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Preventive aspirin treatment of streptozotocin induced diabetes: blockage of oxidative status and revertion of heme enzymes inhibition☆

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Abstract

Some late complications of diabetes are associated with alterations in the structure and function of proteins due to glycation and free radicals generation. Aspirin inhibits protein glycation by acetylation of free amino groups. In the diabetic status, it was demonstrated that several enzymes of heme pathway were diminished. The aim of this work has been to investigate the in vivo effect of short and long term treatment with acetylsalicylic acid in streptozotocin induced diabetic mice. In both treatments, the acetylsalicylic acid prevented δ-aminolevulinic dehydratase and porphobilinogen deaminase inactivation in diabetic mice and blocked the accumulation of lipoperoxidative aldehydes. Catalase activity was significantly augmented in diabetic mice and the long term treatment with aspirin partially reverted it. We propose that oxidative stress might play an important role in streptozotocin induced diabetes. Our results suggest that aspirin can prevent some of the late complications of diabetes, lowering glucose concentration and probably inhibiting glycation by acetylation of protein amino groups.

Abbreviations: AGEs, advanced glycation end products; ALA-D, δ-aminolevulinic dehydratase; ASA, acetylsalicylic acid; PBG-D, porphobilinogen deaminase; STZ, streptozotocin; TBARS, thiobarbituric acid reactive species.

☆ To the memory of our beloved César Polo. Deceased March 9th, 1996.

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1. Introduction

Non enzymatic protein glycation is a complex cascade of reactions leading to the so called advanced glycation end products (AGEs) [1], which accumulate in long living tissue proteins and may contribute to the development of complications in aging [2], diabetes [3] and possibly neurodegenerative amyloidal diseases such as Alzheimers [4].

Glycation of proteins results in inactivation of enzymes and alterations in their structures and functions [5]. Moreover, they may provoke protein crosslinking and aggregation due to advanced Maillard stages [6] or protein oxidative damage catalysed by Amadori products [7]. The autoxidation of glucose or Amadori compounds on protein plays a major role in the formation of glycoxidation products [6] and the generation of free radicals may contribute to the overall complications of diabetes [8]. The binding of AGEs to specific receptors results in enhanced oxidative stress [9].

A frequent coexistence of diabetes and porphyria disease has been reported, mainly porphyria cutanea tarda, in which a higher incidence of diabetes mellitus, as well as glucose intolerance, than in the general population has been observed [10,11]. Furthermore, Yalouris and Raptis [12] have shown that the onset of diabetes overcomes the typical acute symptoms in acute intermittent porphyria.

It has been shown that aspirin (acetylsalicylic acid; ASA) inhibits protein glycation presumably by acetylation of free amino groups [13,14]. We have shown that the activities of several enzymes of heme pathway were diminished in both the diabetic population [15] and in streptozotocin (STZ) induced diabetic mice [16]. Using a method developed for measuring protein glycation in vitro, in a crude preparation of red blood cells, we have demonstrated that δ-aminolevulinic dehydratase (ALA-D, E.C. 4.2.1.24), the second enzyme in the heme pathway, could be glycated by glucose and also by several sugars other than glucose. We then found that ASA was effective in preventing both hemoglobin (Hb) glycation and ALA-D inactivation by glucose [17].

The aim of this work has been to investigate the in vivo effect of short and long term treatment with ASA in STZ-induced diabetic mice. We report here that ASA prevented ALA-D and porphobilinogen deaminase (PBG-D, E.C. 4.3.1.8) inactivation in diabetic mice and blocked the accumulation of lipoperoxidative aldehydes. The status of the antioxidant defense system was also estimated through the activity of the heme protein catalase (E.C. 1.11.1.6). We propose that oxidative stress may play an important role in STZ-induced diabetes and that the impairment of catalase in response to aspirin treatment could play a key role in the diabetic status. Thus, our results favour the hypothesis that ASA could prevent some of the late complications of diabetes, primarily lowering glucose concentration and probably inhibiting glycation by acetylation of protein amino groups.

2. Materials and methods

2.1. Materials

Chemicals were reagent grade and were purchased from Sigma (St. Louis, MO). ASA was obtained from Bayer Lab. (Buenos Aires, Argentina).

2.2. Animals and treatments

Male CF1 mice weighing 25 g were separated in groups of six animals each. Diabetic status was induced with a single dose of STZ i.p. (200 mg/kg of body weight; the drug was dissolved in citrate buffer). ASA feeding (0.16% w/w in the diet) started 30 min thereafter STZ injection and was administered during 7 days (short term treatment) or 45 days (long term treatment). One group of STZ-treated animals received a standard laboratory diet composed of (% of wet weight): 48 carbohydrate, 4 fat, 20 protein, 16 total minerals-cellulose (Asociación de Cooperativas Argentinas, San Nicolás, Buenos Aires, Argentina) during the whole period. Control groups were injected with the vehicle and were maintained under the same feeding conditions. Daily recording of food consumption, weight gain and general clinical state was performed. Food was removed from all animals 16 h before sacrifice. Mice were killed (15, 30 and 45 days after the injection of STZ or vehicle) under ether anaesthesia and the liver and blood samples were processed immediately. All animals received humane care, as outlined in the Guide for the Care and Use of Laboratory Animals.

2.3. Tissue preparation

The liver was perfused with ice cold saline and then removed. A fraction of the whole liver was homogenized (1:10, w/v) in ice cold 0.25 M sucrose. The homogenates were centrifuged at 4°C during 15 min at $15\,000 \times g$. The supernatants were used for measuring ALA-D and PBG-D activities. Another fraction of the whole perfused liver was homogenized (1:10, w/v) in 0.05 M sodium phosphate buffer, pH 7.4 and was directly used for the determination of malondialdehyde or was centrifuged at 4°C during 15 min at $9\,500 \times g$. The resulting supernatant was used for measuring catalase activity.

2.4. Assays

Glucose levels were determined in plasma by using a commercial kit (Wiener Laboratorios, Rosario, Argentina).

Glycosylated hemoglobin (GHb) was measured in red blood cells using the method described by Parker et al. [18] and was expressed as nmol of hydroxymethylfurfural (HMF) per 10 mg Hb.

ALA-D activity was determined by the method described by Batlle et al. [19]. Catalase was measured as described by Chance and Maehly [20] and PBG-D

activity by Batlle et al. [21]. Protein concentration was determined by the method of Lowry et al. [22].

The peroxidation index was estimated by the formation of malondialdehyde (MDA) and determined as thiobarbituric acid reactive species (TBARS) by the method of Niehaus and Samuelson [23].

Enzyme units were defined as the amount of enzyme producing 1 nmol of product (ALA-D and PBG-D) or consuming 1 nmol of substrate (catalase) under standard incubation conditions. Specific activity (Sp. Act.) was expressed as units per milligram of protein.

2.5. Statistical analysis

Data were analyzed statistically using non-paired Student's *t*-test. A probability level of 0.05 was used in testing for significance differences between experimental groups.

3. Results

3.1. Clinical observations

ASA in the diet did not alter the feeding pattern of the animals. The food intake was 4.51 ± 0.40 g per animal per day, therefore the supplied dose of ASA was 200 ± 25 mg/kg body weight per day. This oral dose was used previously by other authors in STZ diabetic animals [24,25].

3.2. Glycemia and GHb

The diabetes status was established by the high glucose levels (> 1.4 g/l) found in animals treated with STZ. High glucose levels were partially restored to basal values after ASA short term treatment and completely after its long term administration. ASA did not produce any effect on glycemia in control animals (Fig. 1A). ASA treatments at shorter intervals (1–5 days) or with lower concentrations (up to 0.10% w/w, during 7 days) were ineffective in reducing plasma glucose levels below 2.0 g/l (data not shown). Under these conditions the biochemical parameters studied were the same as those of the animals treated only with STZ.

The variations observed in glycemia in the different groups show a similar profile to the levels of GHb in blood (Fig. 1), which is considered an accurate and reliable measure of the glycemic status in a diabetic animal [18]. The effect of ASA on GHb was also more striking in the group that received the long term treatment, although a complete restoration to basal levels was not achieved (Fig. 1B).

3.3. ALA-D and PBG-D

Short or long term ASA treatment diminished 15% ALA-D activity in controls (Fig. 2A). Because ALA-D is a zinc-protein [19], this ASA inhibitory effect could be expected and might be ascribed to its metal-chelating properties [26]. The already reported 50% inhibition on hepatic ALA-D activity in diabetic animals [16] was restored to control levels of groups receiving short and long term ASA treatment.

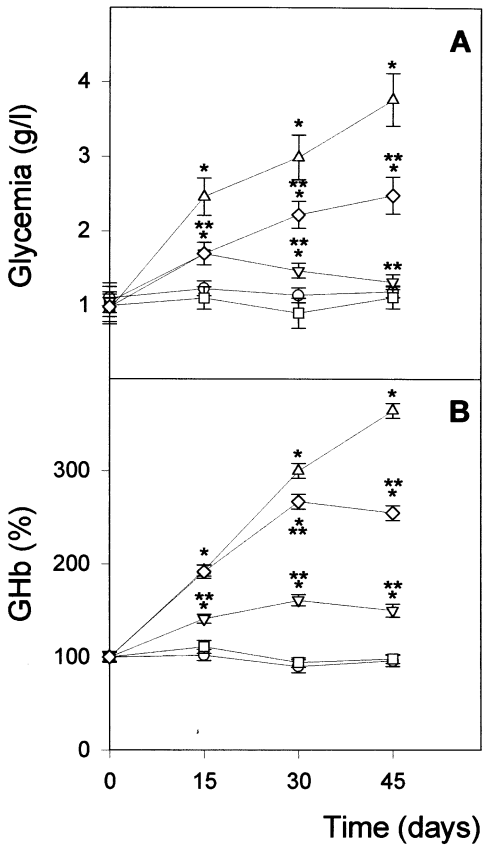

Fig. 1. Glucose plasma levels (A) and GHb production (B) in STZ-induced diabetes mice non treated (△) or treated with ASA (0.16% w/w in the diet) during 7 days (short term treatment) (◇) or 45 days (long term treatment) (▽). Non-diabetic animals were injected with the vehicle and received short term treatment (□) or long term treatment (○) with ASA. Mice were killed 15, 30 and 45 days after being injected with STZ (200 mg/kg, in citrate buffer, a single dose, i.p.) or with vehicle. The data represent mean values ± S.D. of at least six animals and in the case of GHb are expressed as percentage of mean control values (GHb = 35.5 ± 1.7 nmol HMF/10 mg Hb) of animals fed with basal diet and without any other treatment. Glucose mean control value ± S.D. = 1.1 ± 0.2 g/l. Other experimental conditions are as indicated Section 2. *$P < 0.05$ with respect to control group, **$P < 0.05$ with respect to STZ group without ASA.

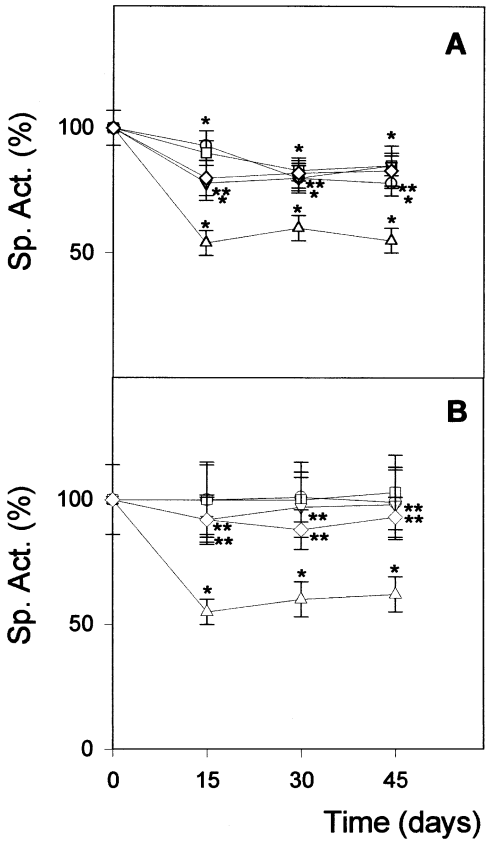

Fig. 2. Effect of short and long term ASA treatment on hepatic ALA-D (A) and PBG-D (B) activities. Mean control value ± S.D.: ALA-D = 22.68 ± 3.35 U/mg protein; PBG-D = 0.526 ± 0.120 U/mg protein. Other experimental conditions and symbols are as indicated in legend to Fig. 1. *$P < 0.05$ with respect to control group; **$P < 0.05$ with respect to STZ group without ASA.

Hepatic PBG-D activity inhibition in STZ-induced diabetic animals [16] was also restored to basal levels at the end of the assayed period in both groups treated with ASA. No effect was detected on this enzyme activity when control animals were treated with ASA either for short or long periods (Fig. 2B).

3.4. Oxidant and antioxidant status

The effect of STZ on hepatic lipid peroxidation was investigated. TBARS levels increased up to 175% along the period of the assay. ASA treatment completely abolished accumulation of TBARS in the long term assay. However, in the short term trial, at day 15 the basal oxidative state was restored but then TBARS gradually increased, reaching a level 50% higher than controls from day 30 onwards. ASA by itself produced no modification in the lipid peroxidation index (Fig. 3A).

To evaluate the state of the antioxidant defense system, we measured catalase activity in all groups. In STZ-induced diabetic mice, catalase activity was 100% increased. ASA gradually reduced the enhanced enzymatic activity after 15 days in the long term treated animals but had no effect when administered for only 7 days. ASA alone produced no changes (Fig. 3B).

4. Discussion

Two different aspects, alterations of enzyme activities and oxidative stress, have been focused on in the present work, to better elucidate the mechanisms for the pathogenesis of some of the complications in diabetes and the role of ASA in vivo to prevent them. STZ-induced diabetes in rodents appeared to be the most suitable

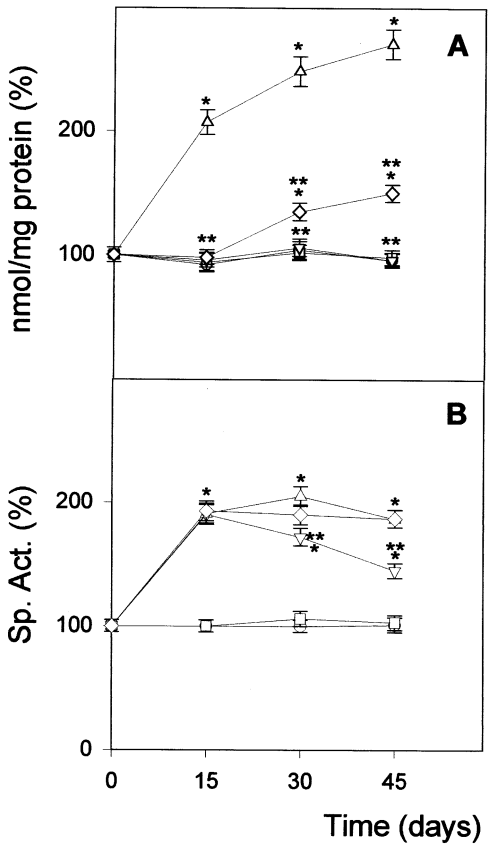

Fig. 3. Effect of short and long term ASA treatment on hepatic TBARS levels (A) and catalase activity (B). Mean control value ± S.D.: TBARS = $135 \times 10^{-3} \pm 24 \times 10^{-3}$ nmol/mg protein; catalase = $1.9 \times 10^3 \pm 0.2 \times 10^3$ U/mg protein. Other experimental conditions and symbols are as indicated in legend to Fig. 1. *$P < 0.05$ with respect to control group; **$P < 0.05$ with respect to STZ group without ASA.

animal model because it reflects the symptoms of both insulin-dependent and non-insulin dependent diabetes. These animals show low endogenous production of insulin and high levels of circulating glucose [27].

Inhibition of the activity of several enzymes in vivo and in vitro in diabetic animals have been reported [28,29]. Studies carried out in a diabetic population have shown that the activities of several heme enzymes, such as ALA-D [11], deaminase and uroporphyrinogen decarboxylase, are diminished in blood [15]. Similar findings were also observed in STZ-induced diabetic mice [16] and rats [11]. In vivo insulin therapy of STZ-diabetic female rats antagonized the effect of the diabetic state on heme synthesis (evaluated through δ-aminolevulinic synthase and ALA-D activities) and on heme degradation (evaluated through the decrease of heme oxygenase activity) [30].

Previously, we investigated the effect of in vitro glycation on ALA-D under different experimental conditions and demonstrated that glucose and other sugars produced enzyme inactivation by glycation and that ASA was effective in preventing both Hb glycation and ALA-D inactivation [17]. We report here that diminution of ALA-D and PBG-D activities in STZ-induced diabetic mice could be prevented by either short or long term ASA treatment. As an additional support to the evidence presented in this study — that glycation of proteins is increased in STZ treated animals and subsequently attenuated by ASA — we demonstrated that both short and long aspirin treatments can partially prevent the formation of glycated hemoglobin.

Since ASA treated animals have lower blood glucose levels, less glycation of proteins can be expected. The 'normalising' effect on blood sugar levels seems to be the primary event, which explains the restoration of ALA-D and PBG-D activities. However, other aspirin effects can not be discarded. It is known that aminoguanidine is able to block the formation of AGEs, but it does not counteract the increase in blood glucose concentrations [31]. We have found that in STZ-induced diabetic mice treated with aminoguanidine (0.2% w/v in drinking water), the inhibition of ALA-D and PBG-D activities was partially prevented (unpublished results).

Acetylation by aspirin has been used as an inhibitor of glycation blocking potential glycation sites (ε-NH_2 groups) [5]. If glycation is considered a contributory factor, inhibition of glycation by acetylation should bring about the corresponding restoration in ALA-D and PBG-D activities. We have demonstrated that treatment with ASA in vivo was effective in preventing both enzymes inactivation observed in diabetic mice, confirming our previous results obtained in vitro [17].

Sajithlal et al. [13] have suggested that under oxidative conditions, glucose reacts with proteins to form potentially reactive end products. Once formed, these AGEs could induce crosslinking of collagen even in the absence of glucose and oxygen. Combination of glucose with transition metals might be necessary for the generation of free radicals and this process might contribute to the overall complications of diabetes [8].

Oxidative stress experimentally induced by STZ leads to a decrease in GSH levels [32]. This effect could be contributing to the diminution of ALA-D and PBG-D

activities in diabetic mice. Both enzymes have essential free sulphydryl groups at their active sites [33].

We have demonstrated herein the association between the diabetic state and lipid peroxidation enhancement. Again, ASA treatment was effective in preventing the increased oxidation state of diabetic animals. Mechanisms that contribute to increased oxidative stress in diabetes may include not only enhanced non enzymatic glycation and autoxidative glycosylation but also metabolic stress resulting from changes in the status of the antioxidant defense system [34]. We have evaluated the behaviour of catalase, one of the key antioxidant enzymes and found that its activity was significantly augmented in STZ diabetic animals and that aspirin only partially reverted these alterations when used for a long term period. In agreement with our findings, other authors have reported that catalase activity was increased in the liver of STZ-induced diabetic rats [35]. In the diabetic status, lipid peroxidation would increase as a consequence of the reaction of unsaturated lipids with different radicals and/or H_2O_2. The enhancement in catalase activity might be a primary defense response, acting as scavenger under the oxidative insult provoked by diabetes.

Aspirin might either inhibit enzymic lipid peroxidation or act as an oxygen radical scavenger [34], blocking the oxidative cellular injury related to the induction of diabetes and even preventing or ameliorating the chemical modification of proteins susceptible to the glycemic stress. These data reinforce the potential therapeutic role of aspirin in the treatment of diabetes. From a clinical perspective, these studies should continue to further evaluate the level of ASA toxicity after prolonged treatment and to better understand aspirin mechanism of action.

Although the data presented here are limited, the importance of these findings is to give evidence that ASA treatment in vivo could lead to an almost complete prevention of some of the secondary effects caused by STZ-induced diabetes mellitus.

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