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Research article

sRAGE Concentration İncreases Following the Treatment of Severe Diabetic Ketoacidosis

Short title: sRAGE and DKA

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What is known?
Type 1 diabetes has become increasingly recognized as a metabolic condition with significant immunology dysregulation playing a major role in its acute and chronic complications before, during and after treatment of diabetic ketoacidosis (DKA). In 1988 we described the presence of subclinical brain edema prior to the treatment of DKA. We later described the same inflammatory phenomena in the lungs of children/adolescents with T1D/DKA.

What this study adds?
In 2003 we described a systemic inflammatory response (SIR), involving inflammatory cytokines and complement, that is expressed with the initiation of DKA treatment. Our present study extends the extent of the inflammatory insult of DKA by reporting on the systemic phenotype of soluble advanced glycosylation end products (sAGE) of the important AGE-RAGE axis in the pathogenesis of diabetic complications.

ABSTRACT
Objective: To determine the time relationships of sRAGE, (a decoy of the AGE-RAGE axis) and D-lactate, (a metabolite of methylglyoxal) in the inflammatory response of DKA. We also determined if RAGE is expressed in the myocardium of a newly diagnosed and untreated young person with fatal T1D/DKA.
Methods: 16 children and adolescents with T1D had blood samples obtained 6-12 hours into treatment; at 3 weeks; and 3 months post treatment. sRAGE and D-lactate at 3 months were baseline.
Results: sRAGE during treatment was 39% lower than the values at 2 weeks ($p = .0036$) and at 3 months ($p = .0023$) post treatment. D-lactate was higher during treatment than at 3 weeks ($p = .04$) and at 3 months ($p = 0.035$).
Conclusion: sRAGE is decreased during treatment compared to concentrations at 2 weeks, and at 3 months post- treatment. The increased D-lactate during treatment is in keeping with the known increased dicarbonyls at this time. The RAGE expression in a young myocardium prior to DKA treatment supports cardiovascular inflammation pre-treatment and at a young age.
Keywords: Diabetic ketoacidosis; D-lactate; Myocarditis; soluble Receptor for Advanced Glycation End-products (sRAGE)

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Introduction

Suboptimal metabolic control caused by the insulin deficiency of type 1 diabetes (T1D) involves varying degrees of metabolic and immunologic dysregulation, resulting in a milieu that mediates oxidative stress [1,2] and inflammation [3]. With significant insulin deficits and poor control, this dysregulation leads to the medical crisis of diabetic ketoacidosis (DKA) and the increased potential of comorbidities. Prior to DKA there is a gradual/dysfunctional increase in an array of inflammatory cytokines, chemokines [4-6], and complement [7], followed by a systemic inflammatory response (SIR) shortly after the initiation of DKA treatment [4,8,9]. The metabolic stress of hyperglycemia, hyperketonemia and increased reactive oxygen species also initiates the non-enzymatic glycosylation of glucose with free amino acids to form the toxic α-dicarbonyls [10, 11]. These precursors/intermediates lead to the formation of advanced glycation end products (AGEs), ligands for the receptor (RAGE) and for soluble RAGE (sRAGE) [12]. RAGE is ubiquitous, and has a major role in the pathogenesis of diabetic cardiovascular comorbidities even in newly diagnosed patients with diabetes [13,14]. sRAGE is a proteolytic, cleaved, secretory isoform, a natural competitor of RAGE and is a (protective) "decoy" that abrogates insults that otherwise occur as the result of AGE ligands transferring to, binding to and activating RAGE [13].
Despite impressive advances in understanding the pathogenesis of the AGE-RAGE axis in acute and chronic medical conditions, uncertainties remain in the pathogenesis of T1D comorbidities and in DKA [15], a relative frequent medical crisis in children and adolescents [16]. The recent article by Rawshani et al., [17] gives reason to reconsider the seriousness of poorly controlled T1D in terms of longevity in children even though DKA is not referred to. The impact of DKA can be deduced because of its

common occurrence when the age of onset is before 10 years, and with the resulting loss of approximately 15 life-years for both women and men. This unfortunate statistic does not fully consider quality of life, including achievement, a factor that is much more difficult to quantify.

This data prompted us to examine the systemic inflammatory marker sRAGE during and after DKA treatment when an increase of toxic and inflammatory factors, such as the dicarbonyls and inflammatory cytokines are expressed [4, 5, 8, 10,11] at the same approximate times. Deleted sentence D-lactate was used as the metabolic marker of flux or catabolism of methylglyoxal (MG) [18], the precursor for the AGE ligands hydroimidazolone-1 (MG-H1), the most abundant human AGE; and N(epsilon)-(carboxymethyl) lysine (CML) [19]. We also studied the myocardial expression of RAGE in an undiagnosed and untreated, fatal case of T1D/DKA [20] to give insight into: 1) the role of treatment in RAGE expression; and 2) the likely developmental sequence of chronic cardiovascular complications of RAGE that result from severe DKA.

Materials and methods

Study design and patients

A prospective longitudinal study design was utilized to study a cohort of children and adolescents with T1D/DKA. The study received Expedited Approval by the IRB at East Carolina University (ECU) Brody School of Medicine since blood samples were only obtained at the time of the routine blood sampling for treatment of DKA and follow up visits. The study was conducted in accordance with the Declaration of Helsinki. A total of sixteen children and adolescents between the ages of 9.5 and 17 years, presenting **with DKA (total CO_2 =/< 12 mmol/L)** were invited to enroll in the study. Informed consent was signed by the legal guardian and assent from the patients 9 years and over when not prohibited by severity of illness. In such cases, patient assent was obtained when clinical improvement permitted. Patients referred from outlying hospitals were stabilized prior to being transported to ECU after consultation with the accepting attending physician in the Pediatric Intensive Care Unit. Treatment was according to previously published guidelines [21] with each patient serving as their own control **and** T3 (3 months) served as the baseline. Transfer of children and adolescents for treatment of DKA was routine in this part of North Carolina at the time of the study.

Study evaluation and analysis

Pretreatment values were obtained for blood pressure (BP), heart rate (HR), complete blood count (CBC), glucose (BG), electrolytes, urea nitrogen (BUN), and creatinine at the referring hospitals. The start of **treatment** was defined as the initiation of continuous intravenous insulin. In addition to the pretreatment BP, BPs were recorded hourly with an automated oscillometric device and appropriately sized BP cuff. BPs were also obtained hourly after initiation of treatment) (T1); at discharge; 2 wks post discharge (T2); and at baseline; 3 mons post discharge (T3) Blood glucoses were obtained hourly, electrolytes, and BUN were measured every two to four hours. A CBC **and** differential was repeated at 24 hrs. None of the patients were known to have hypertension, diabetic retinopathy, nephropathy or coronary artery disease. Exclusion criteria included a history or physical findings suggestive of an acute or chronic infection, emotional or physical disability or autoimmune conditions other than chronic lymphocytic thyroiditis.

Serum samples were analyzed undiluted according to the manufacturer's instructions (Human RAGE ELISA, R&D Systems , Minneapolis, MN USA). The inter-assay coefficient of variation was 7.6%, while the intra-assay coefficient of variation was 3.5%. The analysis covers the pool of circulating both esRAGE and sRAGE and **measured by an enzyme-linked** immunoabsorbent assay.

Serum D-lactate was measured by kinetic spectrophometric assay, using the D-lactate Colormetric Assay kit MAK058 from Sigma (St. Louis, MO USA). It was employed as the end-product of MG catabolism by glyoxalase 1 and 2. In this method, D-lactate is specifically oxidized by bacterial D-lactate dehydrogenase (LDH). To increase the sensitivity of the assay we incubated samples at 37^{o} and the reaction was followed kinetically to achieve maximal sensitivity and linearity. To eliminate interference by the reaction of serum L-LDH with L-lactate, serum was ultrafiltered. The <10 kDA fraction was separated by ultrafiltration through 0.5 ml Amicon Ultra Centrifugal filters spun at 14,000g for 30 minutes in a refrigerated centrifuge at 4^{o}C. The ultrafiltrate was used to measure D-lactate. The limit of detection was 1μM and the reaction was linear up to 15μM. The intra-assay CV at 2μM and 10μM was 5% and 3% respectively. To further ensure specificity, the reaction was performed with and without 1mmol/l L-lactate (upper limit of reference range in serum) and identified < 5% interference ($p<0.05$) in agreement with data in the literature.

Immunohistochemistry (IHC) for myocardial RAGE was studied in the left ventricles of the undiagnosed and untreated case of DKA and the control. Sections were deparaffinized in two changes of xylene and two changes of absolute ethanol for 10 minutes each. Antigen retrieval was performed in 10 mM buffer, pH 6.0 in a microwave for 30 minutes then rinsed in PBS. Sections were blocked in 5% donkey serum for 20 minutes, then PBS for 2 minutes. Rabbit anti-RAGE (1:000) (GeneTex, Irvine CA) was applied for 40 minutes at room temperature in a humid chamber, then unbound antibody removed with 3 changes of PBS for 2 minutes each. The secondary antibody donkey anti-rabbit-Cy3 diluted 1:1,500 was then applied for 40 minutes at room temperature in a humid chamber. Unbound antibody was removed with 3 changes of PBS for 2 minutes each. The sections were then counterstained with Dapl and viewed with an epi fluorescent microscope. All images were documented using the same magnification (200X).

Statistical analysis

Normality of the data was determined by the Sapiro-Wilk test. Since the variables did not show a normal distribution they are described with median +/- interquartile range. Tests for differences between [A (T1), B (T2) and C (T3) (baseline/3 months post-discharge)] in least-square means of RAGE and D-lactate scores were performed with a repeated measures ANOVA model, with the Tukey adjustment for multiple tests applied to the p-values. A correlation analysis of Spearman was used to determine

relationships between variables. Results were significant with p <0.05. Statistical analyses were performed using the SPSS software statistical package for Mac, version 19.0 (SPSS, Chicago, IL, USA).

Results

Table 1 is deleted

The cohort was representative of the middle Eastern coast of North Carolina. The average age of the 16 patients was 13.6 yrs (range: 9.7- 16.9 yrs). The mean duration of T1D for the 10 previously diagnosed patients was 5.7 yrs. (range: 1-12 yrs). Six patients were newly diagnosed with T1D at the time of admission (duration 1 day). There were 7 males and 9 females; 6 Caucasians (C) and 10 African Americans (AA). Patients were within 2 SD of their height for age and had weights within 1.5 SD of their ideal weight for height (data not shown). All patients had uneventful correction of DKA (neurocognitive testing was not performed). All patients had one or more positive islet cell autoantibodies (IAA, IA-2 and GAD65) data not shown.

Chemistries, sRAGE, and D-lactate

T1 chemistry values: glucose 26.6 (range 14.4-46.9) mmol/L and at D/C 10 (5.3-12.2) mmol/L; sodium 135.8 (130-144) mmol/L; potassium 5.2 (3.9-6.7) mmol/L; chloride 100.3 (90-111) mmol/L; total CO2 10.5 (9-11) mmol/L; and BUN 6.4 (4.3-15) mmol/L. None of these admission parameters, other than the increased BG concentration had significant associations with the studied metabolic inflammatory markers (see below).

sRAGE concentration (pg/ml) (data are expressed as medians and inter-quartiles) was lower by 39% at T1 (DKA) 332.18 [257.85 – 506.85] compared with T3 (baseline) 546.20 [390.42-739.19] [$p = 0.0023$]. (Table 1 was table 2). There was a strong negative Spearman correlation coefficient between the decreased sRAGE concentration (T1) and increased BG concentration [r = - 0.59; $p = <.0001$. sRAGE concentration (T3) was higher in: 1) females *vs* males 237.3 [176.4 - 446.2] *vs* 156.5 [76.4 -191.8] pg/mL, $p = 0.04$; 2) Cs *vs* AAs 867.9 [585.0-1,243.1] *vs* 459.7 [356.1-546.2] pg/mL, $p = 0.003$; and for 3) the patients with newly diagnosed T1D/DKA vs previously diagnosed patients DKA 721.6 [585 -768.1] *vs* 549.7 [356.1- 571.8] pg/mL, $p = 0.04$.

D-lactate concentrations (µmol/L) decreased from (T1) 14.1 µmol/L (DKA) *vs* (T3) (baseline) 3.7µmol/L ($p = 0.035$). There was also a negative Spearman correlation coefficient between the increased D-lactate concentration and decreased sRAGE concentration at T1 (Spearman r = – 0.32; $p = 0.05$). Patients newly diagnosed with T1D/DKA have a D-lactate at (T3) (baseline) that was significantly lower *vs* patients who were previously diagnosed with DKA 2.3 [1.2-2.8] *vs* 8.6 [3.2-15.9] µmol/L, ($p = 0.04$) Table 1 was table 2

The young woman with undiagnosed T1D/DKA was found dead in her apartment approximately 24 hours after her death. Ht. was 156 cm; Wt. was 37.3 Kg; and BMI of 15.3 [20]. IHC of myocardial RAGE expression is shown in Figure 1:

(Legend: RAGE was prominently expressed in the DKA myocardium versus the gender and age matched control myocardium.)Should be below Fig 1. and deleted from here.

Discussion

First paragraph was deleted including Refs [22]; [23]. This resulted in numerous subsequent Refs numbers being changed and the total Refs number reduced. These changes are in BOLD and high lited in yellow and now have he correct number.

We believe this is the first longitudinal report of sRAGE during and after the correction of severe DKA. We report a 39% lower concentration of sRAGE during DKA treatment T1 [6-12 hours into treatment] *vs* T3 [3 months post treatment] ($p = 0.0023$), with also a significant increase of sRAGE T1vs T2 [2 weeks post treatment] ($p = 0.0036$). The sRAGE increase continued to the final study point at 3 months, however [T2 *vs* T3] (p = NS) . We hypothesize that the decrease of the sRAGE (decoy) at T1 occurred early in, or possibly prior to, DKA treatment as the result of the significantly increased concentrations of dicarbonyls [10,11], AGE ligand formation followed by sRAGE sequestration. This sequence minimizes or prevents ligand binding, and activation of RAGE. Depending on the extent of the initial ligand binding both mediators of capillary perturbation -MG and MGH1- are candidates to be involved in the pathogenesis of pretreatment subclinical brain edema [22] and interstitial pulmonary edema [23] that occurs in severe DKA. In this regard an additional ligand of sRAGE, malondialdehyde (MDA), which is a highly reactive and a damaging β dicarbonyl, is also elevated during DKA [24], resulting in lipid peroxidation, breakdown of phospholipids, and increased vascular endothelial permeability [25, 26]. These pretreatment subclinical capillary perturbations are relatively common [27] and can progress during DKA treatment, but rarely to the extent of causing signs/symptoms [22, 28]. A decrease of early vascular perturbators at the time of treatment of severe DKA is in keeping with Grossin's [29] and Salonen's [30] hypothesis, that sRAGE has a protective effect. With the decrease of sRAGE and its ligand sequestrations, the residual unsequestered ligand can then activate RAGE.

D-lactate, the steroisomer of L-lactate, the other metabolic marker studied, was formerly viewed to be a metabolic by-product, but is now recognized as an active metabolite in signaling of pro-inflammatory circuits. In particular D-lactate controls T-cell migration [31] and also contributes to the anion gap in the metabolic acidosis of DKA. This marker of MG catabolism was increased at 6-12 hours (T1), and decreased by 2 weeks following treatment (T2) (p = 0.04), with a further decrease at 3 months (T3) ($p = 0.035$). This systemic pattern of D-lactate is in keeping with an early decrease of sRAGE and later subclinical, perturbation of the myocardium [5]. A serious effect of D-lactate is its limited ability to be an effective respiratory substrate in the rat heart and brain due to its altering of mitochondrial energy production [32]. This raises the question: Does a lower D-lactate concentration act synergistically with other DKA perturbations resulting in subclinical cardiac insults?

Salonen's longitudinal study of pre-diabetic children reported a decrease of sRAGE prior to the seroconversion to positive pancreatic autoantibodies. Both ours'and Salonen's DKA study [30] document low sRAGE in relation to DKA insult. The decrease of sRAGE in Salonen's study occurred approximately 30 days before the onset of DKA with no further sRAGE decrease [30]. In contrast, our study identifies a period of sRAGE increase following DKA treatment. We believe these transitions could be influenced by a gradual change in the pH of the milieu. However, low sRAGE concentrations are reported to

occur in various conditions during the acute clinical phase other than DKA. These include: 1) atrial fibrillation [33]; 2) low sRAGE and high cardiac troponin in non-ST segment elevation myocardial infarction [34]; and 3) the autoimmune condition of multiple sclerosis [35].

Our study was not intended to identify a cause and effect relationship between the two inflammatory pathways of the AGE-RAGE axis and the SIR of inflammatory cytokines, but rather was to compare their systemic phenotypes during the treatment of DKA, a time of known myocardial perturbation [5, 36] and post treatment. This difference between the two inflammatory pathways is evident during the 6-12 hour period of treatment (T1) with sRAGE being lower in comparison to the rapid increase in the majority of cytokines possibly initiated by insulin treatment [4,5,8,9]. While interactions between components of the two pathways are likely [37-39], we are unable to confirm interactions in this study. Demographic differences of interest at 3 months (T3), the sRAGE concentration is significantly higher in Cs than in AAs and significantly higher in females than males.

Despite the dichotomy in systemic inflammatory patterns at approximately the same time during treatment the transient systemic decrease of sRAGE and the rapid transient increase of inflammatory cytokines of the SIR [4] likely occurs shortly after (IV) insulin is initiated. In regard to this dichotomy of two systemic inflammatory patterns, including RAGE, it is of note that both inflammatory pathways have significant IHC expression in the teenage brains following fatal DKA/BE [40-42]. This brain expression is like the positive association between these two inflammatory systemic pathways in T2D adults and unrelated to DKA [43].

The significant myocardial IHC expression of the multiligand receptor RAGE in the young woman found "dead-in-bed" with new onset T1D/DKA [20] indicates: early RAGE- mediated cellular activation and a positive feedback initiated by sRAGE-ligand and RAGE interaction. This expression is in keeping with a report of two young fatal T1D/DKA cases (deletion of phrase) both had significant myocardial expression of the inflammatory markers MCP-1 and IL-1 beta [44]. These autopsy IHC studies along with the synthesis of cardiac autoantibodies in uncomplicated severe DKA [36] support the hypothesis that subclinical myocarditis can be initiated by the inflammatory insults of the AGE-RAGE axis and by the inflammatory cytokines in T1D/DKA with eventual progression to diabetic cardiomyopathy in some T1D patients. In keeping with our hypothesis is the study that reported the blocking of RAGE attenuates autoimmune myocarditis [45].

Conclusion

While the limitation of this study is the size of the patient cohort these young T1D patients extend the AGE-RAGE axis, as a contributor to the acute inflammatory insult during the medical crisis and treatment of DKA, a perpetual perpeturator of subclinical inflammation leading to chronic diabetic vascular complications including those of the heart. The inflammation during DKA treatment involves a significant transient decrease of sRAGE, possibly prior to treatment, and a significant transient increase of D-lactate, both metabolic markers of AGE-RAGE activity. Following the dissipation of systemic sRAGE RAGE expression can increase and D-lactate decreases. This pattern supports the hypothesis of sRAGE being a protective "decoy" prior to cell perturbation through its ligand sequestration [10,11, 31].

We believe the concentrations at baseline (T3) of lower sRAGE values for AAs than Cs, and also lower values for males than females and with the opposite relationship reported for inflammatory cytokines during severe uncomplicated DKA treatment [5] are both examples that require further study. The significant myocardial RAGE expression of the young woman who died of undiagnosed and untreated DKA [20] adds to the previously reported myocardial inflammatory cytokines in young patients who died during the treatment of severe DKA [44]. Myocardial expression of RAGE identifies a pathogenesis of the AGE-RAGE unrelated to DKA treatment [20]. Whether RAGE only becomes activated in the myocardium with the life-threatening crisis of severe DKA, whether early myocardial RAGE expression occurs in less severe forms of metabolic/immunologic DKA insults, in addition to the relationship with cardiac function all require the careful follow-up and further study.

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Figure 1. RAGE expression in the myocardium (left ventricle) of a young Japanese woman (age approximately 22 years) who died of undiagnosed (new onset) T1D/DKA prior to treatment. The arrows show positively stained RAGE cells. No stain was present in the age and gender matched control. Magnification x 200

Figure 1 RAGE expression in DKA myocardium

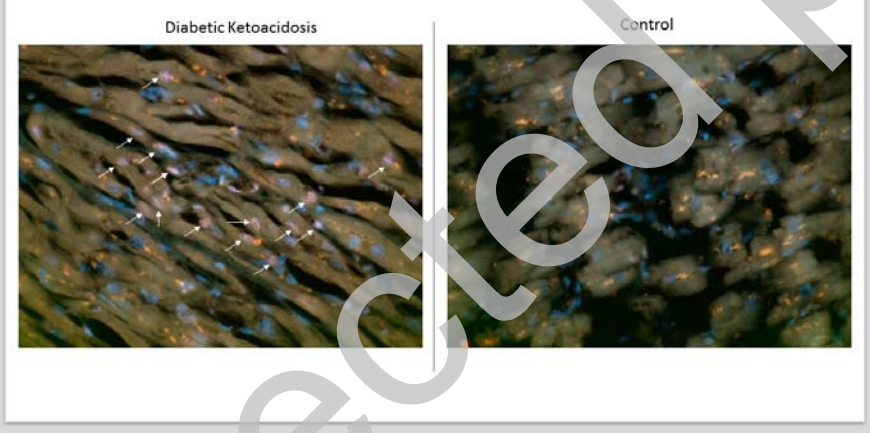

Table 1 sRAGE and D-lactate concentration differences between

DKA, 3 Weeks and 3 Months				
	sRAGE	p-value	D-lactate	p-value
DKA (T1)	332.18 (257.85 – 506.85) pg/ml		14.1 (10.5 – 18.0) µmol/ L	.
	T1 vs T2	.0036		0.04
	T1 vs T3	.0023		0.035
3 Weeks (T2)	521.84 (411.65 -726.55)		5.4 (4.5 - 9.3)	
	T2 vs T3	NS		NS
3 Months (T3)	546.20 (390.42 -739.19)		3.7 (2.5 -12.8)	

Tests for differences between times in least–square means of marker scores performed with a repeated measures ANOVA model, with the Tukey adjustment for multiple tests applied to the p-values.