Inflammatory response of endothelial cells to hepatitis C virus recombinant envelope glycoprotein 2 protein exposure

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*The hepatitis C virus (HCV) encodes approximately 10 different structural and non-structural proteins, including the envelope glycoprotein 2 (E2). HCV proteins, especially the envelope proteins, bind to cell receptors and can damage tissues. Endothelial inflammation is the most important determinant of fibrosis progression and, consequently, cirrhosis. The aim of this study was to evaluate and compare the inflammatory response of endothelial cells to two recombinant forms of the HCV E2 protein produced in different expression systems (*Escherichia coli *and* Pichia pastoris*). We observed the induction of cell death and the production of nitric oxide, hydrogen peroxide, interleukin-8 and vascular endothelial growth factor A in human umbilical vein endothelial cells (HUVECs) stimulated by the two recombinant E2 proteins. The E2-induced apoptosis of HUVECs was confirmed using the molecular marker PARP. The apoptosis rescue observed when the antioxidant* N*-acetylcysteine was used suggests that reactive oxygen species are involved in E2-induced apoptosis. We propose that these proteins are involved in the chronic inflammation caused by HCV.*

Key words: HCV - E2 protein - inflammation - HUVEC

The hepatitis C virus (HCV), a member of the genus *Hepacivirus* in the family Flaviviridae, is a small enveloped virus that possesses a positive-sense single-stranded RNA genome of approximately 9.6 kb (Hoofnagle 2002, Penin et al. 2004, Kaukinen et al. 2013). The genome has a single open reading frame (ORF) (Taylor et al. 2000) encoding a polyprotein precursor of approximately 3,000 amino acid residues that is cleaved by host and viral proteases to generate approximately 10 distinct structural and non-structural proteins (Encke et al. 1998, Penin et al. 2004). One of these proteins is envelope glycoprotein 2 (E2), which undergoes post-translational modification after synthesis and possesses nine-11 potential glycosylation sites (Liu et al. 2001, Whidby et al. 2009). The E2 glycoprotein plays fundamental roles in the initiation of infection at different stages of the replication cycle, including receptor binding, fusion with the host cell membrane and invasion (Bartenschlager & Lohmann 2000, Bartosch et al. 2003, Dubuisson et al. 2008, Lin et al. 2009).

HCV infects approximately 170 million individuals, representing 3% of the world's population (Bian et al. 2009, Burke & Cox 2010, Ruggieri et al. 2013). The World Health Organization estimates that three-four million individuals are infected worldwide every year (Seeff & Hoofnagle 2003). The persistence of the infection and the severity of the resultant inflammation can lead to chronic hepatitis complicated by cirrhosis and/or hepatocellular carcinoma (Ghany et al. 2003, Balasubramanian et al. 2005, Burke & Cox 2010, Kaukinen et al. 2013), making HCV infection one of the most prevalent liver diseases in the world today. HCV infection is responsible for 60% of chronic liver diseases and is the major indication for liver transplants (Lauer & Walker 2001, Whidby et al. 2009).

However, intra-hepatic inflammation appears to be more important than direct viral cytotoxicity in the development of progressive liver injury. Several studies have reported that intra-hepatic inflammation, especially lobular and/or periportal inflammation, is an important determinant of the progression of fibrosis (Zeremski et al. 2007). The cause of endothelial pathology is not well defined, but some hypotheses suggest that several factors may contribute to the inflammatory process, such as nitric oxide (NO), which causes a potential inflammatory lesion in the tissue and increases the expression of chemokines [e.g., interleukin-8 (IL-8)], cytokines and endothelial adhesion molecules, thus amplifying the inflammation cascade (Remick & Villarete 1996, Wald et

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al. 2007). Furthermore, it is believed that HCV proteins, especially the envelope proteins, can be toxic to cells independent of direct viral infection by producing the "innocent bystander" effect (Balasubramanian et al. 2005).

The vascular changes in the cirrhotic livers of patients with chronic hepatitis C have attracted increasing interest because little is known about their relationship with major complications, such as portal hypertension, liver failure and hepatocellular carcinoma; thus, little is known about the prognostic implications of these vascular changes, highlighting the need for a more detailed characterisation of the inflammatory aspects in this scenario. Therefore, the aim of this study was to evaluate and compare the inflammatory response of endothelial cells [human umbilical vein endothelial cells (HUVECs)] to two recombinant forms of the HCV E2 protein produced in different expression systems.

SUBJECTS, MATERIALS AND METHODS

Strains, cell lines and media - *Escherichia coli* DH5α (Invitrogen, USA) was used for the general propagation of plasmids and *E. coli BL21* (*DE3*) was used to express the E2 protein. Bacterial cells were grown under agitation at 37ºC in a low-salt Luria-Bertani medium containing Zeocin at a final concentration of 25 µg/mL. *Pichia pastoris* KM71H$^{(Mut^S)}$ (Invitrogen) was used as the expression host. Yeast cultures were maintained in a yeast extract-peptone-dextrose (YEPD) medium. The media for growth and induction were buffered complex glycerol medium (BMGY) and buffered complex methanol medium (BMMY), respectively, both at pH 4.0 HUVECs (ATCC® CRL-2873™) were grown in RPMI-1640 medium (Sigma Aldrich, USA) containing 10% foetal bovine serum and a mix of antibiotics and antifungals (Sigma Aldrich). The cultures were kept at 37ºC and 5% CO_2 and disassociated from the culture dish using trypsin.

Cloning, expression and purification of E2 protein in E. coli - HCV cDNA was obtained from viral RNA extracted with the QIAmp Viral RNA Mini Kit (QIAGEN, USA), according to the manufacturer's protocol, using pooled sera from individuals with HCV genotype 1a provided by the Laboratory of Clinical Immunology of the Pharmaceutical Science School of Araraquara, São Paulo, Brazil. The HCV sequence was found by comparison using the BLASTn local alignment program and its ORF was entirely sequenced. To express recombinant E2 protein, the soluble form of the protein without the transmembrane domain was selected (residues 384-661). The mature ORF was amplified with the forward primer 5'-GGCCATGGGGGAAACCCACGTCACCGG-3' and reverse primer 5'-GCTCGAGGCTCGGACCTGTCCCTGTC-3' (the underlined bases indicate introduced restriction sites for *NcoI* and *XhoI*, respectively) (Rodríguez-Rodríguez et al. 2009). The pET42a plasmid was used to generate the mature E2 protein ORF flanked by glutathione S-transferase (GST) at the N-terminus and a 6x His tag at the C-terminus. The transformed *E. coli* BL21 were induced for 3 h with isopropylthio-β-galactoside (final concentration 0.4 mM) at 37ºC and 250 rpm when the optical density (OD) at 600 nm reached 0.5. The cells were pelleted, suspended in lysis buffer (10 mM Tris-HCl, 50 mM NaH_2PO_4 and 100 mM NaCl, pH 8.0) and subjected to sonication (5 pulses of 1 min each). The soluble phase was purified using Glutathione Sepharose 4 Fast Flow (GE Healthcare, USA). The binding buffer employed was 10 mM Tris-base, 50 mM sodium phosphate and 100 mM sodium chloride at pH 8.0. The GST-tagged protein was eluted with a two-fold resin volume of elution buffer (10 mM reduced glutathione and 50 mM Tris-HCl, pH 8.0). The fractions containing the purified protein were dialysed against phosphate-buffered saline (PBS) (pH 8.0), quantified using the Pierce™ BCA protein assay kit (Thermo Scientific, USA) and stored at -20ºC. This protein is referred to as E2B in this work.

Cloning, expression and purification of recombinant protein in P. pastoris - The E2 protein ORF was cloned into pPICZαA and the mature ORF was amplified with the forward primer 5'-AAGAATTCGAAACCCACGTCACCGGGGGAA-3' and the reverse primer 5'-AATCTAGATTCTCGGACCTGTCCCTGTCTTCC-3' (the underlined bases indicate introduced *EcoRI* and *XbaI* restriction sites, respectively). The cloning was performed to create a recombinant plasmid containing the E2 protein ORF flanked by the secretion signal peptide (α-factor) at the N-terminus and a 6x His tag at the C-terminus. Before *P. pastoris* transformation, the recombinant plasmid was linearised with *Pme*I endonuclease and introduced into the yeast by electroporation (1.5 kV, 25 µF, 200 Ω) (Cregg 2007). Transformants were cultivated in solid YEPD with 1 M sorbitol and 100 µg/mL Zeocin. The yeast transformants were screened for protein induction in 24-well plates (Boettner et al. 2002). One recombinant yeast colony was selected for protein production and purification. Expression induction for protein purification was performed as described in Generoso et al. (2012), differing only in the use of BMGY and BMMY medium buffered with 100 mM McIlvaine's buffer, pH 4.0. The supernatant was dialysed against PBS buffer (pH 8.0), concentrated using the Labscale TFF System (membrane Pellicon XL50, Millipore, USA) until 10-fold reduction and stored at -20ºC. This protein is referred to as E2Y in this work.

Cell viability - For the determination of cell viability, HUVECs were seeded at 5 x 10^5 cells/mL. The adherent cells were incubated for 24 h at 37ºC and 5% CO_2 with the recombinant proteins (E2B and E2Y) at 250, 125, 62.5, 31.25, 15.63 and 7.81 µg/mL or with 1.0 µg/mL lipopolysaccharide (LPS), 10 ng/mL tumour necrosis factor alpha (TNF-α), 10% sodium dodecyl sulfate (SDS) (positive controls) or RPMI medium and the culture supernatant of *E. coli* BL21 cells (negative controls). After incubation, the cells were incubated with 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (1 mg/mL) for 3 h. The resultant formazan salt was dissolved in acidic 2-propanol and the OD was measured (540/620 nm filters). The OD of the untreated cells was taken as 100% cell viability (Mosmann 1983). The experiments were performed in triplicate.

Cell death - HUVECs were seeded at 5 x 10^4 cells/mL and incubated for 24 h at 37ºC and 5% CO_2 with recombinant E2B and E2Y using the concentrations and controls as described in the *Cell viability* section. The following controls were also added: cells without stimulation (negative) and annexin and propidium iodide (PI) controls. The evaluation of cell death was performed using the Annexin V-FITC Annexin V Apoptosis Detection Kit (BD Pharmingen, USA) according to the manufacturer's protocol. The cells were analysed by flow cytometry (using a FACSCanto flow cytometer, BD Biosciences and FACSDiva software v.6.1.3). In each run, 30,000 cells were analysed and all experiments were performed in triplicate.

N-acetylcysteine (NAC) treatment - The effect of NAC on cells exposed to E2 recombinant proteins was studied with respect to apoptosis (PARP cleavage) and NO and hydrogen peroxide (H_2O_2) production. HUVECs were pre-incubated with 5 mM NAC for 1 h and treated with E2Y, E2B and controls as described above.

PARP cleavage - HUVECs at 5 x 10^5 cells/mL were pre-incubated in the presence or absence of NAC (5 mM) for 1 h and incubated for 24 h at 37ºC and 5% CO_2 with recombinant E2 proteins (E2B and E2Y) using the concentrations and control stimuli described in the *Cell viability* section. HUVECs were lysed in 10 mM Tris (pH 7.4), 1 mM EDTA, 0.5 mM EGTA, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 10 mM $Na_4P_2O_7{\cdot}10H_2O$, 5 µg/mL aprotinin, 5 µg/mL leupeptin and 1 mM PMSF. To evaluate cell apoptosis, 20 µg of lysate protein was electrophoresed in 8% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Hybond ECL). The membranes were blocked with 5% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) and subsequently incubated with rabbit PARP antibody (1:2000 dilution, Santa Cruz Biotechnology, Inc, USA) overnight at 4ºC to detect full-length PARP (116 kDa) and cleaved PARP (carboxyl-terminal catalytic fragment, 89 kDa). After washing with TBS-T for 1 h at room temperature (RT), the membranes were further incubated with a horseradish peroxidase-conjugated rabbit polyclonal antibody (1:2000 dilution; Santa Cruz Biotechnology, Inc) for 2 h followed by 1 h washing (with 3-5 wash buffer changes). Protein bands were visualised with signal reagents. Actin levels were used to control for protein levels and were detected with an antibody against actin (Yang et al. 2004).

NO production - Total NO production was determined in the culture supernatant of HUVECs seeded at 5 x 10^4 cells/mL incubated with E2B and E2Y and controls as described in the *Cell viability* section for 24 h at 37ºC and 5% CO_2. The samples were measured in a NO analyser (Sievers Nitric Oxide Analyzer Overview, model NOA 280i, GE Analytical Instruments, USA), in which the nitrites, nitrates and nitrosothiols present in the supernatant were converted into NO by a saturated solution of vanadium trichloride in 0.8 M HCl at 90ºC. NO was detected by a chemiluminescent reaction in the gas phase between NO and ozone (Archer 1993, Jaiswal et al. 2000).

Arginase activity - Arginase activity was measured using urea. This reaction is based on L-arginine hydrolysis by arginase in cell lysates (Corraliza et al. 1994). Briefly, HUVECs were cultured with recombinant proteins, LPS, TNF-α and culture medium or culture supernatant of *E. coli* BL21 cells using the concentrations and stimuli described in the *Cell viability* section. The cells were lysed using 100 µL of 0.1% Triton X-100 for 30 min under agitation. Subsequently, 50 µL of cell lysate was added to 50 µL of 25 mM Tris-HCl and 25 µL of 100 mM $MnCl_2$ and the final solution was incubated for 10 min at 56ºC for enzyme activation. Next, 50 µL of 0.5 M L-arginine (pH 9.7) was added and the test reaction was incubated at 37ºC for 60 min. The reaction was stopped by adding 400 µL of Stop Solution (96% H_2SO_4, 85% H_3PO_4 and water, at a proportion of 1:3:7 v/v/v). Twenty-five microlitres of 9% α-isonitrosopropiophenone in 100% ethanol was added and the reaction was incubated at 95ºC for 30 min. Finally, the cells were incubated at RT for 10 min and the absorbance was measured using a 540 nm filter. The urea concentration was calculated using a linear equation generated by known quantities of urea. One unit of enzyme activity was defined as the amount of enzyme capable of producing 1 µmol of urea per minute.

H_2O_2 production - HUVECs at 5 x 10^5 cells/mL were incubated for 2 h at 37ºC and 5% CO_2 with recombinant E2B and E2Y using the concentrations and stimuli described in the *Cell viability* section. Approximately 600 ng/mL dihydrorhodamine 123 (DHR) (Sigma-Aldrich) was added and the cells were incubated at 37ºC for 10 min. The cells were washed with PBS (pH 7.2) and centrifuged for 5 min at 300 *g*. The supernatant was discarded and the cells were resuspended in 150 µL of PBS (pH 7.2). The samples were read in the FL1 channel using a FACSCanto flow cytometer (BD Biosciences) and FACSDiva software v.6.1.3. The experiment included a control for spontaneous fluorescence (cells only) and a control for spontaneous production of H_2O_2 (DHR and cells without stimuli) (Walrand et al. 2003).

IL-8, TNF-α and vascular endothelial growth factor A (VEGF-A) production - HUVECs at 5 x 10^4 cells/mL were incubated for 24 h at 37ºC and 5% CO_2 with recombinant E2B and E2Y using the concentrations and controls described in the *Cell viability* section. An additional control using PMA (0.50 µM) was used in the TNF-α detection assay. The negative control consisted of 300 µL of culture medium and 300 µL of PBS (pH 7.2; medium of the protein dilution). Supernatants were collected and centrifuged at 2,860 *g* and 4ºC for 10 min. IL-8, TNF-α and VEGF-A production was measured by ELISA using the kit Human VEGF-A Platinum ELISA (eBioscience Inc, USA), according to the manufacturer's instructions. Cytokine concentrations were calculated using a cytokine calibration curve. The results are expressed in pg/mL.

Statistical analysis - The data were analysed by ANOVA using a 5% level of significance followed by multiple comparisons with the Tukey test and graphic representation of the data. The statistical program GraphPad v.5.00 (GraphPad Software, USA) was used. Values of $p < 0.05$ were considered significant.

RESULTS

The recombinant E2 proteins were expressed in two different expression systems, the *E. coli* Rosetta strain (E2B) and the *P. pastoris* KM71H$^{(Mut^S)}$ strain (E2Y) (Fig. 1). The E2B protein exhibited a molecular weight of approximately 63.5 kDa due to its expression as a fusion protein with GST (26 kDa) and the 6x His tag (1 kDa). The E2Y protein exhibited a molecular weight of approximately 50.0 kDa due to its expression as a fusion protein with the 6x His tag (1 kDa). The N-glycosylation of the E2Y protein was confirmed by protein treatment with a peptide-N-glycosidase, PNGase F (New England Biolabs, USA), according to the manufacturer's protocol. The proteins exhibited different molecular weights (E2B = 36.5 kDa and E2Y = 49 kDa) due to the types of protein processing used in these two systems.

We observed that some concentrations of the recombinant E2 proteins (E2Y: 62.5-250 µg/mL and E2B: 125-250 µg/mL) were slightly cytotoxic to HUVECs (Fig. 2). At 250 µg/mL, the decrease in viability was similar to that obtained when the cells were incubated with LPS or TNF-α. The cellular events provoked by the E2 proteins were evaluated using annexin V and PI assays, which indicated that early apoptosis was the main cause of cell death (Fig. 3). However, even at the highest concentration of E2 protein used in this study, the proportion of apoptotic cells was always lower than 30%.

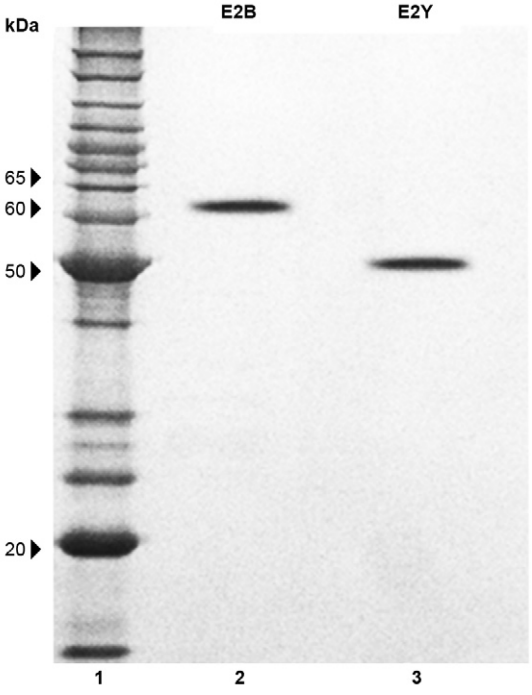

Fig. 1: produced envelope glycoprotein 2 (E2) recombinant proteins (sodium dodecyl sulfate polyacrylamide gel electrophoresis 12%). Channel 1: molecular weight marker [BenchMark™ Protein Ladder (10-220 kDa), Invitrogen]; 2: E2B (approximately 63.5 kDa); 3: E2Y (approximately 50 kDa).

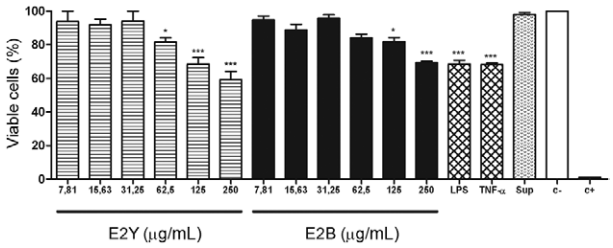

Fig. 2: effect of envelope glycoprotein 2 (E2) recombinant proteins on human umbilical vein endothelial cells viability [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay]. Results are expressed as mean and standard deviation of viable cells. The experiments were performed in triplicate. c-: negative control; c+: culture medium RPMI; LPS: lipopolysaccharide (1.0 µg/mL); Sup: culture supernatant *Escherichia coli* BL21; TNF-α: tumour necrosis factor alpha (10 ng/mL); *: $p < 0.05$ compared to the negative control; ***: $p < 0.001$ compared to the negative control.

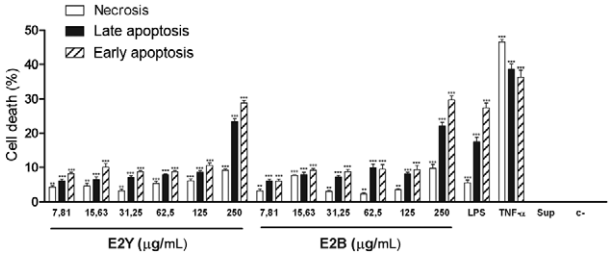

Fig. 3: cytotoxicity of envelope glycoprotein 2 (E2) proteins on human umbilical vein endothelial cells. Results presented as mean and standard deviation of percentage obtained in the assay. In each run, 30,000 cells were analysed and all experiments were performed in triplicate. c-: negative control; early apoptosis: annexin V stained cells; late apoptosis: cells double-positive for annexin V and propidium iodide (PI); LPS: lipopolysaccharide (1.0 µg/mL); necrosis: cells stained with PI; Sup: culture supernatant *Escherichia coli* BL21; TNF-α: tumour necrosis factor alpha (10 ng/mL); ***: $p < 0.001$ compared to the negative control.

To further explore the mechanism of E2 protein-induced apoptosis, we investigated the degradation of PARP, which is thought to be one of the targets of activated caspase-3 or 7 during apoptosis (Yang et al. 2004). Immunoblot analysis revealed that the recombinant E2 proteins induced the degradation of endogenous 116 kDa PARP, as shown by the appearance of 89 kDa fragments (Fig. 4A, B), which were clearly detected in all samples treated with E2 protein or with the control stimuli, TNF-α (Fig. 4E, Line1) and LPS (Fig. 4E, Line 3). These results indicate that PARP cleavage is associated with E2-induced apoptosis in HUVECs. Moreover, the pretreatment of the cells with the antioxidant NAC protected against apoptosis by preventing PARP cleavage (Fig. 4C, D). The same effect was observed for the TNF-α (Fig. 4E, Line 2) and LPS (Fig. 4E, Line 4) controls.

The production of NO by HUVECs was stimulated by E2 protein treatments (Fig. 5). Statistical analysis of the results revealed that there was a statistically significant difference ($p < 0.01$) when compared with the spontaneous control (negative control) or production stimu-

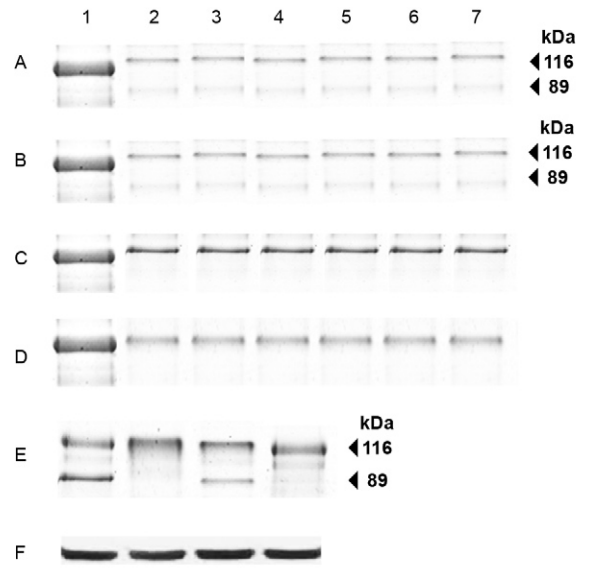

Fig. 4: PARP cleavage in recombinant envelope glycoprotein 2 (E2)-induced apoptosis in human umbilical vein endothelial cells. Cells were pre-treated (C, D) or no (A, B) with *N*-acetylcysteine (NAC) for 1 h and incubated with recombinant E2 proteins, E2Y (A, C) and E2B (B, D) in different concentrations. The experiments were performed in triplicate. Line 2: 7.81 µg/mL; 3: 15.63 µg/mL; 4: 31.25 µg/mL; 5: 62.5 µg/mL; 6: 125 µg/mL; 7: 250 µg/mL; A-D1: lysate of untreated cells; E1: cells without pre-treatment with NAC and treated with tumour necrosis factor alpha (TNF-α); E2: cells pre-treated with NAC and treated with TNF-α; E3: cells without pre-treatment with NAC and treated with lipopolysaccharide (LPS); E4: cells pre-treated with NAC and treated with LPS; F1-4: actin.

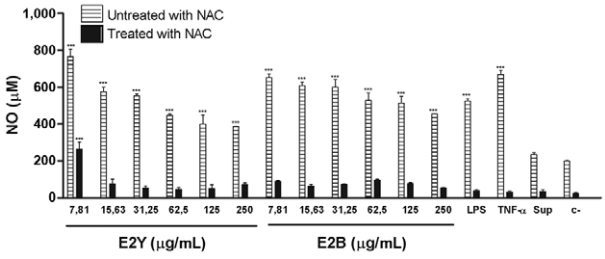

Fig. 5: nitric oxide (NO) production by envelope glycoprotein 2 (E2)-stimulated human umbilical vein endothelial cells. Results are expressed as mean and standard deviation of NO produced in µM. The experiments were performed in triplicate. c-: NO spontaneous production, cells and culture medium; LPS: lipopolysaccharide (1.0 µg/mL); NAC: *N*-acetylcysteine; Sup: culture supernatant *Escherichia coli* BL21; TNF-α: tumour necrosis factor alpha (10 ng/mL); ***: $p < 0.001$ compared to the negative control.

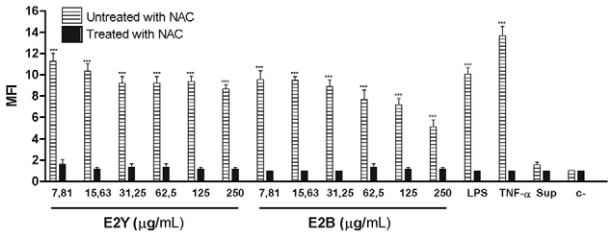

Fig. 6: hydrogen peroxide production by envelope glycoprotein 2 (E2)-stimulated human umbilical vein endothelial cells. Results presented as mean and standard deviation of the mean fluorescence intensity (MFI). The experiments were performed in triplicate. c-: control fluorescence spontaneous, cells without stimulation; LPS: lipopolysaccharide (1.0 µg/mL); NAC: *N*-acetylcysteine; Sup: culture supernatant *Escherichia coli* BL21; TNF-α: tumour necrosis factor alpha (10 ng/mL);***: $p < 0.001$ in relation to negative control.

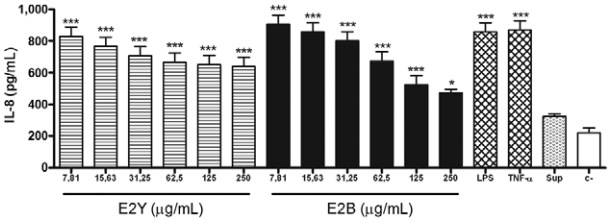

Fig. 7: interleukin-8 (IL-8) production by envelope glycoprotein 2 (E2)-stimulated human umbilical vein endothelial cells. Results presented as mean and standard deviation. The experiments were performed in triplicate. c-: cells and medium and phosphate-buffered saline (pH 7.2). LPS: lipopolysaccharide (1.0 µg/mL); Sup: culture supernatant *Escherichia coli* BL21; TNF-α: tumour necrosis factor alpha (10 ng/mL); ***: $p < 0.001$ compared to negative control; *: $p < 0.05$ compared to negative control.

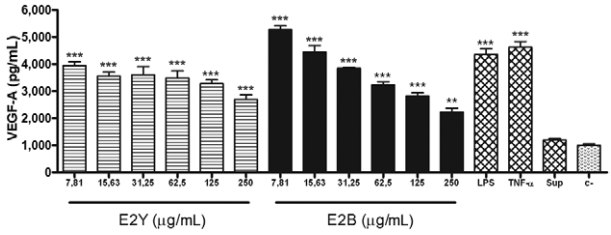

Fig. 8: vascular endothelial growth factor A (VEGF-A) production by envelope glycoprotein 2 (E2)-stimulated human umbilical vein endothelial cells. Results presented as mean and standard deviation. The experiments were performed in triplicate. LPS: lipopolysaccharide (1.0 µg/mL); TNF-α: tumour necrosis factor alpha (10 ng/mL); Sup: culture supernatant *Escherichia coli* BL21; c-: cells and medium and phosphate-buffered saline (pH 7.2); **: $p < 0.01$ compared to the negative control; ***: $p < 0.001$ compared to the negative control.

lated by the bacterial supernatant. The E2 proteins were as effective as the classical stimuli, LPS and TNF-α. One exception was the treatment with 7.81 µg/mL of E2 protein, in which only a slight, but significant elevation in NO was observed relative to the more potent LPS and TNF-α stimuli. The higher production of NO was not the result of increased or decreased arginase activity relative to the negative control (result not shown) because no significant differences were observed when compared with the negative control. Our data also demonstrated that pre-treatment with NAC significantly decreased ($p < 0.01$) the E2 protein-induced NO production.

The production of H_2O_2 was evaluated in HUVECs after exposure to recombinant proteins at different concentrations and control stimuli. The relative production of H_2O_2, calculated as the mean fluorescence intensity, is

presented in Fig. 6. The E2 proteins were able to stimulate the production of H_2O_2 at all of the tested concentrations. Again, the production was similar to that obtained by stimulation with LPS and was inferior to that of TNF-α. Of the two E2 proteins, E2Y was more effective than E2B at concentrations of 7.81 μg/mL ($p < 0.05$). The results of pre-treatment with NAC revealed a significant decrease ($p < 0.01$) in the E2 protein-induced H_2O_2 production.

The E2 proteins were capable of inducing the production of IL-8 compared with non-stimulated cells. The detection of IL-8 production by HUVECs is presented in Fig. 7. There was a statistically significant difference ($p < 0.05$) between all of the stimuli tested compared with the negative control. Unlike the IL-8 results, the E2 proteins were not able to induce the production of TNF-α or LPS by HUVECs. However, 0.50 μM PMA induced HUVECs to produce 173.05 pg/mL TNF-α.

The detection of VEGF-A production by HUVECs in response to control stimuli and recombinant proteins is presented in Fig. 8. The E2 proteins significantly induced ($p < 0.01$) the production of VEGF-A by HUVECs.

The E2 protein-induced production of NO, H_2O_2, IL-8 and VEGF by HUVECs strongly supports the cytotoxicity of these proteins.

DISCUSSION

There is evidence that endothelial cells are directly susceptible to infection by HCV (Fletcher et al. 2012) and that the damage caused by the infection leads to late complications, such as fibrosis, cirrhosis and hepatocellular carcinoma. These late complications are believed to be caused by numerous inflammatory molecules in response to viral infection of the liver (Ming-Ju et al. 2011). Consistent with this hypothesis, we found that E2 proteins were able to induce apoptosis and several inflammatory responses in HUVECs. The putative receptors for E2 proteins in this cellular type have been described previously, including low-density lipoprotein receptor (Agnello et al. 1999), tetraspanin CD81 (Zhang et al. 2004), scavenger receptor class B type 1 (Scarselli et al. 2002), claudin-1 (Evans et al. 2007), occludin (OCLN) (Ploss et al. 2009) and transferrin receptor 1 (TfR1) (Martin & Uprichard 2013).

NO is an inorganic free radical molecule (Furchgott & Zawadzki 1980) that is highly diffusible and reactive (Bredt & Snyder 1992) and is involved in various physiological functions and pathological conditions when produced in excess (Kaufman 1999, Benali-Furet et al. 2005, Deshpande et al. 2012). As a chronic inflammatory disease, hepatitis C induces an increase in NO production (Kane et al. 1997), which may play an important role in the pathogenesis of cirrhosis associated with infection (Hassan et al. 2002). Here, we have demonstrated for the first time that NO production by HUVECs was induced by both recombinant E2 proteins. This NO production may lead to later inflammation in the portal vein and subsequent fibrosis and cirrhosis.

The increased NO production could be the consequence of the increased expression of arginase in HUVECs. HCV infection is associated with the development of hepatocellular carcinoma (Okuda 2007, Tan et al. 2008) and can alter the expression of arginase, thereby stimulating tumourigenesis and hepatocellular carcinoma (Cao et al. 2009). However, this pathway does not appear to be relevant to endothelial cells because arginase expression was not altered by the E2 proteins.

The E2 proteins were also able to induce the production of H_2O_2 by HUVECs. This is additional evidence of the role of E2 in the inflammatory response mediated by HCV. This result is consistent with the findings of Ming-Ju et al. (2011) and suggests the involvement of the E2 protein in H_2O_2 production and the development of inflammation in the hepatic portal vein, with the increased expression of factors related to hepatic fibrosis.

Balasubramanian et al. (2003) reported that the HCV E2 protein was able to stimulate intracellular signalling pathways, leading to the induction of secretion of pro-inflammatory cytokine IL-8. The authors also reported that this production was dose-dependent. IL-8 is also observed in the serum of patients with chronic hepatitis C (Polyak et al. 2001, Akbar et al. 2011), demonstrating a correlation between inflammation, IL-8 serum levels and liver fibrosis (Kaplanski et al. 1997, Mahmood et al. 2002). These findings are evidence that IL-8 may play a role in HCV infection. Consistent with this hypothesis, recombinant E2 proteins stimulated the production of IL-8 in HUVECs. These results are consistent with the work of Balasubramanian et al. (2005). However, the E2 proteins were not able to induce the production of TNF-α by HUVECs. These results are also consistent with the work of Balasubramanian et al. (2005), who reported that HCV proteins can interact with the endothelium and that E2 protein did not induce the production of cytokines such as monocyte chemotactic protein-1, TNF-α and gamma interferon.

Analysis of peripheral blood mononuclear cells and liver biopsy samples of individuals chronically infected by the virus suggests that HCV infection may be able to induce apoptosis, causing damage to the liver while helping the virus to evade the immune system and facilitate viral dissemination (Hiramatsu et al. 1994, Pianko et al. 2001, Chiou et al. 2006). Here, we found that the E2 proteins were also able to induce apoptosis (early and late) as well as necrosis (fewer cells) in HUVECs. Similar results were also observed by Balasubramanian et al. (2005). The glycosylated protein expressed in *P. pastoris* (E2Y) was a more effective inducer of apoptosis as well as necrosis relative to the non-glycosylated protein (E2B), demonstrating the influence of glycosylation on apoptosis. The E2-induced apoptosis of HUVECs was confirmed using the molecular marker PARP. Moreover, the apoptosis induced by recombinant E2 protein was effectively rescued in cells pre-treated with NAC, suggesting that the generation of reactive oxygen species is involved in E2-induced apoptosis in HUVECs. We also suggest that the production of NO, H_2O_2, IL-8 and VEGF-A were not related to cell death induced by high concentrations of the recombinant protein, but are E2-specific effects.

VEGF-A is a potent angiogenic factor that plays a key role in the development of angiogenesis in various tumour types (Toi et al. 2000), including hepatocellular carcinoma (Ng et al. 2001, Poon et al. 2004). VEGF-A has a specific angiogenic effect on endothelial cells

and can be stimulated by HCV infection (Dvorak et al. 1992). Additionally, VEGF-A plays a role in the regulation of several cellular functions, including growth (Nasimuzzaman et al. 2007) and apoptosis (Höglinger et al. 2007). This effect was observed by Hassan et al. (2009) in liver biopsy samples of patients infected with HCV. Hepatic angiogenesis has been described in viral hepatitis, autoimmune liver cirrhosis, primary biliary cirrhosis and hepatocellular carcinoma (García-Monzón et al. 1995, Ker et al. 1999). HCV stimulates the synthesis and secretion of VEGF-A *via* virus-induced oxidative stress (Nasimuzzaman et al. 2007). In our study, the exposure of HUVECs to both recombinant E2 proteins induced the production of VEGF-A. We suggest that oxidative stress, as demonstrated by the production of NO and H_2O_2 in HUVECs in response to E2 proteins, may represent the stimulating factor of VEGF-A production. The literature reports that the HCV core protein is able to stimulate the production of VEGF-A, but there are no data regarding the E2 protein (Hassan et al. 2004, Abe et al. 2012). Therefore, this is the first demonstration that the E2 protein is also able to induce the production of VEGF-A and, consequently, angiogenesis.

HCV is a positive-stranded RNA virus that is unable to integrate its genetic material into the host cell genome. The HCV genome does not contain oncogenes, suggesting that HCV induces hepatocellular carcinoma indirectly by causing chronic inflammation, cell death, proliferation and cirrhosis (Hassan et al. 2009). Here, we provide evidence that endothelial cells, such as HUVECs, are susceptible to E2 HCV envelope proteins.

In conclusion, stimulation with E2 protein induced HUVECs to produce inflammatory and angiogenic factors. Considering that endothelial inflammation is a determinant of fibrosis progression and cirrhosis, we propose that these cellular effects might be involved in the persistence and chronicity of HCV infection. These results may contribute to our understanding of the pathophysiology of hepatitis C and, consequently, to the development of new therapeutic strategies against the interaction of HCV structural proteins and the hepatic endothelium.

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