Oxidative stress in cells infected with bovine viral diarrhoea virus: a crucial step in the induction of apoptosis

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Bovine viral diarrhoea virus (BVDV) belongs to the genus Pestivirus of the family Flaviviridae. Both a noncytopathic (ncp) and an antigenically related cytopathic (cp) BVDV can be isolated from persistently infected animals suffering from mucosal disease. In every case studied so far, the genomic changes leading to the cp biotype correlate with the production of the NS3 nonstructural protein, which, in the ncp biotype, is present in its uncleaved form, NS23. This report shows that, in contrast to ncp BVDV, the cp biotype induces apoptosis in cultured embryonic bovine turbinate cells. Early in the process of apoptosis, cells show a rise in the intracellular level of reactive oxygen species, which is indicative of oxidative stress. This precedes two hallmarks of apoptosis, caspase activation as shown by cleavage of the caspase substrate poly(ADP–ribose) polymerase, and DNA fragmentation. Cells were protected from apoptosis only by certain antioxidants (butylated hydroxyanisole and ebselen), whereas others (N-acetylcysteine, pyrrolidine dithiocarbamate, lipoic acid, dihydrolipoic acid and tiron) turned out to be ineffective. Antioxidants that protected cells from apoptosis prevented oxidative stress but failed to block virus growth. These observations suggest that oxidative stress, which occurs early in the interaction between cp BVDV and its host cell, may be a crucial event in the sequence leading to apoptotic cell death. Hence, apoptosis is not required for the multiplication of the cp biotype of BVDV.

Introduction

The genus Pestivirus of the family Flaviviridae consists of bovine viral diarrhoea virus (BVDV), classical swine fever virus and border disease virus. BVDV occurs worldwide and is an important pathogen of cattle (Houe, 1995; Meyers & Thiel, 1996). Infected animals may show either no symptoms or mild diarrhoea, but severe thrombocytopenia and haemorrhages have also been reported in infected cattle. BVDV isolates can be divided into two biotypes, cytopathic (cp) and non-cytopathic (ncp), according to their effect in cell cultures. In vivo, infection of a pregnant animal with the ncp biotype may result in embryonic death, resorption, stillbirth or induction of non-fatal teratogenic damage, or may lead to the birth of persistently infected calves. Such calves are immunotolerant to the infecting BVDV strain and remain persistently viraemic for the rest of their lives. Persistently infected animals are predisposed to infections with other pathogens, and run a high risk of developing fatal mucosal disease (MD) (for reviews, see Moennig & Plagemann, 1992; Nettleton & Entrican, 1995; Thiel et al., 1996). In animals exhibiting MD, both an ncp and an antigenically related cp BVDV, referred to as a virus pair, can be isolated (Brownlie, 1990). The cp biotype may arise in persistently infected animals by genomic rearrangement of the ncp virus (e.g. insertion of cellular sequences) or rearrangements in the viral genome (for reviews, see Meyers & Thiel, 1996; Rice, 1996). In every case studied so far, the genomic changes leading to the cp biotype are paralleled by the production of the nonstructural protein NS3 (p80). The production of NS3 is thought to be responsible for the induction of cell death in vitro by the cp biotype of BVDV. Moreover, as the presence of the cp biotype of BVDV correlates with MD, cytopathicity may be crucial in the pathogenesis of this fatal disease.

Necrosis and apoptosis are two distinct forms of cell death, the former being a passive process typified by early leakage of the plasma membrane and spillage of the intracellular content, the latter being a highly regulated process that avoids inflammation and damage to the surrounding tissue (Steller, 1995). Apoptosis is an integral part of the developmental programme, and can be induced by a variety of stimuli (e.g. different chemical insults, DNA damage, radiation, growth factor deprivation, cytotoxic T-lymphocytes or virus in-
contrast, nucleotide sequence identity in part of the 5'-UTR between TGAC and TGAN was only 86% (H. P. Stalder, personal communication), indicating that TGAC and TGAN may not be a virus pair (Ridpath et al., 1991). Viruses were passaged and titrated on BT cells as described (Adler et al., 1994) and the titre of the virus stocks was calculated according to Reed & Muench (1938).

**Virus infection.** BT cells seeded in microwell plates (96 wells) or 25 cm² culture flasks at a density of 10⁶ cells per plate or flask were infected with the appropriate BVDV strain in a small volume of culture medium without FCS at an m.o.i. of 1 for 1 h at 37 °C. After adsorption of the virus, the inoculum was removed by washing the cells in culture medium without FCS prior to the addition of complete medium with 2% FCS. Using an m.o.i. of 1, we observed that all cells expressed NS3/NS23 around 20–24 h post-infection as analysed by immunofluorescence staining using an anti-NS3/NS23 antibody.

**Determination of cell viability with crystal violet.** Adherent, viable cells in microwell plates were fixed and stained with 0·75% crystal violet in 50% ethanol, 0·25% NaCl and 1·75% formaldehyde. Stained cells were rinsed with water, lysed in 0·6% SDS, 40 mM HCl dissolved in isopropanol, and the crystal violet intensity quantified at 590 nm in an ELISA reader (Schulze-Osthoff et al., 1994; Adler et al., 1995).

**Immunoblotting of viral proteins.** Cells were washed in PBS and lysed in standard SDS–PAGE sample buffer. The proteins were separated on 10% SDS–polyacrylamide minigels (Bio-Rad) and electroblotted for 1 h at 110 V in a Mini Trans-Blot cell onto nitrocellulose membranes. After blocking with 5% low-fat dry milk, the blot was incubated with a monoclonal mouse anti-NS3/NS23 antibody (49DE9, generated at our Institute) and stained with peroxidase-labelled goat anti-mouse antibody (dilution 1:1000) before ECL detection according to the manufacturer’s protocol.

**PARP cleavage.** Cells were washed in PBS and lysed in PARP sample buffer (62·5 mM Tris/HCl pH 6·8, 6 M urea, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol with bromophenol blue). To effectively dissociate PARP–DNA interactions, samples were sonicated on ice (6 cycles for 5–10 s) and heated for 30 min at 65 °C. Separation of the proteins by SDS–PAGE, blotting and blocking of the membranes was performed as described above. The blot was incubated for 4 h with the anti-bovine PARP antibody (dilution 1:7000) and stained with peroxidase-labelled donkey anti-mouse antibodies (dilution 1:2000) and ECL detection according to the manufacturer’s protocol. To control the protein load per lane, the blot was stained with mouse anti-β-actin (dilution 1:5000) either simultaneously with the anti-PARP antibody or separately after stripping the blot in 100 mM 2-mercaptoethanol, 2% SDS, 62·5 mM Tris/HCl pH 6·7 at 50 °C for 30 min.

**DNA fragmentation.** The fragmentation of cellular DNA was analysed qualitatively by visualizing oligonucleosomal-sized DNA fragments (‘DNA ladder’) or quantitatively by FACS analysis. The DNA laddering was performed as described by Darzynkiewicz et al. (1994) or by Peled-Kamar et al. (1995). Flow cytometric analysis of apoptosis was performed according to Cossarizza et al. (1995). Briefly, adherent and detached cells were collected by centrifugation at 250 g, washed in PBS and the cell pellet (10⁶ cells) was lysed in 500 μl 0·1 M sodium citrate pH 6·5, 1% Triton X-100 and 25 μg/ml propidium iodide (PI). Nuclei were analysed after a 30 min incubation at 4 °C in the dark with a FACSscan flow cytometer (Becton Dickinson) and a minimum of 10⁶ nuclei were analysed.

**Measurements of the cellular redox state.** The cellular redox state was analysed by determination of the oxidation of dichlorofluorescin (DCF) to the fluorescent dichlorofluorescin (DCF) and FACS analysis

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**Methods**

**Reagents.** FCS and cell culture media were purchased from Seromed (Biochrom). FCS was free of BVDV and antibody to BVDV as tested by virus isolation and serum neutralization assay, respectively. Butylated hydroxyanisole (BHA) and ebelsen were dissolved in ethanol and DMSO, respectively, prior to dilution in cell culture medium. Hyb-Bond-C extra nitrocellulose membranes were from Amersham. Peroxidase-labelled anti-mouse antibody was from Kirkegaard & Perry Laboratories (Gaithersburg, MD), anti-bovine poly(ADP–ribose) polymerase (PARP) mouse monoclonal antibody SA-250 was from Biomol (Plymouth, PA) and mouse anti-β-actin (clone AC-74) was from Sigma. All other chemicals were of the highest purity that is commercially available.

**Cells and viruses.** Primary bovine turbinate cells (BT cells) were prepared from foetuses obtained from a local abattoir, and were maintained in MEM supplemented with 7% FCS (2% FCS after virus infection), penicillin (100 IU/ml) and streptomycin (100 μg/ml) at 37 °C in a humidified 5% CO₂ atmosphere. The cells were found to be free of BVDV by immunofluorescence testing. The TGAC (Ridpath et al., 1991), R1935 and SuwaCP cp BVDV strains, and the TGAN (Ridpath et al., 1991) and SuwaNCP ncp viruses were used. The TGAC, TGAN and R1935 virus strains were kindly provided by V. Moennig (Hannover, Germany), whereas SuwaCP and SuwaNCP is a virus pair isolated at our Institute from an animal with MD. Sequencing parts of the 5'-UTR, E2 and NS23 regions of the viral genomes of SuwaCP and NCP revealed the two viral genomes to be identical, except for four point mutations in the NS23 region. Additionally, the size of the viral genome as judged by Northern blotting is identical, excluding a large insertion of viral or cellular RNA (L. Perler & H. P. Stalder, unpublished observations). In

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as described (Bustamante et al., 1997). Adherent and detached cells (10⁶ cells) were collected as described above and resuspended in 500 µl PBS. After addition of 10 µM 2′,7′-dichlorodihydrofluorescein diacetate (2′,7′-dichlorofluorescein diacetate, DCFH-DA; Kodak), cells were incubated for 30 min at 37 °C in the dark, followed immediately by FACS analysis. To separate viable and dead cells, PI (final concentration 10 µg/ml) was added 10 min prior to the end of the incubation. Cell fluorescence was analysed with a FACSScan flow cytometer with the fluorescence of DCFH-DA in channel 1 (FL1; log scale) and of PI in channel 2 (FL2; log scale). A minimum of 10⁶ cells per sample was analysed and the oxidation state (geometric mean) of the viable cells (i.e. by gating for the cells excluding PI, which cannot pass through the intact plasma membrane) and the viability of the entire (ungated) cell population was calculated. For comparison of the different samples, preparations were recorded at single instrument amplification settings.

Results

Infection of BT cells with the TGAC cp BVDV strain, but not with the TGAN ncp strain resulted in cell death in a time-dependent manner, as shown by crystal violet staining of the remaining viable adherent cells (Fig. 1A). Microscopically, dying cells were observed as early as 24 h post-infection. However, since only the viable adherent cells were stained, the observed intensity of crystal violet at 590 nm represents the net effect of cell proliferation and cell death. Therefore, at 24 h post-infection, cell proliferation was still higher than cell death, which resulted in an increased absorption. Additionally, virus-infected cells proliferated at an increased rate independently of the biotype of BVDV, resulting in a higher absorption of crystal violet at 590 nm than that by mock-infected cells. Other strains of cp BVDV also induced cell death, which indicates that this phenomenon is not a strain-specific effect of TGAC (Fig. 1B; see also Fig. 6). However, the kinetics of cell death varied between the different strains, e.g. cell death induced by R1935, in contrast to TGAC (Fig. 1A), was not yet complete at 48 h post-infection (Fig. 1B).

The TGAC-induced cell death occurred via apoptosis, as detected by the appearance of oligonucleosomal-sized DNA fragments (‘DNA laddering’; not shown) and of subgenomic DNA (‘sub-G1 peak’) analysed by PI staining of the cell nuclei and FACS analysis (Fig. 2). Cp BVDV-induced apoptosis was effectively inhibited by the antioxidant BHA in a time- (Figs 1B and 3A) and concentration-dependent manner (Fig. 3B), as detected either by crystal violet staining (Fig. 1B) or by FACS analysis (Fig. 3). Maximal inhibition was seen at 100 µM BHA, whereas concentrations higher than 250 µM were toxic to the cells (not shown). Accordingly, the DNA laddering induced by TGAC was completely prevented by the presence of 100 µM BHA (not shown). However, BHA also partially inhibited cell proliferation of BT cells, and this effect was more pronounced when the cells were infected with virus. Thus, cells incubated in the presence of BHA failed to reach confluency or did so later compared to cells incubated without the antioxidant. This was corroborated by the fact that the absorption at 590 nm after staining cells incubated in the presence of BHA with

![Figure 1: Cp BVDV induces cell death in BT cells. BT cells in 96-well plates were either mock-infected or infected with different BVDV strains at an m.o.i. of 1 as described in Methods. Viable, adherent cells were stained with crystal violet (CV) at 0 h ( ), 24 h ( ) or 48 h ( ) post-infection and the intensity measured at 590 nm. (A) Infection with the TGAC cp strain and TGAN ncp strain (mean ± SD, n = 20). (B) Infection with R1935 cp strain in the absence or presence of 100 µM BHA (mean ± SD, n = 8).

![Figure 2: DNA fragmentation induced by cp BVDV in BT cells. DNA fragmentation in mock- (A) or TGAC- (B) infected BT cells at 30 h post-infection was analysed by PI staining of nuclei followed by FACS analysis. In the FACS histogram, PI fluorescence was detected in FL1 (x-axis). The number of nuclei is given on the y-axis. The percentages indicate the number of nuclei with subgenomic DNA.](image_url)
crystal violet was lower than that of cells incubated in its absence (Fig. 1B).

To investigate whether the effect of BHA on cell viability was due to inhibition of virus replication, we determined the level of expression of the viral NS23 nonstructural protein. Analysis of immunoblots stained with a monoclonal anti-

NS3/NS23 antibody showed that TGAC- and TGAN-infected cells incubated in the presence of BHA contained amounts of NS3 (p80) and NS23 (p125), respectively, which were similar to those found in cells incubated in the absence of BHA (Fig. 4). The proteins other than NS23 and NS3 detected by the monoclonal antibody, especially in the cells infected by TGAC were probably fragments of NS23 produced by the proteolytic activities induced in apoptotic cells. Accordingly, BHA massively reduced the number of these proteolytic degradation products. This indicated that the antioxidant directly interfered with cellular death pathways without inhibiting the expression of viral proteins. Additionally, BHA did not significantly inhibit the production of infectious virus from either TGAC- or TGAN-infected cells as determined by titrations of cell culture supernatants (not shown). Besides BHA, other antioxidants were tested for their effect on apoptosis induced by cp BVDV. Ebselen (a mimic of the cellular enzymes glutathione peroxidases, in particular that of the phospholipid hydroperoxide glutathione peroxidase; Schewe, 1995) at 10 μM inhibited TGAC-induced apoptosis to only about 50% at 24–30 h post-infection (results not shown; see Fig. 6). At later time-points, its inhibitory capacity was again reduced, and at higher concentrations the compound itself was toxic to BT cells. N-acetylcysteine (NAC), another well known antioxidant, did not prevent cp BVDV-induced apoptosis and in fact had a toxic effect itself on both infected and uninfected BT cells, as analysed by crystal violet staining of the viable adherent cells or by FACS analysis (not shown). The cytotoxic effect of NAC was not seen with N-acetylcysteine, which contains a hydroxyl group rather than a sulphydryl group. This excludes unspecific damage such as changes in the osmolarity of the medium by the presence of high concentrations of the reagent. Other antioxidants tested were either toxic to cells or had no effect on cp BVDV-induced apoptosis [e.g. dihydrolipoic acid, lipoic acid, tiron (a superoxide dismutase mimic) or pyrrolidine dithiocarbamate (PDTC)].

Many different proteases, such as serine proteases, calpains and the proteasome pathway are involved in the apoptotic process (Patel et al., 1996). The family of ICE-like (interleukin-1β-converting enzyme) proteases called ‘caspases’ has attracted considerable attention. Activation of caspases during apoptosis results in the cleavage of various critical cellular substrates, such as PARP (considered a hallmark of apoptosis), lamin or protein kinase C δ (Cohen, 1997). To establish whether PARP cleavage is involved in cp BVDV-induced apoptosis, we analysed the expression of PARP by immunoblotting with an antibody which recognizes full-length PARP (116 kDa), as well as the larger cleavage product of 85 kDa. PARP cleavage occurred at 20–24 h post-infection in TGAC-infected (Fig. 5), but not mock- or TGAN-infected BT cells (not shown). After 42 h, the total amount of full-length and cleavage product decreased, reflecting further unspecific proteolytic digestion of fragments not recognized by the anti-PARP antibody. Accordingly, the amount of β-actin, a protein constantly ex-

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Fig. 5. PARP cleavage induced by cp BVDV. TGAC-infected BT cells were incubated in the absence (control) or presence of 100 µM BHA for variable periods of time as indicated in the figure. The cleavage of the full-length PARP (116 kDa) to the larger proteolytic fragment (85 kDa) was followed by immunohistochemistry as described in Methods. Staining of β-actin on the same membrane was used as a control for the protein loading of the individual lanes.

Fig. 6. Cp BVDV induces the production of ROS in BT cells. BT cells were mock-infected or infected with different BVDV strains as indicated in the figures. Cells were incubated in the absence (•) or presence (○) of BHA and ebselen (■), respectively. The intracellular redox state was measured by quantification of DCF fluorescence of viable, PI-negative cells by flow cytometry as described in Methods. The value (geometric mean) of mock-infected cells at 4 h post-infection was set to 100%.

Fig. 7. Intracellular production of ROS is an early event in cp BVDV-induced apoptosis. Mock- (▲) or TGAC-(●, ○) infected BT cells were incubated in medium in the absence (▲, ●) or presence (○) of 100 µM BHA. The intracellular redox state was measured by quantification of DCF-fluorescence of viable, PI-negative cells by flow cytometry as described in Methods. The value (geometric mean) of mock-infected cells at 4 h post-infection was set to 100%.

pressed in intact cells, also decreased after 42–48 h post-infection. Again, 100 µM BHA completely prevented the degradation of both PARP and β-actin in TGAC-infected cells (Fig. 5) but had no influence on the expression of both proteins in mock- or TGAN-infected cells (not shown).

To analyse whether cp BVDV-induced apoptosis was accompanied by increased production of ROS, we loaded BT cells with DCFH-DA. This dye is trapped intracellularly after deacetylation, and DCF green fluorescence was analysed by flow cytometry. The analysis showed that, at 24 h post-infection, TGAC-infected BT cells show massively increased DCF fluorescence (300%) compared to TGAN- (104%) or mock-infected cells (Fig. 6 A; with mock set to 100%). BHA at 100 µM completely prevented the increase in the intracellular oxidative stress (91% DCF oxidation compared to mock-infected cells in the absence of the antioxidant), whereas ebselen, at the maximally tolerated dose of 10 µM, only partially reduced DCF oxidation (to 143%). The change in the intracellular redox state towards a more oxidized state was independent of the BVDV strain used. Thus, BT cells infected with TGAC or SuwaCP, as well as R1935, but not with the ncp strains TGAN or SuwaNCP, showed an increase in DCF fluorescence (Fig. 6B). The extent of the DCF fluorescence depended on the strain of BVDV used to infect the cells. DCF fluorescence was calculated from viable cells, i.e. only from cells with an intact plasma membrane as determined by double-staining with PI. The percentage of viable cells which excludes PI was calculated in the same cell population. By this method, cell viability decreased to a different extent depending on the cp BVDV strain used (Fig. 6C), albeit to a lesser extent than when measured by FACS analysis after staining the nuclei with PI. Again, BHA completely – and ebselen at least partially – prevented a decrease in cell viability as assessed by PI exclusion (Fig. 6C). To test whether the change in the intracellular redox state occurred before or after the first signs of apoptosis, we determined the kinetics of DCF oxidation. In TGAC-infected BT cells, there was no change in the cellular redox state until 10 h post-infection (Fig. 7). However, DCF fluorescence started
to increase 14 h post-infection, with the maximum occurring at 24 h post-infection. This was clearly earlier than other signs of apoptosis, such as DNA fragmentation or PARP cleavage. BHA clearly inhibited this increase in the cellular oxidative stress (Fig. 7) and the decrease in cell viability as determined by PI exclusion (not shown) over the whole time period.

Discussion

The study of cell death induced by BVDV is of particular interest for two main reasons. In vivo, the appearance of cp BVDV in animals that are immunotolerant to this virus is associated with the lethal MD. Although not formally proven, it is generally thought that the failure of these animals to limit the multiplication of cp BVDV may be the crucial pathogenic event, ultimately leading to their death. We have recently provided evidence that cp BVDV may be responsible for another, indirect, type of cell killing. Thus, factor(s) released from macrophages infected with cp BVDV are capable of priming uninfected cells for endotoxin-induced apoptosis (Adler et al., 1997). The unique type of interaction between virus and Gram-negative bacteria may well contribute to the preference of the lesions of MD in the gastrointestinal tract. Another interesting aspect of BVDV-induced cell killing is its apparent correlation with the appearance of the NS3 non-structural protein in infected cells, i.e. the cp strains TGAC, R1935 and SuwaCP, but not the ncp strains TGAN and Suwa NCP, express the NS3 protein. For these reasons, the biological difference between ncp and cp BVDV is as much of interest from the viewpoint of the pathogenesis of a lethal disease as from that of cell biology. In this work, we demonstrate that cp BVDV induces oxidative stress in the early stages of infection of BT cells. Using selected antioxidants that interfere with oxidative stress we were able to prevent cell death that occurred by apoptosis in these cells. Since the protection from cell death was not due to inhibition of virus replication or formation of infectious progeny, our results indicate that cell death and replication of cp BVDV can be dissociated. This is in contrast to work by Levine et al. (1996) where the anti-apoptotic protein Bcl-2 was shown to inhibit Sindbis virus-induced host mortality by inhibiting virus replication rather than by its effect on apoptosis.

Oxidative stress has been suggested to be a mediator of apoptosis induced by a variety of triggers, including virus infections (Buttke & Sandstrom, 1994; Schwarz, 1996; Peterhans, 1997). This was frequently shown either by the addition of ROS or by the anti-apoptotic effect of certain antioxidants. In our system, only BHA (Verhaegen et al., 1995) and ebselen (Ramakrishnan et al., 1996) protected cells from BVDV-induced apoptosis. Other antioxidants, which are known to inhibit cell death in other cells, e.g. NAC (Cossarizza et al., 1995), PDTC (Verhaegen et al., 1995), lipoic acid (Scott et al., 1994) or dihydrolipoic acid (Bustamante et al., 1995), were either toxic to BT cells or else failed to protect them. Differences in the effects of chemically diverse antioxidants have previously been observed in other cell types. Thus, NAC in low millimolar concentrations was shown to induce apoptosis in the human CEM T-cell line (Morse et al., 1995), or dithiocarbamates such as PDTC were reported to trigger apoptosis in thymocytes (Nobel et al., 1995). Moreover, only NAC and PDTC inhibited influenza virus (H3N2 subtype)-induced cell death in J774.1 macrophages (Lowy & Dimitrov, 1997), whereas other antioxidants failed to protect the cells. However, neither NAC nor PDTC protected MDCK cells from apoptosis induced by influenza virus of the H5N9 subtype (Olsen et al., 1996).

Importantly, antioxidants that failed to protect against BVDV-induced cell death also failed to change the intracellular redox status as indicated by DCF fluorescence. This suggests that the protective effect of BHA and ebselen was due to their direct antioxidant action rather than to interference with other pathways, such as the activation of protein kinases (Yu et al., 1997), the release of zinc from metallothionein (Jacob et al., 1998) and the inhibition of nitric oxide synthases (Schewe, 1995). Butylated hydroxytoluene (BHT, an antioxidant homologue to BHA) was reported to reduce the infectivity of Newcastle disease virus in vitro and in vivo (Brugh, 1977), probably by a direct effect on the lipid-containing virus. In contrast, BHA did not interact directly with BVDV, since preincubation of the virus with BHA for 1 h at 37 °C did not reduce virus infectivity (not shown). Additionally, BHT (Gogvadze et al., 1992) and cyclosporine A (CSA) (Schweizer et al., 1993) inhibit ROS-induced Ca²⁺-cycling in mitochondria, an important factor in ROS-induced apoptosis (Richter, 1993). However, BHT was much more toxic to BT cells than was BHA, which is also observed in hepatocytes (Thompson & Moldéus, 1988), and CSA did not inhibit cp BVDV-induced apoptosis (not shown), which excludes a direct effect of BHA on mitochondria and mitochondrial Ca²⁺ homeostasis. However, since BHA and ebselen, which is in particular a mimic of phospholipid hydroperoxide glutathione peroxidase, are both lipophilic antioxidants, their subcellular localization in or close to biomembranes may be important for their effectiveness in inhibiting cp BVDV-induced apoptosis.

The mechanism(s) responsible for the increased level of ROS in BVDV-infected cells is (are) unknown. Oxidative stress is defined as any imbalance between oxidants and antioxidants in favour of the oxidants (Sies, 1997 and references therein). The change in DCF fluorescence, which is indicative of oxidative stress, may thus be due to increased production of ROS, to a decreased capacity of antioxidant defence mechanisms, or to both. The increased activity of NADPH oxidases seems to be a major source of ROS in HL-60 cells and U937 human leukaemic cells induced to apoptosis by irradiation with UV light and by treatment with camptothecin (McGowan et al., 1998). Most of the oxygen taken up by non-myeloid cells is consumed by mitochondria, and 1–2% of the oxygen consumed by mitochondria is reduced to superoxide anion radicals in the respiratory chain (Richter & Schweizer, 1997).
The production of superoxide by mitochondria in L929 fibrosarcoma cells induced to apoptosis by TNF-α was shown to increase dramatically (Henet et al., 1993a). Similar to the apoptosis induced by BVDV, selected antioxidants are capable of inhibiting apoptosis induced by TNF-α (Cossarizza et al., 1995; Goossens et al., 1995; Henet et al., 1993b). The inhibition of cellular protein synthesis observed during infection with cp viruses may also contribute to oxidative stress. Thus, it seems conceivable that the overall inhibition of cellular protein synthesis may reduce the steady-state level of antioxidant enzymes, such as superoxide dismutases or glutathione peroxidases. Moreover, additional mechanisms are known to contribute to a decreased antioxidant capacity, e.g. the extrusion of cytosolic glutathione (Bustamante et al., 1997; Slater et al., 1996) or the downregulation of the transcription of the antioxidant enzyme manganese superoxide dismutase (Briehl & Baker, 1996). DCF, the marker used in this study to analyse the intracellular redox state, may be oxidized by a variety of ROS, among them peroxides, nitric oxide and peroxynitrite (Possel et al., 1997). However, since the latter two compounds were not found to be produced by BT cells (unpublished observations) it seems likely that peroxides may be responsible for the change in DCF fluorescence observed in BT cells infected with cp BVDV.

ROS are part of the cell’s own metabolic regulation (Powis et al., 1995). In contrast to bacteria and parasites, viruses lack the elements of metabolism required for independent replication and, therefore, depend on enzymes in their host cells. Thus, ROS and antioxidative interventions might have consequences for the virus as well as for the host cells. Certain viruses grow better in actively dividing cells than in resting cells, and others, such as herpesviruses, activate in their host cells certain oncogenes which play an important role in cell division (Peterhans, 1994 and references therein). Similarly, BVDV poorly replicates in confluent, non-dividing cells (not shown), and seems to stimulate proliferation of dividing BT cells compared to mock-infected cells (Fig. 1A). The slight (albeit statistically insignificant) decrease in the replication of both cp and ncp BVDV observed in the presence of BHA may relate to the growth-inhibition activity of this antioxidant in BT cells. Accordingly, cp BVDV-infected BT cells in the presence of BHA replicate poorly (Fig. 1B) despite complete inhibition of apoptosis by the antioxidant, and they still show an altered morphology typical of the early stages of cp BVDV infection. This corroborates the finding that antioxidants do not inhibit the induction phase of apoptosis, but rather block downstream events in the execution phase of BVDV-induced cell death.

In summary, our results demonstrate that the cp biotype of BVDV can be prevented from inducing apoptosis by decreasing oxidative stress in the infected cells. As shown with the time-course experiments, the increased intracellular oxidative stress preceded the hallmarks of apoptosis such as PARP cleavage and DNA fragmentation. This indicates that BHA and ebselen affected steps in the mechanism of apoptosis that occur prior to the activation of caspases and the DNase(s) that break(s) down nuclear DNA into the typical oligonucleosomal fragments. Oxidative stress may therefore be a crucial event in the apoptosis induced by cp BVDV.

This work was supported by the Swiss National Science Foundation grant no. 31-39733.93. The generous gift of ebselen from Professor C. Richter (Zurich) is gratefully acknowledged. We thank R. Parham for critically reading the manuscript. Part of this work has been presented at the 4th International Congress of Veterinary Virology in Edinburgh, UK (August 24–27, 1997).

References

hydperoxide-induced Ca$^{2+}$ release from liver mitochondria by inhibiting pyridine nucleotide hydrolysis. Biochemical and Biophysical Research Communications 185, 698–704.


Received 26 October 1998; Accepted 5 January 1999