Published in final edited form as:

Eur J Pharm Sci. 2014 December 18; 65: 112–121. doi:10.1016/j.ejps.2014.09.012.

Melittin-glutathione S-transferase fusion protein exhibits anti-inflammatory properties and minimal toxicity

Jamie E. Rayahin¹, Jason S. Buhrman¹, and Richard A. Gemeinhart^{1,2,3,*}

¹Department of Biopharmaceutical Sciences, University of Illinois, Chicago, IL 60612-7231, USA.

²Department of Bioengineering, University of Illinois, Chicago, IL 60607-7052, USA.

³Department of Ophthalmology and Visual Sciences, University of Illinois, Chicago, IL 60612-4319, USA.

Abstract

Although potent, proteins often require chemical modification for therapeutic use. Immunogenicity, difficult synthesis, and scale-up of these modifications are all engineering obstacles that stand in the way of expanding the use of these therapeutics. Melittin, a peptide derived from bee venom, has been shown to modulate inflammation. Although potentially therapeutic, the native peptide causes cell lysis and toxicity significantly hindering therapeutic application. Based upon the knowledge of the pore formation mechanism, we examined the toxicity and therapeutic effect of a melittin fusion protein with glutathione-S-transferase. The fusion of melittin and glutathione S-transferase results in diminished toxicity of the peptide and retained anti-inflammatory properties at doses that exceed toxic concentration of native melittin. Our results suggest that fusion proteins, particularly those of glutathione-S-transferase, may be facile modifications to control protein activity.

Keywords

Melittin; inflammation; fusion protein; macrophage; glutathione S-transferase; bee venom; protein therapeutics; protein delivery

1. Introduction

Utilization of venoms has a long history in complementary and alternative medicine (Hodgson, 2012). Although sometimes effective in homeopathic doses, and with few exceptions (Cooper, 1996), these venoms act as toxins and cannot be used safely in a therapeutic setting. Of these, the honey bee's venom has drawn particular attention. Since its discovery, melittin, the major component of bee venom, has been examined for its potent

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*Correspondence to: Richard A. Gemeinhart, 833 South Wood Street (MC865), Chicago, IL 60612-7231, USA. rag@uic.edu.

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properties (Bechinger, 1997; Buhrman et al., 2013b; Kwon et al., 2002; Lee et al., 2004; Park et al., 2004). Due to its cell-lytic property, melittin has been examined as a candidate for anti-cancer (Buhrman et al., 2013b; Orsolic, 2012) and anti-bacterial therapies (Asthana et al., 2004; Buhrman et al., 2013b). Melittin's pharmacologic mechanism of action is considered to be pore-formation and subsequent necrotic cell death (Bechinger, 1997; Bechinger and Lohner, 2006; Kloczek et al., 2009; Pratt et al., 2005; Santo and Berkowitz, 2012; Yang et al., 2001). Unexpectedly, melittin has also demonstrated significant anti-inflammatory properties (Kwon et al., 2002; Lee et al., 2004; Park et al., 2004).

The inflammatory reaction is a Janus-faced process with the potential for both protective and adverse outcomes. Acute inflammation is necessary for local trauma or infection to resolve (Medzhitov, 2008; Serhan and Savill, 2005). However, persistent and chronic activation of the immune response can result in tissue damage and pathology (Medzhitov, 2008; Serhan and Savill, 2005). Both exogenous and endogenous stimuli can promote an inflammatory response. In either case, the reaction is a very intricate process with an elaborate array of cytokines and chemical mediators, such as nitric oxide, tumor necrosis factor alpha (TNF- α), and prostaglandins that control its course (Medzhitov, 2008; Nathan, 2002). Dysregulation of any of these signals can cause inflammation and is associated with disease (Karin et al., 2006; Krishnamoorthy and Honn, 2006; Nathan, 2002). The inflammatory response recruits several cell types, however, macrophages are the main immune cells that ubiquitously control the production and release of biochemical signals of inflammation (Mosser and Edwards, 2008). Modification of the macrophage response holds great potential in modulating inflammation (Gordon and Taylor, 2005).

Protein therapeutics have been widely investigated to modulate the inflammatory response of macrophages (Feldmann and Maini, 2003). There are limitations, specifically toxicity, systemic distribution, and limited half-life, that warrant improvements to achieve optimal performance. Modifications of protein therapeutics can influence the mechanism of action, toxicity, and efficacy and oftentimes results in highly efficacious drugs.

Current technologies focus on chemical modifications of the parent protein. These modifications include PEGylation, modification of side chains or reactive groups, and chemical crosslinking (Means and Feeney, 1990). Practical constraints of these modifications, such as immunogenicity of the modification, feasibility, and scale-up, often limit the engineering and medicinal potential of these therapeutics (Basle et al., 2010). Fusion proteins are easy to express and offer another avenue of protein modification. Several biologic therapies have shown improved function and modified activity as fusion proteins, especially in therapeutics modifying the immune system or macrophage function (Boulianne et al., 1987; Czajkowsky et al., 2012; Mohit and Rafati, 2012; Morrison et al., 1984; Sadelain et al., 2013; Shin and Morrison, 1989).

With this in mind, we sought to explore whether a fusion protein of melittin and glutathione S-transferase (GST), a common fusion partner, would maintain anti-inflammatory properties of the peptide, while abrogating its cell-lytic properties, based upon the disruption of protein association and assembly that result in cell lysis (van den Bogaart et al., 2008; Yang et al., 2001). To our knowledge, there have been no studies that demonstrate modifications of the

melittin peptide to control its toxicity and maintain its anti-inflammatory properties. Herein, we compare toxicity and anti-inflammatory properties of GST-melittin fusion protein to native melittin on lipopolysaccharide (LPS) induced inflammation of mouse macrophages. We show, in these studies, that GST-melittin is a non-toxic alternative to native melittin that achieves anti-inflammatory action without lethal response of the native peptide.

2. Materials and Methods

2.1. Expression and purification of recombinant proteins

GST-melittin was purified by a method previously described (Buhrman et al., 2013c). The expression of both GST and GST-melittin was induced in the Rosetta strain (Novagen/EMD Millipore, Billerica, MA). *Escherichia coli* was grown in LB broth at 37°C. After the bacterial density was sufficient, *i.e.* the absorbance at 600 nm reached 0.4 to 0.6, bacterial cells were removed to 25°C and induced overnight (16 hours) with 0.1 mM isopropyl -D-1-thiogalactopyranoside (IPTG). Cells were then centrifuged (3,600×g) for 20 minutes before being resuspended in lysis buffer (50 mM NaH₂PO₄ and 300 mM NaCl at pH 8.0) and lysed by addition of 1 mg lysozyme followed by freezing and thawing 3 times. Lysates were sonicated 3 times in 15-second intervals at 40% intensity and centrifuged (11,300×g for 30 minutes) to separate soluble and insoluble proteins. GST was purified from the soluble fraction by Ni-NTA agarose (Qiagen, Venlo, NLD) according to the manufacturer's instructions. GST-melittin was extracted from the insoluble fraction by the acidic extraction method previously reported (Buhrman et al., 2013c; Buhrman et al., 2012). Briefly, the insoluble lysate was washed with 70 mM TCEP (Thermo-Fisher Scientific) in PBS (pH = 2.3), and GST-melittin was then extracted in PBS with 70 mM TCEP and 1% tween 20 (Thermo-Fisher Scientific). The pH of the extracted protein was raised to 7.4, and the extracted GST-melittin was purified with Ni-NTA (Qiagen) using manufacturer's recommended methods.

2.2. Cell culture

J774A.1 murine macrophages (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gemini, Calabasas, CA, USA) at 37°C in 5% CO₂. Macrophages were harvested by scraping and used between passage 2 and 7. Macrophages were stimulated through treatment with -irradiated LPS derived from *Escherichia coli* serotype 055:B5 (Sigma-Aldrich, St Louis, MO, USA).

2.3. Macrophage viability

The effects of native melittin, GST-melittin, cleaved GST-melittin, and GST on cell viability both with and without LPS stimulation was investigated using CellTiter 96 AQueous One Solution Assay (Barltrop et al., 1991; Cory et al., 1991) of cellular proliferation (Promega, Madison, WI, USA). GST-melittin was cleaved by thrombin (2U) incubation (37°C) for 7 hours. J774A.1 cells were plated at a density of 2×10⁴ cells in a 96-well flat-bottom plate and allowed to adhere for 24 hours. After this incubation period, cells were treated with GST, GST-melittin, cleaved GST-melittin, or native melittin (Sigma Aldrich, St Louis, MO, USA) for one hour after which the appropriate groups were challenged with LPS (2 µg/mL).

After 4 hours the number of viable cells was measured according to the manufacturer's instructions.

2.4. Membrane permeability

J774A.1 cells were plated at a density of 2×10^4 cells in a 96-well flat-bottom plate and allowed to adhere for 24 hours. After this incubation period, cells were treated with 10 ng/ μ L propidium iodide, a cell membrane impermeable nuclear dye, and the specified protein. Phase contrast and epifluorescence micrographs were captured over the course of one hour on an Olympus IX70 inverted microscope.

2.5. Nitric oxide (NO) formation

J774A.1 murine macrophages were plated at a density of 5×10^5 cells/mL in a 24 well plate and allowed to adhere overnight, after which the culture medium was replaced with 500 μ L phenol-free DMEM (Hyclone, Logan, UT, USA) with 10% FBS. Varying concentrations of GST-melittin, native melittin, or GST were added to the culture medium. Cells were pre-treated with each of these components for one hour, after which they were stimulated with LPS (2 μ g/mL). Supernatant media was collected after 24-hour incubation period and nitrite measured using the Griess reagent (Promega, Madison, WI, USA) according to manufacturer's instructions.

2.6. Quantification of inflammatory gene expression

Total RNA was isolated from J774A.1 murine macrophages using TRIzol (Invitrogen, Carlsbad, CA, USA). The absorbance ratio at 260 and 280 nm for each sample was determined spectrophotometrically, and if the ratio was greater than 1.8, the RNA was reverse transcribed into cDNA (Applied Biosystems, Carlsbad, CA, USA). Real time PCR assay was carried out on an Applied Biosystems StepOnePlus™ PCR machine using SYBR® Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). A melting curve analysis was performed after each run to confirm product specificity. All primers were designed to span exon-exon junctions (**Table 1**). Transcripts of -glucuronidase were quantified and used as endogenous control (Schenborn and Groskreutz, 1999). Relative quantities were estimated by the delta-delta-Ct method (Schmittgen and Livak, 2008). The expression of each gene was normalized to untreated cells as control.

2.7. Effect of Peptides on LPS Binding to Macrophages

J774A.1 murine macrophages were plated at a density of 1×10^6 cells/mL in a 12 well plate and allowed to adhere overnight. After which, the culture medium was replaced with 1 mL fresh DMEM (Hyclone, Logan, UT, USA) with 10% FBS. Varying concentrations of GST-melittin (0.3 μ M or 3 μ M) or native melittin (0.3 μ M) were added to the culture medium for one hour. Following this, the macrophages were stimulated with 10 μ g/mL FITC-labeled LPS (Sigma Aldrich, St. Louis, MO, USA). Supernatant media was collected after a 4-hour incubation period and fluorescence was measured at excitation and emission wavelengths of 490 and 550 nm on Turner Quantech fluorometer. LPS binding was compared to that of cells treated with FITC-labeled LPS, but not with the proteins, to determine the extent of inhibition of LPS binding.

2.8. Protein Localization

BSA and GST were amine-labeled with the Alexa-Fluor 430 succinimidyl ester (Invitrogen) according to manufacturer's recommendation. Stoichiometry was set at 1 dye molecule per two protein molecules. GST-melittin labeled on free carboxylic acids using lissamine rhodamine ethylenediamine (Sigma Aldrich) coupled with 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC, Pierce). The stoichiometry was 1:1 protein: EDC and 2:1 protein: rhodamine. Each reaction was allowed to proceed for 2 hours at room temperature, and the resulting labeled proteins were purified from un-reacted dye by Ni-NTA selection. Protein yields were quantified with SDS-PAGE.

J774A.1 murine macrophages were plated at a density of 2×10^5 cells/mL in a total volume of 250 μ L per chamber in a 4-chamber glass bottom dish (In Vitro Scientific). One day after plating, macrophages were treated with labeled bovine serum albumin (BSA), labeled GST, labeled GST mixed with (unlabeled) melittin, or labeled GST-melittin at a concentration of 0.3 μ M. After a 24-hour treatment period in the presence of the labeled proteins, macrophages were washed twice with warm phosphate buffered saline (PBS) and fresh media was re-applied. The nuclei were stained with Hoechst 33258 for 5 min prior to confocal laser scanning microscopy (CLSM) imaging. CLSM images were acquired using a Zeiss LSM 510 META (Carl Zeiss, Germany) with a water immersion 63 $\times$ objective. Excitation wavelengths were 405 nm (Diode 405), 488 nm (argon laser) and 543nm (HeNe laser) for Hoechst 33258, Alexa Flour® 430, and rhodamine, respectively.

2.9. Statistical Analyses

ANOVA was used to test all groups, and post-hoc Tukey analysis was utilized if ANOVA suggested significant differences between the groups. In all cases less than or equal to 0.05 was considered significant.

3. Results

3.1. GST-melittin has no effect on macrophage survival; native melittin kills macrophages at nanomolar concentrations

To address the effect of GST-melittin and native melittin on macrophage survival, J774A.1 macrophages were incubated with a range of concentrations (0-30 μ M) of either GST-melittin or native melittin. Native melittin caused significant reduction in cell metabolism, interpreted as reduced cell viability/cell death, at concentrations as low as 1 μ M and significant inhibition at concentrations below this (**Figure 1A**). We approximated the IC₅₀ for native melittin to be 450 nM (**Figure 1A**). Conversely, GST-melittin alone had no effect on cell survival after a four-hour incubation at concentrations as high as 30 μ M (**Figure 1B**) and an IC₅₀ could not be calculated.

Cellular viability after introduction of inflammatory stimulus, LPS, was also investigated with all treatment groups. Cells were pre-treated with either GST-melittin or native melittin then subsequently treated with LPS (2 μ g/mL) for four hours. Cells treated with GST-melittin show no significant change in viability over four hours with LPS. In contrast, cells

treated with native melittin exhibit reduced IC_{50} of 300 nM when treated with LPS indicating that inflamed macrophages have a slightly greater sensitivity to the toxin.

Phenotypic evidence supports these observations (**Figure 2**). Native melittin's toxicity toward macrophages is detectable within minutes as membrane permeabilization, apparent in the fluorescence micrographs, and cell detachment, swelling, and burst, visible in phase contrast images (**Figure 2**). There is no phenotypic evidence of toxicity or cell permeability when cells **Figure 2** were treated with GST-melittin has latent cell-lytic properties compared to the native peptide.

3.2. GST-melittin is equivalent to native melittin in attenuating nitric oxide synthesis

Following the serendipitous observation of the reduced toxicity of GST-melittin compared to native melittin and the knowledge of previous reports of anti-inflammatory properties of melittin, we became interested in the ability of GST-melittin to act as an anti-inflammatory agent (Kwon et al., 2002; Lee et al., 2004; Park et al., 2007). Therefore, we examined if GST-melittin and native melittin had equivalent effects at reducing nitric oxide synthesis. We chose the approximate IC_{50} dose of native melittin with LPS, 300 nM, as our highest concentration comparing these concentrations to molar equivalent concentrations of GST-melittin.

Nitric oxide synthesis was measured with and without LPS stimulation as total nitrite after twenty-four hours using the Griess reagent. Both GST-melittin and melittin had comparable effects in decreasing nitric oxide synthesis over the treatment period (**Figure 3**). At the same concentration, native melittin and GST-melittin have equal effect at decreasing nitric oxide synthesis in LPS stimulated macrophages ($p > 0.05$). At toxic levels of the native peptide, GST-melittin has the ability to decrease NO production to a greater extent ($p < 0.001$) while not eliciting any toxicity. Both the GST-melittin and the native melittin had no significant effect on nitric oxide synthesis on macrophages that had not been stimulated, with little detectable nitrite measured for unstimulated macrophages (**Figure 3**).

3.3. GST-melittin inhibits inflammatory gene expression in stimulated macrophages

Because GST-melittin decreased production of the inflammatory mediator, nitric oxide, we explored its effect on the expression of inflammatory proteins, iNOS, Cox-2 and TNF- α , at the mRNA level. Melittin and GST-melittin each decrease inflammatory gene expression compared to untreated, unstimulated macrophages. At equal doses (300 nM), inflammatory gene expression after 4 hours is significantly reduced relative to LPS stimulated macrophages that have not been treated (**Figure 4A-C**). In both iNOS (**Figure 4A**) and TNF- α expression (**Figure 4B**), a significant difference in expression of these genes was observed between the GST-melittin treated group and the native protein treated groups, suggesting equivalence of both proteins in this regard. Although stimulated cells treated with GST-melittin have a slightly increased Cox-2 expression compared to those treated with the native peptide, Cox-2 expression is still significantly reduced from the untreated group (**Figure 4C**). These findings suggest that GST-melittin is as efficacious as the native peptide in decreasing iNOS and TNF- α gene expression in J774A.1 macrophages and shows

significant decreases in Cox-2 expression from untreated cells. All other treatment groups showed no statistical differences with the untreated, unstimulated macrophages.

3.4. GST-melittin maintains anti-inflammation at toxic concentrations of native melittin

Because we saw equivalence of the GST-melittin and native melittin in decreasing inflammatory gene expression and nitric oxide formation at non-toxic dose of native melittin, we explored whether or not GST-melittin would maintain anti-inflammation at a 10 fold dose increase. At a 3 μ M native melittin dose, macrophages do not maintain viability with or without LPS stimulation (**Figure 1A**). GST-melittin, on the other hand, maintains cellular viability (**Figure 1B**) and GST-melittin also decreases nitric oxide formation at this concentration (**Figure 3**). At toxic concentrations of the native peptide, GST-melittin continues to decrease inflammatory gene expression compared to untreated inflamed cells (**Figure 5A-C**). Compared to LPS treated macrophages, macrophages transcribed reduced levels of inflammatory genes ($p < 0.001$). For concentrations up to 3 μ M in unstimulated macrophages, there were detectable levels of inflammatory gene mRNA, but these levels were not significantly different from cells in the unstimulated state (**Figure 5A-C**).

3.5. GST and melittin must be fused to maintain survival and anti-inflammation over increased doses

The differences in toxicities and viability of the cells treated with GST-melittin compared to those treated with the native peptide warranted investigation of the effect of fusion of the proteins. After cleavage of the GST from the melittin via a thrombin-cleavable site (Buhrman et al., 2013b; Buhrman et al., 2013c), we show decreases in cellular viability, interpreted as cell death, similar to the native peptide at these concentrations (**Figure 1C**). Similarly, we show that native peptide with the addition of free GST maintains ability to kill (**Figure 1D**). GST alone has no effect on cellular viability with or without LPS treatment (**Figure 1E**). These findings suggest that the activity over increased concentrations of GST-melittin is due to the fusion of these proteins and not the activity after cleavage, which would have resulted in reduced cellular viability, which was not observed.

3.6. GST-melittin has a reduced ability to inhibit LPS binding to cells compared to native peptide

Mechanistically, it has been shown that cationic anti-microbial peptides, such as melittin, have a unique ability to bind anionic LPS and prevent it from interacting with cells (Asthana et al., 2004; Hancock and Diamond, 2000; Srivastava et al., 2012). With this in mind, we investigated the potential of this interaction with the fusion protein. When incubated with FITC-LPS, we confirmed that the native melittin peptide inhibits LPS binding to cells (**Figure 6**). However, at the same concentration and the elevated concentration examined throughout this manuscript, GST-melittin has significantly reduced ability to bind LPS with no statistical significant difference from LPS binding to cells (**Figure 6**). This suggests that the fusion of the peptide to the GST may be inhibiting melittin from neutralizing LPS. LPS neutralization appears to be present to a small degree, but the anti-inflammatory effect is more pronounced than would be expected for this level of LPS neutralization.

3.7. GST-melittin, but not GST, is taken up by macrophages

Native melittin has been shown to interact with the cell membrane (Dempsey, 1990b; Shai, 1999), but has also been shown to facilitate intracellular uptake of small molecules and proteins, due to membrane insertion and pore-formation (Pan et al., 2011; Pan et al., 2012; Pan et al., 2013; Pan et al., 2010). With this in mind, we investigated whether the fusion protein was localized intracellularly or on the membrane after 24 hours of treatment. Macrophages internalized the fusion-protein, GST-melittin, but not GST alone or BSA (**Figure 7**). This suggests that unlike the native peptide, the fusion protein acts intracellularly to exert its anti-inflammatory effects. Interestingly, fusion of both melittin and GST is necessary to elicit such high amounts of protein uptake within the macrophage. While there is uptake of GST, when macrophages are simultaneously treated with native melittin and labeled GST, it is minimal compared to that of the fusion protein (**Figure 7**).

4. Discussion

It is well known that melittin is a cell-lytic and toxic peptide that has anti-inflammatory properties (Esmaeili et al., 2008). It has been shown that melittin, in its native form, at concentrations under 3 μM has anti-inflammatory properties, but validation of the toxicity at these concentrations toward the cell-lines was not apparent (Mosser and Edwards, 2008; Park et al., 2004; Park et al., 2007). We have observed that melittin, in its native form, has toxicity at concentrations in the nanomolar range in macrophages having previously shown similar toxicity in bacteria and cancer lines (Buhrman et al., 2013a; Buhrman et al., 2013b; Buhrman et al., 2013c; Buhrman et al., 2012). This is consistent with several studies in other cell types (Nah et al., 2007; Nishikawa and Kitani, 2011; Soman et al., 2009). But melittin pore-formation capabilities are dependent on membrane characteristics, which can vary by cell type (Dempsey, 1990a; Gordon-Grossman et al., 2012; Raghuraman and Chattopadhyay, 2004; Talbot et al., 1987), suggesting that toxicity will vary between cell types.

For macrophages, the observed overlap between toxicity and anti-inflammatory response for the native peptide makes it an unfavorable pharmacologic modulator of immune response. Therefore, we sought to explore methods to improve the therapeutic response of the melittin peptide while maintaining its therapeutic effects. While examining the use of GST-GSH interactions for controlling the release of melittin (Buhrman et al., 2013b; Buhrman et al., 2013c; Buhrman et al., 2012), we observed the diminished toxicity of GST-melittin fusion protein. We validated this finding in macrophages at fusion protein concentrations up to 30 μM . Our ability to increase the non-toxic concentrations of this peptide by two logs suggests that melittin as a fusion protein may be more therapeutically applicable than the native peptide, and so we sought to explore this idea further by examining GST-melittin's ability to modulate inflammation in J774A.1 macrophages.

Macrophages are the workhorse of the inflammatory response and create and modulate a multitude of signals to respond to stimuli and control pathogenesis (Mosser and Edwards, 2008). Macrophages can release potent chemical signals upon stimulation, one of which, nitric oxide, can lead to excessive tissue toxicity and damage when persistently released (Korhonen et al., 2005). We show that GST-melittin can significantly decrease the release of

this potent chemical signal in macrophages that have been stimulated with LPS. GST-melittin can maintain this inhibition of NO production at toxic doses of the native peptide, reducing NO levels to baseline.

The inhibition of the chemical mediator, NO, can be largely due to the reduced expression of its production machinery, iNOS (Korhonen et al., 2005). We show that GST-melittin has the ability to downregulate expression of this enzyme to the same extent as the native peptide. Additionally, GST-melittin can downregulate expression to near homeostatic levels at increased concentrations, unattainable by the native peptide due to its cell-lytic toxicity. The ability of GST-melittin to downregulate iNOS expression is exceptionally significant, as once the inducible form of this enzyme has been upregulated, it can synthesize significantly more NO than physiologic concentrations produced by the constitutive enzymes over longer periods of time (Moncada, 1999).

Other potent chemical mediators in inflammation due to LPS, such as prostaglandins, are produced by the Cox-2 enzymes (Simon, 1999). Cox-2 is significantly upregulated in macrophages that have been provoked, and results in the majority of prostaglandin synthesis. In turn, the substantial over-production of prostaglandins can result in tissue damage and pain (Davies et al., 1984; Ferreira, 1972). We show that GST-melittin can maintain downregulated Cox-2 expression levels (relative to stimulated cells) at several concentrations. This downregulated Cox-2 expression suggests a potential role of GST-melittin in modulating not only inflammation, but also, the wound healing process as well as pain.

As a third significant marker of inflammation, we explored TNF- α , which is a central regulator in the inflammatory response (Bradley, 2008). We show that GST-melittin, like the native peptide, has the ability to downregulate expression of the TNF- α gene, to near baseline levels. TNF- α is widespread in pathogenesis of diseases beyond just inflammation, and has therefore been an effective target for many diseases (Feldmann and Maini, 2003; Palladino et al., 2003; Tracey and Cerami, 1993).

We have chosen to look at these genes because they are expressed to a large degree in inflammation. However, the implications of the findings of this study are much wider than just inflammation. The mediators and genes studied have roles in other major pathologies, including rheumatic disease and cancer (Dolcet et al., 2005; Tak and Firestein, 2001). Our observations warrant the investigation of the utility of GST-melittin in these pathologies as well.

It is important to note that in the expression of the genes and the production of NO, GST-melittin is only efficacious at higher concentrations when linked as a chimeric fusion protein. When the proteins have been cleaved from one another, the cell-lytic property of melittin is rescued. The fusion of the GST protein onto the melittin peptide is thought to inhibit the aggregation of melittin due to steric hindrance of peptide association. This inhibits formation of secondary alpha-helical structure, which is necessary for pore formation and subsequent cell lysis (Lee et al., 2013; Lin and Baumgaertner, 2000). Our findings suggest that fusion proteins can be used to modify toxicity and action of their parent

proteins. Additionally, this poses significant potential in engineering of fusion proteins of toxic peptides with disease specific cleavable sites to potentially controllably balance their toxic and therapeutic properties. Because melittin and other cationic peptides have been shown to bind LPS and prevent subsequent interaction with cells, we explored this avenue as a potential mechanism for amelioration of the inflammatory response with our fusion protein.

When macrophages are treated with native melittin, there is, indeed, significant inhibition of LPS from interaction with macrophages (**Figure 6**). However, at the same concentration as the native peptide, GST-melittin has a significantly reduced inhibition of LPS from interaction with the cells. Since the LPS is well above the concentration necessary for stimulation (Sweet and Hume, 1996), the approximate 5% reduction in binding resulting from treatment with GST-melittin (0.3 μ M) is not expected to account in whole for the reduced inflammatory response. In fact, this inhibition of LPS, is less than that of the native peptide (at the same concentrations) and not significantly different from cells treated with just the FITC-LPS. However, GST-melittin remains as potently anti-inflammatory as the native peptide. Furthermore, although we see similar inhibition of LPS binding with the native melittin (0.3 μ M) and a higher concentration of GST-melittin (3 μ M), there is a significantly more potent anti-inflammatory response exhibited with treatment of the GST-melittin. This suggests that inhibition of LPS binding to the cells is not the sole factor involved in ameliorating the inflammatory response.

Macrophage internalization of GST-melittin but not of GST or BSA (**Figure 7**) suggests that fusion of the GST to melittin may aid in therapeutic uptake of both melittin and GST into the cell. Although we were focused on the melittin peptide, GST as a therapeutic protein should not be ignored. It has been demonstrated that GST does have anti-inflammatory potential (Bentz et al., 2012; Yang et al., 2008). Additionally, when fused to other cargo, melittin has been shown to facilitate cellular uptake (Pan et al., 2011; Pan et al., 2012; Pan et al., 2013; Pan et al., 2010). Therefore, because macrophages selectively uptake the GST-melittin fusion protein, it is possible that GST may have a dual role in prohibiting melittin's pore-forming capability and simultaneously aiding in the anti-inflammatory response. This could elucidate the similar observed functional response between the native peptide and GST-melittin, but the decrease in inhibition of LPS interaction with cells with the fusion protein.

Other mechanistic hypotheses focus on melittin as the active anti-inflammatory agent. Melittin has traditionally been thought to act within the membrane and through cell membrane-based signaling and has been used to escape endocytosis (Hou et al., 2013; Ogris et al., 2001). It is clear that the cell-permeabilization is diminished with the GST-melittin fusion protein. Other hypotheses that remain as to the mechanism of action and our future studies: (1) melittin-fusion protein monomers within the membrane have the ability to signal through a mechanism similar to the melittin peptide or (2) intracellular melittin signaling through a yet-unknown mechanism. Despite evidence demonstrating the cellular uptake of glutathione S-transferases (Morris et al., 2011; Namiki et al., 2003), our results clearly show that GST is not taken up by cells when melittin is not part of the protein. Further insight into the sub-cellular localization of GST-melittin may more clearly elucidate the role of the

fusion protein in protection against toxicity of the peptide. Delineating the mechanisms for cell entry will allow for the rational design of fusion proteins for intracellular delivery.

Although our findings are promising, they are not without certain potential *in vivo* limitations. Exogenously administered protein therapeutics, such as the GST-melittin, can be immunogenic, which could lead to neutralizing the immunomodulatory activity of GST-melittin. Preliminary antibody testing to the fusion protein as a whole or parts of the fusion protein (either GST or melittin) will be necessary before administration to avoid a potentially immunogenic reaction. Purification of the protein in eukaryotic cells, such as Chinese hamster ovary (CHO) cells, may also reduce likelihood of potential immunogenicity. Careful *in vitro* stability testing as well as immunogenicity testing will help avoid many potential pitfalls of *in vivo* administration of this protein.

5. Conclusions

Utilization of native melittin for anti-inflammation has met challenges due to its toxic properties. Our results demonstrated that compared to the native peptide, GST-melittin has reduced pore-forming capabilities. The reduction in its toxicity has no effect on its anti-inflammatory properties. We have confirmed that GST-melittin and native melittin have similar anti-inflammatory properties at sub-lytic concentrations. The reduced toxicity of the fusion protein allows the use of concentrations of the protein in excess of at least two logs. Anti-inflammatory properties of the protein are augmented at higher concentrations, too toxic to use with the native peptide. Our investigation warrants further mechanistic exploration, but suggests that fusion proteins of other cell-lytic peptides may act similarly. We conclude that GST-melittin is a non-toxic alternative to native melittin for use in macrophage-mediated inflammation due to LPS.

Acknowledgments

This investigation was conducted in a facility constructed with support from Research Facilities Improvement Program Grant (C06 RR15482) from the National Centre for Research Resources of the National Institutes of Health (NIH). This research has been funded, in part, by the University of Illinois at Chicago Center for Clinical and Translational Science (CCTS) award supported by the NCRR (UL1 TR000050, RAG, JSB). JER and JSB were partially funded by the Chancellor's Graduate Research Fellowship. Additionally, the authors thank Dr. Debra A. Tonetti for use of equipment, Yu Zhang for technical assistance, and the anonymous reviewers for their suggestions significantly improving the manuscript.

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Figure 1. GST-melittin has no effect on macrophage survival while melittin kills macrophages at nanomolar concentrations.

Viability of J774A.1 macrophages, as assessed by the MTS assay and presented as the experimental group relative to untreated control. Macrophages were treated in the absence (○) or presence (□) of 2 μg/mL lipopolysaccharide (LPS) stimulation and various proteins: (A) native melittin, (B) GST-melittin, (C) GST-melittin pre-cleaved with thrombin, (D) GST mixed with equimolar native melittin, and (E) GST. Each point represents the mean plus or minus (±) the standard error of the mean of three independent experiments.

Figure 2. GST-melittin has no effect on macrophage permeability while native melittin permeates macrophages at nanomolar concentrations

Representative matched phase contrast (top rows) and epifluorescence (bottom rows) micrographs of J774A.1 macrophages at varying times (columns) indicating morphology and permeability to propidium iodide. Untreated cells melittin (GST-are presented for comparison (untreated; 2 top rows). Cells treated with GST-are presented for comparison (untreated; 2 top rows). Cells treated with GST-melittin; middle 2 rows) do not show typical signs of permeability over one hour. Macrophage permeability and morphology change after treatment with the native melittin (melittin; bottom 2 rows) within 15 minutes of treatment.

Figure 3. Nitric oxide synthesis of J774A.1 macrophages treated with native melittin and GST-melittin

Nitric oxide produced over 24 hours after treatment was measured as its major oxidative metabolite, nitrite. All values are presented as mean plus or minus ($\pm$) the standard error of three independent experiments where † reflects a statistically significant difference ($p < 0.001$) compared to LPS stimulated, untreated (no protein) macrophages and ‡ reflects a statistically significant difference ($p < 0.001$) compared to unstimulated (LPS free), untreated (no protein) macrophages.

Figure 4. Inflammatory gene expression in J774A.1 macrophages treated with native melittin or GST-melittin

Expression of inflammatory markers, iNOS (A), TNF- (B), and Cox- (B), and Cox-2 (C), in macrophages treated with 300 nM native melittin, GST-melittin, or no protein with and without inflammatory stimulus (LPS). mRNA levels were normalized to the expression of the endogenous reference gene, -glucuronidase. Values are presented as mean plus or minus ($\pm$) standard error of the mean of three independent experiments where † reflects a statistically significant difference ($p < 0.001$) compared to LPS stimulated, untreated (no protein) macrophages and ‡ reflects a statistically significant difference ($p < 0.001$) compared to unstimulated (LPS free), untreated (no protein) macrophages.

Figure 5. Inflammatory gene expression in J774.1 macrophages treated with GST-melittin
 Expression of inflammatory markers, iNOS (A), TNF- α (B), and Cox-2 (C), in J774.1 (B), macrophages treated with no protein, 300 nM GST-melittin, or 3 μ M GST-melittin with and without inflammatory stimulus (LPS). mRNA levels were normalized to the expression of the endogenous reference gene β -glucuronidase. Values are presented as mean plus or minus ($\pm$) standard error of the mean of three independent experiments. Statistical significance where $\dagger$ reflects a statistically significant difference ($p < 0.001$) compared to LPS stimulated, untreated (no protein) macrophages and $\ddagger$ reflects a statistically significant difference ($p < 0.001$) compared to unstimulated (LPS free), untreated (no protein) macrophages.

Figure 6. Inhibition of LPS interaction with J774A.1 macrophages

Cells were treated with FITC-LPS after functional concentrations of native melittin (300 nM; Green) and GST-melittin (300 nM, dark blue, and 3 μ M, light blue). FITC-LPS in solution was measured. All values are presented as the mean FITC-LPS remaining in the buffer plus or minus ($\pm$) the standard error of three independent experiments where † reflects a statistically significant difference ($p < 0.05$) compared to FITC-LPS treated native melittin treated macrophages, and ‡‡ reflects a statistically significant difference ($p < 0.01$) compared to FITC-LPS (no protein) treated macrophages.

Figure 7. GST-melittin is internalized by J774A.1 macrophages after 24 hours
Representative pseudocolored confocal micrographs of macrophages treated with BSA (green), GST (green), GST (green) and native melittin, or GST-melittin (green) after 24 exposure. The nuclei (blue; left column), protein (green, middle column), and the pseudocolored composite image (right column) were labeled to indicate their presence within the cells. The scale bar in each image is 10 μ m.

Table 1

Genes and primers used for Quantitative Real Time PCR.

Gene	Protein	Acronym	Primers (5'→3')	Accession number
<i>gusb</i>	glucuronidase, beta	GUSB	FW: GCAAGACATCGGGCTGGTGA REV: TGGCACTGGGAACCTGAAGT	NM_010368.1
<i>nos2</i>	nitric oxide synthase 2, inducible	iNOS	FW: AGCCCCGCTACTACTCCATC REV: GCCACTGACACTTCGCACAA	NM_010927.3
<i>tnf</i>	Tumor necrosis factor	TNF-	FW: AACTTCGGGGTGATCGGTCC REV: TGGTTTGTGAGTGTGAGGGTCT	NM_001278601.1
<i>ptgs2</i>	Prostaglandin-endoperoxide synthase 2	Cox-2	FW: CATGGGTGTGAAGGGAAATAAGGA REV: GGTGAAGTGCTGGGCAAAGA	NM_011198.3