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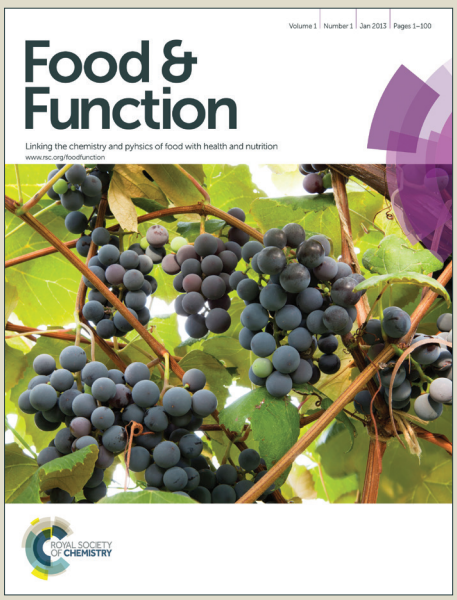

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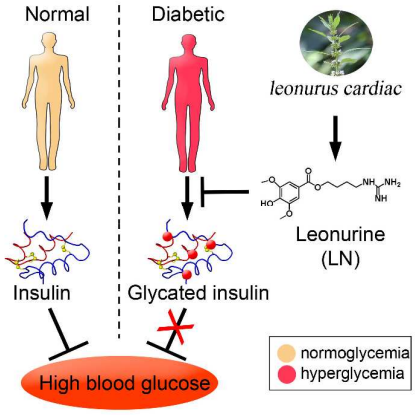

Leonurine inhibits AGEs formation through scavenging the carbonyl species

Inhibitory effect of leonurine on the formation of advanced glycation end products

Lianqi Huang[a], Xin Yang[a], Anlin Peng[b], Hui Wang[a], Xiang Lei[c], Ling Zheng[d] & Kun Huang[a,e]

Long-term hyperglycemia is a typical symptom in *diabetes mellitus* (DM) which can cause high level of protein glycation and lead to the formation of advanced glycation end products (AGEs). The accumulation of AGEs in turn deteriorates DM and its complications. Insulin, the only hormone thatdirectly decreases blood sugar *in vivo*, is vulnerable to glycation which causes the loss of its biological activity. In this study, we used a porcine insulin (PI) - methylglyoxal (MGO) model to investigate the inhibitory effect of leonurine (LN), a natural alkaloid extracted from *herba leonuri*, on AGEs formation. Assays including AGEs-specific fluorescence, fructosamine level and carbonyl group content showed that LN can dose-dependently suppress PI glycation. Significantly decreased cross-linking level on glycated PI was also proven by SDS-PAGE electrophoresis. Further liquid chromatography mass spectrometry study suggested LN may inhibit PI glycation through trapping MGO from reacting with PI. Our results thus indicate LN as a promising anti-glycation agent for the prevention of diabetes and its complications *via* inhibiting AGEs formation.

1. Introduction

Diabetes mellitus (DM) is a global metabolic disease with 382 million diagnosed patients in 2013, which is expected to reach 583 million by 2035.[1] In China, 98.4 million adults are suffering from DM, which means approximately 1 out of 10 adults diagnosed of DM.[1] The typical clinical symptom of DM is long-term hyperglycemia, which is believed to be the direct cause of advanced glycation end products (AGEs) formation.[2] Accumulation of AGEs in turn causes the deterioration of DM and its complications, especially retinopathy, nephropathy, and cardiomyopathy.[3, 4] Therefore, inhibition of AGEs formation has been considered to be a therapeutic strategy for DM and its complications.[5]

AGEs consists of three different types of complex and heterogeneous compounds. Fluorescent cross-linking compounds as pentosidine, non-fluorescent cross-linking adducts like methylglyoxal-lysine dimers (MOLD), and the non-fluorescent, noncross-linking products such as carboxymethyllysine (CML) and pyrraline (a pyrrole aldehyde).[6, 7] Endogenous AGEs arise from non-enzymatic protein glycation (Maillard reaction), which starts with the reaction between carbonyl group of a reducing sugar and the free amino group of a protein to form a reversible Schiff base, then rearrange into more stable Amadori products like fructosamine.[8] Ultimately, the Amadori products further generate dicarbonyl species to form AGEs.[9]

Methylglyoxal (MGO, Fig. 1) is one of the dicarbonyl intermediates generated by glycolysis during protein glycation. Because of its high activity and vast abundance *in vivo*,[10, 11] MGO has been believed to contribute significantly to intracellular AGEs formation.[12-14] Compared to normal population, the concentration of MGO is significantly increased by 2-4 folds in diabetics.[15] *In vivo*, the targets of MGO include arginine, lysine, and cysteine residues of proteins like serum albumin, hemoglobin and insulin.[16, 17]

Recent studies have been focused on small molecular inhibitors that suppress protein glycation. For example, aminoguanidine (AG), tenilsetam and metformin have been demonstrated to be strong anti-glycation agents.[18-20] However, the side effects of these inhibitors are also obvious, for example, as the first well-known glycation inhibitor, AG was withdrawn during its phase Ⅲ clinical trial due to gastrointestinal side effects.[21] Therefore, effective and safe anti-glycation agents are in need.[22]

Leonurus cardiac is a traditional Chinese herbal medicine that has been mainly used for treating nervous system dysfunction, cardiovascular disorders and digestive disorders for centuries. A recent study showed that Leonurine (LN), an alkaloid isolated from *Leonurus cardiac*, has the ability of alleviating diabetic symptoms in *db/db* mice *via* down-regulating NF-κB/IKK pathway.[23] Previous studies have shown that some guanidine group containing compounds, for example AG and metformin, have suppression effect on protein glycation.[24, 25] Arginine, which has a guanidine group in its side chain, is one of protein glycation targets.[26] We thus speculated that guanidine group containing compounds may also interact with MGO, which as a result, scavenges free MGO to inhibit AGEs formation. Based on these facts, we propose LN, which has a guanidine group (Fig. 1), may inhibit protein glycation. To test this hypothesis, in the present study, we investigated the anti-glycation effects of LN and the possible mechanisms by using a porcine insulin (PI) - MGO model.

2. Materials and methods

2.1 Materials

Porcine insulin (PI) was obtained from Wanbang Biopharmaceuticals (Xuzhou, China) and Zinc free PI was prepared by RP-HPLC. Leonurine (LN), trichloroacetic acid

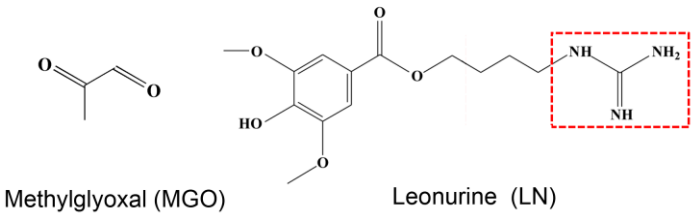

Fig. 1 Structure of leonurine (LN) and methylglyoxal (MGO). Guanidine groups are highlighted in red dotted box.

(TCA) and 2, 4-dinitrophenylhydrazine (DNPH) were purchased from Aladdin-reagent INC. (Shanghai, China). Nitroblue-tetrazolium (NBT), 1-deoxy-1-morpholino-fructose (1-DMF) and aminoguanidine (AG) were obtained from Sigma-Aldrich (St. Louis, USA). All other chemicals were of the highest grade available.

2.2 Sample Preparation and PI Glycation

Zinc-free PI (0.75 mg/ml, 130 μM) was incubated with 2 mM MGO in 100 mM phosphate buffer saline (pH 7.4 containing 0.02% NaF) for 48 h at 37 ℃ as described.[26] Stock solutions of AG and LN were prepared in dimethylsulfoxide (DMSO). AG was used as a positive control with a final concentration of 1 mM, and LN was added in final concentrations of 0.5, 1 and 2 mM. The final concentration of DMSO in the sample was less than 1%. All sample solutions were filtered with a 0.22 μm Millipore filter before use.

2.3 AGEs-specific Fluorescence assay

Glycated samples were diluted 30 times with phosphate buffer saline (pH 7.4) to measure AGEs-specific fluorescence on a Hitachi FL-2700 fluorometer (Hitachi, Tokyo, Japan). The excitation and emission wavelengths were set at 370 nm and 440 nm, respectively.[27, 28] All experiments were performed at least three times.

2.4 Fructosamine Measurement

The concentration of fructosamine was quantified using the nitroblue-tetrazolium (NBT) assay as described.[29] Briefly, 0.1 mg/ml PI samples were incubated with 0.25 mM NBT in carbonate buffer (100 mM, pH 10.4) with 2 M urea at 37 ℃ for 1 h.[30] The absorbance was measured at 530 nm with a synergy™ HT microplate photometer (BioTek, Vermont, USA), with 1-deoxy-1-morpholino-fructose (1-DMF) used as standard curve. A control experiment showed that 2 M urea has little influence on the absorbance (data not shown).

2.5 Protein Carbonyl Content Assay

The content of carbonyl groups was measured as described.[31, 32] A final concentration of 10 mM 2, 4-dinitrophenylhydrazine (DNPH) in 2 M HCl is added to each glycated sample. Mixtures were placed in dark at room temperature for 1 h with vortexing every 10 min. Then, 13% of TCA was added to precipitate proteins and further centrifuged for 10 min at 4℃. The protein pellets were washed once with 13% TCA to remove free DNPH followed by resuspending in 2 N HCl containing 6 M guanidine hydrochloride. Absorbance at 366 nm was measured on a synergy™ HT microplate photometer (BioTek, Vermont, USA).

2.6 SDS-PAGE Electrophoresis

The cross-linking properties of glycated PI, and AG or LN treated samples were studied by denaturing gel electrophoresis.[33, 34] Briefly, 10 μL glycated samples of different groups were mixed with 5 μL loading buffer followed by denaturation at 98 ℃ for 10 min. The denatured samples were separated on a 20% tricine-urea gel and visualized by a fast silver staining kit (Beyotime, Jiangsu, China).

2.7 RP-HPLC and positive ESI-MS analysis

Glycated samples were analyzed by a Hitachi L-2000 HPLC system (Hitachi, Tokyo, Japan) with an Apollo C18 column (Welch Materials, Maryland, USA).[35] Water (A) and acetonitrile (B) were used as mobile phases with a linear gradient 10–90% B for 25 min. Elutes were detected at 215 nm and the molecular weight of each analyte was confirmed by positive ESI-MS.

2.8 Data analysis

All data were expressed as mean ± SEM. Each treatment was repeated at least three times. Data were analyzed by the nonparametric Kruskal-Wallistest followed by the Mann-Whitney test. $P < 0.05$ was considered significant.

3. Results

3.1 Leonurine inhibits the AGEs-specific fluorescence

The formation of AGEs was observed by AGEs-specific fluorescence with excitation and emission wavelength at 370 nm and 440 nm, respectively.[36] PI treated with MGO for 48 h was used as a 100% glycated control, and AG (1 mM) was chosen as the positive control, which significantly inhibited the AGEs-specific intensity to 44.03 ± 2.75% ($P < 0.0001$). PI alone barely affected the fluorescence after 48 h incubation (Fig. 2A), while

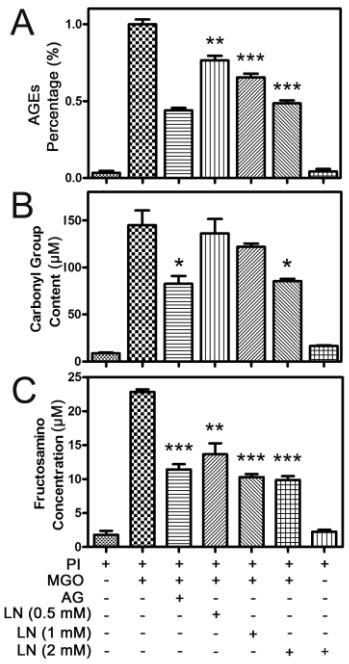

Fig. 2 Chemical characteristics of glycated PI. (A) specific fluorescence assay for the advanced glycated end-products (AGEs); (B) the level of carbonyl group contents; (C) fructosamine content assay. * $P < 0.05$ compared to PI treated with MGO, ** $P < 0.01$ when compared to PI treated with MGO, *** $P < 0.001$ compared to PI treated with MGO.

LN dose-dependently inhibited the intensity of glycated PI. The fluorescence intensity percentage was 76.53 ± 5.06% ($P < 0.05$) in the presence of 0.5 mM LN, whereas in the presence of 1- and 2 mM LN, the percentage was decreased to 65.48 ± 4.16% ($P < 0.001$) and 48.52 ± 3.44% ($P < 0.001$), respectively (Fig. 2A). Meanwhile, LN (2 mM) treated PI (without MGO) suggested that LN had little influence on fluorescence intensity (Fig. 2A).

3.2 Leonurine reduces the levels of carbonyl groups and fructosamine in glycation

Carbonyl content is used as an indicator of the amount of proteins under oxidative stress in a number of human diseases including diabetes.[37] In our experimental setting, protein glycation is the only source that triggers protein oxidative damage; therefore carbonyl content was chosen as an indicator of PI glycation. The carbonyl content of PI exposed to MGO was approximately 16.5-fold higher than that of non-glycated PI group after 48 h incubation, while in AG treated samples (1 mM), it was reduced to 42.97% ($P < 0.05$) compared to MGO-treated PI (Fig. 2B). The anti-glycation effect of LN was dose-dependent. No obvious inhibitory effect was observed with 0.5 mM LN treated samples, but when the concentration of LN was increased to 1- and 2 mM, the level of carbonyl groups was decreased to 84.17% and 59.00% ($P < 0.05$), respectively (Fig. 2B).

Fructosamine is one of the reversible Amadori products formed in the early stage of protein glycation and it can further undergo oxidative cleavage to form AGEs.[38] Thus fructosamine level has also been widely used as a short-term indicator for blood sugar control in clinic.[39] We found that compared to MGO-treated PI samples, 0.5 mM LN treatment significantly decreased the fructosamine level by 40.19% ($P < 0.01$), which was further reduced to 54.90% ($P < 0.0001$) in the presence of 1 mM LN. However, no stronger suppression effect was observed when the concentration of LN reached 2 mM (Fig. 2C).

3.3 Leonurine inhibits the cross-linking of glycated PI

As cross-linked AGEs are formed in the late stage of protein glycation,[38] we performed SDS-PAGE electrophoresis to analyze the cross-linked species. Glycated PI showed two cross-linked bands besides monomers (lane 2, Fig. 3), while non-glycated PI exists only as monomers (lane 1, Fig. 3). AG showed strong suppression on formation of cross-linking structures (lane 3, Fig. 3), which is consistent with a previous report.[40] LN also exhibited impressive dose-dependent suppression on cross-linking (lanes 4-6, Fig. 3). PI treated with LN existed only as monomers, indicating inhibition of LN on the formation of cross-linking species (lane 7, Fig. 3).

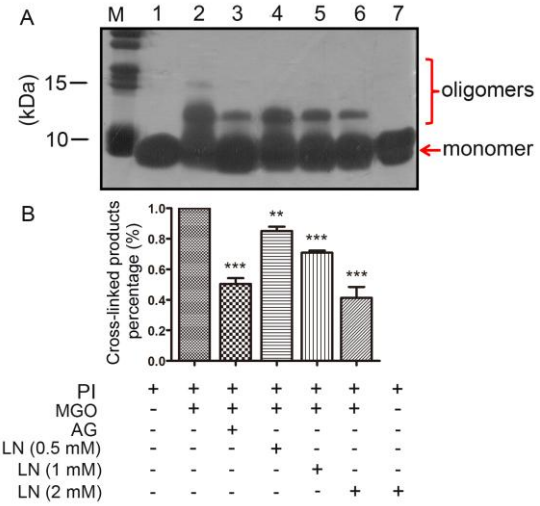

Fig. 3 Study on the cross-linked oligomeric species of glycated PI with SDS-PAGE. (A) As shown in lane 2, PI treated with MGO showed three bands, suggesting formation of cross-linked AGEs. AG suppressed cross-linking (lane 3). LN also dose-dependently inhibits the cross-linking (lanes 4, 5, 6). LN alone did not trigger cross-linking (lane 7). (B) Quantitative analysis of secondary line in (A). PI treated with MGO (lane 2) is used as 100% control. ** $P < 0.01$ when compared to lane 2, *** $P < 0.001$ compared to lane 2.

3.4 Leonurine inhibits PI glycation by trapping MGO

RP-HPLC assay was performed to monitor the glycation products. Results showed that the co-incubation of LN and PI without MGO remained as monomers after 48 h (Fig. 4E & 4F), which is consistent with the SDS-PAGE results (Fig. 3). And multiple new products was monitored in MGO-treated groups after incubation (Fig. 4B), when LN was added to MGO-treated group, the proportion of PI monomers was significantly increased,

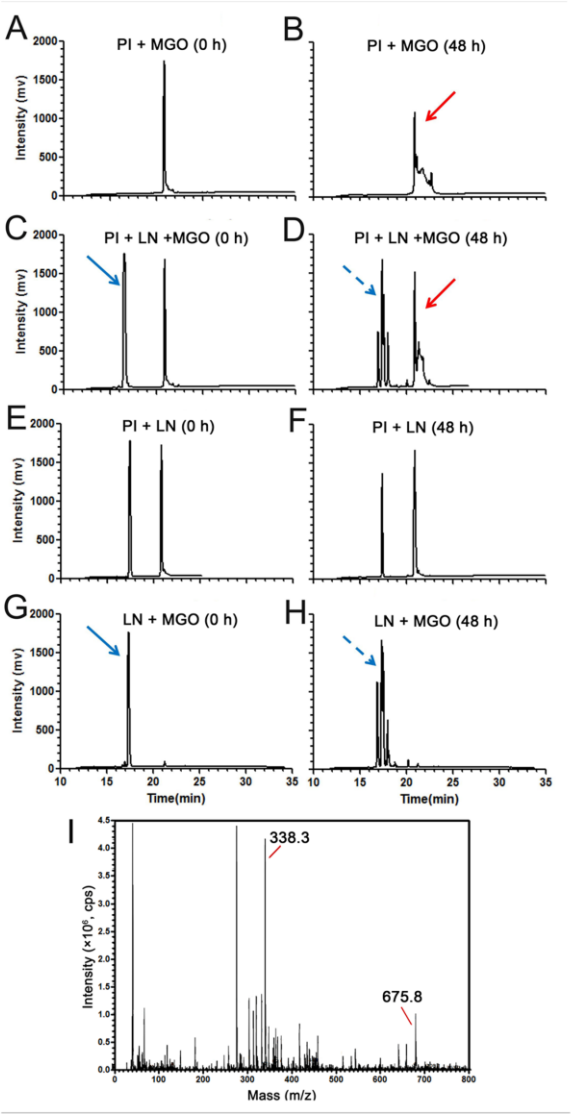

Fig. 4 RP-HPLC and ESI-MS spectra of glycated products. (A-D) Representative RP-HPLC spectra of glycated PI treated with 2 mM MGO in the presence or absence of 2 mM LN at 0 h and 48 h; (E-H) control studies of PI co-incubated with LN (E & F) or LN co-incubated with MGO (G & H) obtained at designated time points; (I) Positive ESI-MS spectrum results of a mixture of LN and MGO. Red arrows, PI and glycated PI; blue arrows, LN before incubation; dotted blue arrows, LN treated with MGO after incubation.

indicating glycation suppression by LN (Fig. 4D & 4B). Moreover, after co-incubating with MGO, LN turned from single peak (Fig. 4C) to multiple peaks (Fig. 4D), which was further confirmed by a control study of MGO treated LN in the absence of PI (Fig. 4G and 4H). Positive ESI-MS results of LN incubated with MGO with or without PI showed peaks on 675.8 ($[M + H]^+$) and 338.3 ($[M + H]^+$) (Fig. 4I), indicating a possible structure formed by two LN with one MGO with a theoretic molecular weight of 675 (Fig. 5B), which suggests LN may inhibit the glycation of PI as a scavenger of free MGO.

4. Discussion

Insulin is the only hormone that directly decreases the level of blood sugar. In diabetes, the glycation of insulin damages its bioactivity, including fail to regulate glucose homeostasis and to stimulate glucose transport *in vivo*.[26, 41, 42] Thus a PI-MGO model was chosen in the present study to assess the inhibition effect of LN on protein glycation. Strong inhibitory effect of LN on AGEs formation was observed by the AGEs-specific fluorescence assay, fructosamine measurement, protein carbonyl content assay, SDS-PAGE electrophoresis (Figs. 2 & 3). We also found dose-dependent inhibition effect of LN that comparable to AG.

Previous studies divide the working mechanisms of AGE inhibitors into three classes: (1) carbonyl trapping agents that attenuate carbonyl stress as AG[22] and flavonoids;[43] (2) metal ion chelators such as (±)-2-isopropylidenehydrazono-4-oxo-thiazolidin-5-ylacetanilide (OPB-9195) suppress glycoxidations;[44]

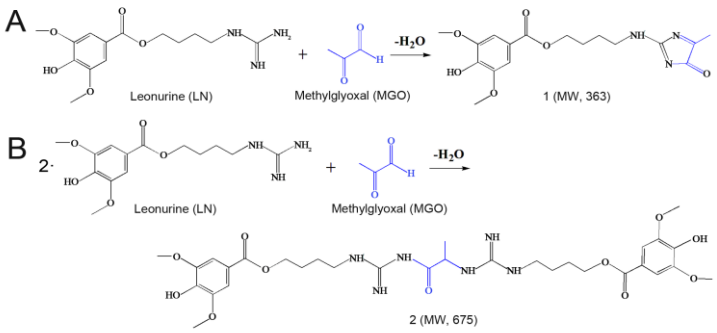

Fig. 5 Possible product structures of LN combined with MGO. (A) MGO modified on the guanidine group; (B) modification of MGO occurred on amino group.

and (3) cross-linking breakers that reverse AGE cross-links as alagebrium chloride (ALT-711).[5, 45] Consistent with our previous hypothesis, guanidine group containing LN could interact with MGO as confirmed by RP-HPLC and ESI-MS, which as a consequence, inhibits the AGEs formation and prevents cross-linking through scavenging the carbonyl species (MGO in this case). Different from our expectation that MGO binds LN in the same way as that of MGO modified arginine (Fig. 5A), the peaks at 675.8 and 338.3 observed by the positive ESI-MS indicated a modification of the amino group (Fig. 5B), suggesting MGO may preferentially modify the amino group rather than the guanidine group.

In summary, LN dose-dependently inhibited the formation of AGEs and suppressed the cross-linked structures through trapping MGO to attenuate the carbonyl stress. These findings support LN to be further considered as an anti-glycation agent for the prevention of diabetes and its complications *via* inhibiting AGEs formation.

Abbreviations

1-DMF	1-deoxy-1-morpholino-fructose
AGEs	Advanced glycation end products
AG	Aminoguanidine
DM	Diabetes mellitus
DNPH	2, 4-dinitrophenylhydrazine
ESI-MS	Electrospray ionisation mass spectrometry
LN	Leonurine
MGO	Methylglyoxal
NBT	Nitroblue-tetrazolium
PI	Porcine insulin
RP-HPLC	Reversed phase high performance liquid chromatography
TCA	Trichloroacetic acid

Conflict of Interest

The authors declare that there are no conflict of interest.

Acknowledgments

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Notes and References

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