Resveratrol Stimulates Sphingosine-1-Phosphate Signaling of Cathelicidin Production

Kyungho Park[1,2,3], Peter M. Elias[1,2,3], Melanie Hupe[1,2,3], Andrew W. Borkowski[4,5], Richard L. Gallo[4,5], Kyong-Oh Shin[6], Yong-Moon Lee[6], Walter M. Holleran[1,2,3,7] and Yoshikazu Uchida[1,2,3]

We recently discovered a regulatory mechanism that stimulates the production of the multifunctional antimicrobial peptide cathelicidin antimicrobial peptide (CAMP). In response to subtoxic levels of ER stress, increased sphingosine-1-phosphate (S1P) production activates an NFκB→C/EBPα-dependent pathway that enhances CAMP production in cultured human keratinocytes. As the multifunctional stilbenoid compound resveratrol (RESV) increases ceramide (Cer) levels, a precursor of S1P, we hypothesized and assessed whether RESV could exploit the same pathway to regulate CAMP production. Accordingly, RESV significantly increased Cer and S1P levels in cultured keratinocytes, paralleled by increased CAMP mRNA/protein expression. Furthermore, topical RESV also increased murine CAMP mRNA/protein expression in mouse skin. Conversely, blockade of Cer→sphingosine→S1P metabolic conversion, with specific inhibitors of ceramidase or sphingosine kinase, attenuated the expected RESV-mediated increase in CAMP expression. The RESV-induced increase in CAMP expression required both NF-κB and C/EBPα transactivation. Moreover, conditioned media from keratinocytes treated with RESV significantly suppressed *Staphylococcus aureus* growth. Finally, topical RESV, if not coapplied with a specific inhibitor of sphingosine kinase, blocked *S. aureus* invasion into murine skin. These results demonstrate that the dietary stilbenoid RESV stimulates S1P signaling of CAMP production through an NF-κB→C/EBPα-dependent mechanism, leading to enhanced antimicrobial defense against exogenous microbial pathogens.

Journal of Investigative Dermatology (2013) **133,** 1942–1949; doi:10.1038/jid.2013.133; published online 18 April 2013

INTRODUCTION

Human epidermis is positioned at the interface with the environment, protecting underlying tissues from exogenous microbial pathogens, mechanical damage, and UV irradiation. These protective mechanisms include the generation of antimicrobial peptides (AMP) that display activity against a broad spectrum of different pathogens, including Gram-negative and Gram-positive bacteria, fungi, and certain viruses (Dunn *et al.*, 2009; Nijnik and Hancock, 2009; Mendez-Samperio, 2010; Schroder, 2010). In addition to its antimicrobial function, the major AMP, cathelicidin antimicrobial peptide (CAMP), is a multifunctional modulator of cytokine secretion/production, angiogenesis, and adaptive immune responses (Lai *et al.*, 2010). Prior studies demonstrated that CAMP expression increases in epithelial tissues, including in epidermal keratinocytes (KCs), after external perturbations, e.g., wounding, suberythemagenic UVB irradiation, oxidative stress, and epidermal barrier abrogation (Aberg *et al.*, 2008; Hong *et al.*, 2008; Kim *et al.*, 2009; Mallbris *et al.*, 2010). However, if these external perturbations become excessive, they instead produce cell cycle arrest and apoptosis by increasing endoplasmic reticulum (ER) stress–induced ceramide (Cer) production (Lei *et al.*, 2008). In contrast, subtoxic perturbations produce lower levels of ER stress, which also increases Cer transiently. Some of the increased Cer, generated following subtoxic stress, is metabolized to S1P, which stimulates CAMP production in epithelial tissues, including the epidermis, via a (to our knowledge) previously unidentified NF-κB- and C/EBPα-mediated pathway (Park *et al.*, 2012). Importantly, this regulatory mechanism operates independently of the well-established vitamin D receptor (VDR)–regulated pathway (Gombart *et al.*, 2005), which instead likely predominates under basal (non-stressed) conditions.

[1]*Department of Dermatology, School of Medicine, University of California, San Francisco, San Francisco, California, USA;* [2]*Department of Veterans Affairs Medical Center, San Francisco, California, USA;* [3]*Northern California Institute for Research and Education, San Francisco, California, USA;* [4]*Division of Dermatology, Department of Medicine, University of California, San Diego, San Diego, California, USA;* [5]*Department of Veterans Affairs San Diego Healthcare System, San Diego, California, USA;* [6]*College of Pharmacy, Chungbuk National University, Cheongju, South Korea and* [7]*Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, San Francisco, California, USA*

Correspondence: Yoshikazu Uchida, Dermatology Service (190), Veterans Administration Medical Center, 4150 Clement Street, San Francisco, California 94121, USA. E-mail: uchiday@derm.ucsf.edu

Abbreviations: AMP, antimicrobial peptide; CAMP, human cathelicidin antimicrobial protein; CRAMP, cathelin-related antimicrobial peptide; Cer, ceramide; ER, endoplasmic reticulum; KCs, keratinocytes; MAP, mitogen-activated protein; mCAMP, murine cathelicidin antimicrobial protein; NOE, N-oleoylethanolamine; PARP, poly(ADP-ribose) polymerase; qRT–PCR, quantitative real-time PCR; RESV, resveratrol; S1P, sphingosine-1-phosphate; VDR, vitamin D receptor

Received 8 January 2013; revised 7 February 2013; accepted 26 February 2013; accepted article preview online 14 March 2013; published online 18 April 2013

Resveratrol (RESV, trans-3,4,5-trihydroxystilbene) belongs to a class of phytoalexins that are synthesized by a restricted number of plants, including berries, peanuts, and red grapes. Notably, the synthesis of RESV in these plants increases in response to external stressors, i.e., infection or UV irradiation (Shakibaei *et al.*, 2009). RESV exerts antioxidant and other anti-inflammatory activities, as well as regulates cellular proliferation, differentiation, Sirt modulation, and mitochondria-initiated apoptosis (Sadruddin and Arora, 2009; Shakibaei *et al.*, 2009). Pertinent to the current studies, RESV also stimulates Cer levels in multiple cell types (Dolfini *et al.*, 2007; Signorelli *et al.*, 2009; Cakir *et al.*, 2011).

We have demonstrated that KCs deploy three metabolic mechanisms that protect against Cer-induced apoptosis (Uchida *et al.*, 2010), i.e., Cer-to-glucosylceramide, Cer-to-sphingomyelin (see also Charruyer *et al.* (2008)), and ceramidase-mediated hydrolysis of Cer to sphingosine. We further showed that subtoxic external perturbations that induce ER stress and increase cellular Cer production also stimulate metabolic conversion of sphingosine to S1P, leading to enhanced CAMP generation (Park *et al.*, 2012). Here, we show that RESV not only increases Cer production but also initiates downstream conversion of Cer to S1P, leading to the stimulation of CAMP production in cultured human KCs. In addition, we show here that topical RESV stimulates S1P signaling of CAMP production in vivo (murine skin). Finally, we demonstrate that pretreatment of KCs with RESV enhances antimicrobial defense against virulent, exogenous *Staphylococcus aureus*. Notably, RESV itself did not induce ER stress, suggesting that RESV directly stimulates S1P signaling of CAMP expression. These studies illuminate yet another important and potentially clinically beneficial biological activity of RESV, i.e., the ability to enhance epithelial innate immunity through exploitation of an ER stress–initiated pathway.

RESULTS

RESV increases cellular levels of S1P in parallel with enhanced CAMP production

Our prior studies demonstrated that subtoxic levels of ER stress, induced either by external perturbations, e.g., UVB irradiation, or by an established pharmacological ER stressor, e.g., thapsigargin, increase not only the levels of cellular Cer but also the conversion of Cer to its distal metabolite sphingosine-1-phosphate (S1P), which then stimulates CAMP production (Park *et al.*, 2012). Hence, we first assessed here whether exogenous RESV stimulates the production of cellular Cer, as well as its downstream metabolites, without inducing excessive ER stress. Lipid quantification showed a modest, but significant, increase in Cer, and large increases in both sphingosine and S1P after treatment of cultured human KCs with exogenous RESV at concentrations $<50\,\mu M$ (Table 1). At these RESV concentrations, indicators of apoptosis (i.e., cell viability and poly(ADP-ribose) polymerase (PARP) cleavage) did not become evident (Figure 1a and b), assuring that these concentrations of RESV are not toxic. Yet, because still higher concentrations ($>100\,\mu M$) slightly decreased cell viability (Figure 1a), we used RESV at concentrations of $<50\,\mu M$ in all subsequent studies.

Table 1. Sphingolipid content in human keratinocytes exposed to endoplasmic reticulum stress

Treatment	Lipid content (pmol per mg protein ± SD)		
	Cer	Sphingosine	S1P
Vehicle	736.3 ± 41.2	19.3 ± 3.2	5.7 ± 0.3
RESV	818.7 ± 41.9[a]	83.4 ± 3.8[a]	9.3 ± 0.3[a]

Abbreviations: Cer, ceramide; RESV, resveratrol; S1P, sphingosine-1-phosphate.
[a]$P<0.01$ ($n=3$) vs. vehicle.
Mean ± SD.

We next determined whether exogenous RESV stimulates CAMP expression *in vitro*. Quantitative RT–PCR (qRT–PCR) analysis revealed a significant increase in CAMP mRNA expression in KCs after 24 hours of exposure to RESV (Figure 1c and d). Consistent with these alterations in CAMP mRNA, western immunoblot analysis showed that CAMP protein levels also increased after RESV treatment (Figure 1e). Finally, ELISA analysis of culture supernatant further demonstrated that RESV also enhanced LL-37/CAMP secretion from KCs (Figure 1f and g). Together, these results indicate that RESV elevates cellular Cer and S1P levels, in parallel with an increase in CAMP/LL-37 production and secretion.

Increased S1P accounts for RESV-mediated enhancement of CAMP production

Our previous studies demonstrated that S1P, but neither Cer nor sphingosine, accounts for the ER stress–induced increases in CAMP production (Park *et al.*, 2012). Hence, we next investigated whether S1P is the Cer metabolite that accounts for the RESV-mediated upregulation of CAMP. Co-incubation of KCs with RESV and *N*-oleoylethanolamine (NOE), a potent inhibitor of ceramidase, the hydrolytic enzyme that generates sphingosine from Cer, significantly attenuated the expected RESV-induced increase in CAMP mRNA and protein expression (Figure 1c and e). In contrast, the addition of NOE alone did not alter CAMP expression. Together, these results suggest that hydrolysis of Cer by ceramidase(s) is required for the RESV-induced stimulation of CAMP expression.

We next determined which distal metabolite of Cer, i.e., sphingosine and/or S1P, is (are) responsible for increased CAMP expression. Inhibition of the conversion of sphingosine to S1P, using dimethylsphingosine, did not stimulate, but instead significantly attenuated, the expected RESV-induced increase in both CAMP mRNA and protein expression (Figure 1d and e), strongly suggesting that sphingosine is not the responsible metabolite.

To elucidate whether S1P is the responsible signal, we next assessed whether RESV stimulates the expression of the sphingosine kinase (SPHK1 isoform) that accounts for ER stress–stimulated CAMP expression (Park *et al.*, 2012).