Inhibition of Lens Crystallin Glycation and High Molecular Weight Aggregate Formation by Aspirin In Vitro and In Vivo

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Previous studies have shown that glycation of lens proteins could be a contributory factor in the development of diabetic and senile cataracts. Acetylation by aspirin (acetylsalicylic acid or ASA) has been used as an inhibitor of glycation which blocks the potential glycation sites (ε-NH₂ groups). If glycation is a contributory factor, inhibition of glycation by acetylation should bring about a corresponding decrease in cataractogenic changes. We relied on in vitro glycation system and streptozotocin-diabetic rats to study the effects of ASA on lens crystallin glycation, thiol oxidation and aggregation. For in vitro studies, sterile lens soluble crystallin preparations from 1-month-old rats were incubated, under nitrogen, with 50 mM glucose and 20 mM ASA up to 15 days at 37°C. To study the in vivo effect in diabetic rats, ASA feeding (200 mg/kg body wt/day) was initiated 1 week prior to streptozotocin administration, and sacrificed on 15, 30, 60 and 90 days after injection. The in vitro data show the inhibitory effect on glycation of ASA with all concentrations that were tested (5, 10, 20 mM ASA); the percentage inhibition increased with increasing ASA concentration and time. For example, with 50 mM glucose and 20 mM ASA incubated for 15 days, there was a significant decrease in glycation (P < 0.05), thiol oxidation (P < 0.05) and aggregation (P < 0.02). Similarly, in in vivo experiments ASA feeding delayed lens opacification by about 30 days with a significant inhibition of lens protein glycation (P < 0.02), while the levels of glucose remaining almost the same (P > 0.5), with a corresponding decrease in HMW aggregates (P < 0.02) and an increase in free protein thiols (P < 0.02) in 90 day diabetic animals. These results strongly suggest that enhanced protein modification by glycation followed by protein unfolding and sulfhydryl oxidation is a critical factor in diabetic cataractogenesis. Invest Ophthalmol Vis Sci 30:1120-1126, 1989

Previous studies in our laboratory and elsewhere have indicated that crystallin glycation could be a contributory factor in the formation of high molecular weight (HMW) protein aggregates that are either disulfide-linked, cross-linked by advanced glycation products, or noncovalently linked. It is believed that glycation initiates or enhances protein unfolding, thus exposing sulfhydryl groups to undergo oxidation especially under increased oxidative challenge. Although this is a reasonable hypothesis for the development of cataract, diabetic cataract in particular, direct involvement of lens protein glycation in the events leading to cataractogenesis is yet to be established. One way of achieving this goal is to inhibit glycation by blocking the glycation sites without affecting the level of glucose. Post-translational modifications are believed to play a significant role in pathogenesis of several tissues including lens proteins. Acetylation by acetylsalicylic acid (aspirin or ASA) of lysine residues inhibits glycation of plasma proteins, and hemoglobin, and lens proteins. Acetylation has also been shown to prevent carbamylation, another post-translational modification of lens crystallins. Rao et al reported that acetylation of ε-amino groups of lens crystallins by ASA may protect against aggregation by blocking glycation, carbamylation or isopeptide bond formation. Use of aspirin as therapeutic agent to reduce diabetic complications was earlier reported by Reid et al. and Cotlier and colleagues. van Heyningen and Harding proposed possible protection against cataract by aspirin, although the evidence for aspirin was disputed by others. We have used acetylation by ASA of potential glycation sites to study its effect on
protein glycation, thiol oxidation and aggregation in an in vitro glycation system and in streptozotocin-diabetic rats.

Materials and Methods

In Vitro Glycation Studies

The purpose of these studies was to demonstrate that in vitro glycation also can lead to HMW protein aggregation and that acetylation by ASA inhibits lens crystallin glycation and prevents HMW aggregation. The in vitro incubation of lens proteins was done according to the method described by Stevens et al. Briefly, lenses were removed from 1-month-old male Spague-Dawley rats, homogenized under nitrogen in 50 mM sodium phosphate buffer, pH 7.0, centrifuged at 6000 g for 60 min, the supernatant dialyzed overnight against the same buffer and sterile filtered through a series of 5, 0.8, and 0.45 µm filters (Gelman Sciences, Ann Arbor, MI). The initial experiments were designed to establish the time and ASA concentration dependence of glycation. For this purpose incubations were done with [14C]glucose and the cpm incorporated per milligram of protein was monitored. Incubation mixture contained 50 mM glucose and 10 mM sodium phosphate buffer, pH 7.0, centrifuged at 6000 g at 60°C for 60 min, the supernatant dialyzed over night against the same buffer and sterile filtered. To determine the time-dependent changes aliquots of 0.5 ml were removed after 1, 3, 5, 10 and 15 days of incubation at 37°C in the presence and absence of 20 mM ASA. After reduction with 100 µl of 1 M NaBH4 (10 min at room temperature and an additional 50 min in ice) the samples were extensively dialyzed against water (with at least two changes) overnight at 4°C. The radioactivity was measured with toluene-based scintillation cocktail (containing triton X-100, POPOP and PPO). To determine the ASA concentration dependence of glycation, incubations were done for a fixed period of 15 days with 0, 5, 10 and 20 mM ASA. ASA concentrations beyond 20 mM resulted in protein precipitation and were not further pursued. After these initial studies, additional lens crystallin incubations were done with 50 mM glucose alone (containing no [14C]glucose), 50 mM glucose and 20 mM ASA, 20 mM ASA alone, and with no additives (ie protein alone) to determine the effect of ASA on HMW aggregation and free thiols. In these experiments aliquots were removed on the 5th and 15th day of incubation and mixed with equal volume of 14 M urea to ensure complete solubilization. Sulphydryl titration with parachloromercuribenzoate (PCMB) was performed immediately whereas affinity chromatographic quantitation of glycated protein and high pressure liquid chromatographic (HPLC) separation of HMW aggregates were done after reduction with NaBH4 (as described elsewhere in the methods).

Studies in Streptozotocin-Diabetic Rats

Seventy male Sprague-Dawley rats, age 1 month, were divided into four groups. Group 1 (15 rats) served as controls. Group 2 (20 rats) were made diabetic by intravenous injection of streptozotocin (65 mg/kg body weight) through tail vein. Group 3 (15 rats) were aspirin controls; these animals were fed powdered rat chow mixed with ASA (Mallinckrodt Chemical Works, St. Louis, MO), 200 mg/kg body weight/day, according to Rendell et al. Group 4 (20 rats) were aspirin diabetic; these animals were fed similar to group 3 but were made diabetic 1 week after starting the aspirin diet. Three to four animals from each group were sacrificed on the 15, 30, 60 and 90 days after injection. Blood was collected for the determination of plasma glucose and glycated hemoglobin (GHb). Lenses were removed and homogenized and separated into water-soluble (WS) and water-insoluble (urea-soluble or US) fractions for the separation of HMW protein aggregates and the determinations of glycated protein and free thiols.

Animal experiments were performed in compliance with ARVO Resolution on the Use of Animals in Research and of the Institutional Committee for Animal Care.

Crystallin Preparation

The water-soluble and the urea-soluble crystallins were prepared according to the modified procedure of Herbrink and Bloemendal as described in detail before.

Quantification of Glycated Crystallins

The glycated proteins in the WS and US fractions were determined by affinity chromatography as described in previous communications using GlycAfin microcolumns from Isolab Inc. (Akron, OH). The columns prepacked with phenylboronate agarose and the developers needed for the chromatography were supplied by the manufacturer. About 0.5 mg of the WS or the US fraction (or the urea-solubilized total protein from the in vitro studies) was used for each determination. For the US fractions all the buffers contained 7 M urea.

Sulphydryl Titration

The concentration of free protein thiols was determined by titration with PCMB according to the modified method of Boyer.
Incubation period (days) Aspirin concentration (mM)

Fig. 1. (A) Time-dependence of the inhibition of \([^{14}C]\)glucose incorporation into lens crystallins by aspirin, in vitro. Lens crystallins were incubated with 50 mM glucose containing \([^{14}C]\)glucose with 20 mM aspirin (•) or without 20 mM aspirin (○) up to 15 days. Before counting for radioactivity, aliquots were reduced with NaBH₄ and dialyzed extensively. (B) Aspirin concentration dependence of the inhibition of \([^{14}C]\)glucose incorporation. Lens crystallins were incubated with 50 mM glucose and 5–20 mM aspirin for 15 days.

Molecular Sieve HPLC Separation of HMW Aggregates

For separation of the HMW aggregates in the WS and US fractions previously reported molecular sieve HPLC methodologies were used. The basic set-up consisted of a Beckman HPLC system, having model 421 controller, dual 110 pumps and model 160 detector with 20 μl flow cell and Hewlett-Packard 3390-A recording integrator. For WS proteins TSK 3000 SW (7.5 × 600 mm) column coupled in series with a TSK 4000 SW (7.5 × 300 mm) column was used. The isocratic mobile phase consisted of 50 mM sodium phosphate, 50 mM NaCl, pH 6.8 with a flow rate of 1 ml min⁻¹. The crystallin components from the urea-solubilized fractions were separated with TSK 2000 SW (7.5 × 600 mm) column coupled with a TSK 3000 SW (7.5 × 300 mm) column. The isocratic mobile phase consisted of 7 M urea, 100 mM sodium phosphate, 100 mM NaCl and 5 mM EDTA at pH 6.5. All chromatograms were run at ambient temperature and absorbance was monitored at 280 nm.

Other Methods

Plasma glucose levels were determined by the glucose oxidase assay of Raabo and Terkildsen provided in kit from Sigma Chemical Company (St. Louis, MO). GHb levels were determined with the Isolab Glyc-Affin System and the method described by the manufacturer was strictly followed. Statistical analysis was done using the student t-test where comparisons were made between untreated diabetic group and aspirin treated diabetic group and also between controls and aspirin treated controls.

Results

Inhibition of Glycation and Aggregation of Crystallins by Aspirin in the In Vitro System

Figure 1A shows the time dependence of in vitro glycation, assessed by cpm incorporated per milligram protein of lens crystallins with 50 mM glucose (containing 10 μCi of \([^{14}C]\)glucose in the presence and absence of 20 mM ASA. The presence of ASA inhibited glycation substantially; the percentage inhibition increased from 35% on day 1 to 46% on day 15 indicating increased modification of potential glycation sites with increasing time. The inhibitory effect of ASA was evident at all concentrations that were tested (Fig. 1B); again, percentage inhibition increased with increasing ASA concentration. Analysis of the incubation mixture for glycated protein, HMW aggregates, and thiol groups on the 5th and 15th day of incubations is shown in Table 1 (also see Fig. 2 for the separation of HMW aggregates in the urea-solubili-

Table 1. Effect of aspirin (ASA) on glycation, thiol oxidation and HMW aggregation of lens proteins in vitro

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Glycated proteins (%)</th>
<th>Thioles (n moles –SH/mg protein)</th>
<th>HMW aggregate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM glucose</td>
<td>7.1 ± 1.5</td>
<td>48 ± 5.0</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>50 mM glucose</td>
<td>19.9 ± 4.0</td>
<td>25 ± 8.0</td>
<td>3.6 ± 0.9</td>
</tr>
<tr>
<td>0 mM glucose + 20 mM ASA</td>
<td>7.1 ± 0.9</td>
<td>42 ± 5.6</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>50 mM glucose + 20 mM ASA</td>
<td>12.9 ± 1.9</td>
<td>41 ± 6.0</td>
<td>2.7 ± 0.5</td>
</tr>
</tbody>
</table>

Incubation period (days)

5 day 15 day

<table>
<thead>
<tr>
<th>Glycated proteins (%)</th>
<th>Thioles (n moles –SH/mg protein)</th>
<th>HMW aggregate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4 ± 0.9</td>
<td>43 ± 4.5</td>
<td>1.2 ± 0.5</td>
</tr>
<tr>
<td>24.7 ± 5.1</td>
<td>18 ± 6.6</td>
<td>20.5 ± 5.0</td>
</tr>
<tr>
<td>7.9 ± 0.8</td>
<td>42 ± 4.2</td>
<td>3.5 ± 1.0</td>
</tr>
<tr>
<td>14.9 ± 1.9</td>
<td>28 ± 6.5</td>
<td>8.4 ± 2.0</td>
</tr>
</tbody>
</table>

0 day values: glycated proteins: 3.0 ± 1.0; thioles 54 ± 4.0; HMW: 1.3 ± 0.2
lized incubation mixture). The glycation data, obtained by the affinity column technique also confirmed that ASA indeed inhibits glycation. Increased glycation has led to increased HMW aggregation and thiol oxidation whereas the inhibition of glycation by ASA produced corresponding inhibition of protein aggregation and protection of protein thiols (15 day incubation: glycated proteins, \( P < 0.05 \); thiols, \( P < 0.05 \); HMW aggregates, \( P < 0.02 \)). Incubation of protein alone did not generate much HMW aggregates. On the contrary, incubation of a mixture of the protein and ASA (without glucose) showed an increase in the protein aggregates, but the increase was relatively small (less than 3-fold) compared to about 17-fold increase in aggregation observed with 50 mM glucose. It is difficult to establish the source of oxidative challenge in our in vitro system. Although the incubation tubes were sealed under nitrogen (following the method of Stevens et al), the residual oxygen in the incubation buffers may oxidize the exposed thiols or oxidation may have occurred by hitherto unknown mechanism.

In Vivo Effect of Aspirin in Streptozotocin-Diabetic Rats

Cataract development: Among the 40 rats that were made diabetic, 20 received ASA-containing diet. In about 90 days, lens opacity was obvious in all the diabetic animals (about 2+), whereas ASA-treated ones were close to normal (Fig. 3). However, by 120 days the ASA-treated diabetic animals developed cataract (about 2+), whereas the untreated ones showed extremely dense opacity (4+ or higher) (data not shown). Thus, acetylation with ASA seems to have delayed the glycation induced lens opacification.

Lens protein glycation: As expected, the plasma glucose levels were higher in the diabetic rats and these levels were unaffected by aspirin treatment \( (P \geq 0.5) \) (Fig. 4A). GHb levels also responded to hyperglycemia in the diabetic animals not receiving ASA; however, a significant decrease in GHb was noticed in the ASA-treated diabetic animals \( (P < 0.002 \) for 30-90 day values), indicating inhibition of hemoglobin glycation (Fig. 4B). Glycated protein levels of the WS and US fractions were significantly reduced by ASA treatment \( (P < 0.05 \) for 60 day values) (Fig. 4C, D). The results of the glycated protein determinations of the control animals were rather inconsistent; however, the general trend was a decrease in glycation with ASA treatment.

HMW aggregate formation: Figure 5B and C show the effect of ASA treatment on lens protein aggregation. As expected from our previous studies there was a steady increase of the HMW aggregate formation in the WS and US fractions of the diabetic and control animals, the former consistently having higher levels of aggregates than the latter. The forma-
tion of both these types of aggregates was significantly reduced (10–40%) in the diabetic animals receiving ASA (soluble, $P < 0.02$ for 30–90 day values; insoluble, 60 day, $P < 0.02$; 90 day, $P < 0.002$). In the controls, on the other hand, after 90 days of ASA administration an increase in the HMW aggregates was noticed (soluble, $P < 0.02$; insoluble, $P < 0.05$), which was consistent with what was seen in the in vitro system (Fig. 2, Table 1).

ASA treatment also resulted in convincingly higher level of free protein thiols than those not receiving this drug (for 60 and 90 day, $P < 0.02$) (Fig. 5A).

**Discussion**

It is well known that ASA can acetylate at the amino groups, such as $\alpha$-NH$_2$ and $\epsilon$-NH$_2$ groups, on proteins.$^8$-$11,13$ A number of reports in the past have indicated ASA therapy having a beneficial effect in preventing cataract in human,$^{17-19}$ presumably by inhibiting glycation, whereas others contradicted such findings by showing increased risk of having cataract by long-term ASA treatment.$^{20,21}$ Most of these studies focused on senile cataract formation. It would have been more meaningful to look at the effect of ASA on the development diabetic cataract, where excessive glycation seems to play a significant role. This was one of the reasons for us to focus on in vitro glycation and on a diabetic animal model. The current studies also were expected to establish a relationship between lens protein glycation and aggregation in cataract development. Each system had a certain advantage. The in vitro system lacked all other factors except the presence of high levels glucose and the resultant increased glycation could be inhibited by ASA to study its effect on protein aggregation. In the in vivo studies, on the other hand, in addition to
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hyperglycemia all their metabolic activities were present and the effect of inhibition of glycation by ASA could be studied in a true in vivo model.

The results of the in vitro studies have convincingly shown that glycation precedes protein unfolding and aggregation. By inhibiting glycation, a corresponding level of inhibition of HMW aggregate formation was seen (Fig. 2, Table 1). The results of the in vivo studies in streptozotocin-diabetic rats complemented those of the in vitro studies. An ASA diet had a significant inhibitory effect on crystallin glycation in the diabetic animals which in turn inhibited the formation of both nondisulfide-linked soluble aggregates and disulfide-linked insoluble aggregates (Figs. 4, 5). This may mean that glycation directly influences protein unfolding, and sulfhydryl oxidation and aggregation is only a secondary event (because the soluble aggregates contain no disulfides). However, significant depletion of thiol groups occurred even during the early period (within 5 days) of incubation in the presence of 50 mM glucose ($P < 0.01$) and also during early days (within 15 days) of hyperglycemia in the diabetic rats ($P < 0.05$). During this period, however, the increase in HMW aggregates was only minimal (Table 1, Fig. 5). Thus, it is possible that even before HMW aggregates become detectable, disulfide-linked, low molecular weight aggregates could be formed.

The inhibitory effect of acetylation by ASA appears to be mediated through a blockage of amino groups that prevents glycation. In fact, the direct effect of acetylation, apart from protecting these amino groups, seems to be a slight increase in the unfolding of the proteins as seen in the control incubations of lens crystallins with ASA (without glucose) and in control rats receiving ASA (Table 1 or Fig. 5). On the contrary, in the diabetic animals where excessive glycation is the main problem, this "adverse effect" of acetylation is overcome by the apparent beneficial effect of glycation inhibition. In essence, it appears that acetylation is less harmful and has a less destabilizing effect on lens protein organization than glycation. These findings may have some bearing on the various contradictory reports, based on clinical experience, on the effect of ASA therapy on cataractogenesis.\textsuperscript{21,24} Glycated lens proteins accumulates rather slowly over the entire life of a nondiabetic rat (or human) and to effectively inhibit glycation ASA has to be administered over a long period of time, which may be undesirable because the adverse effects may overwhelm the beneficial effects. This increases the risk of senile cataract. In the diabetic population, on the other hand, glucose level increases rapidly and cataract develops over a relatively short period of time, which could be delayed by ASA treatment.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig5}
\caption{Effect of aspirin feeding on: (A) soluble lens protein thiols; (B) soluble HMW aggregates; and (C) insoluble HMW aggregates. Line legends are same as in Figure 4. Controls $n = 3$ and diabetics $n = 4$. Values are mean ± SD.}
\end{figure}

However, the dosage used in the current study is excessively high, as is the blood glucose level. ASA is hydrolyzed to salicylic acid, which is not an acetylating agent.\textsuperscript{29,30} Therefore, higher doses are required to bring about protection of amino groups by acetylation, which may also be one of the reasons for only 30–40% protection observed in these in vivo studies (Fig. 5).

It seems that there is a lot of analogy between the effect of ASA on glycation and its effect on carbamylation of lens proteins as investigated by Harding and coworkers.\textsuperscript{14} The carbamylation of lens proteins also
causes conformational changes and cataract. It was shown that ASA reduces the rate of carbamylation of all the lens crystallins whereby decreasing the opacification.

Interestingly, in a recent report Huby and Harding showed protection of nonenzymatic galactosylation of lens crystallins by ASA in an in vitro system, which is in agreement with our findings concerning nonenzymatic glycosylation both in vitro and in vivo. We have chosen to run appropriate controls for all the experiments, which made the interpretation of the data much easier and the conclusions more meaningful.

Key words: diabetes, glycation, acetylation, lens crystallins, protein aggregation

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References
