

Effect of ERK Signaling Pathway on the Level of Soluble RAGE (sRAGE) Peptide in AGE-Induced Oxidative Stress

Farnosh Shemirani, Razieh Yazdanparast*, and Safiyeh Aghazadeh

Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran.

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HIGHLIGHTS

- Prominent activity decrease of intracellular antioxidant enzymes was demonstrated upon AGE-treatment.
- An increase in superoxide anion level was demonstrated upon AGE-treatment.
- The level of sRAGE is negatively associated with the ERK expression in AGE-treated cells.
- The level of sRAGE was shown to significantly elevate in the presence of ERK inhibitor.

Keywords:

Advanced glycation
end products
ERK signaling
pathway
Reactive oxygen
specious
Soluble receptor

ABSTRACT

In the context of diabetes, there is a bidirectional relation between AGEs and ROS which is amplified under hyperglycemic condition. Indeed, the AGE/RAGE interaction, via triggering several signaling pathways underlies such interplay. These damaging signaling cascades could be attenuated by naturally occurring soluble form of RAGE, namely sRAGE. In the present study, the effect of AGEs on the intracellular superoxide anion level and the antioxidant defense system was studied. Moreover, the link between the level of soluble RAGE and the AGE-triggered signaling pathways, namely ERK, was also investigated. Our results demonstrated that the elevation in intracellular superoxide anion level was associated with decrease in antioxidant enzyme activities. Our data also revealed that the level of sRAGE is negatively correlated with the ERK expression in a hyperglycemia model system. Hence, the current study brings about novel evidences suggesting that the level of sRAGE would be under the influence of stress-sensitive signaling pathways.

Introduction

The detrimental effects of persistently elevated glucose levels appear to be the plausible cause of morbidity and mortality of diabetes mellitus (Singh et al., 2014). The intricate series of events, resulting in cellular malfunction in response to high levels of glucose are not thoroughly understood. However, among the several hyperglycemia-evoked metabolic derangements, accumulation of AGEs (advanced glycation end-products) is a key nodal point

that has been implicated in the context of diabetes (Fraser and Hansen, 2005). The formation of AGEs, the heterogeneous product of nonenzymatic glycation of macromolecules, sparks oxidative stress in pathological states including hyperglycaemia of diabetes. Indeed, AGEs are the molecular signature of hyperglycemia and oxidative stress (Goldin et al., 2006).

Oxidative stress is defined as the surplus of prooxidants due to the insufficient removal by antioxidant defense network (Kumawat et al., 2009). Importantly, there is a close bidirectional association between ROS and AGEs in the context of diabetes. Indeed, oxidative stress facilitates the formation of AGEs and on the other side AGEs have great potential in eliciting redox imbalance. The latter is

* Corresponding Author:

Email: ryazdan@ut.ac.ir (R. Yazdanparast)

mediated via the interaction with their cell surface receptor, namely RAGE (Jiang et al., 2004). RAGE (receptor for AGEs), is a multi-ligand receptor which belongs to the immunoglobulin superfamily of cell-surface molecules. RAGE, by acting as a signal receptor for ligands governs a broad array of cellular responses by promoting gene expression, proliferation, migration, and altered cellular signaling. Among the several RAGE-ligand-triggered signaling pathways, MAPKs (mitogen activated protein kinases) have a prominent role in the context of diabetes (Evans et al., 2003).

MAPKs (Mitogen-activated protein kinases), are a family of Serine/threonine kinases and function as the key regulators of fundamental cellular processes. There are three distinct main subgroups within the MAPK family, namely JNK (c-Jun N-terminal Kinases), ERK (Extracellular signal-Regulated Kinase), and p38 group of protein kinases (Johnson and Lapadat, 2002). The p38 MAPK and ERKs are recognized as stress-activated kinases which response to several stress-induced stimuli like ROS and hyperglycemia. Based on multiple studies, the activation of ERK and p38 MAPK increases in diabetic condition and exacerbates the late diabetic complications (Evans et al., 2003).

Regarding the above considerations, finding preventive approaches in blocking the AGE-RAGE axis is an area of intense study in the field of diabetes. In this line, one of the recently discovered compelling inhibitor of AGE/RAGE mediated consequences is sRAGE. Soluble RAGE, a circulating secretory form of RAGE is composed of the extracellular domain of RAGE, which might exert protective role by neutralizing the action of AGEs. Soluble RAGE in the circulation can be formed through different mechanisms. sRAGE can be generated by ectodomain shedding of the membrane associated receptor. Alternatively, sRAGE can be generated via alternative splicing of RAGE pre-mRNA transcripts (Maillard-Lefebvre et al., 2009). Studies on sRAGE are continuously progressed in the last decades. There are a variety of studies implicating that soluble RAGE level is low in diabetic subjects and is negatively correlated with glycemic control (Devangelio et al., 2007; Tam et al., 2011; Motawi et al., 2013). In our previous study (Shemirani and Yazdanparast, 2014), we have shown that the level of sRAGE is inversely associated with the hyperglycemia-induced oxidative stress status. Moreover, we have indicated that improving the intracellular redox status via antioxidant therapy could manifestly raise the sRAGE level, reinforcing the notion that modulation of sRAGE levels may highlight a promising strategy for therapeutic interventions. In the present study, ERK and P38 signaling pathways were opted due to their significant involvement in the pathogenesis of diabetes and the question arises as to whether the level of soluble RAGE would be affected by AGE-associated signaling pathways

in a hyperglycemia model system.

Material and Methods

Chemicals

The cell culture medium (RPMI-1640), fetal bovine serum (FBS), and penicillin streptomycin were purchased from Gibco BRL (Life Technology, Paisley, Scotland). Cell line was obtained from Pasteur Institute of Iran (Tehran, Iran). Anti-Tubulin antibody was from Biosource (Nivelles, Belgium). Nitroblue tetrazolium (NBT), 5, 5-dithiobisnitro benzoic acid (DTNB), nicotinamide adenine dinucleotide reduced (NADH), phenazine methosulphate (PMS), H_2O_2 , and nicotinamide adenine dinucleotide phosphate reduced (NADPH) were obtained from Merck Co. (Germany). EDTA was obtained from Sigma-Aldrich Chemical Co. Ltd. (England). The p38 MAPK inhibitor (SB202190), ERK1/2 inhibitor (PD98059), and antibodies including anti-total ERK1/2, anti-phosphorylated ERK1/2, anti-total p38, and anti-phosphorylated p38 were obtained from Biosource (Nivelles, Belgium).

Cell culture

The human K562 cell line was cultured at 37°C under 5% CO_2 humidified atmosphere in RPMI- 1640 medium supplemented with fetal bovine serum (10%, v/v), streptomycin (100µg/ml), and penicillin (100 U/ml). A hemocytometer and the abilities of the cells to exclude trypan blue were used to evaluate cell numbers and viabilities.

Preparation of AGE-modified albumin

We prepared AGE albumin based on the thermal glycation method (Bhatwadekar and Ghole, 2005). Briefly, BSA (50 mg/ml) and glucose (0.5 M) in phosphate buffer (0.2 M, pH 7.4) were incubated at 50°C for 4 days. Lysine derived AGEs were also prepared by incubating lysine (0.1 M) with BSA (50 mg/ml) at 50°C for 4 days. The control samples were incubated under similar condition but without glucose (0.5 M). All incubations were done under sterile conditions and in the presence of 100 µl chloroform.

Measurement of intracellular ROS

The intracellular reactive oxygen species (ROS) content was estimated by the non-fluorescence dye, DCFH-DA (Lebel et al., 1992). Following rapid diffusion into the cells, DCFH-DA is enzymatically hydrolyzed by the intracellular esterases to form non-fluorescent DCFH. The later reacts with intracellular radicals to produce DCF (dichlorofluorescein) which is a fluorophor. In this experiment, the cells were treated with various

concentrations of AGE-modified albumin (50, 100, 150, 200 mg/l) and high glucose (30 mM). The high glucose-treated cells were incubated for a further 24h while the AGE-treated cells for 24h and 48h. After this time, the cells were incubated with 10 μ M DCFH-DA for 1 h and then, the compound-treated cells were washed twice and then suspended in phosphate buffer saline. Finally, the fluorescent intensity was monitored on a Varian spectrofluorometer with excitation and emission wavelengths of 485 and 530 nm, respectively.

Assay of superoxide anion levels

Superoxide formation was evaluated by monitoring the reduction of cytochrome C. The control and compound-treated cells were harvested by centrifugation and washed once with Hank's balanced salt solution (HBSS). The cells were then resuspended in HBSS (100 μ M), containing cytochrome C (50 μ M) and incubated at 37°C for 30 min. Cytochrome C reduction was monitored at 37°C by recording the absorption at 550 nm for 10 min with 1 min intervals, using microplate reader (Gen 5, BioTek, USA). The amount of superoxide released was calculated using the extinction coefficient of 21 mM⁻¹ cm⁻¹ and expressed as nmol/mg protein (Bagchi et al., 1999).

Measurement of superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity was measured according to the extent inhibition of amino blue tetrazolium formazan in nicotineamide adenine dinucleotide, phenazine methosulfate, and nitroblue tetrazolium system (NADH-PMS-NBT). One unit of enzyme activity was defined as the amount of enzyme which caused 50% inhibition of NBT reduction/mg protein (Kakkar and Viswanathan, 1984).

Measurement of the catalase activity

Catalase (CAT) activity was measured according to the method of Aebi (Aebi, 1984) by following the decrease in absorbance of H₂O₂ at 240 nm for 2 min. Briefly, 200 μ l of cell lysate was added to a cuvette containing 1.995 ml of 50 mM phosphate buffer (pH 7.0). Reaction was started by the addition of 1 ml of freshly prepared H₂O₂ (30 mM). The rate of decomposition of H₂O₂ was measured by spectrophotometric means at 240 nm. The enzyme activity was expressed as $\times 10^{-1}$ k/mg protein, where k represents the rate constant of the first-order reaction of catalase. Protein concentration was determined by the Lowry method.

Immunoblotting

After treatment of K562 cells with AGE-albumin (200 mg/

ml), p38 and ERK inhibitors for different time intervals, proteins were extracted from whole cells by lysing those in lysis buffer containing Triton X-100 (1 %), SDS (1 %), Tris (10 mM, pH 7.4), NaCl (100 mM), EGTA (1 mM), EDTA (1 mM), sodium pyrophosphate (20 mM), Na₃VO₄ (2 mM), NaF (1 mM), sodium deoxycholate (0.5 %), glycerol (10 %), phenylmethylsulphonyl fluoride (1 mM), leupeptin (10 μ g/ml), pepstatin (1 μ g/ml), and aprotinin (60 μ g/ml). Protein concentration of each sample was determined using Lowry method. Equal quantities of protein (50 μ g) were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a hybond-P polyvinylidene difluoride (PVDF) and/or nitrocellulose membranes (Amersham, Bioscience, UK). The filter membranes were blocked in Tris-buffered saline (pH 7.4), containing 0.1 % Tween-20 and 5 % non-fat milk overnight at 4 °C. The blocked blots were incubated with primary antibodies for 2 h at room temperature using antibody dilutions as recommended by the manufacturer in Tris-buffered saline pH 7.4, 0.1 % Tween-20, Followed by 90 min incubation with anti rabbit or -mouse horseradish peroxidase-conjugated secondary antibodies (Biosource, Belgium). Each protein band was detected by an enhanced chemiluminescence (ECL) detection system (Amersham-Pharmacia, Piscataway, NJ) according to the manufacturer's instructions. The band densities were measured using Image J software.

Statistical Analysis

Data were expressed as percent of values of untreated control cells, and each value represents the mean $\pm$ SD (n = 3). The significant differences between the means of the treated and untreated cells were calculated by unpaired Student's t test and P<0.05 was considered significant.

Results

Assessment the effects of AGE-albumin on intracellular redox status in K562 cells

In order to examine to what extent AGE-albumin would affect the intracellular redox status, the intracellular ROS content was measured by DCFH-DA staining approach (Fig. 1). The intracellular ROS content in k562 cells, increased by almost a factor of 5.4 following 48h exposure to AGE-modified albumin (200 mg/l) compared to untreated control cells. However, Pretreatment of the cells with Resveratrol (20 μ M) attenuated ROS production in AGE-treated K562 cells by A factor of 2.9.

Alteration in intracellular superoxide anion levels in AGE-treated K562 cells

Superoxide anion is one of the most important oxygen radicals whose overproduction leaves marks in early

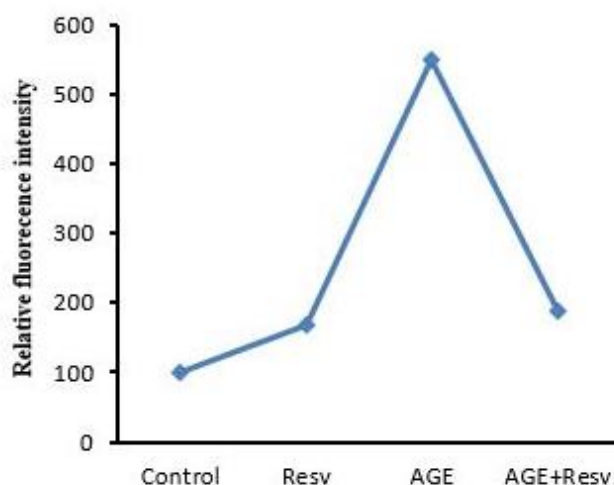


Figure 1. The effect of AGE-modified albumin on intracellular redox status in K562 cells. K562 cells were treated with AGE (200 mg/l) for 48h and relative fluorescence intensity was measured using 2-7 dichlorodihydrofluorescein diacetate (DCFDA) dye. Values correspond to mean $\pm$ SD of three independent experiments. *Significantly different from control cells ($p < 0.05$).

stages, and hence contributes to the future development of diabetic complications. In the present study, the level of intracellular superoxide level obviously increased by almost 46% upon treatment with AGE-albumin. However, pretreatment of K562 cells with resveratrol could counteract the results and decline the superoxide level close to those in control untreated cells (Fig. 2).

Modulation of enzymatic antioxidants activity upon exposure of K562 cells to AGE-modified albumin

Variation in intracellular reactive oxygen species is frequently associated with the alteration in the activity of free-radical quenching enzymes such as superoxide dismutase and catalase. The activity of superoxide

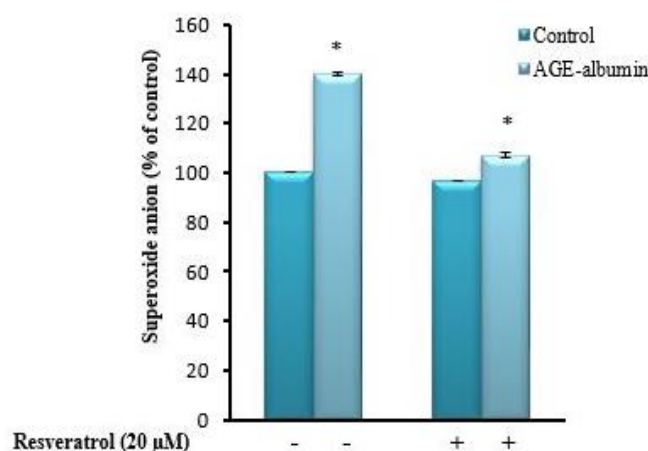


Figure 2. Evaluation of intracellular superoxide anion level upon exposure of K562 cells with AGE-albumin. Cells were treated either with AGE-modified (200 mg/l) albumin for 48h or pretreated with resveratrol (20 μ M) for 1h followed by incubation with AGE-albumin for further 48h. Then, the intracellular production of superoxide anion was analyzed spectroscopically. Values correspond to mean $\pm$ SD of three independent experiments. *Significantly different from control cells ($p < 0.05$).

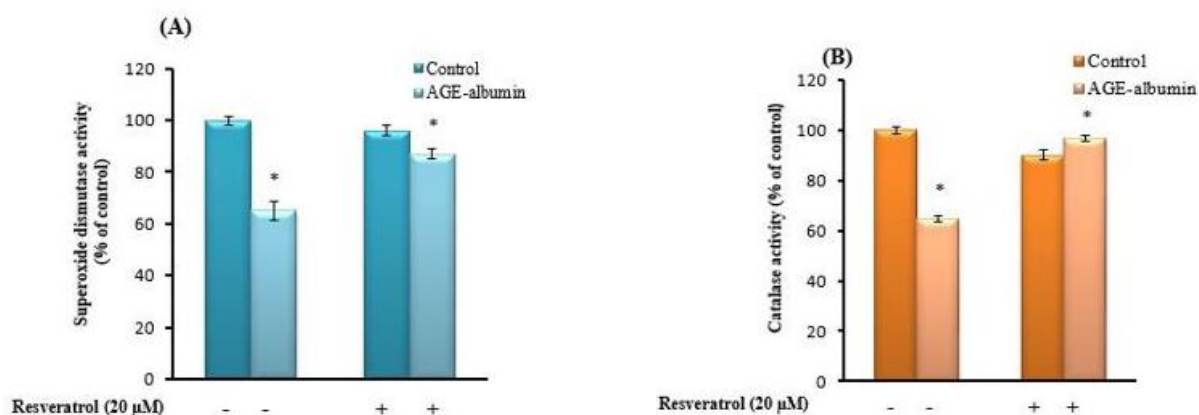


Figure 3. The alteration in the activity of antioxidant enzymes upon exposure of K562 cells to AGE-albumin. Cells were treated either with AGE-albumin (200 mg/l) for 48h or pretreated with resveratrol (20 μM) for 1h followed by incubation with AGE-albumin for further 48h. The activities of SOD (A) and CAT (B) were assessed spectrophotometrically. Values correspond to mean $\pm$ SD of three independent experiments. *Significantly different from control cells ($p < 0.05$).

dismutase diminished by almost a factor of 1.5 under treatment with AGE-albumin. Moreover, our data showed that resveratrol ameliorated AGE-induced redox imbalance and resulted in a 1.3 fold increase in SOD activity (Fig. 3A). In parallel, the 48h exposure of the K562 cells to AGE-modified albumin (200 mg/l) reduced catalase activity by 36% as compared to untreated control cells. However, pretreatment of cells with Resveratrol (20 μM) enhanced catalase activity to 96.7% (Fig. 3B).

Evaluation of ERK expression in AGE-treated K562 cells

Having in mind the activation and momentous participation of ERK and P38 signaling pathways in response to variety of stimuli like hyperglycemia and oxidative stress, we got interested to assess the expression of ERK under the influence of AGE-modified albumin. As demonstrated in Fig. 4, the expression of ERK increased by a factor of 1.6 after 48h exposure to AGE-albumin. However, the expression of ERK in AGE-treated K562 cells in the presence of ERK inhibitor (PD98059) decreased by almost 50%.

Alteration in the level of soluble RAGE in the presence of ERK and/or P38 inhibitors in AGE-treated K562 cells

Regarding our previous study, showing that soluble RAGE and intracellular ROS levels are inversely associated, we intended to examine that how the level of sRAGE would be affected by ROS-sensitive signaling pathways, namely ERK and P38. The present study revealed that the level of sRAGE is associated with the ERK expression in AGE-

treated K562 cells. As presented in Fig. 5, the level of soluble RAGE decreased by a factor of 1.25 upon treatment with AGE-albumin. Whereas, in the presence of ERK inhibitor (PD98059) the level of sRAGE raised meaningfully close to that of control untreated cells. Whereas pretreatment of K562 cells with P38 inhibitor (SB202190) didn't exert any influence on the level of sRAGE.

Discussion

In both types of diabetes, the late diabetic complications are caused by persistent elevations of glucose (Evans et al., 2002). Hyperglycemia is regarded as a nodal trigger for the damaging complications of diabetes. The momentous role of glycemic disequilibrium in etiology of diabetes is applied to its feature for favoring the glycation reactions. (Bonnetfont-Rousselot et al., 2004). The formation of AGEs, the heterogeneous and stable product of protein glycation, fuels the engine of cellular stress and tissue dysfunction. (Daffu et al., 2013). Based on many studies, there is an association between AGEs and ROS, in which they exacerbate the effects of one another in the context of diabetes.

In our previous study (Shemirani and Yazdanparast, 2014), we have demonstrated that there is a significant build-up in both intracellular protein oxidation and free radical generation followed by AGE-albumin treatment. In the present study, we have primarily assessed the intracellular alterations of the superoxide anion level under AGE-induced oxidative stress. Our results revealed that the intracellular superoxide anion production significantly increased upon exposure of K562 to AGE-albumin.

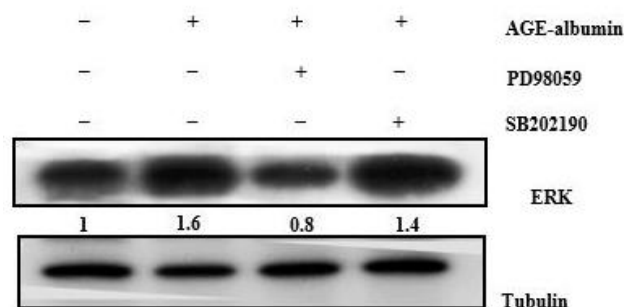


Figure 4. The effect of AGE-albumin on the level of intracellular ERK in K562 cells. Cells were treated with AGE-albumin (200 mg/l) and the protein content of ERK was determined by immunoblotting, using the relevant monoclonal antibody against it.

Furthermore, such elevation in the intracellular ROS content could be attributed to inefficacy of antioxidant defense system. In this line, we intended to examine the activity of superoxide dismutase and catalase, the prominent intracellular free-radical quenching enzymes. Our data have shown that the activity of SOD and CAT meaningfully decreased in AGE-induced redox imbalance in K562 cells.

Regarding the above results, it could be deduced that

the negative outcomes of AGEs and oxidative stress are amplified by one another, as AGEs can beget further ROS, possibly via glycosylation of antioxidant enzymes or through specific AGE receptors (Kalea et al., 2009). One of the most common mechanisms via which AGEs can elicit their damaging impacts on cellular functions is mediated through its receptors, namely RAGE. Receptor for advanced glycation end products (RAGE) are the most distinguished receptors for AGE (Neeper et al.,

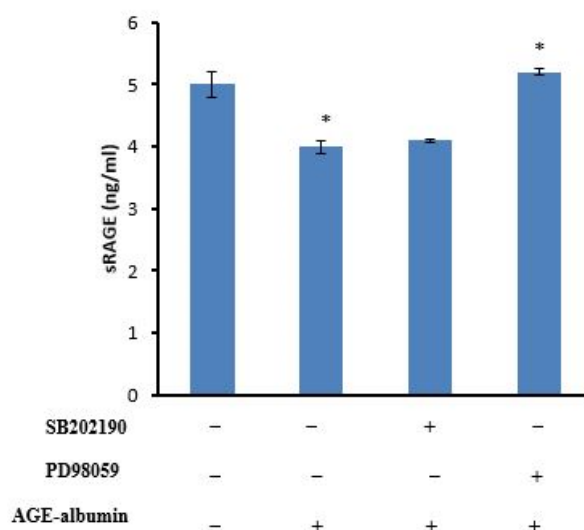


Figure 5. The modulation of the level of soluble RAGE upon treatment of K562 cells with AGE-albumin in the presence of ERK and/or P38 inhibitors. Cells were treated either with AGE-albumin for 48h or pretreated with ERK inhibitor (PD98059) and/or P38 inhibitor (SB202190) followed by further 48h incubation. The level of circulating form of RAGE (sRAGE) was measured in the culture medium of the treated cells using an ELISA kit. The estimation of concentration of sRAGE in each sample was done using the relevant calibration graph. The results are expressed in ng/ml. Values correspond to mean $\pm$ SD of three independent experiments. *Significantly different from control cells ($p < 0.05$).

1992). Numerous studies have elucidated that the AGE-RAGE interaction evokes several downstream signaling cascades which are accounted for the most of the late diabetic complications (Lander et al., 1997; Huttunen et al., 1999; Taguchi et al., 2000;). Indeed, an amplifying loop supervenes among the AGEs, AGE-RAGE axis, and ROS which is the basis of designing new therapeutic interventions (Daffu et al., 2013).

In this line, soluble form of the RAGE (sRAGE) is a potential therapeutic approach through acting as a decoy for RAGE ligands. In our previous study, we have proved that amelioration of the intracellular redox status via resveratrol resulted in a significant increase in sRAGE level, reinforcing a close association between oxidative stress and the level of sRAGE. In this study, we aimed to examine the relation between the level of sRAGE and stress-sensitive signaling cascades like ERK and p38. Our data have shown that the expression of ERK increased in AGE-treated K562 cells, followed by a decrease in sRAGE levels. Interestingly, pretreatment of AGE-treated cells with ERK inhibitor resulted in a meaningful increase in sRAGE level. Moreover, no noticeable change has observed either in P38 expression or the level of sRAGE upon AGE treatment in the absence or presence of P38 inhibitor. Hence, our data for the first time demonstrated that the level of soluble RAGE is affected by ERK signaling pathway.

Conclusion

In conclusion, the present study besides highlighting the amplifying and bidirectional relation between AGEs and ROS in the pathology of diabetes, accentuates the role of sRAGE as a promising therapeutic approach. The possible involvement of signaling cascades in modulating the sRAGE level promises to be an important and favorable field for drug design beyond the current treatments for diabetes.

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Competing Interests

All authors declare that there is no conflict of interest in this study.

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