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Antimicrobial effects of plant defence peptides expressed by bovine endothelial cells on intracellular pathogens

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Abstract

Background: The actions of plant antimicrobial peptides (PAP) against intracellular pathogens are poorly known. It has been reported that wheat puroindolines show antibacterial activity against *Staphylococcus epidermidis* endocyted by macrophages. In this work, we evaluated the intracellular antimicrobial activity of PAP γ-thionin and thionin Thi2.1 produced by bovine endothelial cells against intracellular *Staphylococcus aureus* and *Candida albicans.* We used three host-pathogen models: 1) bovine mammary epithelial cells (BMEC)-*S. aureus*, 2) bovine endothelial cells (BEC)-*S. aureus* and 3) BEC-*C. albicans*, and evaluated the effect of conditioned media from BEC producers of PAP (γ-thionin and thionin Thi2.1). **Results:** In the first model, conditioned medium (CM) containing Thi2.1 completely inhibited *S. aureus* intracellular after 24 hrs treatment. In the second model, CM from BEC containing γ-thionin has a better effect killing intracellular *S. aureus* for 12-24 hrs incubations than CM from endothelial cells producers of Thi2.1; this was related with an increase of nitric oxide production (~2 times) in BEC infected and treated for 12 hrs with CM containing γ-thionin, which negatively correlates with bacterial viability. In the third model, CM containing Thi2.1 showed a more potent intracellular fungicidal activity (~85% of inhibition) at 24 hrs treatment than CM containing γ-thionin (~35% of inhibition). **Conclusions:** This work shows new effects of PAP to control intracellular bacterial or fungal infections.

Keywords: antimicrobial peptides, *Arabidopsis thaliana* thionin, *Capsicum chinense* γ-thionin, *Candida albicans*, intracellular pathogens, *Staphylococcus aureus*

INTRODUCTION

Infectious diseases caused by bacterial and fungal pathogens are one of the leading causes of morbidity and mortality worldwide; most of them have evolved to parasitize mammalian cells, which include epithelial and endothelial cells that form the natural cellular defence barriers (Alonso and García del Portillo, 2004). Intracellular colonization allows microorganisms to evade the immune system and the antimicrobial treatment, favouring the increase of resistant microbial strains, which constitutes a severe health problem (Radtke and O'Riordan, 2006).

Staphylococcus aureus is one of the most important human pathogens worldwide. It can be found as a part of the normal animal flora. However, infections by *S. aureus* are a worldwide public health concern and represents a major problem for the dairy industry; moreover, the existence of highly virulent

methicillin-resistant *S. aureus* (MRSA) strains constitutes a serious public health problem (Kerro-Dego et al. 2002; Sinha and Herrmann, 2005; Garzoni and Kelley, 2009). This bacterium has the ability to colonize the skin and mucous membranes (Sinha and Herrmann, 2005; Garzoni and Kelley, 2009). *S. aureus* can also persist intracellularly into bovine mammary epithelium causing mastitis, whereas in humans the invasion of endothelial cells allows it to infect almost any organ (Kerro-Dego et al. 2002; Sinha and Herrmann, 2005).

C. albicans is an opportunistic fungal pathogen that may be present as a normal component of the human microflora. It is responsible for a variety of diseases in the immunocompromised or immunosuppressed host ranging from superficial to systemic mycoses (Cotter and Kavanagh, 2000). During systemic infections, vascular endothelium plays a critical role, since *C. albicans* is able to adhere, penetrate and damage endothelial cells to gain access to other tissues (Filler et al. 1995).

C. albicans can exist in two morphologically distinct forms, budding blastospores or hyphae. The hyphal form may help to *C. albicans* to invade the endothelial cells and is considered as one of the *C. albicans* virulence factors (Cotter and Kavanagh, 2000). Thus, systemic candidiasis constitutes the second most common cause of death from hospital-acquired infections, only overcome by MRSA strains. Candidiasis involves blood dissemination of *C. albicans* to multiple organs that favours cell invasion by this fungus (Hadley et al. 2002; Grubb et al. 2009).

Difficulties to control *S. aureus* and *C. albicans* infections have made necessary to find new therapeutical alternatives. In this sense, the use of antimicrobial peptides (AP) is an attractive option. AP have been isolated from microorganisms, invertebrates, plants, amphibians, birds, fish, and mammals (including humans), where they represent a defence system against invading pathogenic microorganisms (Dawson and Liu, 2008). AP actions include direct inhibition of microbial pathogens combined with immune system stimulation (Nicolas, 2009).

Antimicrobial peptides from plants (PAP) are classified based on the identity of their amino acid sequence and the number and position of cysteines forming disulfide bonds. So far, 10 families have been described in plants, (García-Olmedo et al. 2001; Lay and Anderson, 2005). These include lipid transfer peptides (LTPs), thionins, defensins, hevein and knottin like proteins, as well as antimicrobial proteins isolated from *Macadamia integrifolia* (MBP-1) and *Impatiens balsamina* (Ib-AMP). All these AP exert their effect at the plasma membrane of the microorganisms that they attack, although their action mechanisms vary depending on the family.

Thionins were the first PAP whose antimicrobial activity against plant pathogens was demonstrated *in vitro* (García-Olmedo et al. 2001). This class of molecules has been found in various plant tissues, such as the seed endosperm, the stem and roots; they present a three-dimensional structure that can be represented by gamma letter (γ), where the vertical portion consists of a pair of antiparallel α-helices and the short horizontal arm consists of an antiparallel β-sheet (Thevissen et al. 2007). This structure is stabilized by six to eight disulfide-linked cysteins (Thomma et al. 2002; Padovan et al. 2010). They are involved in protection against plant pathogens and are toxic to bacteria and phytopathogenic fungi (Vignutelli et al. 1998; Zasloff, 2002; Stec, 2006). It has been suggested that toxicity requires the electrostatic interaction of the thionins with the negative charges of the membrane, causing the formation of pores (Thevissen et al. 2007). *Arabidopsis thaliana* expresses the thionin Thi2.1, which is constitutively induced after pathogen attack (Epple et al. 1995). Zárate et al. (2007) have described that Thi2.1 transcript is regulated by jasmonic acid and ethylene during *Bemisia tabaci* infestation. This AP is composed of 44 amino acids stabilized by six disulfide-linked cysteins (Table 1).

Otherwise, plant defensins are cationic PAP with an approximate molecular weight of 5-7 kDa, composed of 45 to 54 amino acids. They are structurally and functionally related to defensins described in mammals and insects. These AP have been found in all studied plants, even it is hypothesized that they are ubiquitous in the plant kingdom. Plant defensins possess a pattern of eight conserved cystein residues, and exhibit a predominant antifungal activity (Carvalho and Gomes, 2009). In *Capsicum chinense* Aluru et al. (1999) have obtained the cDNA from a defensin denominated γ-thionin. This defensin is 50 amino acids long and is stabilized by four disulfide-linked cysteins (Table 1).

Table 1. Characteristics of AP used in this work.

Class	Name	Source	Residue number (madure peptide)	Hydropathy	Disulfide bridges[a]	Construction (pTracer- cDNA-fusion protein)[b]	Produced as a prepeptide	Cell line that produces AP	Activity	Reference
Plant defensins	γ-thionin	*Capsicum chinense*	50	-0,790	4	pTracer-γ- thionin[c]	Yes	BVE-γ-thionin	*Candida albicans* HeLa cells *F. oxisporum*	(Anaya-López et al. 2006a)
Plant thionins	Thi2.1	*Arabidopsis thaliana*	44	-0,570	3	pTracer-Thi2.1-V5 epitope-Hys 6X	Yes	BVE-Thi2.1	*E. coli* *S. aureus* *C. albicans* Mammal cells	(Loeza-Ángeles et al. 2008)

[a]Putative pattern determined by using Ceroni et al. (2006).
[b]pTracer was obtained from Invitrogen.
[c]Not produced as fusion protein.

Through biotechnological approaches, defensin γ-thionin from *C. chinense* and thionin Thi2.1 from *A. thaliana* have been expressed in bovine endothelial cells (BEC) in our working group; which showed activity against bacteria, fungi and tumor cells (Anaya-López et al. 2006a; Loeza-Ángeles et al. 2008). Using this model, we have demonstrated that conditioned medium (CM) from BEC producers of defensin γ-thionin from *C. chinense* shows activity against *C. albicans*, and CM from BEC producers of thionin Thi2.1 from *A. thaliana* inhibits the growth of *S. aureus, Escherichia coli* and *C. albicans.*

In spite of the growing knowledge related to the PAP effects, their actions against intracellular pathogens are poorly known. In this sense, Capparelli et al. (2007) have reported that wheat puroindolines show antibacterial activity against *Staphylococcus epidermidis* endocyted by macrophages.

The actions of defensin γ-thionin from *C. chinense* and thionin Thi2.1 from *A. thaliana* against internalized pathogens in mammal cells are unknown. In the present work we used the following host-pathogen models in order to explore the intracellular effects of conditioned media of BEC producers of γ-thionin and thionin Thi2.1: 1) bovine mammary epithelial cells (BMEC)-*S. aureus*, 2) BEC-*S. aureus* and 3) BEC-*C. albicans*. The invasion ability of *S. aureus* and *C. albicans* has been previously demonstrated in these cells (Anaya-López et al. 2006b; Oviedo-Boyso et al. 2008). Besides their antimicrobial activity, it has been reported that AP have immunomodulatory roles. However, these effects have not been studied in PAP. One of these activities could be the production of nitric oxide (NO), which plays many roles in the immune system, and also can be produced by endothelial or epithelial cells to kill pathogens directly (Bogdan, 2001). In order to explore a possible mechanism by which PAP used in this work could be attacking intracellular pathogens, we analyzed NO production by host cells in response to conditioned media of BEC producers of PAP.

MATERIALS AND METHODS

Organisms

The American Type Culture Collection (ATCC) *S. aureus* subsp. *aureus* 27543 strain (isolated from a case of clinical mastitis) and *C. albicans* ATCC 10231 strain were used to carried out the assays. Bacteria were grown at 37ºC overnight in Luria-Bertani broth (LB, Bioxon, México) and colony forming units (CFU) were adjusted by measuring the optical density at 600 nm. *C. albicans* was grown overnight at 30ºC in YPD broth (2% dextrose, 2% Bacto peptone, 1% yeast extract; Difco).

Conditioned media of antimicrobial peptide-producer cell

To obtain the conditioned media, immortalized bovine endothelial cells BVE-E6E7 (Cajero-Juárez et al. 2002) producers of γ-thionin (BVE-γ-thionin) and thionin Thi2.1 (BVE-Thi2.1) were grown at confluence in p100 Petri dishes (Costar). Culture medium was replaced with 5 ml of Optimem medium (Gibco) without serum and antibiotics. Then, cells were cultured during 24 hrs. Conditioned media were clarified by centrifugation (10 min, 1200 x *g*). Protein concentration was determined by Bradford method (Bradford, 1976). 3.125 µg/ml of protein in conditioned medium (CM) were used in all experiments based on the antimicrobial activity reported previously (Loeza-Ángeles et al. 2008). The CM of BVE-E6E7 non-transfected (BVE-NT) was used as a control. The characteristics of the PAP used in this work are shown in Table 1.

Cell cultures

BVE-E6E7 was used to perform infection assays with *S. aureus* and *C. albicans*. Cells were routinely cultured as reported previously (Loeza-Ángeles et al. 2008).

Primary bovine mammary epithelial cells (BMEC) were used to carry out infection assays with *S. aureus*. Their isolation and culture was performed as described previously (Anaya-López et al. 2006a).

Infection assays

To perform infection assays with *S. aureus*, BEC or BMEC were grown at confluence on 24 well plates. Polarized monolayers of BMEC were created on plates coated with 6-10 µg/cm^2 of rat-tail type I collagen (Sigma). BEC or BMEC confluent monolayers were infected with a multiplicity of infection (MOI) of 30:1 bacteria per cell. For this, cells were washed three times with phosphate buffer saline (PBS, pH 7.4), inoculated with bacterial suspensions in LB broth from overnight cultures, and incubated for 2 hrs in 5% CO_2 at 37ºC. After infection, BEC and BMEC monolayers were washed three times with PBS and incubated in culture medium without serum supplemented with 50 µg/ml of gentamicin for 2 hrs at 37ºC to eliminate extracellular bacteria.

Subsequently, BEC and BMEC monolayers were washed three times with PBS, and incubated with the different conditioned media for 2, 6, 12 and 24 hrs. CM from BVE-NT was employed as control. Negative control consists in 50 µg/ml of gentamicin during all the times evaluated. Finally, cells were detached with trypsin-EDTA (Sigma) and lysed with 250 µl of sterile distilled water. Cell lysates were diluted 100-fold, plated on LB agar for triplicate and incubated overnight at 37ºC. The CFU number was determined by the standard colony counting technique.

To achieve infection assays with *C. albicans*, BEC were placed on 24 well plates. 2.5 X 10^5 yeast cells/well from an overnight culture were used as inoculum and were incubated with BEC for 4 hrs in 5% CO_2 at 37ºC. Then, infected cells were washed three times with PBS and incubated with 1 ng/ml of amphotericin B by 2 hrs. BEC were incubated with the different conditioned media for 2, 12 and 24 hrs. CM from BVE-NT was employed as control. Negative control consists in 1 ng/ml of amphotericin B during all the times evaluated. Monolayers were detached with trypsin-EDTA (Sigma) and lysed with 250 µl of sterile distilled water. BEC lysates were diluted 100-fold, plated in triplicate on YPD agar and incubated overnight at 37ºC. The number of CFU was determined by the standard colony counting technique.

Determination of nitrite concentration

Nitric oxide (NO) secreted by BEC and BMEC to culture medium was evaluated by measuring the nitrite concentration (NO_2^-) in cell-free media using the Greiss reaction (Gutiérrez-Barroso et al. 2008). NO was estimated in infection assays described above as well as in non-infected cells. Nitrite concentration in conditioned media from BEC producers of PAP was also estimated. After infection assays, medium was filtered through 0.22 µM membranes (Millipore) to eliminate bacteria or fungi.

Data analysis

Experiments were running on triplicate and data were compared by one-way ANOVA or Student's *t* test using the software StatPlus for Mac (v. 2009). Correlations were calculated using Pearson's coefficient. The results are reported as means ± the standard errors (SEM).

RESULTS

Effect of conditioned media against internalized *S. aureus* in BMEC

In this work, we evaluated the effects of conditioned media from BEC producing γ-thionin and thionin Thi2.1 against *S. aureus* and *C. albicans* internalized in mammal cells. Previously, we have demonstrated that CM from BVE-γ-thionin does not have cytotoxic effects against BMEC or BEC (data not shown). However, CM from BVE- Thi2.1 decreased the viability of BMEC or BEC after 24 hrs treatment (Loeza-Ángeles et al. 2008). For this reason, we performed all experiments until 24 hrs. In the first host-pathogen model tested, we analyzed the activity of conditioned media against *S. aureus* internalized in BMEC. According to CFU recovered, we showed that CM from BVE-Thi2.1 reduces significantly (~80%) intracellular viability of *S. aureus* at 2 hrs of treatment, considering the effect of CM from BVE-NT as 100% (Figure 1). Interestingly, no CFU were recovered at 24 hrs. Otherwise, CM of BVE-γ-thionin caused a reduction of ~80% in CFU recovered at 2 hrs; this decrease remained constant in all evaluated times (Figure 1).

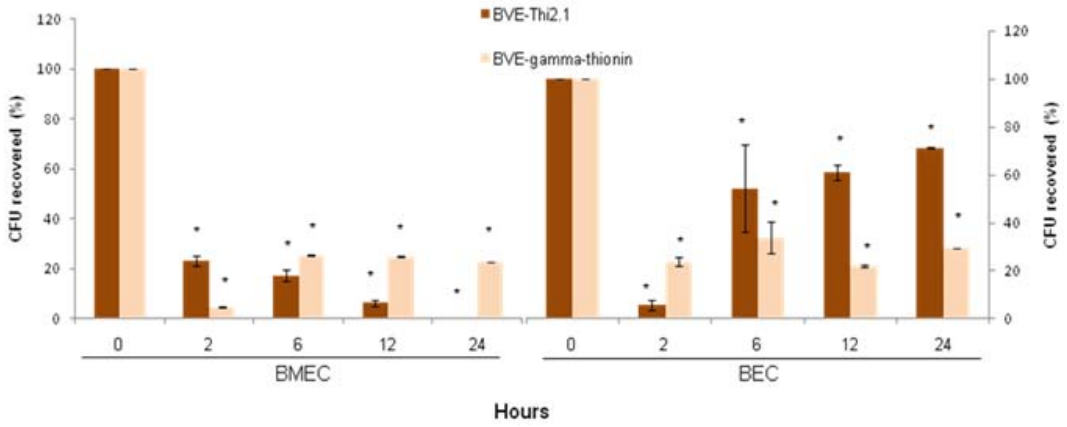

Fig. 1 Number of *Staphylococcus aureus* internalized in BMEC and BEC in the presence of conditioned media. BMEC or BEC were infected during 2 hrs with *S. aureus*, and then were treated with gentamicin for 2 hrs and incubated with 3.125 µg/ml of protein of diverse conditioned media for different times. Bars are the percentage of recovered CFU after BMEC or BEC lysis considering as 100% the effect of CM from BV

Effect of conditioned media against internalized *S. aureus* in BEC

In Figure 1, we also showed the effect of conditioned media from BVE-γ-thionin and BVE-Thi2.1 against *S. aureus* internalized in BEC. CM from BVE-Thi2.1 caused an inhibition in bacterial CFU recovered (~90%) during the first 2 hrs, but after 6 hrs an increase in CFU recovered was observed; in this case the inhibition was only ~30-50%. In other hand, CM of BVE-γ-thionin inhibited *S. aureus* CFU recovered (70-80%) during 12-24 hrs treatments.

Effect of conditioned media against internalized *C. albicans* in BEC

Using the host-pathogen model BEC-*C. albicans*, we analyzed the effect of conditioned media from BVE-γ-thionin and BVE-Thi2.1 against internalized yeast. We observed that CM from BVE-Thi2.1 reduced the number of CFU recovered (~40%) during incubations of 12-24 hrs (Figure 2). A similar behaviour was detected with CM from BVE-γ-thionin. Curiously, we detected an induction of *C. albicans* intracellular proliferation during conditioned media incubations of 2 hrs, being more evident with CM from BVE-Thi2.1.

Effect of conditioned media on NO production

In order to explore if intracellular antimicrobial effects of conditioned media from BVE-γ-thionin and BVE-Thi2.1 are direct responses against pathogens, or indirect ones by means of the regulation of host cell innate immune response, we analyzed NO production in the host-pathogen models described, in response to conditioned media of BEC producers of PAP. Table 2 shows NO production by BMEC infected with *S. aureus*. The basal NO production in the presence of the different conditioned media was not modified, which suggests that conditioned media do not regulate this process. However, upon infection with *S. aureus* NO production raised significantly at 6 and 24 hrs (~2.5 fold) in respect to basal production. Furthermore, NO production was not modified significantly when conditioned media from cells that express PAP were added to infected cells, which supports the idea that the increase in NO production is attributable mainly to infection; only at 12 hrs of treatment we observed a significant increase in NO production as result of conditioned media addition. In spite of this, the reduction in the number of CFU recovered from BMEC treated with CM from BVE-Thi2.1 (Figure 1), does not correlate with NO production (Pearson's coefficient -0.04365 for all the times evaluated), whereas there is a positive correlation in the number of CFU recovered and NO production in BMEC treated with CM from BVE-γ-thionin (Pearson's coefficient 0.61176 for all the times evaluated). This result suggests that this peptide could carry out the antimicrobial activity through the activation of defence mechanisms different from NO production.

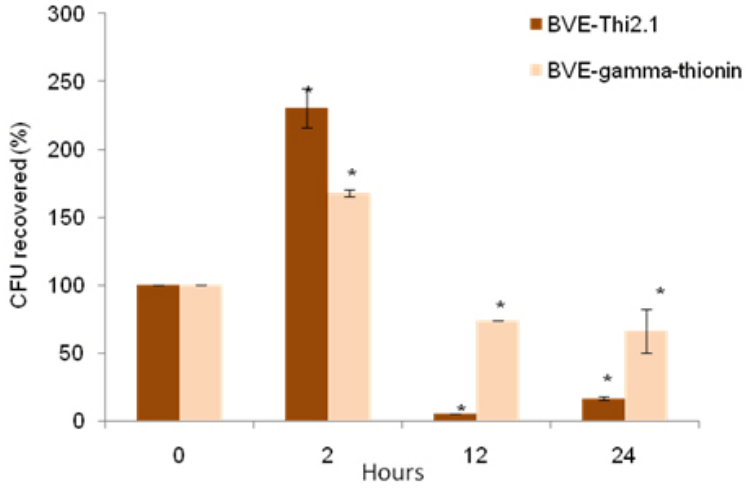

Fig. 2 Number of *Candida albicans* internalized in BEC in the presence of conditioned media. BEC were infected during 4 hrs with *C. albicans*, and then were treated with amphotericin B for 2 hrs and incubated with 3.125 µg/ml of protein of diverse conditioned media for different times. Bars are the percentage of recovered CFU after BEC lysis considering as 100% the effect of CM from BVE-NT. *Each bar*represents the mean ± SEM forthree independent experiments. The symbol * indicates significant changes ($P < 0.05$) in relation to the respective CM at 0 hrs.

Table 3 shows NO production of BEC infected with *S. aureus* or *C. albicans.* Basal NO production in BEC was not modified in the presence of conditioned media in a similar way as observed for BMEC. Also, infection with *C. albicans* alone or in presence of conditioned media did not modify NO production. However, infection of BEC with *S. aureus* induces the release of NO at 2, 12 and 24 hrs in relation to basal production, which suggests that this increase is due to the pathogen. Additionally, NO production was stimulated in BEC infected with *S. aureus* and treated with CM from BVE-γ-thionin at 6, 12 and 24 hrs, and with CM from BVE-Thi2.1 at 6 and 24 hrs. The reduction in the number of *S. aureus* recovered in BEC treated with CM from BVE-γ-thionin (Figure 1) is oppositely related to NO production (Pearson's coefficient -0.73074 for all the times evaluated), whereas for the effect of CM from BVE-Thi2.1 there is a positive correlation in the number of CFU recovered and NO production (Pearson's coefficient 0.8295 for all the times evaluated). These data suggest that antibacterial activity detected in BEC in the presence of CM from BVE-γ-thionin could result from NO production. In the other host-pathogen models employed there is no negative correlation. Thus, the diminution in the number of *S. aureus* recovered in BMEC or the reduction of *C. albicans* recovered in BEC in the presence of conditioned media may depend on the activation of other host cell defence mechanisms.

Table 2. NO production by BMEC.

	Basal NO_3 (µM ± SEM)* Conditioned medium:			**Infected with *S. aureus* (µM ± SEM) Conditioned medium:**		
Time (hrs)	**BVE-NT**	**BVE-γ-thionin**	**BVE-Thi2.1**	**BVE-NT**	**BVE-γ-thionin**	**BVE-Thi2.1**
2	1.35 ± 0.28	1.35 ± 0.33	1.38 ± 0.30	1.48 ± 0.39	1.27 ± 0.20	2.33 ± 0.64[a]
6	1.37 ± 0.31	1.36 ± 0.32	1.38 ± 0.29	3.10 ± 0.77[a]	3.05 ± 0.60[a]	2.48 ± 0.69[a]
12	1.36 ± 0.32	1.36 ± 0.32	1.37 ± 0.30	1.33 ± 0.08	3.73 ± 0.56[ab]	3.55 ± 1.03[ab]
24	1.36 ± 0.32	1.37 ± 0.31	1.37 ± 0.30	3.41 ± 0.76[a]	4.19 ± 0.57[a]	3.68 ± 0.64[a]

*Data are presented as mean ± SEM of three independent experiments of NO secreted by BMEC, measured as nitrite concentration. [a]Means differ significantly ($P \leq 0.01$, calculated by one-way ANOVA) at the measured time points in relation to the same conditioned medium without infection (basal). [b]Means differ significantly ($P \leq 0.01$, calculated by one-way ANOVA) at the measured time points in relation to the effect of conditioned medium from BVE-NT in the same experimental group.

DISCUSSION

The antimicrobial activity of several commonly used antibiotics may be compromised when pathogens reside intracellularly, due to the poor drug distribution or its inhibition by intracellular milieu (Briken, 2008). Therefore, the need to find out novel compounds against intracellular infections is increasing. In this sense, AP produced by different organisms can be promising candidates to treat these kind of infections based on many of them may recognize and inactivate cellular targets *in vitro,* through translocation across the plasma membrane in a nonlethal manner (Nicolas, 2009). In this way, Capparelli et al. (2007) have reported the intracellular activity of wheat puroindolines against *Staphylococcus epidermidis* in macrophages.

The first aim of the present paper was to evaluate the effect of PAP γ-thionin and thionin Thi2.1 produced by bovine endothelial cells against the intracellular pathogens *S. aureus* and *C. albicans*, for which we used three host-pathogen models: 1) BMEC-*S. aureus*, 2) BEC-*S. aureus* and 3) BEC-*C. albicans*. Intracellular survival persistence of *S. aureus* into bovine mammary epithelium and endothelium, as well as *C. albicans* systemic dissemination through endothelium have been widely documented (Kerro-Dego et al. 2002; Sinha and Herrmann, 2005; Grubb et al. 2009). In all assays we used 3.125 µg/ml of protein in conditioned media, previously reported as bactericidal or fungicidal (Anaya-López et al. 2006a; Loeza-Ángeles et al. 2008). This amount of protein does not reduce the host cell viability at the times tested (data not shown). Both PAP might be able to cross cytoplasmic membrane accordingly to their hydropathy index (Table 1). In the first model employed, we detected a more potent inhibitory effect against *S. aureus* internalized into BEMC using CM from BVE-Thi2.1 (Figure 1) than CM from BVE-γ-thionin. We compared the effect of these conditioned media in relation to the effect of CM from non-transfected cells, in order to discard the effects of other products besides PAP secreted by BVE-cells. These results are in agreement with previous reports that demonstrated that CM from BVE-Thi2.1 directly kills *S. aureus*, and those that showed a lack of antibacterial activity in CM from BVE-γ-thionin (Anaya-López et al. 2006a; Loeza-Ángeles et al. 2008). Furthermore, we observed that CM from BVE-Thi2.1 increased its activity during the course of time. This effect could also be the consequence of the activation of defence mechanisms in BMEC by CM from BVE-Thi2.1 but not by CM from BVE-γ-thionin, suggesting that different PAP regulate diverse protection mechanisms in mammal cells alone or in combination with pathogens. In addition, the defence mechanisms activated by CM from BVE-Thi2.1 in BMEC do not comprise NO production (Table 2). In this sense, Zughaier et al. (2005) have demonstrated in macrophages that several AP neutralize endotoxin induction of NO, whereas an overnight treatment with a combination of AP and bacterial components significantly enhanced the release of reactive oxygen species (ROS) compared with cells challenged with endotoxin or AP alone. In the second model used (Figure 1, BEC-*S. aureus*), CM from BVE-γ-thionin has a better effect killing intracellular *S. aureus* for 12-24 hrs incubations than CM from BVE-Thi2.1. Because CM from BVE-γ-thionin does not have antibacterial activity, this effect can be attributable to the regulation of defence mechanisms by host cells. In this sense, CM from BVE-γ-thionin significantly stimulates the production of NO in BEC (Table 3). However, the regulation of other innate immune defence mechanisms by PAP cannot be excluded, such as endogenous AP production (Nicolas, 2009). Accordingly, Zughaier et al. (2005) have reported that macrophages primed overnight with a human defensin, and bacterial components significantly enhanced the release of reactive oxygen species (ROS). In addition, other immunomodulatory effects of defensins besides ROS production have been reported, for example the production of proinflammatory chemokines and cytokines in dendritic cells (Hölzl et al. 2008). Interestingly, CM from BVE-Thi2.1 has a more potent bactericidal activity in BMEC than in BEC. This difference could result from the specific modulation by CM of BVE-Thi2.1 of elements of innate immune response of host cell; however further research is necessary in order to explore other innate immune responses regulated by PAP in our models. In this regard, there are few works that explore the regulation of mammal innate immunity by PAP. Recently, Palumbo et al. (2010) have demonstrated that puroindolines A and B from wheat seeds are able to reduce infection with *Listeria monocytogenes* in a mouse model. Furthermore, these AP modulate the inflammatory response of infected mice. Previously, Capparelli et al. (2007) showed that puroindolines A and B kill intracellular *Staphylococcus epidermidis*, as a strategy to prevent acne. Puroindolines are small cationic AP that contain a conserved cysteine backbone formed by ten Cys, they are tryptophan-rich and have a tertiary structure similar to non-specific lipid transfer proteins (ns-LTPs) (Bhave and Morris, 2008). The conserved cysteine backbone is also present in Thi2.1 and γ-thionin (Table 1). In agreement with our results, work from Brinch et al. (2009) reports the bactericidal effect against intracellular *S. aureus* of plectasin, a defensin-like antimicrobial peptide produced by the fungus *Pseudoplectania nigrella.*

Table 3. NO production by BEC.

	Basal NO_3 (µM ± SEM)* Conditioned medium:			Infected with *S. aureus* (µM ± SEM) Conditioned medium:			Infected with *C. albicans* (µM ± SEM) Conditioned medium:		
Time (hrs)	**BVE-NT**	**BVE-γ-thionin**	**BVE-Thi2.1**	**BVE-NT**	**BVE-γ-thionin**	**BVE-Thi2.1**	**BVE-NT**	**BVE-γ-thionin**	**BVE-Thi2.1**
2	1.12 ± 0.06	0.96 ± 0.04	0.97 ± 0.01	4.29 ± 0.53[a]	1.54 ± 0.52[b]	2.37 ± 0.44[b]			
6	1.60 ± 0.07	1.04 ± 0.15	0.99 ± 0.1	1.55 ± 0.25	2.01 ± 0.49 [b]	4.83 ± 1.24[b]	1.19 ± 0.42[†]	1.20 ± 0.41[†]	1.24 ± 0.35[†]
12	1.28 ± 0.004	1.33 ± 0.37	0.95 ± 0.07	7.30 ± 2.17[a]	11.99 ± 0.53[ab]	5.37 ± 1.93[a]	1.25 ± 0.34	1.27 ± 0.32	1.29 ± 0.31
24	1.22 ± 0.01	1.35 ± 0.15	1.02 ± 0.08	10.68 ± 0.2[a]	13.89 ± 0.52[ab]	7.82 ± 0.44[ab]	1.31 ± 0.33	1.28 ± 0.23	1.25 ± 0.38

*Data are presented as mean ± SEM of three independent experiments of NO secreted by BEC, measured as nitrite concentration. [†]NO production at 2 hrs. [a]Means differ significantly ($P \leq 0.01$) at the measured time points in relation to the effect of the same conditioned medium without infection (basal). Calculated by one-way ANOVA. [b]Means differ significantly ($P \leq 0.01$) at the measured time points in relation to the effect of conditioned medium from BVE-NT at the same experimental group. Calculated by one-way ANOVA.

Our data point out the relevance to study the effect of PAP to control intracellular bacterial infections and to modulate host innate immune response. In addition, these results interestingly show that modulation of innate response, such as production of NO, depends also on the pathogen, because BEC infected with *C. albicans* and treated with conditioned media do not increase the level of NO. This effect can be mediated by different innate immune signal pathways, such as the type of Toll-like receptor involved, which are different for fungi and bacteria (Means et al. 2009; Mogensen, 2009). In BEC infected with *C. albicans* we observed that CM from BVE-Thi2.1 has a more potent fungicidal activity at 24 hrs treatment than CM from BVE-γ-thionin. According to previous reports, this CM can achieve these effects by direct killing of *C. albicans* (Anaya-López et al. 2006a; Loeza-Ángeles et al. 2008). In Figure 2, we also showed that after two hours of infection there is a higher number of intracellular *C. albicans* in BEC. Consistent with our results, it has been previously reported that *C. albicans* organisms are capable of stimulating the synthesis of cytokines, chemokines and adhesion molecules by human umbilical vein endothelial cells (HUVEC); in addition, the amount of cytokines synthesized by HUVEC in response to *C. albicans* is closely associated with the level of host cell invasion by this organism (Villar et al. 2005). However, further research is necessary in order to explore if CM from BVE-Thi2.1 and BVE-γ-thionin regulate other host innate immune responses in our models and if these effects are dose-dependent. In conclusion, this works shows the feasibility of using PAP to control intracellular bacterial or fungal infections, by direct killing or by means the modulation of host innate immune response.

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