Bacterial infection of endometrial stromal cells influences bovine herpesvirus 4 immediate early gene activation: a new insight into bacterial and viral interaction for uterine disease

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Abstract

Experimental infection with the γ -herpesvirus bovine herpesvirus 4 (BoHV-4) rarely establishes disease, yet BoHV-4 is commonly associated with uterine disease in cattle. Uterine disease involves co-infection with bacteria such as *Escherichia coli*, which stimulate the production of prostaglandin E₂ (PGE₂) by endometrial cells. BoHV-4 replication depends on immediate early 2 (IE2) gene transactivation and, in the present study, PGE₂, *E. coli* or its lipopolysaccharide upregulated the IE2 gene promoter in uterine cells. Bacterial co-infection is important for BoHV-4 uterine disease.

Reproduction (2008) **136** 361–366

Introduction

Abortion may follow infection with a variety of α -, β - and γ -herpesviruses, but viral causes of uterine disease are seldom investigated in cattle. Although postpartum metritis affects up to 40% of cattle, causing considerable infertility and economic loss, it has been assumed that most diseases are of bacterial origin, and virus isolation or serology is rarely considered (Sheldon & Dobson 2004). The first isolation of bovine herpesvirus 4 (BoHV-4) from a case of bovine metritis was reported in the USA (Park & Kendrick 1973). Later, several other isolates were obtained from cows with reproductive disorders from different countries, including Italy (Castrucci *et al.* 1986). In Belgium, BoHV-4 seroprevalence was associated with postpartum metritis and chronic infertility of cattle (Czaplicki & Thiry 1998). Postpartum metritis has also been associated with BoHV-4 in the USA (Frazier *et al.* 2002), Spain (Monge *et al.* 2006), and Serbia (Nikolin *et al.* 2007). There is a lacuna in the knowledge about the direct correlation between viral infection and uterine pathology.

The BoHV-4 immediate early (IE) genes are expressed immediately during cell infection, do not require prior viral protein synthesis for their expression, and their expression is mediated by the pool of transcription factors made by the cell, already present at the moment of infection and able to transactivate at the

transcriptional level of the IE promoters. BoHV-4 IE2 protein (replication and transcription activator homologous, Rta) encoded by open reading frame 50 (ORF 50) is well conserved among γ -herpesviruses (Zimmermann *et al.* 2001). Rta expression plays a primary role in initiating viral lytic replication, not only during reactivation of latently infected non-permissive cells but also during *de novo* infection of permissive cells (van Santen 1993, Donofrio *et al.* 2004). Viruses are restricted to using the metabolic and biosynthetic pathways of the cells that they infect. These pathways vary between cell types, lineage, stage of differentiation, and with the state of cell activation. There are many examples of viruses that replicate in specific cells and at particular stages of cell growth, differentiation, or activation. This includes the reactivation of cytomegalovirus when host cells differentiate into macrophages, initiation of papillomavirus replication by keratinocytes, and replication of minute virus in testicular cells. The key mechanism mediating these effects is the regulation of viral gene expression at the transcriptional level by host cell factors. In previous studies (Donofrio *et al.* 2007a, 2007b), the interaction, tropism, and outcome of BoHV-4 challenge of endometrial epithelial and stromal cells were investigated. In the present report, to further investigate the mechanisms associated with endometrial tropism, a molecular switch involving the

viral *IE2* gene has been associated with extracellular stimuli belonging to the intrauterine microenvironment, and not as bacterial contamination.

Results

Activation of BoHV-4 promoter in BES cells

To be able to quantify transactivation of the *IE2* promoter in bovine endometrial stromal (BES) cells, a luciferase reporter construct was developed by sub-cloning the BoHV-4 *IE2* promoter in front of the pGL3 Luciferase reporter vector (Promega) ORF to generate pIE2prom-Luc (Fig. 1a). BES cells were co-transfected with pIE2prom-Luc or pGL3 empty vector as a negative control, and pRK-Renilla (Promega) to normalize the efficiency of

Figure 1 (a) Diagram showing the genomic region containing BoHV-4 *IE2* gene (not to scale), where the exon II (white box) contains most of the *IE2* ORF, except the translation initiation codon contained in the exon I (white box) together with the 5' UTR. The splicing donor (SD) and splicing acceptor (SA) sites are indicated. During splicing, the intron is removed and the two exons (I and II) are joined together to generate the *IE2* ORF. Sense and antisense primers used for the PCR amplification of the promoter region sequence (1143 bp) are indicated by arrows, spanning the full promoter containing the TATA box and the first 15 non-coding nucleotides (UTR) of the first exon. The KpnI and NheI restriction sites have been introduced into the 5' end of the primers, for sub-cloning the amplicon in front of the luciferase ORF (grey box) contained into the pGL3 vector. Numbers indicate nucleotide position in the BoHV-4 full genome published sequence (GeneBank accession number: AF318573; Zimmermann *et al.* 2001). (b) *IE2* promoter activity in BES cells at different time post transfection with pIE2prom-Luc or pGL3 empty vector. The data represent the mean relative luciferase units after normalizing with the cotransfected *Renilla* activity. Each reaction was done in quadruplicate, and each point represents the average $\pm$ s.d. from three experiments (* $P < 0.001$).

transfection. Cells were harvested at different times after transfection and luciferase activity was measured. The *IE2* promoter was activated within 12 h of transfection and it reached the maximum level by 48 h (Fig. 1b). The observation that 24 h was in the middle of the linear range of the promoter activation was used to select 24 h after transfection as the optimal time to analyze the *IE2* promoter activity in subsequent experiments.

Escherichia coli stimulates the BoHV-4 *IE2* gene promoter

The capability of bacteria to stimulate the BoHV-4 *IE2* gene promoter was investigated using *E. coli*, because they are the most frequently bacterial isolates from the uterus of postpartum cattle with uterine disease (Olson *et al.* 1984, Sheldon & Dobson 2004). Two strains of *E. coli* isolated from the uterus of postpartum cattle were used: *E. coli* 154 is a pathogenic strain associated with severe metritis and *E. coli* 361 was from a clinically normal animal. The bacteria were heat killed as described previously (Herath *et al.* 2006) and used at a range of concentrations spanning those present in the uterus of postpartum cattle (Dohmen *et al.* 2000, Williams *et al.* 2007). BES cells were challenged for 6 h with the bacteria, starting 24 h after transfection with pIE2prom-Luc or pGL3 empty vector and pTK-Renilla. Both strains of *E. coli* increased *IE2* promoter activity in a concentration-dependent manner (Fig. 2a and b). Much of the host's immune response and pathology associated with *E. coli* is attributable to the bacterial endotoxin, lipopolysaccharide (LPS). LPS exerts its effect by binding to the TLR4/CD14/MD2 receptor complex, present in many cell types, including endometrial cells (Herath *et al.* 2006). To test whether LPS contributed to the activation of the *IE2* promoter by *E. coli*, BES cells were treated for 6 h with *E. coli*, in the presence of different concentrations of the LPS inhibitor polymyxin B (PMB; Sigma), 24 h after transfection with pIE2prom-Luc or pGL3 empty vector and pTK-Renilla. PMB reduced the stimulatory effect of LPS (Fig. 2c and d).

Specific stimulation of the BoHV-4 *IE2* gene promoter by LPS

To further determine the role of LPS in *IE2* activation, the experiment was repeated with pure (O55:B5, Sigma) and ultrapure LPS (O111:B4, Invivogen) from different *E. coli* serotypes, at concentrations reflecting the range measured in the uterine lumen of postpartum cows (Williams *et al.* 2007). Both forms of LPS stimulated the *IE2* promoter in a concentration-dependent manner (Fig. 3a and b). A GFP reporter construct for *IE2* was used to confirm these observations (data not shown). Both forms of LPS increased BES fluorescence, indicating activation of the *IE2* promoter. To determine whether the response was

Figure 2 Transiently transfected BES cells with pIE2prom-Luc (black bars) or pGL3 (gray bars) empty vector for 24 h. The cells were then stimulated for 6 h with 10^3 , 10^4 , 10^5 , 10^7 , or 10^9 CFU/ml *E. coli* isolate 361 (a) or isolate 154 (b). The results are expressed as percentage of induction, where 100% is the untreated control (—). Inhibition of the effect of *E. coli* isolate 361 (c) or isolate 154 (d) by increasing concentration of the LPS antagonist, polymyxin B (PMB). The data represent the mean relative luciferase units after normalizing with the cotransfected *Renilla* activity. Each reaction was done in quadruplicate, and each point represents the average $\pm$ s.d. from three experiments. Values differ significantly from *E. coli*-untreated control (in a and b, $*P < 0.001$) and PMB-untreated control (in c and d, $*P < 0.001$).

associated with LPS and not contamination of the compounds, BES cells were treated with PMB before being challenged with LPS. The IE2 response to LPS was reduced by PMB (Fig. 3c and d), with the greater reduction for O111:B4 probably reflecting the greater purity of this preparation compared with O55:B5.

PGE₂ stimulates the BoHV-4 IE2 gene promoter

Prostaglandin E_2 (PGE_2) may also be involved in the activation of the IE2 promoter when there is co-infection of the uterus with BoHV-4 and *E. coli*. *E. coli* stimulates BES cell secretion of PGE_2 and 6 μ M PGE_2 reactivated BoHV-4 replication in persistently infected bovine

Figure 3 Transiently transfected BES cells with pIE2prom-Luc (black bars) or pGL3 (gray bars) empty vector for 24 h. The cells were then stimulated for 6 h with increasing concentrations of LPS O111:B4 (a) or O55:B5 (b). The results are expressed as percentage of induction, where 100% is the untreated control (—). Inhibition of 100 ng/ml O111:B4 (c) or O55:B5 LPS (d) activity by increasing concentration of PMB. The data represent the mean relative luciferase units after normalizing with the cotransfected *Renilla* activity. Each reaction was done in quadruplicate, and each point represents the average $\pm$ s.d. from three experiments. Values differ significantly from LPS-untreated control (in a and b, $*P < 0.001$) and PMB-untreated control (in c and d, $*P < 0.001$).

macrophages (Donofrio *et al.* 2005, Herath *et al.* 2006). Hence, BES cells were treated for 6 h with different concentrations of PGE₂ (Sigma), 24 h after transfection with pIE2prom-Luc and pTK-Renilla. The *IE2* promoter was stimulated by 0.4 µM PGE₂, with maximal stimulation by 2–10 µM PGE₂ (Fig. 4a).

Independent and co-stimulatory effect of LPS and PGE₂ on the BoHV-4 *IE2* gene promoter

To explore whether *IE2* activation by LPS and PGE₂ is independent, BES cells were challenged with LPS, PGE₂, or their combination, 24 h after transfection with pIE2prom-Luc and pTK-Renilla, and 12 h after treatment with a pan-cyclooxygenase (COX) inhibitor (250 µM indomethacin; Sigma) or vehicle. LPS and PGE₂ independently stimulated *IE2* expression as expected. However, the presence of indomethacin did not block the effect of LPS (Fig. 4b), so LPS may activate *IE2* independent of PGE₂. The combination of LPS and PGE₂ appeared to exhibit a co-stimulatory effect of *IE2*.

Discussion

Bos taurus is particularly prone to uterine infection and metritis after parturition, (Sheldon & Dobson 2004). The most commonly recognized uterine pathogen is *E. coli* (Olson *et al.* 1984, Dohmen *et al.* 2000, Sheldon *et al.* 2002, Sheldon & Dobson 2004, Williams *et al.* 2007). However, BoHV-4 is also consistently associated with metritis. Bacteria may stimulate BoHV-4 replication of the virus following the recruitment from the bloodstream to the site of inflammation of macrophages persistently infected with BoHV-4. This theory may explain how BoHV-4 can be isolated from healthy animals in the absence of inflammation.

Assuming *IE2* is the molecular master switch for BoHV-4 replication, the capability of endometrial cells to transactivate the *IE2* promoter was previously investigated by transient transfection of an EGFP-labeled construct containing the *IE2* gene promoter and electroporated into endometrial epithelial and stromal cells, BT, BEL, MDBK, and BEK cells. EGFP started to accumulate robustly as soon as 24 h after electroporation in the cytoplasm of stromal cells, in contrast to the other cell types where weak visible green cells appeared not before 3 days post electroporation (Donofrio *et al.* 2007a, 2007b). The present study provides evidence that bacterial co-infection is important for the activation of the *IE2* gene promoter in BES cells, necessary to activate the BoHV-4 lytic replication associated with uterine disease. Bacterial contamination of the uterine lumen is ubiquitous in postpartum cattle (Sheldon & Dobson 2004); BoHV-4 metritis is consistently associated with bacterial co-infection (Park & Kendrick 1973, Castrucci *et al.* 1986, Mehrotra *et al.* 1986, Czaplicki & Thiry 1998, Frazier *et al.* 2002, Monge *et al.* 2006, Nikolin *et al.* 2007). Bacterial infection stimulates PTGS2

Figure 4 Transiently transfected BES cells with pIE2prom-Luc (black bars) for 24 h. (a) The cells were then stimulated with increasing concentrations of PGE₂ for 6 h. The results are expressed as percentage of induction, where 100% is the untreated control (—). (b) In a parallel experiment, BES cells were untreated or pre-treated with 250 µM indomethacin and then stimulated with PGE₂, LPS, or a combination of LPS and PGE₂. The data represent the mean relative luciferase units after normalizing with the cotransfected *Renilla* activity. Each reaction was done in quadruplicate, and each point represents the average $\pm$ s.d. from three experiments (* $P < 0.001$).

(COX2) expression and production of PGE₂ in bovine endometrial cells (Herath *et al.* 2006). The role of PGE₂ on *IE2* activation is particularly important in the uterus, as *E. coli* or LPS induces PTGS2 expression and PGE₂ production by BES cells (Herath *et al.* 2006). Indeed, LPS stimulates sufficient PGE₂ secretion (~ 0.3 µM) to activate *IE2* expression. Furthermore, BoHV-4 also induces PTGS2 protein expression and PGE₂ secretion in BES cells (Donofrio *et al.* 2007a, 2007b).

Here, we show that *E. coli*, LPS, and PGE₂ activate the BoHV-4 *IE2* gene promoter, and this probably involves PGE₂-dependent and -independent pathways. These observations provide a plausible mechanism underlying the rapid activation of viral replication in the bovine endometrium associated with uterine disease. Indeed, there could be a positive feedback loop between PGE₂ production and viral replication, initiated by the bacterial co-infection. This mechanism could be

important in other tissues other than the uterus, where bacterial and viral infections coexist. Identification and exploration of the underlying mechanisms of viral–bacterial synergism will provide targets for prevention and treatment using drugs and vaccines.

Materials and Methods

Reagents

Purified LPS (from *E. coli*, O55:B5 and O111:B4), PGE₂, indomethacin, and PMB were purchased from Sigma–Aldrich. Heat-killed *E. coli* isolate 361 and 154 were isolated from a normal animal and case of clinical bovine endometritis associated with pyrexia, respectively, as described (Sheldon *et al.* 2002).

Endometrial cell isolation and primary cultures

Six bovine uteri from post-pubertal, non-pregnant, BoHV-4 serum-negative animals with no evidence of genital disease were collected at a local abattoir immediately after slaughter and kept on ice until further processing in the laboratory. The physiological stage of the reproductive cycle for each genital tract was determined by observation of the ovarian morphology. Genital tracts with an ovarian stage I corpus luteum were selected for endometrial tissue and cell culture, and only the horn ipsilateral to the corpus luteum was used.

The endometrium was cut into strips and placed into serum-free RPMI-1640 (Sigma), supplemented with 50 IU/ml penicillin, 50 µg/ml streptomycin, and 2.5 µg/ml amphotericin B (Sigma), working under sterile conditions. The strips were then chopped into 1 mm³ pieces and placed into HBSS (Sigma) and used as previously described (Fortier *et al.* 1988) with the following modifications. Briefly, tissue was digested in 25 ml sterile-filtered digestive solution, which was made by dissolving 50 mg trypsin III (Roche), 50 mg collagenase II (Sigma), 100 mg BSA (Sigma), and 10 mg DNase I (Sigma) in 100 ml phenol red-free HBSS. Following a 1.5-h incubation in a shaking water bath at 37 °C, the cell suspension was filtered through a 40 µm mesh (Fisher Scientific, London, UK) to remove undigested material and the filtrate was resuspended in phenol red-free HBSS containing 10% FBS (Sigma) and 3 µg/ml trypsin inhibitor (Sigma) (washing medium). The suspension was centrifuged at 100 *g* for 10 min and, following two further washes in washing medium, the cells were resuspended in RPMI-1640 containing 10% FBS, 50 IU/ml penicillin, 50 µg/ml streptomycin, and 2.5 µg/ml amphotericin B. The cells were plated at a density of 1 × 10⁵ cells in 2 ml per well using 24-well plates (Nunc, London, UK). To obtain separate stromal and epithelial cell populations, the cell suspension was removed 18 h after plating, which allowed selective attachment of stromal cells (Fortier *et al.* 1988). The absence of immune cells in the uterine cell cultures was confirmed by RT-PCR for the CD45 pan-leukocyte marker, as previously described (Herath *et al.* 2006). The culture media were changed every 48 h until the cells reached confluence. All cultures were maintained at 37 °C, 5% CO₂ in air, in a humidified incubator.

Plasmids

The *IE2* promoter region of the BoHV-4 genome (nucleotide 61 391–62 534; GenBank accession number AF318571; Zimmermann *et al.* 2001) was generated by PCR, using total DNA isolated from BoHV-4-infected MDBK cells as template and a pair of *IE2* promoter primers (sense: 5'-AAACCCGGTACCG-CCAGTGCCAAGCTTTTAAG-3'; antisense: 5'-GGGAAGTAGC-TAGCCTGTTGTTCTGCTCCCTTTTA-3') containing an artificial KpnI site on the 5' end and a NheI site on the 3' end respectively.

One microgram sample DNA was amplified over 35 cycles, each cycle consisting of denaturation at 94 °C for 1 min, primer annealing at 55 °C for 1 min, and chain elongation with High Fidelity PCR Enzyme Mix (Fermentas, St Leon-Rot, Germany) at 72 °C for 2 min. PCR amplification was carried out in a final volume of 50 µl, containing 0.2 mM deoxynucleoside triphosphate and 0.25 µM of each primer. In the first cycle, the samples were denatured at 94 °C for 5 min, and in the last cycle, the extension step was increased to 7 min. The amplicon was column purified, KpnI, NheI digested, and sub-cloned into pGL3 Basic vector (Promega) to generate pIE2prom-Luc. pTK-Renilla were obtained from Promega. All constructs were sequenced to guarantee the fidelity of the PCR products.

Transient transfection

Confluent BES cells in 24-well plates were co-transfected with 0.5 µg pIE2prom-Luc or 0.5 µg pGL3 empty vector, as a negative control and 0.05 µg pRK-Renilla to normalize the efficiency of transfection, using lipofectamine (LTX) transfection reagent (Invitrogen) as suggested by manufacturer. Transfection mixture was prepared in DMEM without serum and antibiotics and left on the cells for 6 h at 37 °C, 5% CO₂ in air, in a humidified incubator. After 6 h, the transfection mixture was replaced with complete medium (RPMI-1640, 10% FBS, 50 IU/ml penicillin, 50 µg/ml streptomycin, and 2.5 µg/ml amphotericin B) and left to recover for 18 h at 37 °C, 5% CO₂ in air, in a humidified incubator. After 24 h of transfection, cells were treated with different reagents for period.

Luciferase reporter assay

Luciferase reporter assay was performed with a Dual Luciferase Reporter Assay System kit (Promega) with minor modifications. Following treatments, cells were washed with PBS, lysed with 100 µl lysis passive buffer by freeze-thawing at –80 °C. According to the manufacturer's specifications, 10 µl of the cell lysate was added to 50 µl LAR, and luciferase activity was determined with a Perkin–Elmer Victor³ Multilabel Counter (Perkin–Elmer, Milan, Italy). Individual assays were normalized for *Renilla* luciferase activity with a second reading, adding 50 µl Stop & Glo substrate.

Statistical analysis

Experiments were performed with four replicates at each time point and each experiment was repeated three times. Statistical differences were tested by ANOVA.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

Sheldon is a BBSRC Research Development Fellow (Grant No. BB/D02028X/1). Donofrio was supported by a Royal Society International Incoming Short Visit.

Acknowledgements

We would like to thank the Italian Ministry of University and Scientific Research (Italian National Grant MIUR, PRIN 2005, 2005078885), the Fondazione Cariparma (Cassa di Risparmio di Parma, Italy).

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Received 21 April 2008

First decision 21 May 2008

Accepted 23 June 2008