

PROCESS FOR INHIBITING PATHOLOGICAL COLLAGEN CROSS-LINKING IN DIABETES PATIENTS

The present invention relates to the treatment of glucose-mediated collagen cross-links in diabetes-mellitus patients by means of arginine, spermidine, creatine or agmatine.

Diabetic conditions accelerate and alter the cross-linkage of long-lived proteins, such as collage (S. L. Schneider and R. R. Kohn, *J. Clin. Invest.* 66, 79 (1980), 67, 1630 (1981)). The above knowledge was the basis for intensive research, aiming at a non-enzymatic glycosylation of the above proteins. More recent studies suggest that advanced, non-enzymatic glycosylation products form the glucose-derived collagen cross-links (compare V. Monnier, A. Cerami, *Science* 211, 491 (1981) and M. J. C. Kent, N. D. Light, A. J. Bailey, *Biochemical J.* 225, 745 (1985)). The reaction diagram at the center of the accompanying FIG. 1 reflects a reaction mechanism for the above unusual cross-linkage.

Glucose reacts with the amino groups of proteins in a reversible, nucleophilic addition to form a Schiff's base adduct (aldimine), which then transforms into the more stable and still reactive Amadori product (compare H. B. Mortensen and C. Christophersen, *Clin. Chim. Acta* 134, 317 (1983)).

The resultant Amadori product then goes through a number of additional slower reactions with the amino groups of other proteins, forming glucose-derived, inter-molecular cross-links, such as 2(2-furoyl)-4(5)-(2-furanyl)-1H-imidazol, the recently described advanced glycosylation product (S. Pongor, P. C. Ulrich, F. A. Bencsath, A. Cerami, *Proc. Natl. Acad. Sci. USA* 81, 2684 (1984)).

Over longer periods of time, advanced glycosylation products accumulate continuously on long-lived proteins, such as collagen (M. Brownlee, H. Vlassara, A. Cerami in: *Diabetes Complications, Scientific and Clinical Aspects*, M. J. C. Grabbe, Ed. (Pitman, London 1986)).

The above age-dependent accumulation of advanced glycosylation products is accelerated in the collagen of diabetic persons on account of their long-term exposure to elevated glucose level (V. Monnier, R. R. Kohn, A. Cerami, *Proc. Natl. Acad. Sci. USA* 81, 583 (1984)). Based on the assumption that a glucose-mediated formation of protein cross-links could be prevented if the reactive carbonyls of the early glycosylation products (ketoamines) could be blocked pharmacologically, Brownlee and co-workers examined the influence of a nucleophilic hydrazine compound (aminoguanidine, $\text{H}_2\text{N}-\text{C}(=\text{NH})-\text{NH}-\text{NH}_2$) on the course of the above reaction (M. Brownlee, H. Vlassara, A. Kooney, P. Ulrich, A. Cerami, *Science* 232, 1629 (1989)). They demonstrated that aminoguanidine prevents the in-vitro formation of advanced glycosylation products and the glucose-induced collagen cross-links (see FIG. 1, lower left). Their results also demonstrated that aminoguanidine, administered to rats, inhibits the diabetes-induced accumulation of advanced glycosylation products and an abnormal protein cross-linking in the connective tissue of arterial walls. The effect of aminoguanidine on the formation of advanced glycosylation products was evaluated by measuring specific fluorescence, as was described before for collagen.

Concerning albumin glycosylation, it was observed that aminoguanidine prevents the formation of an advanced albumin glycosylation product, whereas the Amadori reaction takes place more or less unchanged.

The effect of aminoguanidine on in-vitro collagen cross-linking was determined by means of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of cyanogen bromide cleavage products of native collagen fibrils. In the course of time, the gel pattern of the cyanogen bromide cleavage products derived from collagen, which was incubated with glucose, showed higher values for cross-linked peptides with a high molecular weight. The presence of aminoguanidine in the incubation mixture reduced significantly the amount of cross-linked peptides with a high molecular weight.

The in-vivo effect of aminoguanidine was demonstrated on non-diabetic and alloxan-diabetic rats. Intraperitoneal injections of aminoguanidine were administered daily. The amounts of the resultant fluorescent advanced non-enzymatic glycosylation products were determined. The extent of cross-linking of the connective tissue of the aorta was lower in the animals treated with aminoguanidine than in the untreated animals.

Collagen solubility, a further parameter for cross-linking, decreased to normal values in the animals treated with aminoguanidine.

Like most hydrazines, aminoguanidine also has an extremely high toxicity. It can therefore not be used for practical purposes.

It is the object of the present invention to provide substances which inhibit the formation of cross-links in collagen proteins by a mechanism similar to that of aminoguanidine and which are non-toxic.

The present invention comprises the use of arginine, particularly of L(+)-arginine ($\text{HCOOC}-\text{CH}(\text{NH}_2)-(\text{CH}_2)_3-\text{NH}-\text{C}(\text{NH})-\text{NH}_2$) of agmatine, which is the decarboxylation product of arginine ($\text{CH}_2(\text{NH}_2)-(\text{CH}_2)_3-\text{NH}-\text{C}(\text{NH})-\text{NH}_2$), of spermidine (N-(3-aminopropyl)-1.4-butanediamine, $\text{H}_2\text{N}-(\text{CH}_2)_3-\text{NH}-(\text{CH}_2)_4-\text{NH}_2$) or of creatine (N-amidinosarcosine, $\text{H}_2\text{N}-\text{C}(\text{NH})-\text{C}(\text{CH}_3)-\text{CH}_2-\text{COOH}$), at certain high dosage levels significantly above those at which these substances are found endogenously or normally ingested exogenously.

Arginine is a non-toxic amino acid, which is contained in all proteins of the daily diet. Spermidine and creatine are found in the human body in significant amounts. But none of these is thus present in the human body at levels found to be effective according to the present invention.

The free base of L-arginine was used for the below investigations.

The following in-vitro studies were carried out:

1. Fluorescence test according to the method by V. Monnier et alii (see above).
2. SDS-PAGE of glucose-incubated, isolated collagens, with and without L-arginine, using aminoguanidine as control substance.
3. Incubation tests with various collagen preparations, interstitial collagens and basal-lamina collagen.

The following in-vivo experiments were carried out, using both Swiss mice and KK mice with spontaneous diabetes:

4. Solubility tests.
5. SDS-PAGE of isolated collagens.