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4-Thiaproline reduces heart lipid peroxidation and collagen accumulation in the diabetic db/db mouse

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Summary. Collagen accumulation is a main pathological feature of diabetic cardiomyopathy. The underlying mechanisms seem to be increased cross linking by reactive carbonyles. The purpose of the study was to decrease the collagen content of total ventricular tissue by the oral administration of thiaproline, which could reduce collagen due to its functions as a proline analogue, blocking collagen production and as a free oxygen radical scavenger, blocking reactive carbonyles and oxygen species and subsequently collagen cross linking.

Thiaproline was administered to genetically diabetic db/db mice and compared to untreated animals. Total ventricular collagen as expressed by hydroxyproline was significantly lower in the treated group (means 0.23 micromoles/100 mg tissue in the treated vs 0.35 micromoles/100 mg tissue in the untreated group, $p < 0.001$). Significantly more collagen could be eluted in the treated group ($p < 0.001$) and carboxymethyllysine was significantly reduced in the treated group ($p < 0.001$). Di-tyrosine and glycemic control did not differ between the groups. Glutathione was significantly increased in the TP treated experimental group ($p < 0.001$) and lipid peroxidation products were significantly decreased (means 0.221 absorbance in the treated group versus 0.321 absorbance in the untreated diabetic group) correlating with total ventricular collagen content ($r = 0.87$, $p < 0.01$).

We conclude that thiaproline reduced total ventricular collagen content by inhibiting collagen cross linking as reflected by increased solubility of collagen and expressed by higher elution quantity of collagen. Thiaproline, and/or its metabolites induced increase of heart glutathione which may well have been scavenging reactive carbonyles derived from lipid peroxidation and advanced stage nonenzymatic glycosylation as shown by decreased total ventricular carboxy-methyllysine and lipid peroxidation products paralleling reduced heart collagen content.

It remains to be shown that the successful reduction of heart collagen by thiaproline is paralleled by improved functional properties.

Keywords: Amino acids – Thiaproline – Collagen – Diabetes – db/db mouse

Introduction

Diabetic cardiomyopathy presents with prominent myocardial fibrosis. The mechanism(s)leading to collagen accumulation, however, are not fully elucidated yet. Several factors have been incriminated but nonenzymatic glycosylation of connective tissue proteins with subsequent oxidation is a widely accepted concept. This glycoxidation hypothesis implies a role of advanced stage nonenzymatic glycosylation products for the development of connective tissue changes of micro-and macroangiopathy and tissue collagen accumulation (Baynes, 1991). Reactive carbonyles as deoxyglucosone, methylglyoxal and the lipid peroxidation products malondialdehyde and hydroxyalkenals are aldehydes mediating cross linking of collagen by reaction with free amino groups. This increased cross linking renders connective tissue proteins highly insoluble and may well contribute to tissue collagen accumulation representing diabetic long term complications (Schnider and Kohn, 1981). Decreased solubility of proteins in the diabetic state is an unequivocal finding and therapeutic strategies aim to block reactive carbonyles to prevent cross linking (Brownlee et al., 1986). Recent reports, however, have shown that glucose itself may be responsible for collagen accumulation in tissues by increasing collagen synthesis at a transcriptional level (McClain et al., 1992).

In a recent publication we reduced kidney collagen accumulation in diabetic db/db mice by the oral administration of thiaproline, a compound inhibiting collagen synthesis and/or cross linking (Lubec B et al., 1994).

Based upon this observation we used this compound for the experimental reduction of heart collagen and provide additional evidence for a proposed mechanism of action.

Materials and methods

Animals

20 black female db/db mice manifesting diabetes mellitus at the age of approximately 6 weeks (Shaw's farm, UK) and 20 age matched inbred nondiabetic mice as counterparts were used in the experiments. All studies were performed in accordance with the regulations of the American Physiology Society. These animals were divided into 4 groups of 10 animals each. 10 normal control mice and 10 db/db mice received a daily dosage of 4 thiaproline (Sigma) 30 mg/kg body weight/per day from the 11th to the 23rd week of life. 10 normal control mice and 10 db/db mice were given tap water during the observation period.

Untreated db/db mice had a daily fluid intake of 5.2 ± 1.4 ml, treated db/db mice 5.8 ± 1.9 ml. All the groups had free access to mouse cake (Altromin). The food intake did not differ between the treated and untreated db/db mice and between treated and untreated normal mice.

The body weight of the db/db mice at the start (untreated animals 47 ± 8.2 g, treated 48 ± 9.1 g) and at the end of the experiment (untreated animals 69 ± 10.1, treated animals 68 ± 9.2 g) did not differ significantly.

The animals were sacrificed at the end of the 23rd week by neck dislocation. Fructosamine levels were determined at sacrifice using a commercially available kit (Fructosamine kit, Hoffmann-La Roche). The basic routine clinical laboratory including glucose, urea, GOT, GPT, creatinine, yGT, creatine kinase, LDH, was determined on a Kodak Ektachem autoanalyzer and showed no differences between the groups. Further details on the experimental animals are given in a previous report (Lubec B et al., 1994).

Total ventricular tissue collagen

a) Total ventricular tissue collagen (TVTC) was evaluated by the determination of trans 4 hydroxyproline after hydrolysis of weighed total ventricular tissue using 6 N HCl incubating 16 hrs at 105° Celsius in a thermoblock. The method of Woessner was applied on the hydrolyzates evaporated and redissolved in distilled water (Woessner, 1986).

b) TVTC was determined by the Sircol assay, a specific dye binding test method. Aliquots of ventricular tissue were homogenized in a Potter in an ice bath in a solution of 0.05 M acetate buffer pH 4.1 containing 0.005 M EDTA, 1 mg pepsin per 100 mg of heart weight, incubated at 25°C for 72 hrs and finally spun down in a centrifuge at 4,000 g. The supernatant was used for the Sircol collagen assay (Sircol collagen assay Kit, Oubis LtD, UK), sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis and determination of glutathione.

Determination of collagen solubility was performed as given in previous publications (Lubec et al., 1990). Briefly, homogenized total ventricular tissue was incubated for 72 hours with pepsin in a 0.05 M acetate buffer pH 4.1 containing 0.005 M EDTA at room temperature. The eluted material was centrifuged and the supernatant was subject to hydrolysis as given above and hydroxyproline determined according to the method given above.

SDS-polyacrylamide gel electrophoresis was run according to the principle of Laemmli (1970). Eluted collagen was applied onto the gel in equal amounts and the pattern evaluated for high molecular weight material (indicating increased cross links) or low molecular weight bands (reflecting collagen split products).

N-epsilon-carboxymethyllysine was determined in hydrolyzates of total ventricular tissue by an HPLC method given in detail in a previous publication (Weninger et al., 1992). Briefly, samples were derivatized with o-phthalaldehyde and run on HPLC using a gradient system.

Dityrosine determination was performed on reversed phase chromatography using an Ultrasphere XL-ODS C18 column (Beckmann). Solvent A was 12 mM sodium phosphate buffer pH 7.2 + 1% tetrahydrofurane + 2% acetonitrile. Solvent B was 12 mM sodium phosphate buffer pH 7.2 + 50% acetonitrile. The gradient run was given in a previous paper (Lubec G et al., 1994). A Waters 600 E system controller, a Waters 715 WISP autosampler and a 470 Fluorescence detector were used.

Standard o-phthalaldehyde derivatization as given previously (Lubec G et al., 1994) was applied. Di-tyrosine standard was obtained from S. Anderson (Oregon State University, Dpt of Chemistry, USA).

Determination of glutathione

Glutathione (in its reduced form) was determined in pepsin eluates (extracts) as described above and assayed following the enzymatic method of Anderson (Anderson, 1989) using a commercially available kit (GSH 400, Bioxytech). The principle of the assay is the formation of thioethers between a patented reagent and all mercaptans present in the sample. The second step, beta elimination under alkaline conditions, specifically transforms the substitution product obtained with glutathione into a chromophoric thione

whose maximal absorbance is at 400 nm. The assay was run according to the suppliers' informations.

Evaluation of lipid peroxidation products

Lipid peroxidation was evaluated in heart hydrolyzates, which were evaporated to dryness on a Pierce Reactitherme and redissolved in the reaction buffer of the commercially available assay LPO 586 (Colorimetric assay of lipid peroxidation, Bioxytech, SA, Bonneuil sur Marne, France).

The assay evaluates reactive aldehydes as malondialdehyde and 4 hydroxy-2 (E) nonenal, the main decomposition products of peroxides derived from unsaturated fatty acids and released esters (Esterbauer and Cheeseman, 1990). The principle of the method is that a chromophoric reagent reacts with malondialdehyde and 4 hydroxyalkenals at 45°C and the stable product can be read at 586 nm. The assay was performed according to the supplier's instructions.

Statistical handling of data

Comparison of groups was performed using the Mann Whitney U test, correlations were calculated using the linear regression coefficient (SAS User's Guide, 1985).

Results

The results as means and standard deviation are given in Table 1.

Thiaproline had no effect on the parameters of collagen, solubility, SDS-PAGE, CML, in nondiabetic treated and untreated mice representing the control group.

Glucose was considerably increased in the diabetic mice, but there was no significant difference between untreated and treated animals both in the non diabetic and the diabetic group.

Comparable results were found for plasma fructosamine in the diabetic mice and fructosamine was significantly elevated in the diabetic panels.

Total ventricular collagen was significantly decreased in the treated db/db panel as compared to the untreated diabetic group ($p < 0.001$). Hydroxyproline levels in the treated diabetic group did not differ significantly from the non diabetic groups. This finding of reduced collagen content in treated mice was confirmed by Sircol collagen assay results.

Elutable collagen as reflected by hydroxyproline in the treated diabetic group was about twice that in the control groups ($p < 0.01$) and about three times that in the non treated diabetic group ($p < 0.001$).

The ratio of eluted/total hydroxyproline was significantly elevated in the treated diabetic group ($p < 0.01$).

SDS polyacrylamide gel electrophoresis

There were no differences in the patterns of eluted collagens between the groups ruling out collagenolytic degradation of eluted collagens and big differences in cross linked material (Fig. 1).

Table 1. Means and standard deviation of parameters evaluated

Group	Total ventricular collagen hydroxyproline μmol per 100 mg tissue	Total ventricular collagen hy sircol assay mg collagen/100 mg tissue	Eluted collagen μmol/100 mg tissue	CML nmol/100 mg tissue	di-Tyrosine ng/100 mg tissue	GSH nmol/100 mg tissue	LPO products (extinction)
db/db untreated	0.35 ± 0.06	2.14 ± 0.40	0.20 ± 0.06	0.52 ± 0.09	2.9 ± 0.3	30.6 ± 1.4	0.321 ± 0.04
db/db thiaproline treated	0.23 ± 0.04	1.19 ± 0.30	0.41 ± 0.09	0.40 ± 0.08	2.6 ± 0.4	36.4 ± 2.0	0.221 ± 0.03
normal mice untreated	0.24 ± 0.05	1.09 ± 0.28	0.31 ± 0.06	0.34 ± 0.08	2.6 ± 0.4	30.4 ± 2.1	0.201 ± 0.03
normal mice thiaporline treated	0.24 ± 0.06	1.10 ± 0.31	0.30 ± 0.07	0.30 ± 0.07	2.7 ± 0.3	33.6 ± 2.1	0.206 ± 0.04

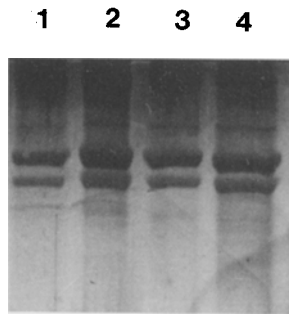

Fig. 1. The pattern of SDS polyacrylamide gel electrophoresis showing the identity of collagen extracted with pepsin (100 μg protein applied onto wells) in all groups tested. Lane *1* collagen extracted from untreated control mice, lane *2* from thiaproline treated control mice, lane *3* from diabetic untreated mice, lane *4* from thiaproline treated diabetic mice

CML levels were significantly lower in the treated db/db experimental group ($p < 0.001$) as compared to all other groups.

Dityrosine levels did not differ significantly between the study groups.

Total ventricular glutathione showed significant differences between groups: Highest values were detected in the treated normal control group differing significantly from the untreated control group ($p < 0.01$) and two experimental groups ($p < 0.01$ for both). The treated experimental group showed significantly higher levels of reduced GSH than their untreated diabetic mates ($p < 0.01$).

Total ventricular lipid peroxidation products were highest in the untreated experimental group differing significantly from the treated experimental group ($p < 0.01$), the untreated control goup ($p < 0.01$) and the treated control group ($p < 0.001$). The untreated control group differed significantly from the treated control group ($p < 0.01$).

There was a significant correlation between heart lipid peroxidation products and collagen content ($r = 0.87$, $p < 0.01$). There were no other significant correlations between the parameters listed at the $p < 0.05$ level.

Discussion

Total ventricular collagen was significantly reduced by the experimental treatment of diabetic mice with thiaproline. This proline analogue is being incorporated into collagen chains in place of proline. The incorporation of thiaproline leads to an impaired helical conformation of collagen with the consequence of its rapid degradation (Lubec B et al., 1994), which would be one explanation for reduced heart collagen content. Furthermore, the incorporation of thiaproline was reported to stop the ribosomal elongation of the collagen peptides to be synthesized (Busiello et al., 1979), which in turn could serve as an explanation for collagen reduction. A third mechanism for the reduction of total heart collagen could have been the oxygen radical scavenging properties of thiaproline (Hayase et al., 1991). Free oxygen radical medi-

ated increased cross linking leading to collagen accumulation (i.e. fibrosis) in diabetes may well have been counteracted by thiaproline as the marker for free oxygen radical involvement and glycoxidation, carboxymethyllysine, was reduced in the thiaproline treated group. Fourthly, blocking of reactive carbonyles responsible for excess cross links by thiaproline might have taken place as more collagen could be eluted from total ventricular tissue after thiaproline treatment and total ventricular collagen content correlated with lipid peroxidation carbonyles. Scavenging of reactive carbonyles, derived from advanced stage nonenzymatic glycosylation as e.g. deoxyglucosone (Hayase et al., 1991) or from lipid peroxidation as e.g. malondialdehyde and hydroxynonenals, could have taken place: we found decreased lipid peroxidation products in the thiaproline treated group. Increased lipid peroxidation has been reported to be a significant factor for the development of diabetic long term complications (Gallaher et al., 1993; Gallou et al., 1993; Lung et al., 1993; Ghiselli et al., 1992). Scavenging could have been mediated by thiaproline directly or indirectly by its metabolites: The condensation of thiols with carbonyles forming semimercaptals is a basic reaction in organic chemistry and biochemistry. Thiaproline is known to be hydrolyzed to cysteine in vivo spontaneously and enzymatically (Cavallini et al., 1956) and cysteine is a potent active oxygen radical scavenger (Roberts, 1992).

In addition, thiaproline derived cysteine is the major precursor for glutathione production, another potent naturally occurring oxygen free radical scavenger representing a major detoxification system of the body (Meister and Anderson, 1983; Meister, 1988, 1991). Compatible with this interpretation we found significantly increased total ventricular glutathione in the thiaproline treated diabetic group and in the thiaproline treated control panel. The fact that cross links were not affected by a "lathyritic" activity of thiaproline in the heart tissue of control mice could be either due to a higher collagen turnover described in diabetes (Ihm et al., 1992) or due to low reactivity with protein bound carbonyles of normal cross link precursors contrasting high reactivity of elevated free carbonyl (aldehydes) described in the diabetic state (Hayase et al., 1991).

Eluted collagen from treated and untreated diabetic mice did not differ on SDS-PAGE, however. The reason might be that only normally cross linked collagen is eluted and that the eluted collagen does not reflect cross linked collagen in the tissue, remaining insoluble and unextractable.

Influences on the outcome by glycemic control was ruled out by comparable parameters in the diabetic panels.

A toxic effect mediating decreased collagen synthesis is highly improbable as the body weight, fluid, food uptake and clinical laboratory parameters did not differ between the diabetic groups.

Increased collagenolytic activity in thiaproline treated diabetic mice as underlying mechanism for reduced collagen accumulation would have been seen on SDS-PAGE gels but no low molecular weight collagenous material indicating split products was found.

The finding of comparable di-tyrosine levels in all groups examined in the study let us suggest that hydroxy-radical attack is most probably not involved

in diabetic pathobiochemistry as shown by identical findings of o-tyrosine, another in vivo parameter for hydroxy radical attack, in a previous paper (Capeillere-Blandin et al., 1991; Khaidar et al., 1994); it rules out the mechanism of hydroxy radical attack for free radical cross linking and the action mechanism of thiaproline for its treatment.

Concluding, we suggest that the reduction of aldehydes as derived from advanced stage nonenzymatic glycosylation and from lipid peroxidation may have been blocked by thiaproline or its thiol metabolites resulting into decreased collagen cross linking and therefore decreased heart collagen content.

Although we could reveal that thiaproline reduces heart collagen accumulation, a significant feature of diabetic cardiomyopathy, it remains to be shown that the successful reduction of heart collagen is also accompanied by improved mechanoelastic functional properties.

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