Antiaggregant Effect of Taurine Chloramines in the Presence of Serum Albumin

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The effects of taurine chloramine derivatives on initial aggregation of isolated platelets suspended in buffered saline were studied. Inhibition of ADP-induced aggregation in pure cell suspension depended on the structure of chloramine antiaggregants. The most effective of them was N,N-dichlorotaurine; its concentration needed for 50% inhibition of aggregation was about 0.1 mM. Weaker antiaggregants N-chloro-N-methyltaurine and N-chlorotaurine in a final concentration of 0.5 mM reduced platelet aggregation by only 10%. The studied chloramines considerably differed by their characteristics (velocity of the reaction with sulfur-containing groups of atoms). N,N-dichlorotaurine exhibited the weakest reactivity with methionine thioester group. In turn, the velocity constant with reduced glutathione was by 2-3 orders of magnitude higher than that of other chloramines. Antiaggregant effect of taurine chloramine derivatives was 2-fold higher in the presence of serum albumin, presumably due to special interactions of taurine chloramines in complex with albumin with platelets.

Key Words: *chloramine; N,N-dichlorotaurine; serum albumin; platelets; aggregation*

Pathological thrombosis caused by platelet aggregation and intravascular blood hypercoagulation is a frequent cause of death. High therapeutic efficiency of covalent antiaggregants (platelet inhibitors) preventing intravascular clotting was demonstrated in recent studies [8-11,13]. These antiaggregants inhibit platelet functions via chemical modification of molecular targets in cells. Acetylsalicylic acid (aspirin) reacting with prostaglandin H_2-synthase [9], and thienopyridines [10,11], whose metabolites react with sulfhydryl group of ADP receptors, are widely used in clinical practice.

We previously proposed to use chloramine derivatives of biogenic compounds, mainly of amino acids and taurine, as covalent antiaggregants [1-3,5]. Their specific feature is inhibition of platelet functions in all mechanisms of cell activation. Amino acid chloramines are natural substances. They are formed in the body in reactions between amino acids and hypochlorite produced by myeloperoxidase in activated neutrophils. Chloramine group is active in the chloramine antiaggregants. It oxidizes sulfur-containing groups (thiol and thioester groups) in the platelet plasma membrane proteins.

Normally, less than 10% of antiaggregant reacts with the targets in the blood, while >90% is involved in secondary processes: utilization in various reactions or binding to other structures [12]. It is therefore interesting to study the capacity of covalent antiaggregants to react with different blood components, primarily serum albumin [14]. Amino acid chloramines should react with sulfhydryl group and surface thioester groups of serum albumin methionine [5]. The possibility of binding of these antiaggregants by serum albumin is not yet studied.

We studied the antiaggregant characteristics of taurine chloramines and changes in their activities under the effect of serum albumin.

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MATERIALS AND METHODS

Initial aggregation of isolated platelets (formation of small aggregations) was studied. The possibility of binding of the studied chloramines with serum albumin was studied by differential spectrophotometry.

Amino acids, taurine, ADP, BSA (A2153; all reagents from Sigma), and sodium hypochlorite (NaOCl, Aldrich) were used in the study. Taurine chloramine derivatives were obtained in the reaction between NaOCl and amino acids; molar concentrations of amino acid were higher than NaOCl concentration by ~10%. N,N-dichlorotaurine was obtained by adding taurine to NaOCl solution (1:2 final molar concentrations). The formation of chloramine derivatives was controlled spectrophotometrically by the presence of 253-255-nm peak for monochloro derivatives and 302-nm peak for N,N-dichlorotaurine in the absorption spectrum. The concentrations of NaOCl and taurine chloramine derivatives were evaluated by iodometric titration. Albumin was dissolved in phosphate buffer (139 mM NaCl, 32 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). Chloramine and albumin absorption spectra were recorded on a DU-720 spectrophotometer (Beckman-Coulter).

Experiments were carried out on isolated rabbit platelets. Rabbit blood was stabilized with acid sodium citrate (85 mM tri-substituted sodium citrate, 71 mM citric acid, 11 mM glucose, pH 4.5), 5:1 v/v, and centrifuged at 460*g* for 15 min. For platelet isolation, EDTA (1 mM) was added to the supernatant (platelet-rich plasma) and centrifuged at 1850*g* for 7 min. Sodium citrate (0.1 volume of 3.8% solution, pH 7.4) was added to the precipitate and incubated at 37°C for 5 min. Platelet suspension was then 10-fold diluted with buffer solution (10 mM HEPES, 134 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.5 mM Na_2HPO_4, 0.5 mM glucose, pH 7.4) and stored at ambient temperature. The studied compounds were incubated with platelet suspension for 5 min. Before registration, platelet-depleted (2%) plasma, $CaCl_2$ (1 mM), and aggregation inductor ADP (10 μM) were added to the platelet suspension.

Aggregation of isolated platelets with the formation of small aggregations was studied by kinetic nephelometry. This method is based on measurement of the intensity of light scatter at small angles as a function of time [4]. The platelet sample in a rectangular cuvette of the aggregometer was illuminated with He-Ne laser (λ=632.8 nm). Light scattered within 0.5-7.0° was separated using a combinations of diaphragms. Changes in light scatter intensity (ΔI) recorded 50 sec after addition of the aggregation inductor served as the quantitative measure of platelet aggregation capacity. In studies of the amino acid chloramine derivatives this value was standardized to the control.

In order to evaluate the velocity constants, 1.0 ml sulfur-containing compound in a concentration of 4.0 mM was rapidly added to the cuvette with chloramine solution (1.0 ml, 4.0 mM), so that the final concentration of compounds was 2 mM. After agitation, the kinetics of reduction of optical density of chloramines in the absorption spectrum maxima at λ=254 nm (for monochloramine derivative) and λ=302 nm (for N,N-dichlorotaurine) was recorded. The velocity constant of the initial reaction of chloramines with sulfur-containing compounds was estimated by the known formula (by common laws of chemical reaction kinetics): $k=\Delta C/\Delta t(C_1 \times C_2)$, $\Delta C=\Delta D/\varepsilon l$, where C_1 and C_2 are concentrations of reagents, ε is molar absorption coefficient for chloramine, l is optic path length (in cm), and Δt is time during which the optical density of the reaction mixture decreased by 10-15% (ΔD). The equations for estimation of velocity constants were solved using Excel 2000 software.

The results of measurements of platelet aggregation and reaction velocity constants are presented as the arithmetic means, their range was described as the mean square error in the mean.

RESULTS

Taurine chloramine derivatives attract special attention among chloramines. These compounds are stable and can be prospective agents for prevention of platelet-mediated thrombosis [3]. We studied the effects of N,N-dichlorotaurine, N-chlorotaurine, and N-chloro-N-methyltaurine on the initial aggregation of isolated platelets. Suppression of isolated platelet aggregation in buffer solution, induced by addition of ADP, was most pronounced after addition of N,N-dichlorotaurine (Fig. 1). The concentration of N,N-dichlorotaurine inhibiting aggregation by 50% (C_{50}) was 0.2 mM by active chlorine; 10% reduction of platelet aggregation was attained after addition of N-chloro-N-methyltaurine and N-chlorotaurine in concentrations of 0.5 mM.

Intactness of sulfur-containing groups in plasma membrane proteins is essential for platelet aggregation, primarily the integrity of the sulfhydryl group of the ADP receptor [7]. Therefore it is important to know the reaction capacity of chloramines towards sulfur-containing groups. To this end, the velocity constants for reactions of the studied chloramines with methionine and reduced and oxidized glutathione were evaluated spectrophotometrically. For methionine (chloramines react with thioester group) the constants for N,N-dichlorotaurine, N-chloro-N-methyltaurine, and N-chlorotaurine were 0.220±0.062, 15.3±3.2, and 21.7±2.7 $M^{-1} \times sec^{-1}$, respectively. Hence, N-chloro-N-methyltaurine and N-chlorotaurine react with thioester group at virtually the same velocity, while N,N-di-

chlorotaurine exhibits low reaction capacity. Reaction velocity constants for N,N-dichlorotaurine, N-chloro-N-methyltaurine, and N-chlorotaurine with reduced glutathione (with sulfhydryl group) reached $>10^3$, 3.20±1.04, and 5.90±1.12 $M^{-1}\times sec^{-1}$, respectively. Velocity constant for the reaction of N,N-dichlorotaurine with oxidized glutathione was 0.0040±0.0005 $M^{-1}\times sec^{-1}$ and much lower than the constant for N-chlorotaurine (0.60±0.004 $M^{-1}\times sec^{-1}$). Hence, N,N-dichlorotaurine is characterized by much higher capacity to the reaction with the sulfhydryl group. Presumably, this capacity determines more pronounced antiaggregant activity of N,N-dichlorotaurine with isolated platelets.

Sulfur-containing groups are present not only in the platelet plasma membrane, but also in plasma proteins, primarily in serum albumin molecule. Human serum albumin molecule contains 1 sulfhydryl group and 6 thioester groups, while the molecule of BSA often used in experiments has 1 sulfhydryl and 4 thioester groups, and therefore serum albumin can modify the antiaggregant effect of chloramines.

We compared antiaggregant effects of taurine chloramine derivatives on the initial aggregation of isolated platelets suspended in buffered saline and in the presence of BSA (0.34 mM). Antiaggregant effects of N,N-dichlorotaurine and N-chlorotaurine sharply increased in the presence of BSA (Fig. 1). Preliminary data indicate that albumin exhibits a similar effect on inhibition of platelet aggregation with N-chloro-N-methyltaurine. This potentiation of the antiaggregant effects of taurine chloramines by albumin can be explained by the formation of albumin—taurine chloramine derivative complexes providing more effective reaction of chloramines with platelets.

In order to clear out this possibility experimentally, we studied changes in absorption spectrum of serum albumin after its addition to chloramine solution. These studies are possible only for N,N-dichlorotaurine, because its absorption band with the peak at λ=302 nm not completely overlaps with the protein absorption band. Chloramine in a final concentration of 1 mM (2 mM by active chlorine) was added to BSA solution (0.07 mM) in phosphate buffer and the absorption spectra were recorded. Differential absorption spectra were calculated from spectrophotometer electron tables.

According to chemical analysis data, only 25-30% active chlorine from the added chloramine was detected by the iodometric method. Presumably, the greater part of chloramine in the mixture reacts with the sulfur-containing groups of the protein, but this does not agree with characteristics of the differential absorption spectrum (difference between absorption spectra of albumin — chloramine mixture and pure

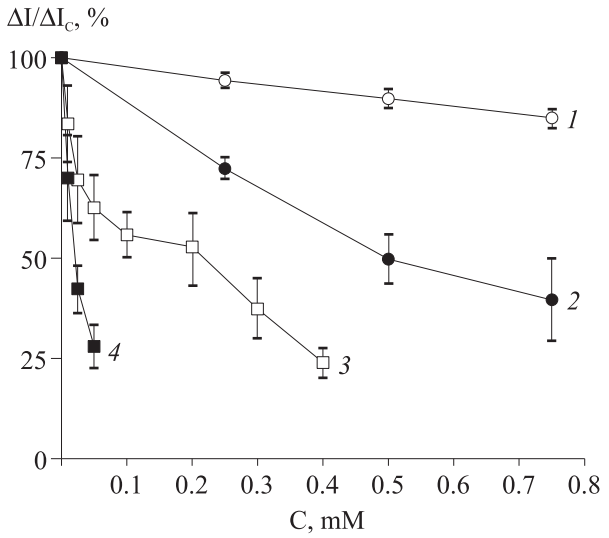

Fig. 1. Concentration relationships for the effects N-chlorotaurine and N,N-dichlorotaurine on initial aggregation of isolated platelets in buffered saline and in the presence of BSA (0.34 mM). ΔI, ΔI_C: changed intensity of diffuse light (aggregation degree) recorded 50 sec after addition of ADP in experimental and control samples, respectively. C: active chlorine concentration. *1*) effect of N-chlorotaurine on platelets in saline; *2*) effect of N-chlorotaurine on platelets in the presence of BSA; *3*) effect of N,N-dichlorotaurine on platelets in saline; *4*) N,N-dichlorotaurine effect on platelets in the presence of BSA.

protein). The N,N-dichlorotaurine absorption band at wavelengths >295 nm was retained in the differential spectrum obtained directly after preparation of the mixture (Fig. 2, curve *2*). Chloramine absorption band was weaker than that for free chloramine by no more than 10-15%. These results can be attributed to the fact that the greater part of N,N-dichlorotaurine in the mixture is bound to albumin. This explanation is confirmed by the results of the analysis of the rest part of the differential absorption spectrum. A structured band of 265-290 nm appeared in the spectrum directly after chloramine addition (Fig. 2). Poorly pronounced peaks in this band and in a shorter wavelenght area were well reproduced. Among these peaks were those of 253, 256, 267, 273, 277, 280, 285, 287, 290, and 293 nm. The majority of them appeared in the differential spectrum obtained by 1-nm shifting of the albumin absorption spectrum towards the longer wavelenghts. These data and published reports [6,7] suggest that N,N-dichlorotaurine transfers the albumin molecules into a different state modifying the microenvironment of aromatic amino acid residues: phenylalanine (peaks at 255-279 nm), tyrosine (peaks at 280-285 nm), tryptophan (290-295 nm). There are good grounds to assert that serum albumin really binds N,N-dichlorotaurine and conformation changes. The differential spectrum at wavelengths shorter than 295 (protein) changed significantly after 2-h incubation of albumin with N,N-dichlorotaurine, while chloramine absorption virtually

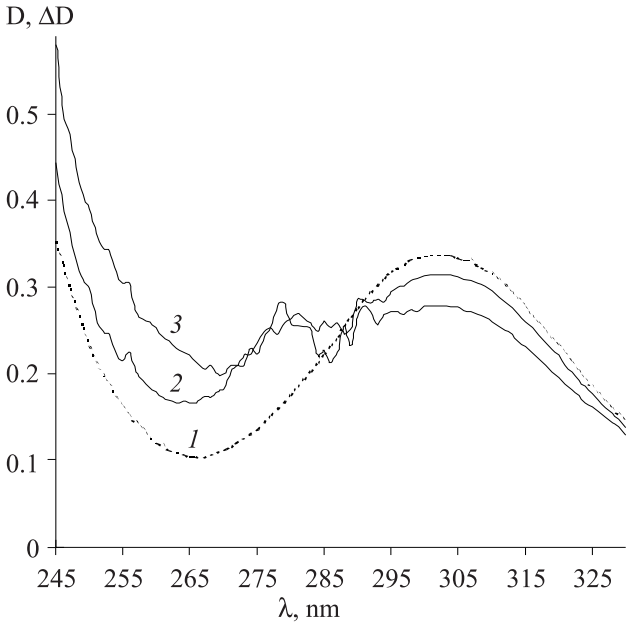

Fig. 2. Absorption spectrum for N,N-dichlorotaurine 2 mM solution (*1*) and differences between absorption spectra for N,N-dichlorotaurine (2 mM) mixture with albumin (0.07 mM) and albumin absorption spectrum recorded directly (*2*) and after 2-h incubation of the mixture (*3*). D: optical density (*1*); ΔD: optical density difference (*2*; *3*).

did not change (Fig. 2). This indicates that the modification of protein status under the effect of chloramine develops with time.

Hence, the efficiency of antiaggregant treatment of isolated platelets with various taurine chloramine derivatives depends on their structure. N,N-dichlorotaurine is characterized by the highest antiaggregant activity, which was presumably caused by high velocity of the reaction with platelet sulfhydryl groups. Antiaggregant effect of N,N-dichlorotaurine and N-chloro-N-methyltaurine is potentiated in the presence of serum albumin. According to spectrophotometry data, serum albumin binds N,N-dichlorotaurine, which causes changes in protein conformation. Presumably, high antiaggregant efficiency of taurine chloramines in the presence of serum albumin is a result of specific interactions with the protein-chloramine complex with platelets.

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