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Proteome wide reduction in AGE modification in streptozotocin induced diabetic mice by hydralazine mediated transglycation

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The non-enzymatic reaction between glucose and protein can be chemically reversed by transglycation. Here we report the transglycation activity of hydralazine using a newly developed MALDI-TOF-MS based assay. Hydralazine mediated transglycation of HbA1c, plasma proteins and kidney proteins was demonstrated in streptozotocin (STZ) induced diabetic mice, as evidenced by decrease in protein glycation, as well as presence of hydralazine-glucose conjugate in urine of diabetic mice treated with hydralazine. Hydralazine down regulated the expression of Receptor for Advanced Glycation End products (RAGE), NADPH oxidase (NOX), and super oxide dismutase (SOD). These findings will provide a new dimension for developing intervention strategies for the treatment of glycation associated diseases such as diabetes complications, atherosclerosis, and aging.

Glycation is a proteome wide phenomenon resulting from a series of chemical reactions between proteins and reducing sugars leading to formation of heterogeneous Advanced Glycation End products (AGEs). The level of AGEs increases profoundly in diabetes due to chronic hyperglycemic condition. AGEs interact with Receptor for AGEs (RAGE) leading to oxidative stress and activation of pro-inflammatory pathways, which is believed to be the major cause of glycation associated diseases such as diabetic complications, aging, obesity, inflammation, polycystic ovarian syndrome, ischemic cardiovascular disease, neurodegenerative disorders and cancer^{1,2}. Reducing AGE levels has been considered as an intervention strategy for the treatment of glycation associated diseases^{3,4}. Some of the molecules that reduce AGE levels include aminoguanidine⁵, OPB-9195⁶, ALT-946⁷, however, these molecules have not been approved by FDA due to toxic side effects. On the other hand several FDA approved drugs like metformin⁸, aspirin⁹, diclofenac¹⁰ show antiglycation activity. All these drugs mainly inhibit the AGE formation; however efforts towards reversing AGE formation are minimal. Interestingly cells have evolved an enzymatic reaction known as deglycation mediated by fructosamine-3-kinase (FN3K)^{11,12} through which AGE formation can be reversed. Deglycation can be achieved chemically by transglycation, where in sugar moiety of Schiff's base/Amadori product is transferred to nucleophiles like free amino acids, polyamines¹². Glutathione mediated transglycation has been demonstrated *in-vivo*¹². Therefore, deglycation and transglycation are important protective mechanisms against glycation. Development of transglycating agents for preventing or reversing glycation has been hindered due to lack of high throughput assays. Currently available assays for identification of transglycating compounds are based on NMR analysis, which limits a high throughput screening¹². In this study we have developed mass spectrometry based transglycation assay, using this assay transglycation activity of hydralazine was discovered. Furthermore, hydralazine mediated transglycation was demonstrated in streptozotocin (STZ) induced diabetic mice as evidenced by decreased HbA1c, plasma protein glycation, and formation of hydralazine glucose conjugates in urine. Consequently decreased expression of Receptor for Advanced Glycation End products (RAGE), NADPH oxidase (NOX), and Super oxide dismutase (SOD) was observed with hydralazine treatment. These findings suggested transglycation can be used as an intervention strategy for the treatment of glycation associated diseases such as diabetic complications, atherosclerosis, and aging.



Results

In our previous study we have developed a MALDI-TOF-MS based insulin glycation inhibition assay¹³, where in insulin (m/z 5808) was glycated with glucose and the intensity of glycated insulin (m/z 5970) was monitored in presence or absence of drugs. This assay was modified to study transglycation by monitoring release of free insulin (m/z 5808) using glycated insulin (m/z 5970) and nucleophilic drugs as reactants. Using this assay, hydralazine, an anti-hypertensive and vasodilating drug, was identified as a transglycating agent (Fig. 1a). The transglycation of insulin was also evidenced by formation of hydralazine glucose conjugate (m/z 323.13) in a MALDI-TOF-MS analysis (Fig. 1 b). Further, the effect of hydralazine on protein glycation was studied both *in vitro*, as well as *in vivo* using STZ induced diabetic mice model system.

Glycation leads to protein crosslinking⁴ and formation of fluorescent AGEs¹⁴. Hydralazine inhibited glycation induced *in vitro* HSA protein cross-linking as analyzed by SDS-PAGE analysis (Fig. 2a). In

addition, the drug decreased AGE fluorescence emission at 440 nm suggesting that it inhibits AGE formation (Supplementary Fig. 1). Hydralazine inhibited the AGE formation in a concentration dependent manner and its inhibition was more pronounced compared to aminoguanidine at the same concentration (Fig. 2b). Hydralazine mediated inhibition of *in vitro* HSA glycation was also studied by LC-MS^E analysis, a data independent acquisition wherein all the eluted peptides are fragmented¹⁵. This method allowed label free analysis and quantification of even the low intense AGE modified peptides. Previously LC-MS^F has been used to characterize post translational modification namely for demidation¹⁶, phosphorylation¹⁷, and glycation^{18,19}. Glycated HSA showed more number of AGE modified peptides than unglycated HSA. The number of AGE modified peptides decreased in presence of hydralazine and aminoguanidine. Hydralazine was more potent than aminoguanidine in inhibition of AGEs as observed by decreased number of AGE modified peptides in LC- MS^E analysis (Fig. 2c). The representative MS/MS annotated

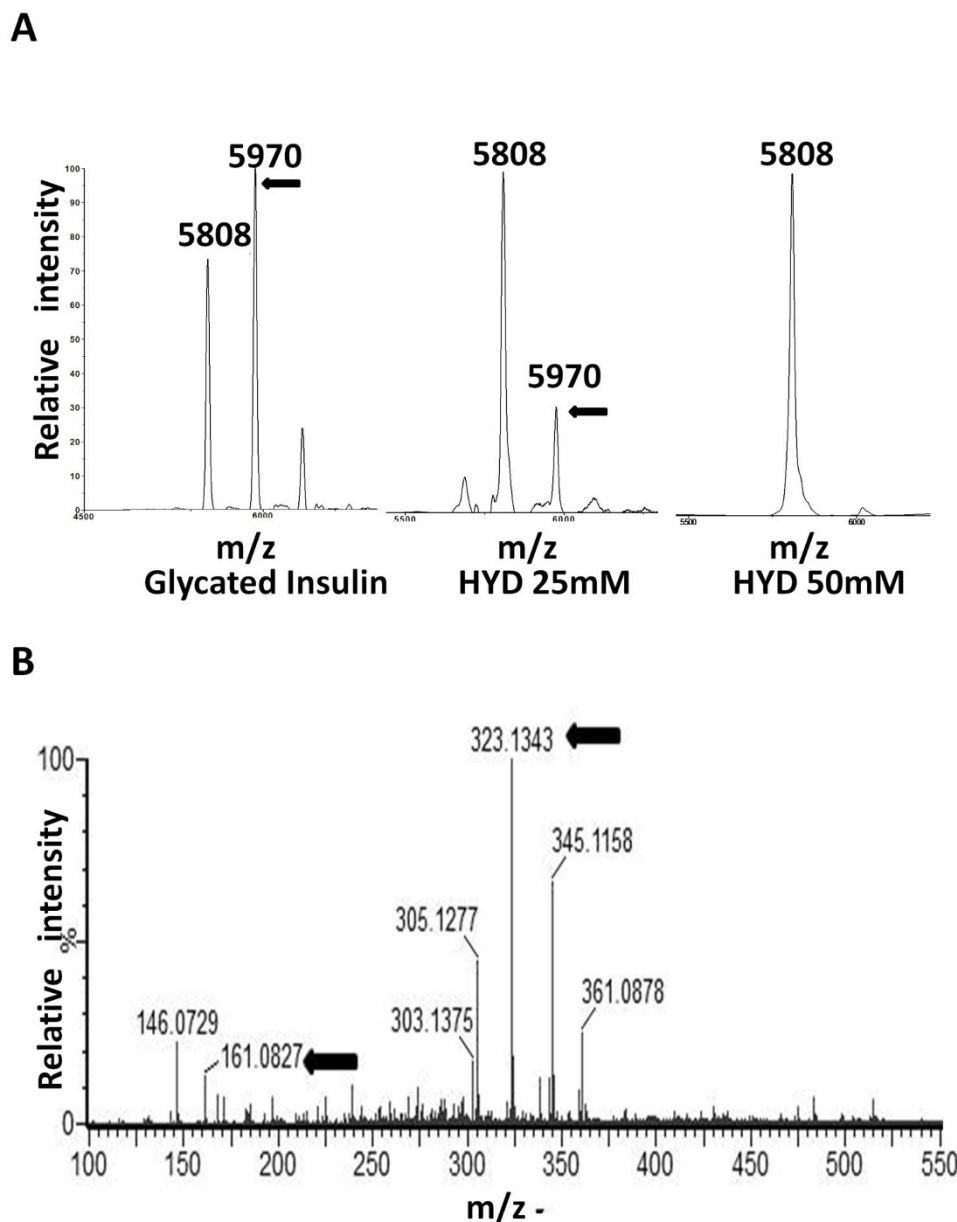


Figure 1 | *In vitro* transglycation activity of hydralazine (A) Glycated insulin (m/z 5970) was incubated with either 0 mM, 25 mM or 50 mM hydralazine for three hours at 37°C and formation of unglycated insulin (m/z 5808) was monitored by MALDI-TOF-MS. Hydralazine showed concentration dependent transglycation of glycyated Insulin. (B) MALDI-TOF-MS analysis of hydralazine-glucose conjugate formed during transglycation assay where m/z 161.08 and m/z 323.13Da are hydralazine and hydralazine-glucose conjugate respectively.

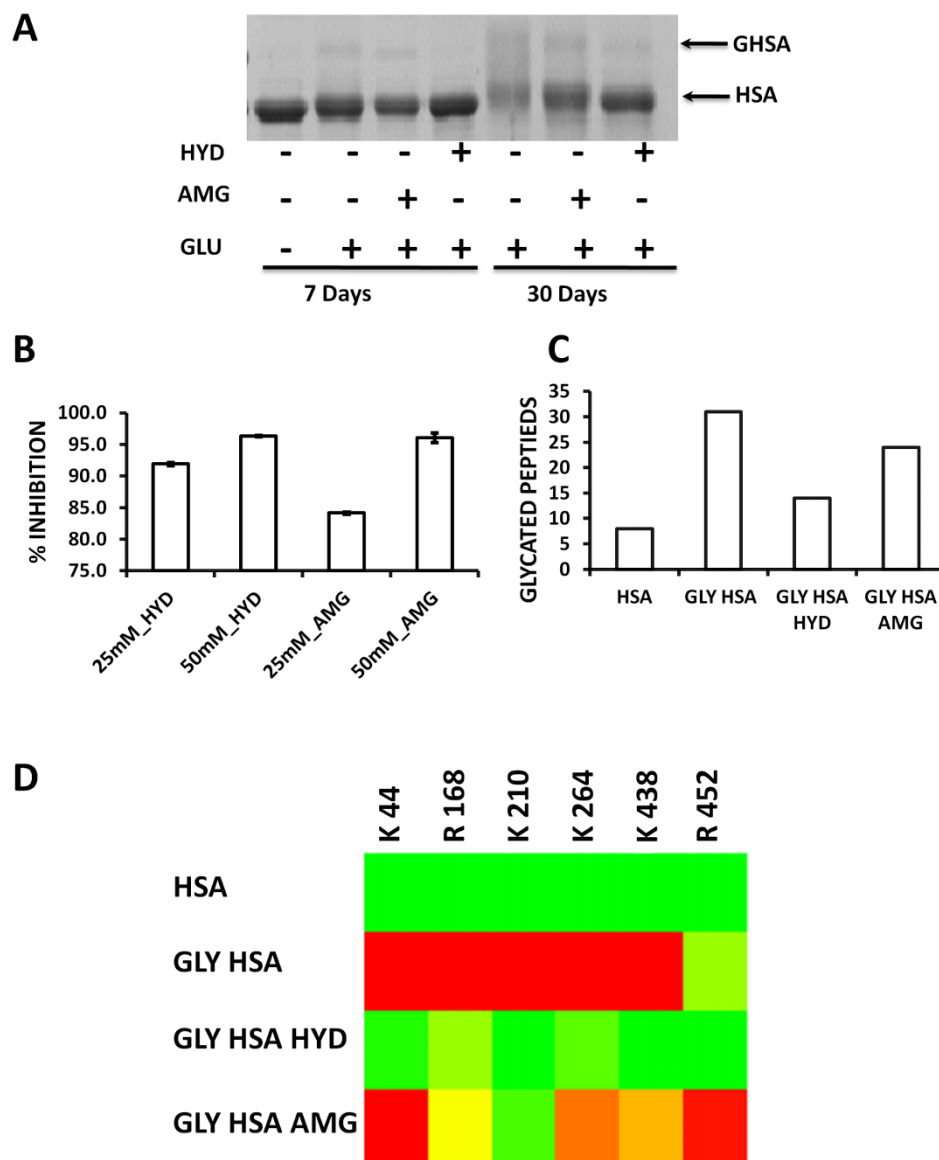


Figure 2 | *In vitro* antiglycation activity of hydralazine. HSA was glycosylated *in vitro* in presence or absence of 25 mM and 50 mM of hydralazine or aminoguanidine at 37°C for 7 or 30 days. (A) SDS-PAGE analysis depicting inhibition of glycation induced HSA crosslinking by hydralazine. (B) Hydralazine and aminoguanidine showed glycation inhibition in a concentration dependent manner as observed by decrease in AGE fluorescence emission at 440 nm. The per cent glycation inhibition was calculated by using the formula $(C-T)/C \times 100$, where C and T are fluorescence emission of glycosylated HSA without or with inhibitor respectively. The bar graph represents the mean values with standard deviation ($n = 3$). (C) LC-MS^E analysis depicting the number of AGE modified peptides in HSA, glycosylated HSA, glycosylated HSA in presence of hydralazine (50 mM) and aminoguanidine (50 mM). The values in the bar graph represent the number of AGE modified peptides in at least two replications. Glycosylated HSA had more number of AGE modified peptides than unglycosylated HSA. In presence of Hydralazine and aminoguanidine the number of AGE modified peptides were decreased. (D) Heatmap analysis of AGE modified GSAR containing peptides. The extent of AGE modification of GSAR containing peptides of glycosylated HSA was maximum. Hydralazine and aminoguanidine treatment decreased the AGE modification of GSAR containing peptides. The values in the Heatmap represent the average cumulative intensity ratio (CIR) of AGE modified peptides containing Glucose Sensitive Amino acid Residues (GSARs) to their unmodified form ($n = 2$). Heat map was generated using Matrix2png online software.

spectra have been shown in supplementary Fig. 2. Furthermore, the extent of decrease in AGE modification by hydralazine was studied. In a recent study, the extent of glycation of eight glucose sensitive peptides of human serum albumin was monitored for early diagnosis of Type 2 diabetes²⁰ (supplementary Table 1). In this study, a similar approach was used albeit with a slight modification, which is described in Material and Methods. As expected the cumulative intensity ratio (CIR) of AGE modified peptides containing Glucose Sensitive Amino acid Residues (GSARs) was highest in glycosylated HSA than non-glycosylated HSA. In presence of hydralazine and aminoguanidine,

the CIR of GSAR peptides decreased and this decrease was more in hydralazine treatment (Fig. 2d).

Furthermore, transglycation by hydralazine was demonstrated *in vivo* in STZ induced diabetic mice. Glycation associated parameters such as glycosylated hemoglobin (HbA1c), fructosamine, plasma AGEs were monitored. STZ induced diabetes led to increase in HbA1c (8.1%), which was decreased significantly in mice treated with hydralazine (Fig. 3a). The HbA1c decreased with time, and was reversed to near normal levels (4.4%) within 15 days of hydralazine treatment (300 mg/L). However, treatment of aminoguanidine for 15 days,



even at a higher concentration (2.4 g/L) failed to decrease HbA1c (8.4%) (Fig. 3a). A similar trend was also observed in plasma fructosamine levels (Fig. 3b). These results suggested that the immediate decrease in glycation can be achieved by transglycation rather than glycation inhibition. Hydralazine also decreased AGE modification of plasma proteins. Serum albumin and few abundant plasma proteins showed enhanced AGE modification in diabetic condition, which were transglycated by hydralazine treatment as reflected by decreased AGE modification in western blotting (Fig. 4a). The decrease in AGE modification was similar to a relatively higher concentration of aminoguanidine treatment (Fig. 4a). This trend was also evident in plasma AGE levels quantified by ELISA (Fig. 4b). Further, LC-MS^E analysis of serum albumin and few abundant plasma proteins revealed relatively lesser number of AGE modified peptides in hydralazine treatment than aminoguanidine treated and diabetic mice (Fig. 4c and Supplementary Fig. 3).

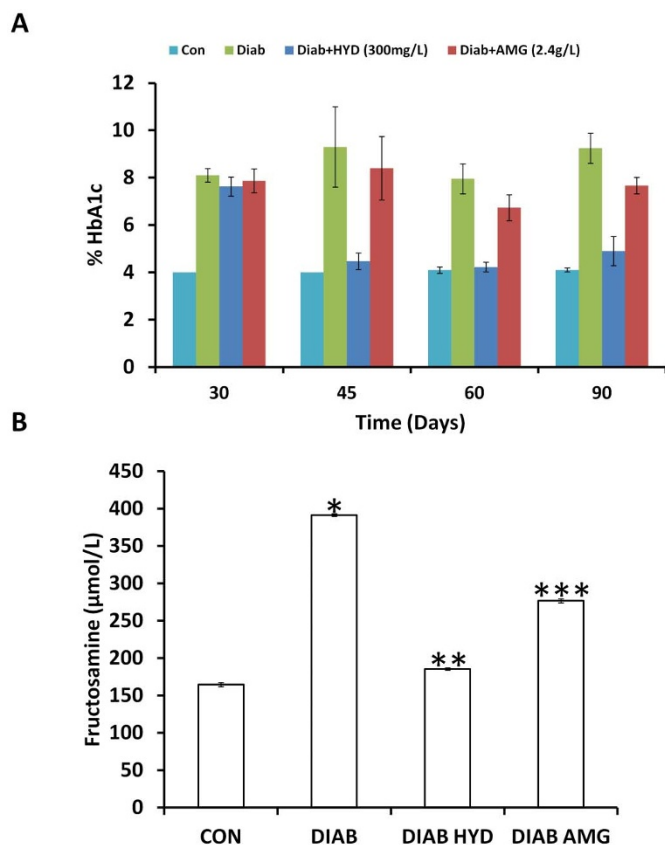


Figure 3 | *In vivo* transglycation activity of hydralazine. Male BALB/c mice were injected with 50 mg/kg body weight of STZ for five consecutive days to induce diabetes. The induction of diabetes was confirmed after 30 days by measuring the blood glucose levels and HbA1c. Animals having blood glucose level of above 6.12 ± 1.7 mmol/L with ≥ 7.0 HbA1c were treated with either hydralazine (300 mg/L) or aminoguanidine (2.4 g/L). (A) Bar graph depicting the HbA1c of control mice, diabetic mice, and diabetic mice treated with hydralazine or aminoguanidine at 30th, 45th, 60th and 90th day of experiment. Hydralazine decreased the HbA1c by 45th day (15 days of drug treatment) and was almost equal to that of control mice. The values represent mean \pm SE, $n = 6$, P -value < 0.05 . (B) Bar graph depicting the plasma fructosamine levels of control mice, diabetic mice, and diabetic mice treated with hydralazine or aminoguanidine at 90th day of experiment. Fructosamine level was measured by using the NBT assay. The reduction of NBT to formazon was measured at 540 nm and levels were calculated using the standard fructosamine. Fructosamine levels were decreased with hydralazine treatment (The values represent mean \pm SE, $n = 6$, P -value < 0.05).

The extent of *in vivo* AGE modification was studied as described above (Fig. 2d). However, in case of mouse, the GSARs were identified by homology based sequence alignment with human serum albumin (Supplementary Table 1c). Heatmap analysis of AGE modified GSAR containing peptides of serum albumin suggested that the extent of modification was highest in diabetes, and was decreased with hydralazine treatment (Fig. 4d).

Accumulation of AGEs in the kidney is associated with development of nephropathic condition²¹. Hydralazine and aminoguanidine decreased AGE levels and AGE modification of kidney proteins in diabetic mice as measured by ELISA and western blot respectively (Fig. 5a and 5b). AGEs are also known to induce increased expression of RAGE, NADPH oxidase and SOD²². Hydralazine and aminoguanidine treated diabetic mice kidney showed decreased expression of these proteins (Fig. 5c).

Hydralazine mediated transglycation was also evidenced by analysis of hydralazine glucose conjugate in urine using high resolution accurate mass spectrometer. To analyze the hydralazine glucose conjugate, retention time (RT 6.3 min) was determined by spiking its labeled form (hydralazine ¹³C-glucose conjugate) as an internal standard (Supplementary Fig. 4). The fragmentation pattern of unlabeled and labeled hydralazine glucose conjugate has been shown in Supplementary Fig. 5. The fragmentation was exactly identical except for few fragments that showed an increase in mass by 1Da e.g. m/z 306.1278 and m/z 198.0853 due to presence of ¹³C label in those fragments (supplementary Fig. 6, 7). A similar fragmentation pattern was observed in urine sample spiked with labeled conjugate (Fig. 6a). Extracted ion chromatogram (XIC) of selected fragment ion (m/z 305.1244) was used to develop standard curve (Supplementary Fig. 8). Hydralazine glucose conjugate was identified and quantified by monitoring the XIC of its selected fragment ion (m/z 305.1244). About 304.22 ± 20.35 pg of hydralazine glucose conjugate per milliliter of urine was detected in diabetic mice treated with hydralazine (Fig. 6b). This drug conjugate was not detected in control mice treated with hydralazine perhaps due to its lower concentration. Hydralazine treatment showed beneficial effects in terms of decreased levels of plasma cholesterol, triglycerides, blood urea nitrogen, and creatinine levels, as compared to diabetic mice and aminoguanidine treated diabetic mice (Table 1).

Discussion

In this study we have shown that hydralazine inhibits AGE formation by transglycation. The possible mechanism by which hydralazine causes transglycation is depicted in Fig. 7. The reaction between glucose and protein leading formation of Schiff's base and Amadori product is reversible in nature. It is possible that hydralazine interferes at the level of Schiff's base or Amadori product formation and removes the protein bound glucose by virtue of its nucleophilic nature. A similar mechanism was proposed in case of glutathione mediated transglycation¹².

Previous studies have employed various strategies to inhibit AGE accumulation²³. These approaches are broadly divided into chemical and cellular approaches. Chemical approaches involve inhibition of Amadori products²⁴ and AGEs²⁵, chemical quenching of reactive dicarbonyls²⁶, cross-link breakers²⁷. While, cellular approaches include enzymes such as, aldose reductase²⁸, which converts reducing sugars to corresponding sugar alcohols; aldehyde dehydrogenases and glyoxalases²⁹, which convert reactive dicarbonyls to their inactive forms; matrix metalloproteases that are involved in the release of soluble RAGE (sRAGE) from mRAGE, which prevents AGE interaction with mRAGE³⁰. Another such enzyme, namely fructosamine-3-kinase is involved in deglycation¹¹, which inspired the discovery of small molecule mediated chemical deglycation called transglycation¹². Biomolecules such as glutathione, cysteine, and polyamines have shown to have transglycation activity¹². Synthetic molecules such as

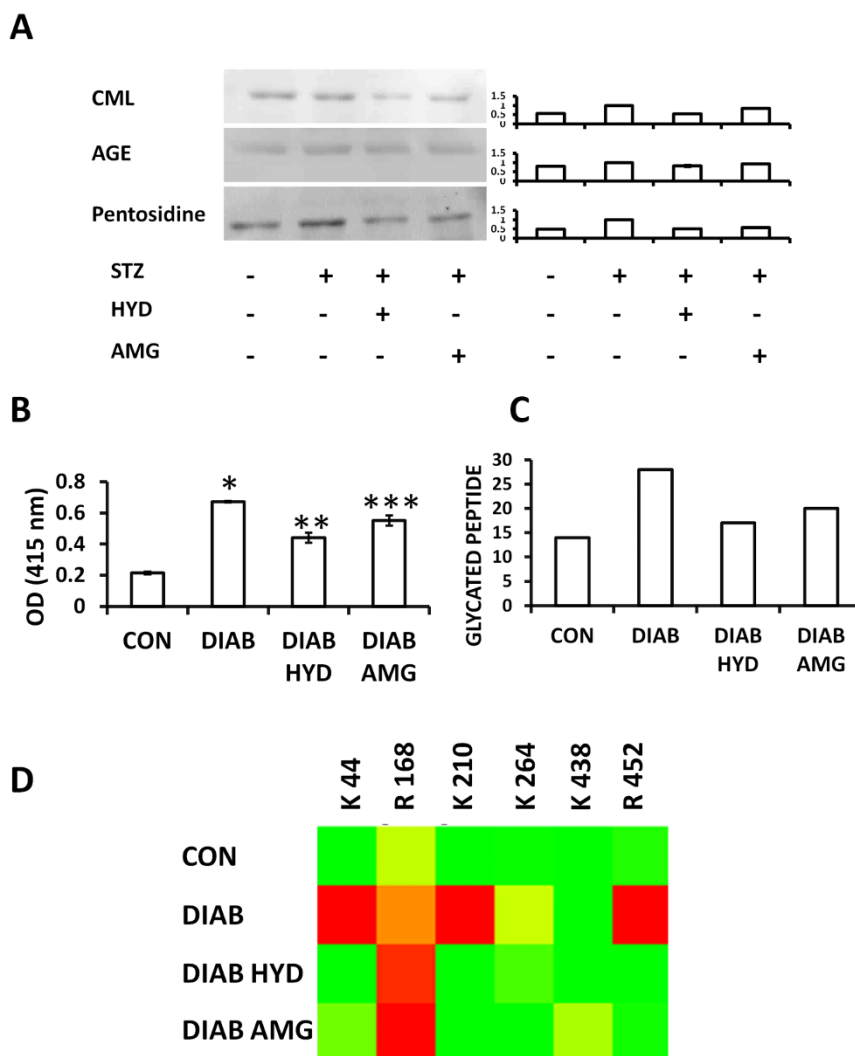


Figure 4 | Hydralazine mediated *in vivo* reduction of AGE modification of plasma proteins. (A) 5 μ g of plasma proteins were separated on 10% SDS-PAGE and transferred onto the PVDF membrane. Plasma proteins were probed for AGE modification by western blot analysis using antibodies against CML (top panel), AGE (middle panel), and pentosidine (bottom panel) antibodies. Hydralazine decreased the AGE modification as measured by densitometric analysis, which is shown in the bar graph. (B) Plasma AGE levels were measured by ELISA. Each well of a 96-well microtiter plate coated with 0.1 ml of 20 μ g/ml of plasma was blocked with 0.5% gelatin. Subsequent reactions were performed with 0.1 ml of anti-AGE antibody (0.5 μ g/ml), secondary antibody conjugated to alkaline phosphatase and PNPP (p-nitro phenyl phosphate) substrate respectively. The absorbance of each well was recorded at 415 nm with a microplate absorbance reader. Mean of A_{415} were used to calculate the relative AGE levels in the plasma. The bar graph depicts decrease in relative AGE levels upon treatment with hydralazine and aminoguanidine. (The values represent mean \pm SE, $n = 6$, P -value < 0.05). (C) Bar graph depicts the decrease in number of AGE modified peptides of serum albumin by LC-MS^E analysis in presence of hydralazine (HYD) and aminoguanidine (AMG) ($n = 2$) (D)) Heatmap analysis of extent of AGE modification of GSAR containing peptides of mouse serum albumin. The extent of AGE modification of GSAR containing peptides of glycated mouse serum albumin was maximum. Hydralazine and aminoguanidine treatment decreased the AGE modification of GSAR containing peptides. The values in the Heatmap represent the average cumulative intensity ratio (CIR) of AGE modified peptides containing Glucose Sensitive Amino acid Residues (GSARs) to their unmodified form ($n = 2$). Heat map was generated using Matrix2png online software.

isoniazid and aminoguanidine show poor transglycation activity³¹. Peptide derivative like N-acetyl carnosine with transglycation activity is being used in the eye drop formulation for the treatment of diabetes induced cataract³², although this is an encouraging indication for use of glycation inhibitors, yet there are no FDA approved drugs. In view of this, discovery of hydralazine as a transglycation agent has a great significance in controlling AGE levels and for the treatment of AGE induced diseases.

Previous studies have shown hydralazine inhibits glycation of low density lipoproteins and prevents foam cell formation in murine macrophages³³. The drug was shown to inhibit *in vivo* AGE formation, and improved renal damage in a type 2 diabetic nephropathy rat model³⁴. These studies supported our finding of hydralazine as a transglycating agent and AGE inhibitor. AGE accumulation is

associated with up-regulation of RAGE expression and activation of AGE-RAGE axis leading to oxidative stress. In earlier studies hydralazine treatment resulted in decreased oxidative stress and lipid peroxidation³⁵, perhaps this can be attributed to inhibition of AGE formation. Recent studies involving hydralazine as an acrolein scavenger³⁶ support its ability of transglycation. Therefore, the drug has been suggested for the treatment of atherosclerosis³⁷, nervous system trauma³⁸ and Alzheimers³⁹. In this study for the first time we have unequivocally demonstrated the transglycating ability of hydralazine by *in vitro* and *in vivo* experiments including insulin transglycation by MALDI-TOF-MS, analysis of hydralazine-glucose conjugate, as well as proteome wide decrease in AGE modification of plasma proteins and AGE levels in plasma and kidney. The decrease in AGE modification and AGE levels was quite significant in comparison

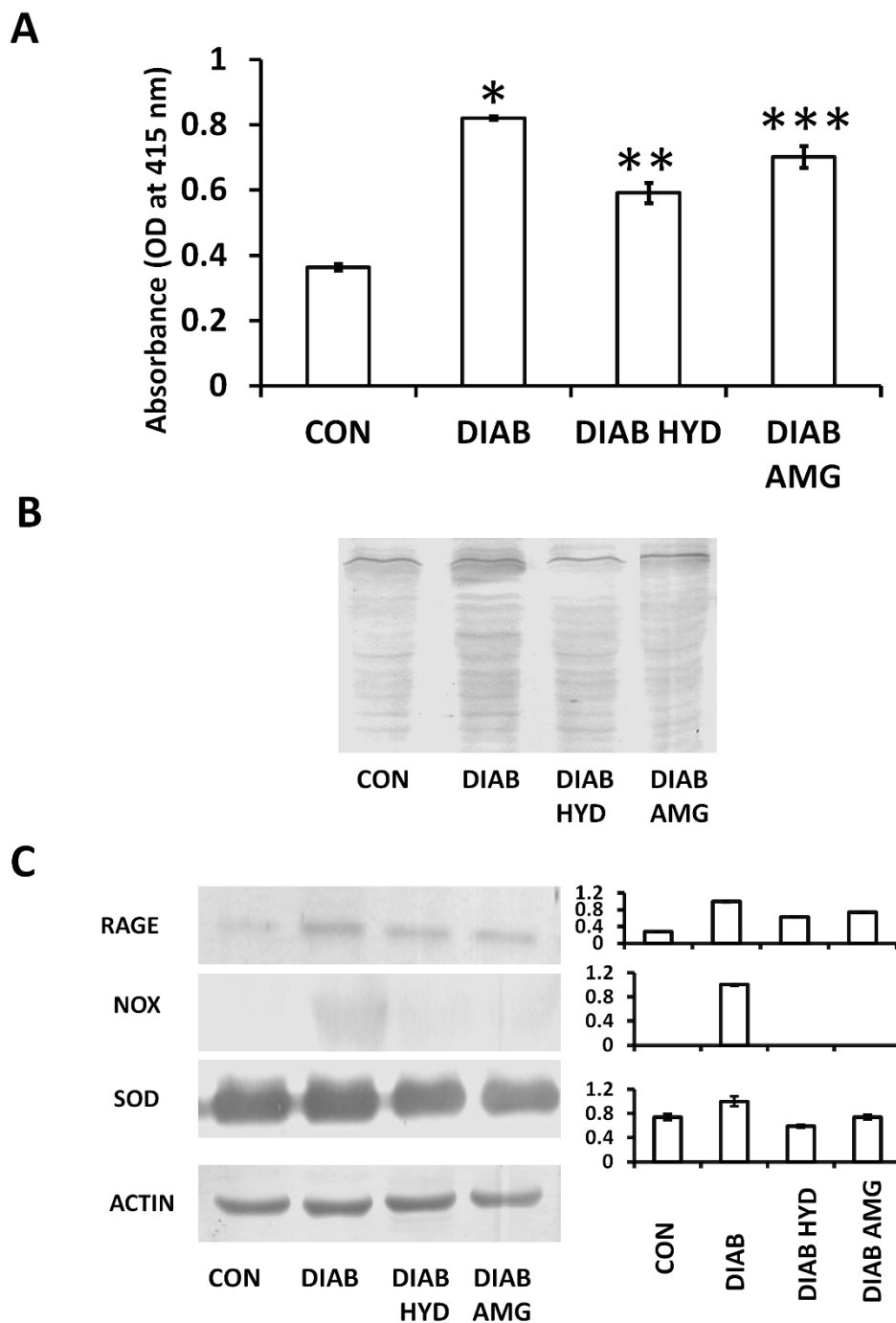


Figure 5 | Effect of hydralazine mediated transglycation on kidney proteins. (A) Kidney AGE levels were measured by ELISA. Each well of a 96-well microtiter plate coated with 0.1 ml of 20 $\mu\text{g/ml}$ of kidney proteins, was blocked, subsequent reactions were performed with 0.1 ml of anti-AGE antibody (0.5 $\mu\text{g/ml}$), secondary antibody conjugated to alkaline phosphatase and PNPP (p-nitro phenyl phosphate) substrate respectively. The absorbance of each well was recorded at 415 nm with a microplate absorbance reader. Mean of A_{415} were used to calculate the relative AGE levels in the kidney. The bar graph depicts decrease in relative AGE levels upon treatment with hydralazine and aminoguanidine. (The values represent mean \pm SE, $n = 6$, P -value < 0.05). (B) 50 μg of kidney proteins were separated on 12% SDS-PAGE and transferred onto the PVDF membrane. AGE modification was probed using AGE antibodies by western blot analysis. Hydralazine decrease the AGE modification of kidney proteins (Lane DIAB-HYD) compared to STZ induced diabetic kidney proteins (Lane DIAB). (C) Western blot analysis of 20 μg of kidney proteins indicating the expression of RAGE, NOX and SOD. Hydralazine and aminoguanidine treatment decreased the expression of these proteins (Lanes DIAB-HYD and DIAB-AMG) as shown by densitometric analysis in the bar graph.

with a relatively higher dose of aminoguanidine treatment. Furthermore, hydralazine was able to reduce HbA_{1c} and AGE levels quite faster when compared to aminoguanidine suggesting transglycation is a better intervention strategy. As hydralazine is already FDA approved drug for the treatment of hypertension, it has the potential to be repositioned for treatment of glycation induced diseases.

Methods

All chemicals were procured from Sigma-Aldrich unless otherwise mentioned. Hydralazine was generous gift from Dr. M. K. Gurjar, Director (R and D), Emcure, Pharmaceuticals, and Pune. Antibodies for CML, RAGE, NOX, and SOD were from Abcam (UK), pentosidine antibody from Cosmo Bio (Japan) and AGE antibody, secondary antibody-biotin conjugate, secondary antibody-HRP were purchased from Merck Millipore, (India), GOD-POD assay kit purchased from Beacon Diagnostics Pvt. Ltd, (India).

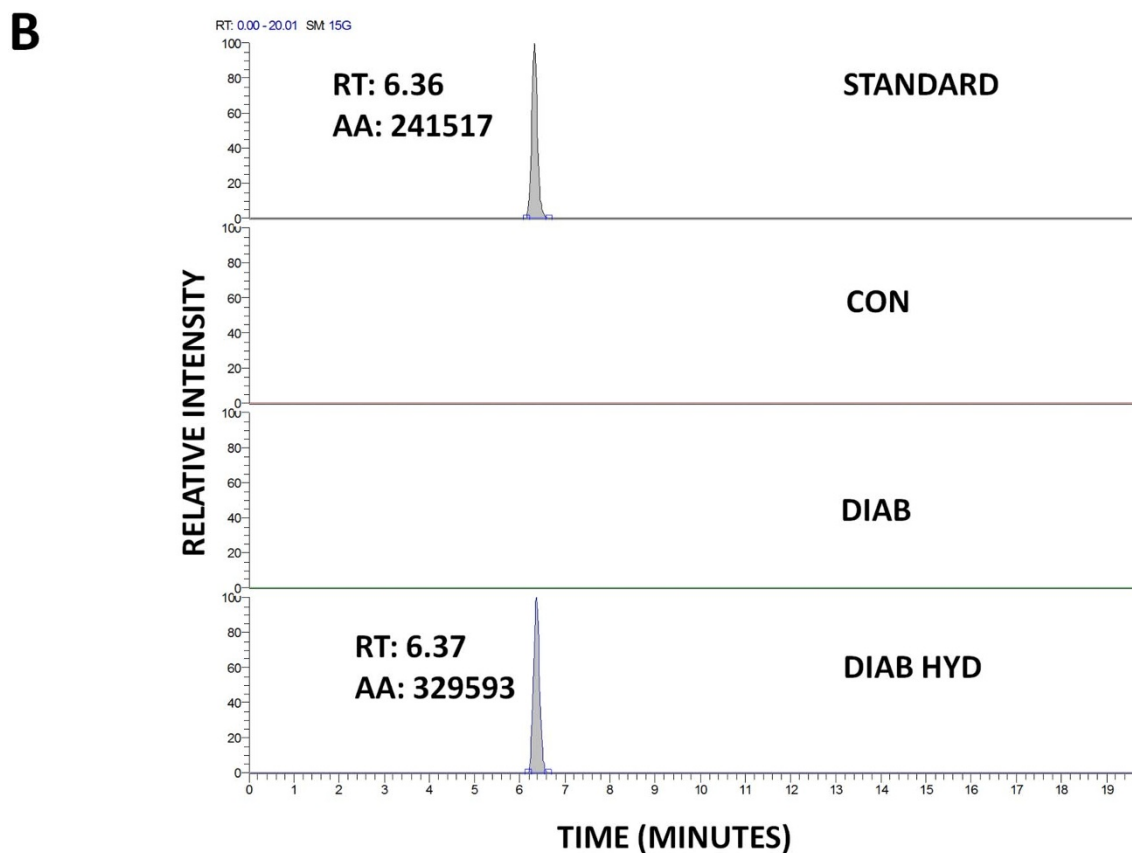
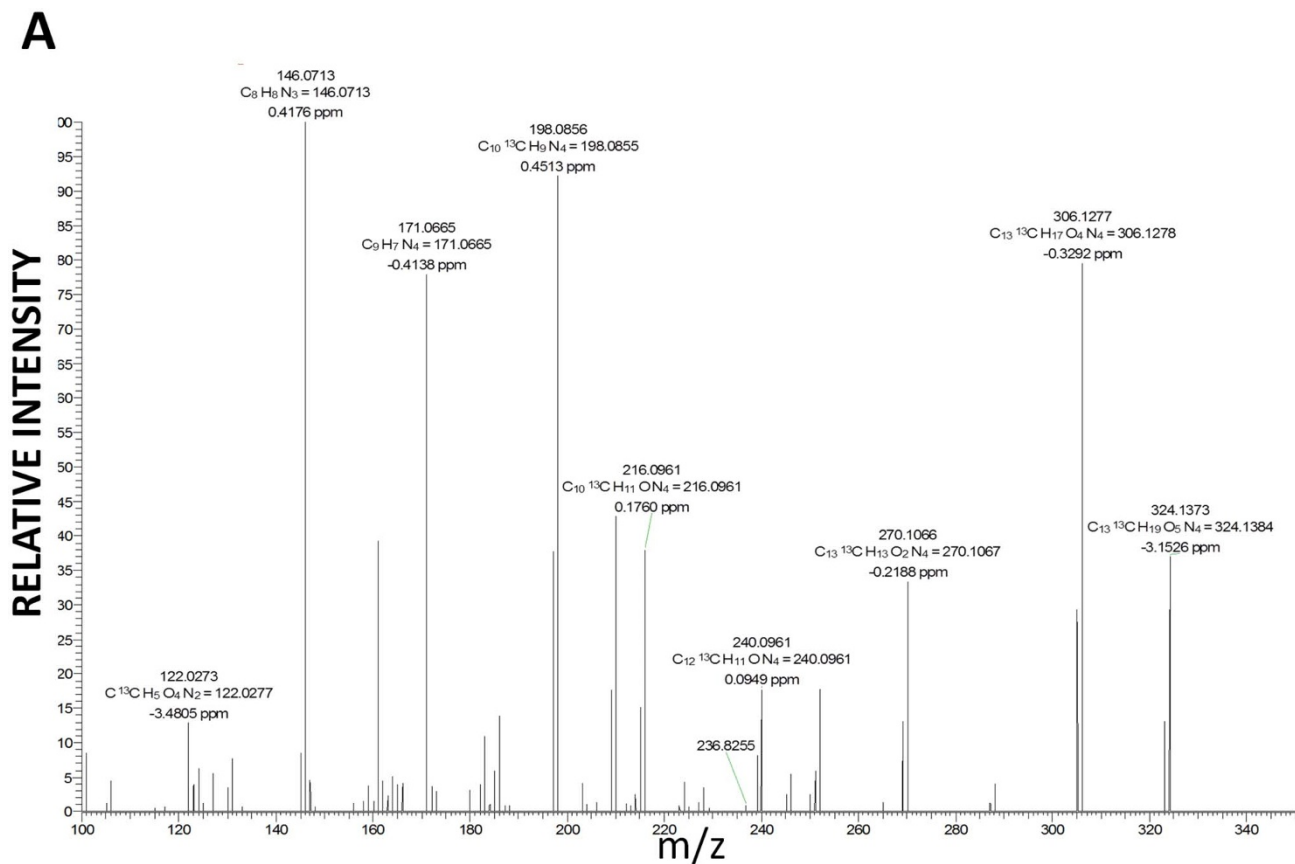


Figure 6 | Detection of hydalazine-glucose conjugate in urine of diabetic mice treated with hydalazine (A) MS/MS fragmentation of hydalazine glucose conjugate in the urine of hydalazine treated diabetic mice, which was spiked with its labeled form. (B) Extracted ion chromatogram (XIC) of selected fragment ion (m/z 305.1244) of hydalazine-glucose conjugate (m/z 323.1335, RT 6.3 min) of synthetic standard, as well as in urine of control and diabetic treated with hydalazine.



Table 1 | Blood urea nitrogen (BUN), creatinine, cholesterol and triglyceride levels in plasma of control, diabetic and diabetic treated with hydralazine and aminoguanidine mice. (The values represent mean \pm SE, n = 6)

Treatment	BUN (mg/dl)	Creatinine (mg/dl)	Cholesterol (mg/dl)	Triglycerides (mg/dl)
Control	23.53 \pm 5.64	0.29 \pm 0.03	71.00 \pm 12.13	107.25 \pm 10.01
Diabetes	38.48 \pm 15.12	0.37 \pm 0.04	118.25 \pm 37.05	138.75 \pm 21.54
Diabetic + hydralazine	18.33 \pm 4.58	0.27 \pm 0.02	68.75 \pm 14.05	96.75 \pm 18.00
Diabetic + aminoguanidine	24.47 \pm 4.29	0.32 \pm 0.05	69.33 \pm 14.29	112.00 \pm 24.24

MALDI-TOF-MS based insulin transglycation assay. Insulin was glycosylated as described earlier¹³. The glycation of insulin was monitored for 7–10 days till the relative intensity showed 50% on MALDI-TOF-MS (Voyager, ABSCIEX). The excess glucose was removed by dialysis against MilliQ water, lyophilized and stored at -80°C until use. Removal of glucose was confirmed by GOD-POD assay (Beacon). The transglycation assay was performed by incubating 50 μl of 1 mg/ml glycosylated insulin with 50 μl of 25 mM and 50 mM of hydralazine at 37°C for three hours and analyzed by MALDI-TOF-MS.

Glycation of human serum albumin (HSA). *In vitro* glycation of pure HSA was done as described⁴⁰ with slight modifications. Briefly, The reaction was carried out by incubating 200 μl of 50 mg/ml HSA in 0.01 M phosphate buffer (pH 7.4) and 100 μl 0.5 M D-glucose containing 5 mM sodium azide as a bacteriostat with or without 100 μl of 25 mM and 50 mM (final concentration) of hydralazine at 37°C for 30 days. HSA glycation was monitored by fluorescence spectroscopy¹⁴ and LC-MS^E^{18,19}.

Animal experiments and sample preparation. *Ethics statement.* The animal experiments were approved by Institutional Animal Ethics committee of National Centre for Cell Sciences, India. The experimental protocols were carried out in accordance with the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), India.

Male BALB/c mice were injected with 50 mg/kg body weight of streptozotocin (STZ) in 50 mM citrate buffer pH 4.5 for five consecutive days to induce hyperglycemia, whereas control mice were injected with 50 mM citrate buffer⁴¹. The induction of diabetes was confirmed after 30 days by measuring the blood glucose levels with glucometer (Bayer, Germany) and HbA1C level by using HbA1C kit (Bayer Germany). Animals having blood glucose level above 6.12 ± 1.7 mmol/L with ≥ 7.0 HbA1C were selected for further studies. For each treatment eight animals were grouped into control, diabetic and diabetic treated with either hydralazine (300 mg/L)⁴² or aminoguanidine (2.4 g/L)⁴³ for 60 days after induction of diabetes.

Hydralazine and aminoguanidine was made available through drinking water as described^{43,44}. Glucose and HbA1c levels were monitored on 30th, 45th, 60th and 90th day (0th, 15th, 30th and 60th day after initiation of drug treatment, respectively). Animals were euthanized at the end of study after 60 days of drug administration. Blood samples were collected and were immediately analyzed for blood glucose and HbA1c. Urine was collected in the last week of the experiment by maintaining them in Nalgene metabolic cages after acclimatization for 24 h. Collected urine was stored at -80°C till analysis⁴⁴. Plasma was obtained by EDTA treatment, which was then centrifuged at 1500 g for 5 min, and the supernatant was stored at -80°C until further use. Total cholesterol, triglycerides, creatinine, and blood urea nitrogen (BUN) concentrations were determined in plasma, and protein and creatinine concentrations in urine with an automatic analyzer (Synchro CX7, Beckman Coulter Inc., Fullerton, CA). Kidney tissue was collected by snap freezing into liquid nitrogen. Later the tissue was perfused with cold phosphate buffer saline to remove blood stains prior to homogenization. The tissue was homogenized to fine powder in liquid nitrogen and the protein was extracted in buffer consisting of 7 M urea, 2 M thiourea, 2% CHAPS, 1% DTT, 40 mM Tris, and centrifuged at 14,000 g for 30 min at 4°C . The supernatant was collected and stored in aliquots at -80°C . Protein concentration was determined by using Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

NBT Colorimetric fructosamine assay. Plasma fructosamine level was measured by using the NBT assay as described previously⁴⁵. 200 μl of 0.75 mM NBT was added to a 96-well microplate containing 10 μl of the plasma. The reduction of NBT by

fructosamine group was measured at 540 nm after incubation at 37°C for 30 min using an iMark microplate absorbance reader (Bio-Rad, Hercules, CA).

LC-MS^E, protein identification, database search, and PTM analysis. Prior to LC-MS^E analysis plasma proteins and HSA were digested with trypsin after reduction and alkylation with 100 mM DTT and 200 mM iodoacetamide respectively. Two microliters (100 ng/ μL) of digested peptides was spiked with 100 fm of enolase, was analyzed by using online nanoACQUITY UPLC coupled to a SYNAPT HDMS (Waters Corporation, Milford, MA) by MS^E¹⁸. After MS^E analysis, data were analyzed with Protein Lynx Global Server software (PLGS version 2.4 Waters Corporation, Milford, MA). A preliminary search of processed samples was performed for protein identification against the UniProt mice database containing 24382 protein entries. Glycation modification sites were identified by subjecting PLGS search against subset of protein databases that were identified in the preliminary search as described^{18,19}. The false positive rate was set to 4% in the PLGS workflow. The PLGS identified glycation modifications were validated by following criteria. (a) accurate shift in precursor ion mass due to glycation (b) MS/MS spectra of glycosylated peptides should have at least seven fragments (c) the MS/MS spectra was analyzed for fragment mass error and fragment retention time error (d) glycosylated peptides should exist in at least two replications. An example of annotation of glycation modification has been shown in the supplementary data.

Extent of AGE modification of serum albumin. The extent of glycation modification was determined by analyzing the AGE modification of glucose sensitive peptides of serum albumin as described²⁰ with a slight modification (supplementary Table 1). We have monitored the AGE modification of all the peptides containing the Glucose Sensitive Amino acid Residues (GSARs) (K44, R168, K210, K264, K438, R452) since trypsin digestion can generate different peptides containing these amino acid residues. The Cumulative Intensity Ratio (CIR) of AGE modified peptides containing GSAR to their unmodified form was monitored to quantify the extent of AGE modification. The CIR was used because the same GSAR can undergo various AGE modifications e.g. CEL, CML etc., and therefore it becomes important to consider the intensity of all the AGE modified peptides. The Cumulative Intensity Ratio (CIR) of AGE modified peptides containing GSAR to their unmodified form was monitored to quantify the extent of AGE modification.

Enzyme-linked immunosorbent assay. Each well of a 96-well microtiter plate was coated with 0.1 ml of 20 $\mu\text{g}/\text{ml}$ of plasma/kidney protein sample in 50 mM carbonate buffer (pH 9.6) and incubated overnight at 4°C . The plate was washed three times with PBS containing 0.05% Tween 20 (Buffer A). Each well was blocked with 0.5% gelatin, washed with Buffer A, and reacted with 0.1 ml of anti-AGE antibody (0.5 $\mu\text{g}/\text{ml}$). Then the wells were washed with buffer A, incubated with secondary antibody conjugated to alkaline phosphatase (Bangalore Genei, India) for 30 min. Thereafter the plate was washed and incubated in dark with PNPP (p-nitro phenyl phosphate) for 15 min, and the reaction was stopped by addition of sulphuric acid (0.5 M, 10 μl per well). The absorbance of each well was recorded at 415 nm with an iMark microplate absorbance reader (Bio-Rad, Hercules, CA).

Western blotting. Plasma and kidney proteins were separated on 10% and 12% SDS-PAGE respectively and transferred onto the PVDF membrane. The membranes were blocked with 5% skimmed milk powder dissolved in TBS (20 mM Tris-HCl (pH 7.5), 0.15 M NaCl). Plasma proteins were probed for AGE modification by western blot analysis using antibodies against AGE, CML, and pentosidine antibodies. While

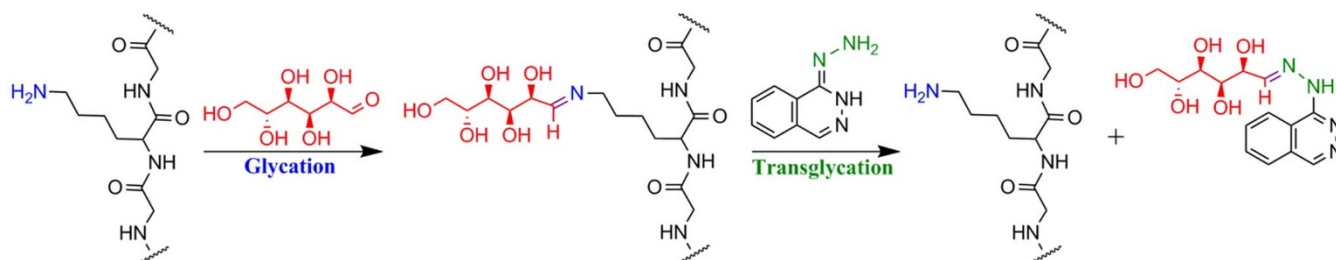


Figure 7 | Probable Mechanism of hydralazine mediated transglycation. Hydralazine may interfere at the level of Schiff's base or Amadori product formation during glycation reaction and removes the protein bound glucose by virtue of its nucleophilic nature.



kidney proteins were probed for AGE modification, RAGE, NOX and SOD. Antibodies against actin served as loading control. Antibody dilution was adopted according to the manufacturer instructions. Corresponding biotinylated secondary antibody was used in 1 : 2000 dilution followed by incubation with streptavidin HRP conjugate. Immunoreactive bands were visualized using DAB system (Sigma) and documented by Bio-Rad G-800 densitometer.

Synthesis of hydralazine glucose and hydralazine ¹³C-glucose conjugates.

Hydralazine glucose conjugate (aldehyde-D-glucose phthalazin-1-ylhydrazine) was prepared by incubating 0.51 mmole of D-glucose and hydralazine hydrochloride with sodium acetate (1.02 mmole) in water: methanol (1 : 3)⁴⁶. The reaction mixture was kept at boiling water bath for 10 min. Further the reaction was continued at room temperature for 16 hours. The product was filtered, washed with methanol and dried to obtain yellow, needle-like crystals of hydralazine glucose conjugate with quantitative yield. The product was characterized by FT-IR (Fourier Transform Infrared spectroscopy) Vmax 3306 (broad, NH and OH) 1647 (C=N) and 1563 cm⁻¹(Ph) and HRMS (high resolution mass spectrometry) with accurate mass of 323.1384. (Supplementary Figure 9). The same reaction conditions were used to synthesize hydralazine-¹³C-glucose conjugate.

Urine analysis for the identification of Hydralazine glucose conjugates. To analyze hydralazine glucose conjugate in urine, urinary proteins were removed by ultrafiltration with 3 kD molecular weight cut-off filters. The filtrate was collected and kept in -80 °C until use. The urinary metabolites were separated and analyzed as described earlier⁴⁷. In brief 2 μl of sample was separated by Accela UPLC (Thermo Scientific) consisting of binary solvent system and a reverse phase Hypersil GOLD C18, 8 μm, 4.6 × 150 mm. The binary solvent system included 99.9% water and 0.1% formic acid (mobile phase A) and 99.9% acetonitrile and 0.1% formic acid (mobile phase B). Hydralazine glucose conjugate was eluted with a flow rate of 0.5 ml/min using sequential gradient of mobile phase B for 1–15% (0–3 min), 15–50% (3–6 min) and finally 50–95% (6–9 min). The UPLC was connected to online high resolution mass spectrometer Q- Exactive (Thermo Scientific), a hybrid quadrupole orbitrap mass analyzer. The mass spectra were acquired in high resolution (30000 FWHM) mode by using Xcalibur and data was processed by Quant software (Thermo Scientific). The method consisted of full scans and targeted MS/MS of selected precursor ion at a defined mass and retention time, with the following instrument parameters; microscans 1, AGC target 2e⁵ and Maximum injection time 120 ms. The retention time was determined by analyzing both labeled (¹³C) and unlabeled synthetic hydralazine glucose conjugate.

Hydralazine glucose conjugate was analyzed by constructing the XIC of selected fragment ions namely m/z 305.1244 and m/z 306.1278 of unlabeled and labeled (¹³C) synthetic hydralazine glucose conjugates (m/z 323.1350 and m/z 324.1384) respectively. The standard curve was developed by plotting the log₁₀ value of area under curve (AUC) of the selected fragment (m/z 305.1244) XIC against log₁₀ value of various concentrations of synthetic hydralazine glucose conjugate ranging from 40 fg to 4 ng. All the XIC were extracted with an accuracy of 3 ppm. An optimum concentration of 500 pg/ml of labeled hydralazine conjugate was spiked as an internal standard into the urine samples. Hydralazine glucose conjugate from urine sample was identified and quantified by comparing the XIC of selected fragment ion (m/z 305.1244).

Statistical analysis. All experiments were performed in triplicates. Statistical analysis was performed by Student's t-test. Data are expressed as means ± SD. A p-value < 0.05 was considered as statistically significant.

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Author contributions

M.J.K. conceived the idea, designed, supervised the study and written the manuscript. S.K.K. developed MALDI based assay for transglycation and carried out the animal experiments. S.B., S.B.G. and S.D.B. have carried out western blotting and proteomic analysis including protein extraction, digestions and mass spectrometric acquisitions. M.G.J. and A.B.D. were involved in mass spectrometric data analysis, M.L.S. helped in animal experiments and carried out biochemical analysis, H.V.T. and H.S.P. synthesized labeled hydralazine glucose conjugate and designing metabolite analysis. S.K.K., S.B.G. and M.G.J. carried out metabolite analysis. R.B. designed the animal experiment and reviewed the manuscript. All authors reviewed the manuscript.

Additional information

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