Direct reaction of taurine with malondialdehyde: evidence for taurine as a scavenger of reactive carbonyl species

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Though taurine has been reported very useful in preventing oxidative stress and various age-related diseases, the detailed biochemical mechanism of its biological functions is not well understood. Direct reaction of malondialdehyde (MDA) with taurine was studied using spectrofluorometry, spectrophotometry and liquid chromatography online with mass spectrometry (LC/MS). The results indicated that taurine reacted readily with MDA at supraphysiological conditions to yield mainly two products: a fluorescent 1,4-dihydropyridine and non-fluorescent enaminal derivatives. Taurine also significantly inhibited the formation of lipofuscin-like fluorescence induced by MDA-modified bovine serum albumin. These findings suggested that taurine effectively reduces carbonyl stress due to the amino group in its molecular structure, and we propose that it should be the mechanism related with the pathophysiological functions of taurine in the biological system.

Keywords: malondialdehyde, carbonyl-amino reaction, taurine

Introduction

Reactive carbonyl species (RCS), including malondialdehyde (MDA), 4-hydroxynonenal (HNE), methylglyoxal (MGO) and other unsaturated carbonyl intermediates of oxidative stress and glycation, can readily attack almost all macromolecules causing deleterious effects in the biological system.^{1–3} These processes, especially carbonyl-amino cross-linking, have been widely documented to be responsible for the formation of age pigment-like fluorophores and

various acute and chronic diseases, such as atherosclerosis, osteoarthritis, diabetes mellitus, and neuronal degenerative diseases.^{1,2,4–6}

Nevertheless, under normal physiological conditions, the biological effects of the RCS are controlled *in vivo* by a sophisticated enzymatic and non-enzymatic defense system. The former mainly includes aldehyde dehydrogenases to speed the conversion of aldehyde to carboxylic acid, aldo-ketoreductases to convert RCS to alkenes, and glutathione transferases to conjugate RCS with GSH to form Michael adducts.^{4,5} The latter contains a variety of endogenous or exogenous low molecular weight chemicals, such as carnosine, taurine, pyridoxamine, histamine, hydralazine, dihydralazine and aminoguanidine.^{4,5,7,8}

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As one low molecular RCS scavenger, taurine is one of the major free intracellular amino acids found in high concentrations (3–100 mM) in most types of animal tissues, especially in cardiac and skeletal muscle.⁹ Although it is not an essential amino acid of the human body, taurine has been reported very useful in preventing the toxicity of lipid peroxidation¹⁰ and various age-related diseases, such as atheroma,¹¹ hypertension,¹² diabetes,¹³ Alzheimer's disease,¹⁴ and even in retarding the process of biological aging.¹⁵ However, the mechanism of its biological functions is not well understood. Some recent studies suggest that taurine can reduce oxidative stress, mainly by scavenging oxygen free radicals.^{16,17} On the contrary, Aruoma and co-workers¹⁸ demonstrated early that taurine performs neither as a classical oxygen free radical scavenger nor as an antioxidant in a standard model system. Nevertheless, Ogasawara and co-workers¹⁰ have suggested that taurine may react with MDA (10). However, the reaction mechanism and the chemical structure of possible adducts remain unknown.

For these reasons, we investigated the mechanism and products of the reaction of taurine with MDA, and its preventive effect on MDA modification of bovine serum albumin (BSA).

Materials and methods

Materials and stock solutions

1,1,3,3-Tetramethoxypropane (TMP) was obtained from Fluka Chemie AG (Buchs, Switzerland). Taurine and other chemicals (analytical grade) were from Sangon Biotech Co., Ltd (Shanghai, China). Water was produced using a Milli-Q Plus purification system (Millipore, Bedford, MA, USA).

Taurine stock solution (50 mM) was prepared by dissolving 0.063 g of taurine into 50 ml of 0.2 M phosphate buffer solution (PBS; pH 7.4). A fresh MDA stock solution (10 mM) was prepared by hydrolyzing TMP, which was modified according to a method described by Li *et al.*¹⁹ Thus, 0.084 ml (0.5 mmol) TMP was mixed with 1.0 ml 1.0 M HCl, and shaken at 40°C for about 2.5 min. After the TMP was fully hydrolyzed, the pH was adjusted to 7.4 with 6.0 M NaOH, and the stock solution was finally diluted to 50 ml with 0.2 M PBS (pH 7.4). The concentration of MDA in the stock solution was estimated by absorbance at 267 nm using $\epsilon_{\text{MDA}} = 31,500 \text{ M}^{-1}\text{cm}^{-1}$.

Fluorescence analysis of the reaction mixture of taurine and MDA

Fluorescence spectra of the reaction mixture was

detected with a Perkin-Elmer LS 50B spectrofluorometer (Norwalk, NJ, USA).

Reactions of taurine with MDA at different concentrations

To investigate reaction kinetics, taurine (1.0 mM) was incubated with different concentrations of MDA in 0.2 M PBS at 37°C (pH 7.4). Reaction products were diluted 1000-fold in 0.2 M PBS pH 7.4 before spectrofluorometric measurements.

Similarly, MDA (1.0 mM) was incubated with different concentrations of taurine in 0.2 M PBS at 37°C (pH 7.4). Reaction products were diluted 200-fold in 0.2 M PBS pH 7.4 before spectrofluorometric measurements.

LC/MS analysis of the reaction products of taurine and MDA

To separate the principal reaction products of taurine with MDA and assay their molecular weight, taurine (1.0 mM) was incubated with MDA (4.0 mM) in 0.2 M PBS at 37°C for 72 h, and the reaction products were analyzed by LC/MS. LC/MS experiments were carried on an LC/MS-2010 quadrupole mass spectrometer interfaced with an electrospray ionization (ESI) source (LC/MS-ESI). The positive electrospray ionization mode was applied for detection. Separation was done by a Thermo Hypersil-Keystone Hypurity C₁₈ (150 mm × 2.1 mm, 5 μm) analytical column in the following conditions. The volume ratio of methanol: acetonitrile:CF₃COOH-H₂O (0.02%) was 5:5:90. The flow rate was 0.22 ml/min and the injection volume was 5 μl . The wavelength of the SPD-M10Avp diode array detector (DAD) was set in the range of 200–450 nm. The ESI/MS source was set as follows: the temperatures were maintained at 250°C, 250°C, and 200°C for the probe, CDL, and block, respectively. The voltages were set at 4.5 kV, –30 V, 25 V, 150 V, and 1.5 kV for the probe, CDL, Q-array 1,2,3 bias, Q-array radio frequency, and detector, respectively. The flow rate of nebulizer gas was 1.5 l/min. The ions used for selected ion monitoring were chosen by scanning in the positive ion mode from $m/z = 0$ –500.

Effect of taurine on the MDA-modified BSA

BSA (10 g/l) was incubated with MDA (1.0 mM) in the absence or presence of different concentrations of taurine (taurine and MDA were added simultaneously to the reaction system) in PBS. The reaction was conducted at 37°C for 24 h, 48 h, 72 h, 96 h respectively. Modified protein was precipitated by adding isometric 20% trichloroacetic acid (TCA) and centrifuged (3000 g) for 3 min. In order to remove fluorescent adducts from the reaction of taurine with

Figure 1 Typical fluorescence spectra of product 1 of taurine reacted with MDA at 0 h (dotted line) and at 72 h (solid line). The tiny peaks at 0 h are an indication of Raman scatter peaks of water

MDA fully, the pellet was washed 3 times and precipitated with 20% TCA and collected after centrifugation. Finally, the precipitated protein was redissolved in 0.2 M PBS (pH 7.4), and the concentration was adjusted to 10 g/l. The sample was diluted 75-fold before the fluorescence was read at an excitation wavelength of 395 nm and an emission wavelength of 460 nm.

Results

After MDA mixed with taurine, a lipofuscin-like blue fluorescence (excitation 395 nm; emission 460 nm) appeared (Fig. 1 dotted line), and increased with time (Fig. 1 solid line).

A concentration-dependent fluorescence increase was observed with fixed starting concentration of

Figure 2 Product fluorescence altered with different concentration of taurine and MDA after incubation in 0.2 M PBS (pH 7.4) at 37°C for 5 days. (A) Different concentrations of MDA with 1.0 mM taurine. (B) Different concentrations of taurine with 1.0 mM MDA. Results are mean values of triplicates $\pm$ SD

Figure 3 LC/MS analysis of the reaction mixture after 72 h incubation and proposed structure of products. (A) The DAD chromatogram and proposed product structures of taurine reacted with MDA. (B) The mass spectra corresponding to the total ion chromatogram at the retention time 2.339 min and the proposed structure of product 1. (C) The mass spectra corresponding to the total ion chromatogram at the retention time 5.410 min and the proposed structure of product 2

taurine (1.0 mM) or MDA (1.0 mM) (Fig. 2). After reacting for 72 h, the fluorescence intensity of adduct reached a plateau. When the starting concentration of MDA was fixed at 1.0 mM, the fluorescence yield from 4 mM or 8 mM taurine were similar to that from 10 mM, indicating a possible saturation of taurine in the reaction (Fig. 2B).

LC/MS was employed to identify the reaction products. After the reaction mixture was incubated for about 72 h, a diode array detector (DAD) chromato-

gram showed the peaks of the products were at 2.339 min (product 1) and 5.410 min (product 2; Fig. 3A). The mass spectra corresponding to the retention time of product 1 showed two main peaks: m/z 260 [Mp_1+H] $^+$ and m/z 244 [Mp_1-CH_3] $^+$ (Fig. 3B). The mass spectra corresponding to the retention time of product 2 showed two main peaks: m/z 180 [Mp_2+H] $^+$ and m/z 109, respectively (Fig. 3C). The possible molecular structures of products 1 and 2 are illustrated in Figure 3B,C according to the information obtained above and the

Figure 4 UV-absorption spectrum of products of taurine with MDA

knowledge in the related field. The formation mechanisms of product 1 were similar to those of the other fluorescent dihydropyridine derivatives which were clearly elaborated by Kikugawa²⁰ and confirmatively reviewed by Esterbauer and his co-workers.¹

The UV-absorption spectrum from LC/MS showed that the maxima of product 1 were at 238, 261, and 390 nm (Fig. 4), the UV-absorption spectrum of product 2 was at 275 nm (Fig. 4).

As shown in Figure 5, when BSA (10 g/l) was incubated with 1.0 mM MDA at 37°C for different time intervals, the fluorescence intensity increased steadily. After 96 h incubation, the lipofuscin-like fluorescence increased by 27 times as compared with the BSA control. However, the addition of different concentrations of taurine (0.5, 1.0, 2.0, 4.0, and 8.0 mM) inhibited the formation of lipofuscin-like fluorescence by 7%, 17%, 29%, 45% and 63%, respectively (Fig. 5). The consequences also indicated that the inhibitions were in direct proportion to the taurine concentration and incubation time (Fig. 5). Our results suggested that taurine might inhibit AGEs/ALEs formation by reacting with MDA, thus preventing the formation of MDA-protein adducts.

Figure 5 Protective effect of taurine on chemical modification of BSA by MDA. BSA (10.0 g/l) was incubated with MDA (1.0 mM) in the absence or presence of different concentrations of taurine (0.5 mM, 1.0 mM, 2.0 mM, 4.0 mM and 8.0 mM) in 0.2 M PBS (pH 7.4) at 37°C for 24 h, 48 h, 72 h, 96 h. The lipofuscin-like fluorescence was measured at excitation 395 nm, emission 460 nm. Results were mean values $\pm$ SD of three parallel tests

Discussion

Though a recent study did not observe depletion of taurine to MDA at room temperature for 2 h,²¹ taurine was found to react directly with MDA in supraphysiological conditions (Fig. 1). As high concentrations of MDA may form acetaldehyde under certain conditions,²² two molecules of MDA and one molecule of acetaldehyde were proposed to react with one molecule of taurine to form dihydropyridine adducts.^{23,24} The proposed structures of products suggested that the molecular proportion of taurine:MDA was 1:3 in product 1 (Fig. 3B), and 1:1 in product 2 (Fig. 3C). Further assay by LC/MS confirmed the speculation that two products in this reaction were a 1,4-dihydropyridine adduct and an enamine derivative, respectively (Fig. 3). This reaction mechanism was also similar to that of MDA with other amine compounds, such as glucosamine,⁷ histamine⁸) and pyridoxamine.²⁵

Though MDA is frequently reported to react with amino compounds forming fluorescent products such as dihydropyridinium derivatives,^{26–30} the contribution of MDA to protein carbonyls still remains controversial.³¹ In our experimental conditions, MDA-modified BSA showed lipofuscin-like fluorescence at 460 nm when excited at 395 nm, which was consistent with the fluorescence of dihydropyridine. The results demonstrated that taurine inhibited the development of fluorescence of MDA and BSA adducts, which implied a preventive effect on the formation of AGEs/ALEs. Although the MDA concentration (1.0 mM) added to BSA was higher than the average physiological level, it is reasonable to consider that biological tissues or cells may accumulate chemicals on their membrane, which may raise the MDA concentration to such a 'higher' level in the micro-environment.

The advantages of taurine on preventing age-related diseases have been widely recognized. However, its reaction mechanisms remain confused.¹⁶ Our study showed that taurine reacted directly with MDA and formed two adducts. From the molecular structure of taurine, we can clearly observe that the functional group with responsibility for reducing carbonyl stress is the amino group. While the sulphonic acid end may be inert to free radical oxidation or to other chemical reactions, the amino group is the main functional group in the small molecule of taurine.¹⁸ Considering that the amino-carbonyl reaction is a crucial 'garbage cleaning' process following oxidative and glycation stresses, the high amounts of taurine in cardiomyocytes and

neurons, suggests that more profound investigation and appropriate interpretation of this process are needed.

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