Rage Ectodomain Shedding is a Conserved and Differentially Regulated Process

Alexander E. Braley
University of Miami, abraley@med.miami.edu

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RAGE ECTODOMAIN SHEDDING IS A CONSERVED AND DIFFERENTIALLY REGULATED PROCESS

By

Alexander E. Braley

A THESIS

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RAGE ECTODOMAIN SHEDDING IS A CONSERVED AND DIFFERENTIALLY REGULATED PROCESS

Alexander E. Braley

Approved:

Sapna K. Deo, Ph.D.
Associate Professor, Biochemistry and Molecular Biology

Ralf Landgraf, Ph.D.
Associate Professor, Biochemistry and Molecular Biology

Barry Hudson, Ph.D.
Research Assistant Professor, Endocrinology, Diabetes and Metabolism

M. Brian Blake, Ph.D.
Dean of the Graduate School
The Receptor for Advanced Glycation End-products (RAGE) is a multi-ligand cell signaling receptor present on most cell types. Upregulation of RAGE is seen in a number of pathological states including various cancers, inflammatory and vascular disease, dementia and diabetes. Several studies have shown RAGE ectodomain shedding to be but little is known about the regulation and functional effects.

Here we show evidence that human and mouse ectodomain shedding is inducible by several diverse shedding stimulators and the basic mechanism conserved. It is also shown here that RAGE shedding likely goes through different pathways, as ionomycin but not PMA shedding is inhibited by PI3K and PMA but not ionomycin shedding induced by PKC inhibitors. Preliminary support is established for ADAM17 cleavage of RAGE in addition to ADAM10. Honing in on the cleavage mechanism of rage, we show that one cleavage site is likely responsible for all RAGE ectodomain shedding. Finally, we show that cleavage increases cell invasion and migration, important for cancer associations.

RAGE ECD shedding is a conserved and tightly regulated mechanism involving distinct signaling pathways. Shedding of RAGE regulates key cellular properties including adhesion and migration. Our data suggest that proteolysis of RAGE is a major regulatory process and may emerge as a novel therapeutic target for cardiovascular diseases.
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Chapter 1: Introduction

RAGE (receptor for advanced glycation endproducts) is a 403 amino acid transmembrane, multi-ligand receptor expressed in most cells, but more highly expressed in heart, lung, and skeletal muscle.\(^\text{1,9}\) This protein is a type-1 transmembrane receptor, consisting of an intracellular, transmembrane and extracellular domain. The extracellular domain is made up of a variable-type domain and two constant-type domains, named so for their similarity to immunoglobulin domains.\(^\text{9}\) The variable domain is the site of most reported ligand bindings.\(^\text{9}\) This protein was originally discovered in bovine lung as a receptor for advanced glycation end products, which are the products of non-enzymatic reactions between the amino group of a protein and sugar molecules by way of the Maillard Reaction.\(^\text{9,14}\) Conditions are favorable for the formation of AGEs in the blood when there is an extremely high level of blood glucose, as present in diabetics, also during inflammation and oxidative stress. RAGE was later found to bind to a wide range of ligands including S100(A,B,P), HMGB1, Collagen I & IV, MAC-1, and others.\(^\text{9}\) Rage signaling is generally proinflammatory and enhanced signaling leads to cellular dysfunction.\(^\text{9}\) Up-regulation is associated with the development of a range of diseases ranging from diabetes, various types of cancer, and various vascular diseases.\(^\text{9}\) Importantly, RAGE has been implicated in the pathogenesis of both atherosclerosis and cardiovascular disease. Our lab has shown in the past that RAGE signal activation leads to a pro-inflammatory state that leads to many cellular responses and phenotype changes that are implicated in cardiovascular disease development and progression.\(^\text{8,15,16}\) The mechanism behind RAGE’s association with these diseases is not well-understood.\(^\text{9}\)
RAGE is capable of signaling through several pathways including MAPK, RHO GTPase, and Protein Kinase C (PKC). This signaling can affect proinflammatory gene regulation, apoptosis, and migration/invasion. Increased RAGE expression is associated with advanced development of invasive and metastatic tumors. RAGE signaling has been shown to be essential for migration in a C6 glioma model and has been shown to increase atherosclerotic lesion size.

In 1999, RAGE was found to have a soluble variant that can be formed by alternative splicing; this variant, named RAGEv1, is formed by exclusion of exon 10 that leads to a premature stop codon and the truncated form is secreted. This soluble form went on to be the subject of many studies exploring the role of endogenous sRAGE. Much more recently, in 2008, it was discovered that RAGE undergoes proteolytic cleavage by matrix-metalloproteases. This occurs near the cell surface and results in the release of the ectodomain in a process known as “ectodomain shedding.” Ectodomain shedding is a process that is shared by many transmembrane proteins such as cell adhesion molecules, receptors, immunoglobulins, and enzymes. The first definitive example of ectodomain shedding thoroughly characterized is that of TNF-alpha, which was found to be shed by TACE/ADAM17. Ectodomain shedding has multiple functions. It is a useful for generating soluble protein, because it allows for a reservoir of protein to be kept on the surface ready for quick release, as opposed to synthesizing soluble proteins de-novo. Additionally, if the protein has a function on the membrane (receptor, adhesion, etc.), then shedding can serve to rapidly down-regulate the activity of the protein and/or can quickly diminish signaling effects inside the cell. Also of note, improper regulation of this shedding is seen in certain pathological states.
It is known that RAGE ectodomain shedding contributes far more to the native levels of sRAGE than does alternatively spliced sRAGE.\textsuperscript{9} Despite this, the mechanism of RAGE shedding, how it is regulated, and the signaling pathways activated are not well understood. It has been suggested that RAGE ectodomain shedding is primarily controlled by signaling through the PKC pathway, and ADAM10 has been implicated in the actual cleavage. Unfortunately, there is no consensus sequence for ADAM10, along with other ADAMs such as ADAM17/TACE; as such it is not easy to determine which protease(s) may or may not be responsible for cleaving RAGE. Full understanding of the cleavage of RAGE is important since many of the interactions are not unique to RAGE regulation. Any attempt to alter this process therapeutically may elicit undesirable pleiotropic effects or perhaps lead to less than efficacious results if a more comprehensive understanding of this process is not attained. We feel of important consequence, there may be differential regulation of RAGE ectodomain shedding, and these different mechanisms of shedding may include more than one signaling pathway and may also involve more than one protease. Additionally, we suspect that the differential regulation of RAGE ectodomain shedding may allow for additional cellular responses after shedding, perhaps leading to stimulators or inhibitors of shedding that also inhibit or activate RAGE signaling, perhaps even leading to a complex regulation of RAGE production levels. Digging deeper may even reveal different compounds even shift the splice variant expression ratios. In addition many ectodomains that undergo cleavage do so by more than one sheddase. We hypothesize that RAGE undergoes cleavage by yet identified partners, which may be activated by different signaling pathways.
Chapter 2: Methods

Generation of Stable Cell lines

HEK-293 and C6 cells were obtained from American Type Culture Collection (ATCC), and used to generate stable cells expressing human RAGE, mouse RAGE, RAGEv4 (RAGE splice variant 4) and empty vector (mock) by inserting into pcDNA3.1 vector and transfecting using Fugene 6 and antibiotic selection with G418 or zeocin as previously described (Hudson 2008)(Kalea 2010). Stable expression was confirmed by Western blot using anti-RAGE polyclonal antibodies. HIS-tagged RAGE was generated by inserting in-frame a 6HIS-2HA tag in between the N-terminal signal sequence and the rest of the RAGE cDNA as done previously by Sakaguchi et al.13

Cells were maintained in Dulbecco’s Modified Eagle Medium (Gibco) supplemented with 10% fetal bovine serum from (Atlanta Biologicals), along with Penicillin/Streptomycin (Gibco), and MycoZap (Lonza) antibiotics. All cell counting was performed using Countess by Invitrogen according to manufacturer’s instructions.

Ectodomain Shedding Assays

Adherent cells were removed from culture flask using trypsin-EDTA (Gibco) and seeded at 250,000 cells per well in a 6 well plate, and grown for 2-3 days until 70% confluence was reached. The cells were then serum starved overnight and the following day incubated for one hour with fresh serum-free DMEM prior to experiments. All media changes were performed with sterile PBS (Corning) rinse. Shedding assays were performed in 2mL of serum-free DMEM with or without shedding compounds for a period of one hour. Media was then removed and cell fragments removed by
centrifugation at 16,000g for 15 minutes. PMA, APMA, Ionomycin, Cantheradan, CalyculinA, and Orthovanadate were all acquired from Sigma. Pervanadate was generated by mixing 100mM Hydrogen Peroxide and 100mM Sodium Orthovanadate pH 10, at 1:1 as established previously.\textsuperscript{18}

**Western Blots**

Cell lysate was collected immediately after experiment by rinsing in ice cold PBS followed by lysis with Mammalian Protein Extraction Reagent (Thermo Scientific) with protease (Sigma) and phosphatase inhibitors (Sigma). Cells were de-adhered using a cell scraper and lysis proceeded for 20 minutes on ice with occasional agitation. Insoluble debris was then removed by centrifugation. Protein concentration was determined by Bradford assay using bovine serum albumin as a standard. SDS-PAGE gels were performed using Invitrogen NuPAGE equipment in MOPS buffer (Invitrogen) for 2 hours at 100V. Western blotting was performed using Invitrogen NuPAGE transfer apparatus and buffer and Immobilon-FL membranes (Millipore). Transfer was performed on ice for 2 hours at 25V. Membranes were rinsed with Tris Buffered Saline (Corning) before being blocked in 1:1 Odyssey Blocking Buffer (Li-Cor):TBS for one hour at room temperature on plate shaker. Primary Antibody incubation was performed at appropriate concentration in 1:1 Odyssey:TBS with 0.1% Tween-20 (Sigma) and 0.02% Sodium Dodecyl Sulfate (Sigma) overnight at 4°C on plate shaker. Membranes were washed 3 times for 5 minutes with TBS-T. IRDye 600RD and 800CW secondary antibodies were supplied by Li-Cor and were used 1:5000 for 1 hour at room temperature shielded from light on plate shaker. Membranes were washed 3x for 5 minutes in TBS-T and one additional wash in TBS
prior to visualization. Membranes were visualized on a Li-Cor Odyssey Infrared Imaging system and images analyzed using Li-Cor application software version 3.025.

All primary antibodies were diluted 1:1000 and secondary antibodies 1:5000 (unless otherwise noted) in 1:1 Odyssey:TBS containing 0.02% SDS and .1% Tween-20. RAGE monoclonal antibodies were purchased from Millipore, and RAGE polyclonal antibody was generated as previously described at 1:2000 dilution. Anti-actin antibody was purchased from Millipore. Anti-His antibody obtained from Millipore. ADAM10 and ADAM17 antibodies were obtained from Millipore. Antibody optimizations according to Li-Cor suggestions lead to the determination that 0.02% SDS and 0.1% were optimal for consistent reduction of background and increase in signal.

ELISA

Human and mouse Rage ELISAs were performed using the DuoSet human and mouse RAGE ELISA kits acquired from R&D and performed according to the manufacturer’s instructions. Capture antibody (100ul per well) was diluted in PBS and incubated overnight at room temperature. Plates were blocked for 2 hours at room temperature (RT) with constant agitation using 300ul per well of Reagent Diluent 2 (RD2) obtained from R&D diluted as indicated 1:100 in Ultrapure Water (Invitrogen). Sample incubation was performed for 2 hours at RT with agitation using 50ul of sample and 50ul of working concentration of reagent diluent. RAGE standards were generated using a serial dilution in RD2 of individual aliquots of the RAGE standard (25,000 pg/ml) provided in the DuoSet kit, resulting RAGE standard curve had a high of 5000 pg/ml and low of 78 pg/ml. RAGE detection was performed using RAGE detection antibody diluted as
instructed in RD2 and incubated for 2 hours at RT with constant agitation. Streptavidin-HRP was obtained from R&D and diluted 1:100 in RD2 and 100ul per well was incubated at RT with constant agitation while protected from light. For visualization, 100ul per well of OneStep Reagent from Thermo Scientific was incubated shielded from light for 5 minutes. Reaction was quenched with 50ul of 2N H₂SO₄ (Sigma). ELISAs were read using a BioRad iMark 1.04.02 at 450nm subtracting from 595nm background. Data was analyzed using Microplate Manager Version 6.1.

**Knockdown**

HEK-293 cells were transfected with either scramble, ADAM10, or ADAM17 siRNA using lipofectamine (Invitrogen). Two days after transfection cells were serum starved and stimulated with PMA. Knockdown was confirmed using western blot using anti A10 antibody and A17 antibody and qPCR.
Chapter 3: Results

Rage is shed constitutively in mouse and human models

HEK-293 cells stably expressing either human or mouse full length (membrane bound) RAGE were incubated overnight in serum free media and were found by western blot and ELISA to constitutively shed RAGE into the culture medium without any stimulation (Figure 1). The mRAGE band, as previously described, is slightly smaller than the hRAGE band. The characteristic dual bands caused by differing glycosylation states were visible in both human and mouse RAGE bands. Importantly, as HEK-293 cells express very little rage, the mock transfected lane had no visible band. This, along with a propensity to be easily transfected, makes the cell line a good host for RAGE ectodomain shedding studies.

Rage ectodomain shedding can also be stimulated, which is also conserved

Ectodomain shedding is a phenomenon that occurs in a wide range of substrates of different types with many different biological consequences. Shedding can occur in cell adhesion molecules, receptors, growth factors, enzymes and others. There are several pharmacological compounds which induce ectodomain shedding in ways that can help to elucidate the potential factors involved in the process that leads up to shedding. Ectodomain shedding of RAGE is known to be stimulated by a variety of activators, notably the PKC activator Phorbol Myristate Acetate (PMA), and the calcium ionophores calcimycin and ionomycin. We observed shedding via western blot and ELISA after one hour stimulation with PMA and ionomycin but also we found that RAGE shedding was induced by metalloprotease activator 4-aminophenylmercuric acetate (APMA) and fetal
bovine serum (FIGURE 2). Since soluble RAGE is present in mice as well, we wanted to see if mouse rage would exhibit similar shedding patterns. When stably expressed in HEK cells, we observed a very similar shedding pattern of mRAGE when stimulated with PMA, ionomycin, APMA, and FBS (Figure 2). This suggests that the mechanism behind shedding is conserved between human and mouse. Importantly, serum is shown to be a good source of endogenous RAGE shedding stimulation, which is important for physiological relevance.

Phosphatase inhibition is another way of activating sheddases and we were interested in seeing the effects on RAGE shedding. Cantharidin and Calcinin A are compounds that inhibit the pp1/pp2 family of phosphatases. Cantharadin has been shown to affect ectodomain shedding of L-selectin through a P38 dependent mechanism. Interestingly, RAGE shedding was observed by ELISA and western blot when human RAGE expressing HEK cells were treated with cantharadin, calculinA for one hour. We also observed induced RAGE shedding when cells were treated with the tyrosine phosphatase inhibitor pervanadate(Figure 3). Taken together this hints at a regulation mechanisms outside of the current view of PKC dependence.

**RAGE shedding induced by ionomycin is not affected by PKC inhibition**

Since we suspected RAGE shedding to be possible independent of PKC signaling, we sought to test the effect of PKC inhibitors Go6976 and GFX109203x on RAGE induced shedding with PMA or Ionomycin. As expected, PMA induced shedding was markedly reduced with inhibitors Go6976, GFX109203, and metalloproteinase inhibitor BB94.
Interestingly, ionomycin induced RAGE ectodomain shedding was not affected by PKC inhibition by Go6976 or GFX109203. Ionomycin induced shedding was successfully abrogated by both BB94 and the calcium chelator EGTA. Interestingly, calcium chelation with EGTA had no effect on PMA induced signaling, indicating a lack of calcium dependence (Figure 4). It is important to note, that Go6976 and GFX109203 are potent inhibitors specific to PKCα and PKCβ1 but not PKCδ.

**PI3K associated with Ionomycin, but not PMA stimulation**

To further probe the potential differences in regulation of RAGE ectodomain shedding, we used the two most common RAGE shedding inducers PMA or Ionomycin with or without inhibitors of various pathways such as p38/MAPK, MEK, SAP/JNK, and PI3K. Each inhibition assay performed after an hour of pre-incubation with each respective inhibitor before an hour long incubation with inhibitor and shedding agonist. We found that inhibition of the p38/MAPK, MEK, and SAP/JNK pathways with SB, U0126, and SP respectively had no significant effect on RAGE shedding induction with PMA or Ionomycin. Surprisingly, we did note a significant inhibition of ionomycin induced shedding with PI3K inhibition but did not see a similar inhibition of PMA induced shedding (FIGURE 5). This further supports the assertion that differential regulation of RAGE shedding exists.

**RAGE cleavage site narrowed down using non-cleavable RAGE variant**

The full length RAGE protein is around 50-55 kDa, and the cleaved form of RAGE is about 45 kDa, leading to the conclusion that the cleavage site must be close to the
extracellular surface of the cytoplasmic membrane. Several attempts have been made to identify the precise location of cleavage, but it remains elusive\textsuperscript{10}.

In 2009 several splice variants of mouse RAGE were identified. Of the products that were not subject to nonsense mediated decay, MRAGE\textsubscript{v4} was of particular interest to us as it was missing exon 9. This exon, although very small (9 residues), is located on the extracellular region proximal to the membrane and may contain the cleavage site. We took this variant and stably expressed it in HEK-293 cells and detected in lysate a protein product that is slightly smaller than mRAGE\textsubscript{v1} and is recognized by several RAGE antibodies [Figure 6]. Excitingly, no RAGE shedding was observable via western blot or RAGE ELISA [Figure 6]. We were astounded to find that no RAGE shedding was observed even when stimulated with PMA, ionomycin, APMA, or serum [Figure 6]. This indicates for the first time that mRAGE\textsubscript{v4} is an endogenously expressed non-cleavable RAGE variant. This strongly supports the notion that exon 9 contains the cleavage site.

**Rage shedding accomplished by one cleavage site**

The consistent presence of double bands for sRAGE has been attributed to differential glycosylation; and the presence of faint bands when using polyclonal antibodies has been assumed to be nonspecific antibody binding, but the presence of an alternative cleavage site has not been definitively ruled out. One could argue that although our monoclonal antibody probed western blots show only one sRAGE band, the particular epitope recognized by this antibody might not be present in an alternatively cleaved sRAGE molecule and would hence go undetected. We decided to address this concern using an N-terminal histidine tag conjugated to full length RAGE transiently expressed in HEK
cells. If multiple cleavage sites exist, but create products lacking the epitope recognized by our monoclonal antibody, there should still be multiple bands when probing with a histidine antibody. As expected, stimulation with PMA, ionomycin, APMA, and serum revealed one HIS reactive band that lined up perfectly with the RAGE reactive band (Figure 6). This bad size was observed to be 60KD, which is a result of the 6xHIS-G+HA tag along with 45 kDa sRAGE. This provides strong reason to believe that there is only one cleavage site of RAGE.

**Knockdown of ADAM10 & ADAM17 both inhibit RAGE shedding**

To address the question of if the RAGE cleavage enzyme is only ADAM10, we used siRNA for ADAM10 and ADAM17 followed by shedding analysis and confirmation of knockdown. Surprisingly, we saw a reduction in both basal shedding after 4 hours as well as PMA stimulation(Figure 6). Importantly, this effect was witnessed with only a 50% knockdown of both ADAMs (Figure 6). The knockdown of A10 reduced RAGE shedding more than A17 did, but this hints that ADAM17 may play at least some role in cleaving RAGE.
Chapter 4: Discussion

Mechanisms of RAGE shedding

Constitutive RAGE shedding has been demonstrated by RAGE expressing cell lines as well as in-vivo models. Here, we demonstrate, by western blot and ELISA, that Human Endothelial Kidney (HEK) cells stably expressing both mouse and human isoforms of full-length membrane bound RAGE also shed the ectodomain with and without exogenous stimulation. This suggests for the first time that the mechanism behind RAGE shedding is conserved between humans and mice. When considering how various compounds simulating different signaling pathways all managed to induce RAGE shedding indicates probable regulation by multiple pathways and presumably multiple shedding enzymes. Previous studies have shown that RAGE shedding can be activated by PMA and calcimycin among other molecules. Zhang et al. also suggested that matrix metalloproteinases ADAM 10 and MMP9 are responsible for cleaving RAGE upon stimulation. Indeed Zhang’s interpretation of his data is seemingly contrary to our findings, where we show calcium influx mediated by the calcium ionophore ionomycin is independent of PKC signaling. Upon closer inspection of Zhang’s 2008 FASEB paper shows that even in ADAM10−/− cells there was still observable to exhibit constitutive shedding, albeit diminished. Our experience shows that a considerable amount of RAGE needs to be present in media to be detectable on even the best western blot, which means that any band without the presence of the purported metalloproteinase in-and-off itself argues against sole responsibility of shedding belonging to ADAM10. In addition, their conclusion that ADAM17 knockdown had no effect on RAGE shedding was not conclusive as they offered no quantification of the westerns. As many studies in the past
were limited to precipitation of media followed by quantification of western blots, (both can be increase the opportunities for confounding variables) we felt that a thorough examination of the shedding stimulators was warranted with a much more consistent and reliable ELISA based method.

Furthermore, the consistent nature of ELISA, which provides actual concentrations of protein as opposed to merely providing the ability to see relative levels of one experiment, allows for more rigorous evaluation of inhibitor studies, whose effects can easily be masked if inhibition does not result in profound consequences. The poor sensitivity of western blot detection of RAGE requires a large amount of media to be concentrated, which the very nature of the procedure provides a significant opportunity for error that may make evaluation of findings difficult at best. Compounding with blotting’s inability to confidently quantitate across gels between experiments (necessary to properly employ statistics), ELISAs are clearly less prone to experimental and investigator error. With further studies using ELISA based approaches in addition to blotting may prove to alleviate the problem of conflicting results behind the mechanism of RAGE shedding. Our results, though clear, may not contain the full story, however, as there are many other inhibitors to be examined and surely signaling contributors to be identified. Our preliminary knockdown results show some reason to believe ADAM17 may be involved in RAGE shedding, although the experiment would need to be repeated for validation. In the future we are interested in examining the various shedding compounds with a stable knock down of ADAM 10, ADAM17, and MMP9 as well as testing some of the other shedding compounds with pathway inhibitors to better tease out the signaling interactions.
Cleavage site of RAGE

Although the receptor for advanced glycation end products was discovered in 1992, it wasn’t until 2008 that it was determined that sRAGE can be formed by proteolytic cleavage. Since then, despite several attempts, the cleavage site has not been determined. In addition, the presence of many bands present on RAGE probed westerns are shown through glycosylation studies using glycopeptidase F treated samples to most likely not be bona-fide RAGE protein, we understood that the argument could be made that an additional cleaved product of RAGE may not include glycosylation sites and/or may lack the epitopes recognized by the most popular RAGE antibodies. Here, using an N-terminal tag, thoroughly dismiss any potential concerns concerning multiple cleavage sites of hRAGE. That being said, we have yet to repeat the N-terminal tagged experiment in a mouse cell with mouse RAGE, so there still may be a small chance that a non-conserved additional cleavage point exists.

Non-cleavable endogenous mRAGE

Many previous studies have identified splice variants of RAGE, many focused on finding an endogenous DN-RAGE, but an endogenously expressed non-cleavable alternatively spliced variant had hitherto not been found. Here we show that the 9 missing residues proximal to the transmembrane region render mRAGE\_v4 incapable of being cleaved. This is especially of note because unlike most other identified mRAGE splice variants, v4 is relatively highly expressed in several tissues. These tissues (expressed as a ratio of mRAGE v4:v1) include heart (21%), kidney(12%), and lung(41%), but does not appear to be present in brain tissue. This is especially interesting given the associations of
RAGE with the development of Alzheimers.\textsuperscript{17} As our shedding experiments of mRAGE\textsubscript{v4} were performed in HEK cells, it’s possible (although unlikely) that some novel shedding mechanism that exists only in mice is capable of cleaving mRAGE\textsubscript{v4}. In order to prove this is not the case, further studies will have to show similar results in a mouse model and perhaps even utilize a similar approach to our N-Terminal HIS tagged RAGE as seen in Figure 7. It should also be pointed out that as of yet a human splice variant incapable of being cleaved has not been found.\textsuperscript{7}
FIGURES

Figure 1.

![Image of Figure 1 showing Western Blot results for conditioned media and cell lysate samples comparing mock, human RAGE, and mouse RAGE.](image)

**Figure 1.** RAGE ectodomain shedding is constitutive and is conserved between human and mouse RAGE. 293 cells transfected with RAGE or mock, were incubated for 24 hrs in serum free media. A. Conditioned media and B. Cell lysate was subjected to WB using anti-RAGE polyclonal antibodies.

Figure 2.

![Image of Figure 2 showing Western Blot results for human RAGE protein levels before and after treatment with PMA, ionomycin, and APMA.](image)

**Figure 2.** RAGE ectodomain shedding is inducible by diverse inducers of ectodomain shedding. RAGE expressing cells were either untreated (NS) or treated with shedding inducers including PMA (20nM), Ionomycin (1uM), APMA (29uM) or 10% FBS for 60 mins. Conditioned media was subjected to A. WB using anti-RAGE polyclonal antibodies or B. & C. Or quantified using the RAGE human ELISA. Data are means of n=3 independent experiments.
Figure 3. RAGE ectodomain shedding is inducible by diverse inducers of ectodomain shedding. RAGE expressing cells were either untreated (NS), or treated with shedding inducers including PMA (200 nM), ionomycin (1 μM), APMA (20 nM), 10% FBS, Protein phosphatase inhibitors (Cantharadine, calyculin A and sodium pervanadate) for 60 mins. Conditioned media was subjected to A: WB using anti-RAGE polyclonal antibodies or B & D. O* quantified using the RAGE human ELISA. Data are means of n=3 independent experiments.

Figure 4. RAGE ectodomain shedding induction is abrogated by PKC inhibitors with PMA induction but not by ionomycin induction. RAGE expressing cells were either untreated (NS), or pretreated with inhibitors for one hour, followed by incubation with nothing, PMA (200 nM) or ionomycin (1 μM) or PMA or ionomycin with a range of inhibitors including Go6976 (1 μM), GF109203x (1 μM), BB94 (1 μM), or EGTA (1 μM) for 60 minutes. Data are means of n=3 independent experiments.
Figure 5. RAGE ectodomain shedding induction is abrogated by PI3K inhibitors with ionomycin induction but not by PMA induction. RAGE expressing cells were either untreated (NS), or pretreated with inhibitors for one hour followed by incubation with nothing, PMA (200 nM) or ionomycin (1 μM) or PMA or Ionomycin with a range of inhibitors including SB203580, SP600125, U0126(MEK), or PI3K inhibitor for 60 minutes. Data are means of n=3 independent experiments.

Figure 6. An alternatively spliced isoform of RAGE does not undergo ectodomain shedding. A. RAGE WB of conditioned media/cell lysate from mRAGE and mRAGEv4 stable 293 cells. B. RAGE ELISA of mRAGE and mRAGEv4 cells treated with inducers of ectodomain shedding including PMA, Ionomycin, APMA and serum.
Figure 7.

**Figure 7.** GE ectodomain shedding has one cleavage site. Cells transiently expressing N-Terminal his tagged RAGE were treated with various shedding compounds and concentrated media was subjected to western blot with anti-RAGE antibody and anti-Histidine antibody. The lack of any other bands when probing for histidine strongly suggests that there is only one cleavage site for RAGE. Data is representative of n=3 independent experiments.

Figure 8

**Figure 8.** RAGE ectodomain shedding is affected by both A17 and A10 knockdown basally and with PKC stimulation. HEK293 cells expressing human RAGE were transduced with either scramble, A10, or A17 siRNA prior 2 days prior to shedding assay. A. Western blot verification of knockdown using anti ADAM10, ADAM17, RAGE, or Actin antibodies. B. qPCR validation of knockdown using ADAM10 or ADAM17 primers. C. RAGE ELISA of RAGE shedding assay using knocked down cells.
Reference List


