·Basic Research·

Effect of carnosine on steroid-induced modification of lens α-crystallin

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Abstract

• AIM: To explore whether carnosine can protect α -crystallin modification and decrease chaperone by a steroid, and whether carnosine could directly react with a steroid.

• METHODS: Bovine lens α -crystallin was separated by size-exclusion chromatography on a Sephacyl S-300 HR column. α -Crystallin was incubated with different concentrations of prednisolone-21-hemisuccinate (P-21-H) with or without carnosine for different times. The chaperone activity of α -crystallin was monitored using the prevention thermal aggregation of α -crystallin. The modified α -crystallin was examined by SDS-PAGE and fluorescence measurements. The absorbance spectra of solutions of carnosine and P-21-H were investigated.

• RESULTS: P-21-H decreased the chaperone activity of α -crystallin in a concentration- and time-dependent fashion. Carnosine only worsened this effect. The tryptophan fluorescence intensity of α -crystallin modified by P-21-H was significantly decreased compared with unmodified crystallin, whereas its non-tryptophan fluorescence was increased with a shift to longer wavelengths in a time- and dose-dependent manner, suggesting that new fluorophores were possibly formed. Carnosine readily reacted with P-21-H thereby inhibiting steroid-mediated protein modification as revealed electrophoretically. The increased absorbance was time-dependent, suggesting adducts may be formed between carnosine and P-21-H.

• CONCLUSION: Carnosine reacts with P-21-H, which suggests carnosine's potential as a possible anti-steroid agent.

• KEYWORDS: carnosine; α -crystallin; molecular chaperone; steroid

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INTRODUCTION

α-Crystallin, a major structural protein in the lens, has a chaperone-like function, binding unfolding lens proteins and protecting various proteins against aggregation induced by heating, chaotropic agents, reduction and chemical modification [1, 2]. Carnosine, a naturally-occurring dipeptide (β-alanyl-L-histidine), appears to possess anti-glycating, anti-oxidant and free-radical scavenging activity[3,4]. The potential biological and therapeutic significance of carnosine against human age-related cataract has been reported[5].

Corticosteroids therapies taken for prolonged periods of time are risk factors for cataract. Prednisolone-protein adducts have been identified in human cataracts [6]. Based on our previous studies of decreased chaperone activity of α-crystallin [7, 8], the aim of present study is to further investigate the possibility whether carnosine can prevent steroid-induced modifications and whether carnosine can protect α-crystallin against decreased chaperone activity induced by a steroid, all of which are pertinent to cataract.

MATERIALS AND METHODS

Materials Bovine lenses were obtained from a local abattoir. Sephacryl S-300HR was purchased from Pharmacia (Milton Keynes, UK). Carnosine, prednisolone-21-hemisuccinate (P-21-H) and all the other chemicals were obtained from Sigma Chemicals (Poole, Dorset, UK).

Isolation of crystallins The α-crystallin and β-crystallin were isolated from bovine lenses by Sephacryl S-300 HR gel permeation chromatography (100×2.7cm) using the method previously described [9]. The supernatant was then loaded

onto the column and eluted at a rate of 30mL/h. The fractions corresponding to α-crystallin and β-crystallin were pooled and dialyzed against three changes of distilled water over 24 hours at 4℃, then freeze-dried. The purity was demonstrated by SDS-PAGE, and the protein stored at -20℃ until required.

Incubation of α−crystallin with P−21−H All incubations were carried out in 50mmol/L sodium phosphate buffer (containing 0.5g/L NaN_3), pH 7.0 at 37℃ unless otherwise stated. In the preliminary experiment, α-crystallin samples (30mg) were dissolved in 15mL of 25mmol/L P-21-H with or without 50mmol/L and 100mmol/L carnosine. The solutions were then filtered through sterilized Millipore filters (0.2μm) into sterilized 30mL glass vials and incubated at 37℃ in a shaking water bath. At time 0 and after 10 and 21 days, samples (3mL) were removed from each of the solutions and dialyzed against five changes of distilled water over 24 hours using micro-dialysis tubing (molecular weight cut-off 12-14ku) to remove excess glycating agents. The sample was then freeze-dried and analyzed by SDS-PAGE. The β-crystallin heat-induced aggregation assay was used to determine the chaperone ability of the modified and unmodified α-crystallin.

In other experiments, the 10mmol/L P-21-H was used in these incubations up to 8 days. In an attempt to investigate whether the reaction between carnosine and a steroid was responsible for part of the yellowing in the earlier experiments, carnosine was incubated with 25mmol/L P-21-H over 20 days without any protein.

SDS−PAGE SDS-PAGE was carried out following Laemmli's discontinuous buffer system by using a Bio-Rad Mini-Protean II dual slab mini gel apparatus according to the manufacturer's instructions. Samples (up to 10μL) were added to each well and electrophoresis was carried out using 120g/L separating gels and 40g/L stacking gels at a constant voltage of 200 volts.

β −crystallin heat −induced aggregation assays The chaperone activity of α-crystallin was assessed by its ability to prevent the increase in turbidity upon heating solutions of β-crystallin as described previously [9]. Bovine β-crystallin (1.87mg) was dissolved in 6mL of degassed 0.05mol/L sodium phosphate buffer, pH 6.7 and the solution was then passed through a Gelman 0.2μm pore-sized sterilised Millipore filter into a sterilized glass container. Light scattering resulting from heat-induced aggregation was monitored continuously at 360nm, for 55 minutes. Chaperone function was represented as the percentage of protection afforded by α-crystallin against the scattering produced by the β-crystallin control aggregation after 55 minutes. All assays were repeated in triplicate.

Tryptophan and non −tryptophan fluorescence measurements The loss of protein tryptophan fluorescence, an indicator of conformational change or tryptophan modification, is a marker of crystallin integrity. The tryptophan fluorescence in α-crystallin solution after incubation with or without carnosine was measured by using a Perkin-Elmer LB 50 B luminescence spectrophotometer as described previously [10]. The excitation wavelength was set to 280nm, slit width 5nm, and the fluorescence emission was recorded between 280-550nm. The non-tryptophan fluorescence spectra were obtained with excitation wavelength at 340nm. α-Crystallin solutions (0.04g/L) in 0.05mol/L sodium phosphate buffer (pH 6.7) were used unless otherwise stated.

Absorbance readings of the α-crystallin samples were measured at various wavelengths (220-400nm) on a UVIKON 930 Kontron spectrophotometer. α-Crystallin solutions (0.4g/L) in 0.05mmol/L sodium phosphate buffer (pH 6.7) were used unless otherwise stated. Absorbance readings of the preparations of carnosine with sugars or P-21-H after incubations were also measured at various wavelengths (220-400nm).

Statistical Analysis Results were reported as the mean±SD, unless otherwise specified, of at least three separate experiments. Statistical significance of differences was examined using a student's *t* test where relevant.

RESULTS

Incubation of α−Crystallin with P−21−H and Carnosine

Cross −linking and chaperone activity In preliminary experiments, α-crystallin was incubated with 25mmol/L P-21-H over 20 days causing a remarkable precipitation of α-crystallin. After 10 days incubation, part of the α-crystallin has become water-insoluble resulting in very faint bands in the presence of P-21-H, and none with carnosine (data not shown). A concentration- and time-dependent colour change was observed in the presence of carnosine. At 24 hour, a colour change of preparations was observed in the presence of 100mmol/L carnosine. At longer incubation periods (over 72 hours), it became milky yellow with visible precipitation. Surprisingly, the solution of α-crystallin with 25mmol/L

P-21-H but without carnosine also became only light yellow after 6 days incubation, less yellow than with carnosine.

To investigate the effect of carnosine on loss of chaperone activity, α-crystallin was incubated with the lower concentration of P-21-H (10mmol/L) for up to 8 days. No significant colour change of solution was observed in the presence of 10mmol/L P-21-H and 100mmol/L carnosine even after 8 days incubation. At time 0, there was no modification of α-crystallin preparations and no significant change in chaperone activity (Table 1). A time-dependent decrease in chaperone activity of α-crystallin with P-21-H was observed up to 8 days incubation. However, in the presence of carnosine, the α-crystallin preparations showed significant further decreases in chaperone activity.

Our observation suggests that there is no protection of carnosine against a P-21-H-induced decrease in chaperone activity of α-crystallin. Indeed, contrary to F6P study above, carnosine speeds up the loss of chaperone function.

Tryptophan and non-tryptophan fluorescence measurements

The absorbance of the P-21-H-treated α-crystallin samples at 250nm decreased in the presence of carnosine from time zero to 10 days incubation, and continued decreasing to 20 days, due to the precipitation of α-crystallin as previously observed (data not shown). Furthermore, a significant absorbance at about 250nm was shown in the presence of 25mmol/L P-21-H and carnosine only, and it was in a time-dependent manner, suggesting the adducts may be formed between carnosine and P-21-H.

Thus the tryptophan fluorescence of modified α-crystallin was completely lost after 10 days and 20 days incubation whereas non-tryptophan fluorescence was slightly increased with a shift to longer wavelengths, indicating formation of new fluorophrores (data not shown).

In the lower concentration of P-21-H(10mmol/L) experiment, the non-tryptophan slightly increased in intensity with significant decrease of tryptophan fluorescence, suggesting a conformational change of α-crystallin. The non-tryptophan fluorescence was increased slightly in the presence of carnosine with peak at approx. 525nm; indicating new fluorophores are possibly formed (data not shown).

Table 1 The effect of 10mmol/L P-21-H and carnosine on chaperone activity of α-crystallin. Percent protection against aggregation at time 0,4 and 8 days

α–crystallin preparations	0d	4d	8d
α	97.8±1.5	94.0±5.2	92.3±6.8
α + P-21-H	94.8±2.7	90.0±6.1[a]	86.1±3.2[b]
α + P-21-H + 50 mmol/L C	92.2±4.0	78.2±5.7[c]	67.4±4.6[d]
α + P-21-H + 100 mmol/L C	90.9±4.0	78.5±4.4[e]	44.2±5.3[f, g]

α: α-crystallin; P-21-H:10mmol/L prednisolone-21-hemisuccinate, C:carnosine; [a]P=0.44253, α*vs* α+P-21-H at 4 days; [b]P=0.22486,α *vs* α+P-21-H at 8 days; [c]P=0.0242, α *vs* α+P-21-H+50mmol/L C at 4 days; [d]P=0.00638, α *vs* α+P-21-H+50mmol/L C;at 8 d;[e]P=0.01732, α *vs.* α+P -21-H+100mmol/L C at 4 d; [f]P=0.00065, α *vs* α+P-21-H+ 100mmol/L C at 8 days; [g]P=0.00472, α+P-21-H+50mmol/L C *vs* α+P-21-H+ 100mmol/L C at 8 days

DISCUSSION

The current results presented here provide evidence to support the hypothesis that carnosine can protect the loss of chaperone activity of α-crystallin by glycation, in addition to describing the effects of carnosine on prevention cross-linking. This effect may exhibit therapeutic potential. Corticosteroids can react with lens proteins to form stable adduct which results in loss chaperone activity of α-crystallin and inactivation of enzymes[4,11,12].

Carnosine was previously identified as an anti-oxidant and anti-glycation agent. It inhibits crosslinking of proteins [13, 14], protects against inactivation of enzymes induced by glycation, oxidation and a steroid [4, 15, 16]. α-Crystallin acts as a molecular chaperone to prevent the aggregation and inactivation of other proteins. Specific areas important for chaperone binding and function have been identified throughout the N-terminal-region, connecting peptide and C-terminal extension [17]. The C-terminal domain of this protein (homologous to domains present in small heat-shock proteins) is implicated in chaperone function, although the domain itself has been reported to show no chaperone activity. However, quaternary structure of α-crystallin is necessary for its chaperone-like activity [18]. Glycation and steroid-induced loss of chaperone activity is possibly due to structural alterations of crystallin and further modification of C-terminal domain.

The present results showed that the tryptophan fluorescence intensity of α-crystallin modified by P-21-H was significantly decreased, whereas its non-tryptophan fluorescence was increased with a shift to longer wavelengths in a time-and dose-dependent manner, suggesting that new fluorophores were possibly formed. Carnosine may dissociate thermal-induced aggregates through its promoting hydration [19] or disaggregate glycated α-crystallin [13]. Amino acid sequence of carnosine is similar to Lys-His, whereas the amino groups of lysine residues are the primary targets by glycation. Therefore, carnosine have

potential to react with sugars and steroid.

The results described in this paper suggest that carnosine reacts directly with P-21-H and results in formation of new adduct, which are responsible for the protective effect of chaperone activity. Carnosine may intracellularly suppress the deleterious effects of a reactive carbonyl group by reacting with P-21-H to form protein-carbonyl-carnosine adducts similar to the reported aldehyde adducts [20] and carnosine-glyceraldehyde 3-phosphate adduct [21]. This work points to a novel property of carnosine acting as an anti-steroid compound by formation adducts of carnosine-P-21-H. Its protection against steroid-induced inactivation of esterase and catalase has been described recently[4,16].

However, the higher concentration of carnosine at longer time incubation had a deleterious effect on chaperone activity of α-crystallin. It may point to the possibility that carnosine reacts with a steroid extensively and results in accumulation of new adduct, which in turn, further reacts and aggregates crystallin. This maybe deleterious to the ability to protection against thermal induced aggregation of α-crystallin presented as chaperone activity. The potential biological and therapeutic significance of there observation need to be further explored.

To conclude, this is the first report describing protective effect of carnosine on steroid-induced decreased chaperone activity of α-crystallin, additionally including anti-cross-linking mediated by a steroid and possible adducts formation between carnosine and steroid.

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REFERENCES

1 Harding JJ. Viewing molecular mechanisms of ageing through a lens. *Ageing Res Rev* 2002;1(3):465-479

2 Horwitz J. Alpha-ceystallin. *Exp Eye Res* 2003;76(2):145-153

3 Hipkiss AR. Carnosine, a protective, anti-ageing peptide? *Int J Biochem Cell Biol* 1998;30(8):863-868

4 Yan H, Harding JJ. Carnosine protects against the inactivation of esterase induced by glycation and a steroid. *Biochim Biophys Acta* 2005;1741(2):120-126

5 Babizhayev MA, Deyev AI, Yermakova VN, Semiletov YA, Davydova NG, Kurysheva NI, Zhukotskii AV, Goldman IM. N-Acetylcarnosine, a natural histidine-containing dipeptide, as a potent ophthalmic drug in treatment of human cataracts. *Peptides* 2001;22(6):979-994

6 Bucala R, Manabe S, Urban RC, Cerami A. Nonenzymatic modification of lens crystallins by prednisolone induces sulfhydryl oxidation and aggregate formation: *in vitro* and *in vivo* studies. *Exp Eye Res* 1985;41(3):353-363

7 van Boekel MA, Hoogakker SEA, Harding JJ, de Jong WW. The influence of some post-translational modifications on the chaperone-like activity of alpha-crystallin. *Ophthalmic Res* 1996;28(Suppl 1):32-38

8 Yan H, Liu B, Yu L, Wang WN. Effect of glucocorticosteroid on chaperone activity of alpha-crystallin. *Chin Ophthal Res* 2005;23(1):37-39

9 Yan H, Harding JJ, Hui YN, Li MY. Decreased chaperone activity of alpha-crystallin in selenite cataract may result from selenite-induced aggregation. *Eye* 2003;17(5):637-645

10 Derham BK, Harding JJ. Effects of modifications of alpha-crystallin on its chaperone and other properties. *Biochem J* 2002;364(3):711-717

11 Manabe S, Bucala R, Cerami A. Nonenzymatic addition of glucocorticoids to lens proteins in steroid-induced cataracts. *J Clin Invest* 1984;74(5):1803-1810

12 Hook DW, Harding JJ. The effect of modification of alpha-crystallin by prednisolone-21-hemisuccinate and fructose 6-phosphate on chaperone activity. *Dev Ophthalmol* 2002;35:150-160

13 Seidler NW, Yeargans GS, Morgan TG. Carnosine disaggregates glycated alpha-crystallin: an in vitro study. *Arch Biochem Biophys* 2004;427(1):110-115

14 Yan H, Harding JJ. Carnosine inhibits modifications and decreased molecular chaperone activity of lens α-crystallin induced by ribose and fructose 6-phosphate. *Mol Vis* 2006;12:205-214

15 Hipkiss AR, Michaelis J, Syrris P. Non-enzymatic glycosylation of the dipeptide L-carnosine, a potential anti-protein-cross-linking agent. *FEBS Lett* 1995;371(1):81-85

16 Yan H, Harding JJ. Protective effect of carnosine against inactivation of enzymes induced by glycation and a steroid: relevance to cataract. *Am J Ophthalmol* 2005;139:S55

17 Derham BK, Harding JJ. α-Crystallin as a molecular chaperone. *Prog Retin Eye Res* 1999;18(4):463-509

18 Avilov SV, Aleksandrov NA, Demchenko AP. Quaternary structure of alpha-crystallin is necessary for the binding of unfolded proteins: a surface plasmon resonance study. *Protein Pept Lett* 2004;11(1):41-48

19 Yeargans GS, Seidler NW. Carnosine promotes the heat denaturation of glycated protein. *Biochem Biophys Res Commun* 2003;300 (1):75-80

20 Hipkiss AR, Brownson C, Carrier MJ. Carnosine, the anti-ageing, anti-oxidant dipeptide, may react with protein carbonyl groups. *Mech Ageing Dev* 2001;122 (13):1431-1445

21 Seidler NW, Shokry SS, Nauth J. Properties of a glycation product derived from carnosine. *J Biochem Mol Biol Biophys* 2001;5:153-162