 °C/s), 20 s annealing step at 60 °C (20 °C/s) for the CVB3 RNA-strand specific primer pair EV1 (5′-CCCTGAATGCGGCTAATCC-3′) and EV2 (5′-ATTGTCACCATAAGCAGCCA-3′) (Verstrepen et al., 2001) and a combined polymerisation-extension step at 72 °C for 20 s (20 °C/s). To melt all primer dimers every cycle was terminated at 82 °C for 50 s (20 °C/s). In order to verify specificity and identity of the PCR products a melting curve was routinely performed after all cycles by heating at 94 °C (20 °C/s), following cooling to 50 °C (20 °C/s) for 1 min and again heating the samples to 94 °C (0.2 °C/s) to determine the specific melting temperature of all PCP products. As internal DNA standard a defined copy number of the linearized plasmid pBKH3 was used (Knowlton et al., 1996). For data analysis corresponding software from Roche was applied.

3. Results

3.1. The fatty acid synthase is overexpressed in CVB3-infected human cells

FAS was found to be overexpressed in CVB-3 infected HeLa and HepG2 cells (Fig. 1a and b). More precisely, 2D electrophoresis followed by matrix-assisted laser desorption/ionisation-mass spectrometry (MALDI) revealed two different isoforms thereof in both cell lines tested. On 2D-gels both of these isoforms showed a molecular weight of approximately 270 kDa and IEP's of approximately 6.3 and 6.5, respectively. The latter differences might be due to a varying degree of phosphorylation (see Section 4). The data obtained by tryptic digests and MALDI experiments allowed a clear classification of the protein, i.e. all non-trypsin derived peaks matched with aminoacid sequences of FAS. An example of

five independent experiments performed is shown in Fig. 1c and d.

3.2. Cerulenin inhibits CVB3 replication

Guinea and Carrasco (1990) found for the first time that a continuous phospholipid synthesis is required for efficient poliovirus genome replication. Fogg and Molla and their coworkers demonstrated that membrane altering compounds, such as cerulenin, inhibit RNA replication but not the protein expression of poliovirus *in vitro* (Fogg et al., 2003; Molla et al., 1993). Cerulenin is an antifungal antibiotic isolated from culture filtrates of the fungus *Cephalosporium caerulens* (Tomoda et al., 1984). This drug is a potent inhibitor of FAS in various microorganisms and animal tissues (Funabashi et al., 1989; Moche et al., 1999). Furthermore, the effect of cerulenin on a variety of animal viruses is well documented, e.g. human immunodeficiency virus (Bu et al., 1989; Pal et al., 1988), Epstein-Barr virus (Li et al., 2004) and Mayaro virus (Pereira and Rebello, 1998).

Within this framework we investigated the influence of cerulenin on CVB3 replication. To this end, CVB3-infected HeLa cells were treated with cerulenin as described in Section 2. As shown by microscopy, CVB3-infected cells which were not treated with cerulenin showed a clear onset of the cytopathic effect (Fig. 2c), characterized by shrinking of the cells and the subsequent disintegration of the cellular assembly (Wessely et al., 1998a,b). This was in contrast to CVB3-infected and cerulenin-treated cells (Fig. 2b) which remained almost unaffected (Fig. 2a). Virus yield was determined after different time intervals, i.e. 0.5 h, 2.5 h, 4.5 h and 6.5 h. Fig. 3 shows the result of the virus titer determination in function of cerulenin treatment. The progeny of CVB3 decreased approximately 100-fold upon cerulenin treatment in a final concentration of 45 μM after 6.5 h.

3.3. Repressed CVB3 replication is due to inhibition of the cellular β-ketoacyl acyl carrier protein synthase by cerulenin

It is well known that cerulenin induces apoptosis via accumulation of malonyl-CoA (Pizer et al., 2000). Cerulenin at a concentration of less than 150 μM had no cytotoxic effects on HeLa cells, as based on cellular growth (not shown). To further exclude cytotoxic effects we introduced two additional compounds, i.e. C75 and TOFA, both interfering with fatty acid synthesis.

For these studies the effects of C75 and TOFA were tested on CVB3 replication. C75 is a synthetic inhibitor of FAS (Kuhajda et al., 2000). Like cerulenin, C75 interferes with the binding of malonyl-CoA to the β-ketoacyl acyl carrier protein synthase of FAS, thus inhibiting long-chain fatty acid elongation (Kuhajda et al., 1994). C75 showed no detectable cytotoxic effect on HeLa cells at a concentration of 150 μM (not shown). TOFA inhibits the acetyl-CoA carboxylase, the rate-limiting enzyme in long-chain fatty acid synthesis (Halvorson and McCune, 1984). The mode of action of these three compounds and their chemical structures are depicted in Fig. 4.

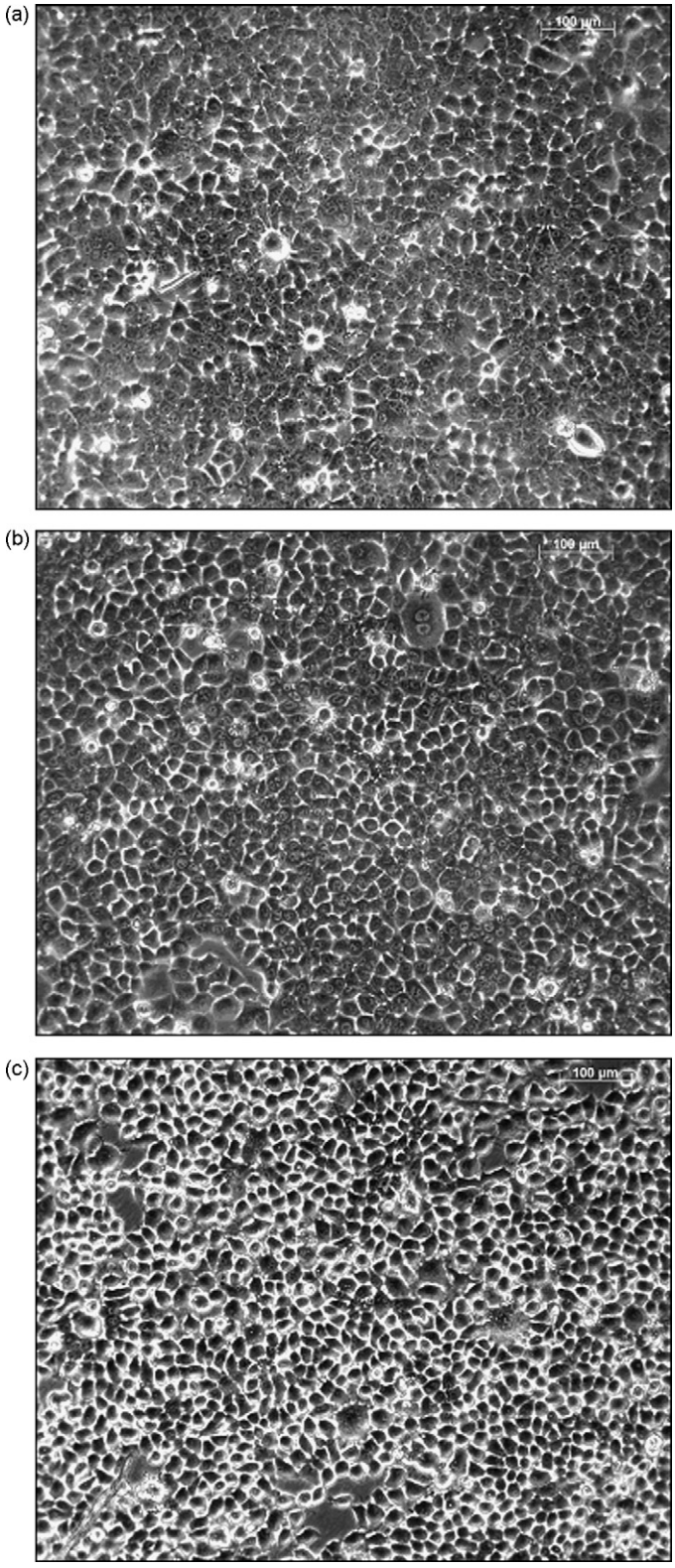

Fig. 2. Delayed cytopathic effect of infected HeLa cells due to cerulenin-treatment. (a) Cells 4.5 h after mock infection and treatment with DMSO as control. (b) Cells 4.5 h after CVB3-infection and treatment with cerulenin. (c) Cells 4.5 h after CVB3 infection and treatment with DMSO.

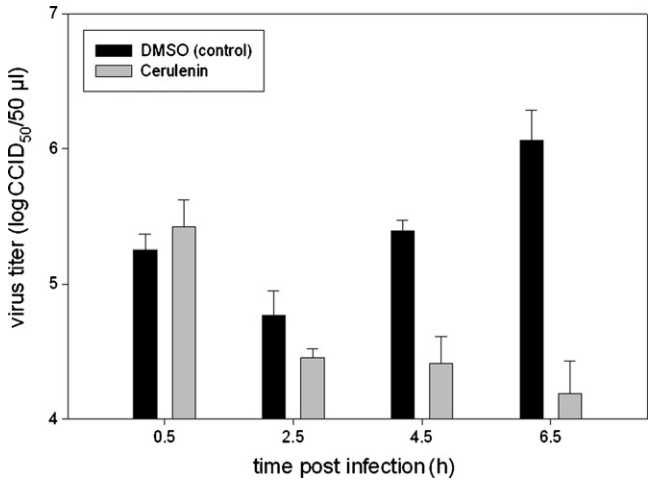

Fig. 3. Influence of cerulenin on CVB3 yield. Virus titers were measured by $CCID_{50}$ analysis according to Reed and Muench (1938). Average titers and standard deviations are shown as $\log_{10}$ values of $CCID_{50}$ units/50 µl medium. A marked decrease of the virus progeny to approximately 100-fold was detected in the cerulenin-treated HeLa cells.

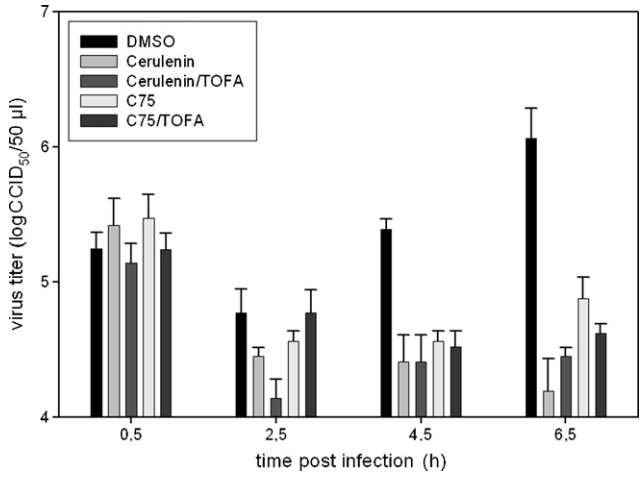

Fig. 5. Influence of cerulenin, cerulenin/TOFA, c75, and C75/TOFA on viral replication. Virus titers were measured by $CCID_{50}$ analysis. Average titers are depicted as $\log_{10}$ values of $CCID_{50}$ units/50 µl medium. A marked decrease of the virus progeny was detected in HeLa cells in the presence of inhibitors.

It is well known that a simultaneous application of TOFA to the cerulenin- or C75-treated cells extensively reduces the cytotoxicity of the latter two drugs, because the toxic malonyl-CoA is not accumulated (Pizer et al., 2000). As shown in Fig. 5, C75 had a similar effect on viral replication as cerulenin, whereas TOFA had no inhibitory effect on the virus production (not shown). A decrease in the virus yield also occurred in infected cells treated either with cerulenin/TOFA or C75/TOFA. This proved that the inhibition of FAS by cerulenin or C75 was not based on a non-

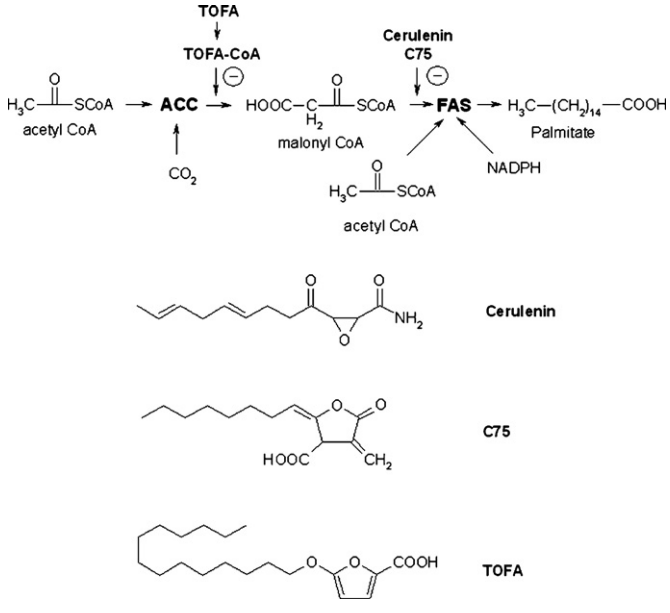

Fig. 4. Inhibitors of fatty acid synthesis pathway. TOFA as an inhibitor of the acetyl-CoA carboxylase (ACC) arrests the synthesis in the initiation reaction. Consequently, the accumulation of malonyl-CoA within the cell, which is induced by inhibitors of the β-ketoacyl acyl carrier protein synthase cerulenin and C75, will be circumvented. According to Pizer et al. (2000), modified. The chemical structures of the fatty acid synthesis inhibitors cerulenin, C75 and TOFA are shown below.

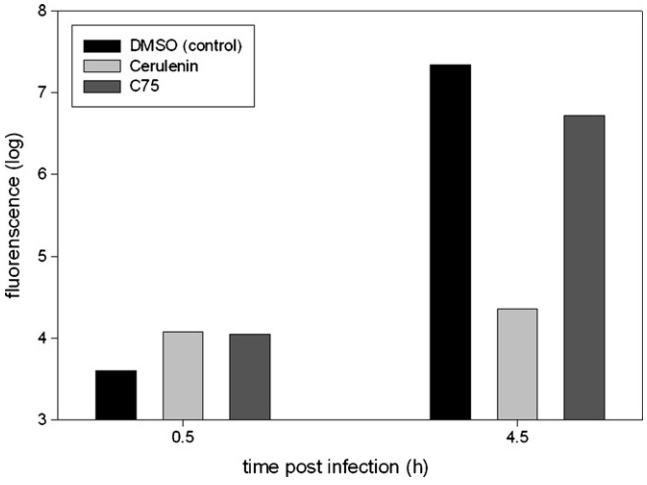

Fig. 6. Real time PCR interpretation of CVB3-VP1 cDNA in infected HeLa cells treated with cerulenin and C75. A decrease of CVB3-VP1 RNA was detected in the treated cells in contrast to the control cells.

specific cytotoxic effect caused by the highly reactive epoxy group of cerulenin.

These results were supported by real time PCR experiments determining the synthesis of VP1 (Fig. 6). In the control cells, the VP1 DNA conspicuously increased 4.5 h after infection, whereas in cerulenin-treated cells only a slight increase of VP1 could be detected. This showed that viral RNA replication was strongly inhibited by cerulenin. C75 was less efficient, which was corroborated in cerulenin- or C75-treated cells by virus titer analysis (see Fig. 5). This indicated that cerulenin was a more potent inhibitor of viral replication than C75.

4. Discussion

Recently it has been shown that the mitogen-activated protein kinase (MAPK) p38 is activated by CVB3 infections (Si et al., 2005; Rassmann et al., 2006). Interestingly, studies using Epstein-Barr virus (EBV)-infected cells have shown that the

expression of FAS is induced upon infection by a p38 stress MAPK-dependent mechanism (Li et al., 2004). Here we demonstrated that FAS is up-regulated in CVB3-infected cells. This result is in accordance to Guinea and Carrasco (1990), who described an increase of phospholipid synthesis due to poliovirus infection. The occurence of two FAS spots may indicate a nonphosphorylated and a phosphorylated form of FAS (Beausoleil et al., 2004). It is known, that the phosphorylated protein is less active than the nonphosphorylated form (Beausoleil et al., 2004). Remarkably, FAS was up-regulated in both CVB3-infected HeLa and HepG2 cells, indicating a universal response to a CVB3 infection. Treatment of the cells with fatty acid synthesis inhibitors resulted in a strongly impaired viral replication when compared to non-treated cells.

Cerulenin and C75 are inhibitors of the β-ketoacyl acyl carrier protein synthase subunit of FAS (Fig. 4). In contrast to C75, cerulenin treatment led to a much stronger suppression of the viral replication. It has been shown that C75 is a relatively inefficient inactivator of FAS with a dissociation constant of approximately 16 mM (Rendina and Cheng, 2005). Therefore, the β-ketoacyl acyl carrier protein synthase remains active during inhibition by C75, albeit to a much lower extent when compared to the non-inhibited protein. Thus, we could exclude unspecific reaction mechanisms of cerulenin. To further exclude, that the inhibition of the virus progeny is a result of cytotoxic effects provoked by malonyl-CoA accumulation we used TOFA as additional inhibitor of fatty acid synthesis (Fig. 4). The treatment of infected cells with TOFA had no detectable effect to viral progeny (not shown). Interestingly, a combination of cerulenin/TOFA and C75/TOFA strongly reduced the cytotoxicity of cerulenin and C75 (Pizer et al., 2000), whereas the viral progeny remained suppressed (Fig. 5). Therefore, the inhibition of viral replication by cerulenin and C75 was not based on cytotoxic effects.

Positive-strand RNA viruses modify and utilize rearranged intracellular membranes to create structures to support replication of their genomes (Egger et al., 2002). Fogg et al. (2003) suggested that the initial uridylylation of the genome associated viral protein VPg of poliovirus is disturbed when such structures are defective due to treatment of cells with cerulenin or guanidine hydrochloride. Therefore, these membrane structures are important for the successful replication of CVB3. It is conceivable that FAS is directly involved in the above mechanism, since cerulenin and C75 strongly reduce the number of CVB3 progeny in HeLa cells. However, TOFA, which inhibits the synthesis of malonyl-CoA (see Fig. 4) does not influence viral multiplication. Furthermore, it is known, that guanidine hydrochloride does not only modify membrane structures but it also inhibits the viral 2C protein and therefore the growth of many picornaviruses at concentrations between 0.1 mM and 2.0 mM (Shimizu et al., 2000). Guanidine hydrochloride reversibly blocks viral RNA synthesis without affecting the replication complex formation and viral polyprotein processing (Barton et al., 1995; Barton and Flanegan, 1993, 1997). Taken together, the effect of cerulenin on CVB3 replication can be interpreted by inhibition of the β-ketoacyl acyl carrier protein synthase subunit of FAS.

It is suggested, that VPg is uridylylated by the viral RNA-polymerase 3D. Paul et al. (2003) have reconstituted the VPg uridylylation *in vitro* using polyA, RNA polymerase 3D, and UTP. As reported, in HeLa cell extracts the uridylylation of poliovirus VPg is inhibited by treatment with cerulenin (Fogg et al., 2003). This strongly supports our hypothesis, that the β-ketoacyl acyl carrier protein synthase plays an essential role during the replication of the CVB3 genome *in vivo*.

The inhibition of fatty acid synthesis by cerulenin leads to an inhibition of poliovirus replication but it has no influence on the translation of the viral polyprotein (Fogg et al., 2003) which could be confirmed also in our laboratory for CVB3. The cytopathic effect of cerulenin-treated cells was detected approximately 2 h later than in untreated infected cells, whereas the viral titer in both set-ups was constantly low (Fig. 3). Viral proteins formed in the absence or presence of cerulenin may elicit a cytopathic effect. Simultaneously, cerulenin inhibits the replication of CVB3. This also results in a reduced viral protein synthesis which then accounts for the delayed cytopathic effect.

We might envisage FAS as a potential therapeutic target for an antiviral therapy. In contrast to cellular proteins, viral proteins, such as the RNA polymerase or proteases are exposed to a high mutation rate, which render the viruses resistant against antiviral drugs. If the above compounds should be taken into consideration as anti-picornavirus agents, cerulenin and C75 should be applied simultaneously with TOFA so as to reduce cytotoxicity (Heiligtag et al., 2002).

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