

Investigation of the Mechanisms Involved in the High-Dose and Long-Term Acetyl Salicylic Acid Therapy of Type I Diabetic Rats

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ABSTRACT

Diabetes mellitus has been classified as a conformational disease because of changes induced in the structure and function of proteins due to hyperglycemia. In this study, we investigated the effect of high-dose and long-term use of acetyl salicylic acid (ASA) on the streptozotocin-induced diabetic rats as a model of type I diabetes, with consideration on the structure and/or function of proteins. The *N*-[methylnitrosocarbamoyl]-D-glucosamine (streptozotocin)-induced diabetic rats together with the normal rats were studied for 5 months with and without receiving 100 mg/kg ASA in drinking water. All rats were investigated from different aspects such as heat shock protein (HSP) 70 level, serum glucose and insulin concentration, advanced glycated end product (AGE) and glycated hemoglobin (HbA1c) formation, lipid profile, high-density lipoprotein (HDL) functionality (paraoxonase1 and lecithin cholesterol acyltransferase ac-

tivities), and the antioxidant system. In addition, the in vitro effect of ASA on the structure of albumin as a model protein was studied in the presence of glucose by spectroscopic techniques such as fluorometry and circular dichroism. The results show that ASA therapy causes a decrease in the glucose level and AGE and HbA1c formation, improves the lipid profile, HDL functionality, and the antioxidant capacity, induces serum HSP70, and overall decreases mortality of diabetic rats in comparison with the group without treatment. The conformation of glycated bovine serum albumin is different from the native form, and ASA retains the conformation of this protein similar to the native. The improving effect of ASA on diabetic rats is mostly due to its role as a chemopreventive agent in the structural conservation and protection of proteins involved in diabetes pathogenesis.

Chronic hyperglycemia increases the nonenzymatic glycation of inter- and intracellular proteins in diabetes mellitus. It is well established that the function of a protein depends on its native structure; because glycation can alter the structural and thus the functional integrity of a variety of proteins, this modification could be involved in the pathogenesis of some complications of diabetes (Brownlee, 1995).

Heat shock proteins (HSPs), also called stress proteins, are a highly diverse, evolutionarily conserved family of proteins containing both constitutively expressed and stress-inducible members. HSPs are up-regulated in response to thermal stress and a wide variety of other physical and chemical

insults. HSP70 is one of the major stress protein families found in a variety of organisms, and these molecules play central roles in protein folding as molecular chaperones (Craig et al., 1994). Treatments that induce HSPs have been associated with the development of a resistant state where the organism is protected from subsequent toxicity, suggesting that increased HSP expression constitutes an important cellular defense mechanism (Tolson and Roberts, 2005). Some studies have shown that expression of these chaperones that are required to maintain the integrity of protein structure is reduced during the course of diabetes, and such alterations may result in the onset of complications of diabetes (Kurucz et al., 2002; Atalay et al., 2004).

Due to the high possibility of proteins glycation and their structural changes, elevating chaperone capacity in diabetic patients is reasonable for protection of proteins against these changes and conservation of native structure. Therefore, ap-

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ABBREVIATIONS: HSP, heat shock protein; ASA, acetyl salicylic acid (aspirin); AGE, advanced glycated end product; HbA1c, glycated hemoglobin; LCAT, lecithin cholesterol acyltransferase; HDL-c, high-density lipoprotein-cholesterol; CD, circular dichroism; TC, total cholesterol; TG, triglyceride; LDL-c, low-density lipoprotein-cholesterol; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; FRAP, ferric-reducing ability of plasma; PON1, paraoxonase1; BSA, bovine serum albumin; STZ, *N*-[methylnitrosocarbamoyl]-D-glucosamine (streptozotocin); NSAID, nonsteroidal anti-inflammatory drug.

plying the process or compounds that elevate the level of chaperoning activity in the diabetic models may be a useful way for treatment of this conformational disease.

Acetyl salicylic acid (aspirin or ASA) is a nonsteroidal anti-inflammatory drug that has been in general use since the end of the last century. ASA is absorbed intact in the gastrointestinal tract. As the drug circulates in the plasma, it is readily hydrolyzed to salicylic acid and acetate ion (Kelly, 1970). After oral administration of aspirin in human, only 68% of the ingested dose reaches the systemic circulation as aspirin, where it is converted into salicylate, spontaneously or by nonspecific esterases. The half-life of aspirin has been reported to be approximately 12.6 and 20 min in rat and human, respectively, and 6 days in the *in vitro* condition (Bakar and Niazi, 1983; Jackson and Morrow, 2001). According to our previous study, pseudo-first order rate constant and the half-life of aspirin hydrolysis in 0.05 M Tris buffer, pH 7.4, were obtained at 0.0195 ± 0.0023 k/h and 35.79 ± 3.39 h, respectively (unpublished data).

Low-dose aspirin treatment due to block of thromboxane synthesis by acetylating platelet cyclooxygenase is a standard therapeutic approach in diabetes mellitus for primary and secondary prevention of long-term vascular complications (Colwell, 2004). In addition, multiple animal models and human studies have indicated the salutary effects of ASA as an anticataract (Rao et al., 1985; Shastri et al., 1998) and antiglycating agent in diabetes (Urios et al., 2007). On the other hand, it was reported that high-dose aspirin therapy before heat treatment results in induction of HSPs in the rat skin (Ghavami et al., 2002), but the effect of ASA as a chaperone inducer or as a protector of protein structure was not studied in diabetes mellitus.

Thus, we decided to examine whether high-dose and long-term use of aspirin would ameliorate the conformational changes of proteins and/or affect their activity in diabetic rats. We investigated the effect of ASA, administered in drinking water for 5 months on diabetic rats from different aspects such as inducing heat shock proteins (HSP70) and protective effect on protein structure and function and hence its effect on serum glucose and insulin level, AGE and glycated hemoglobin (HbA1c) formation, lipid profile, HDL functionality (paraoxonase1 and LCAT activities), and the status of the antioxidant defense system. In addition, the *in vitro* effect of ASA on the structure of albumin as a model protein was studied in the presence of glucose by spectroscopic techniques such as fluorometry and circular dichroism (CD).

Materials and Methods

Materials

Aspirin (acetylsalicylic acid), streptozotocin, and Fe II-tripyridyl-triazine [2,4,6-Tri(2-pyridyl)-s-triazine] were purchased from Sigma-Aldrich (Steinheim, Germany), Fluka (Steinheim, Switzerland), and Sigma Chemical Co. (St. Louis, MO), respectively. Unless otherwise stated, all other reagents were purchased from Merck Chemical Co. (Darmstadt, Germany).

In Vivo Studies

Design of *In Vivo* Study. Male Wistar rats, 8 weeks old and weighing 240 ± 20 g, were housed under controlled temperature conditions with a 12-h light/12-h dark cycle. After 3 weeks, they were divided into four groups of seven to nine animals. Two groups (named G2 and G4) received injections *i.p.* with streptozotocin (50

mg/kg body weight in Na^+ -citrate buffer, pH 4.5) (Mendez and Ramos, 1994). If the blood glucose level was <270 mg/dl after 4 days, the injection was repeated once, and thereafter only rats with blood glucose levels ≥ 270 mg/dl were included in our experiments. Control rats (groups G1 and G3) received injections with vehicle alone. The experimental protocol was approved by the Animal Ethical Committee in accordance with the guidelines for the care and use of laboratory animals prepared by Tarbiat Modares University.

The treatment of diabetic and healthy groups (groups G4 and G3) with 100 mg/kg ASA in drinking water began after 1 week (time 0 in the figures and tables). The dose of aspirin was chosen for its ability to induce HSP in rats (Ghavami et al., 2002), and the drinking water was replaced two times a day. The study continued up to the end of month 5. After that, all animals were killed, and their organs were stored at -70°C for further studies.

Blood samples were collected from the orbit vein every 30 days for 5 months. EDTA-treated whole-blood samples were saved for HbA1c determination, and sera samples, prepared by 15-min centrifugation of blood at 5000g to separate clot, were stored at -70°C for further studies. Urine samples collected through metabolic cages every 30 days for 5 months. For this purpose, rats were kept in separate metabolic cages for 6 h.

Determination of Fasting Serum Glucose, Lipid Profile, and Insulin Levels. Sera glucose, total cholesterol (TC), and triglyceride (TG) levels were measured by enzymatic colorimetric methods (Pars Azmune, Tehran, Iran), using AutoAnalyzer Model Selectera 2. After precipitation of non-HDL-c lipoproteins by phosphotungstic acid and magnesium chloride in the sera, HDL-c was determined by an enzymatic colorimetric method (Pars Azmune). LDL-c was calculated according to the Friedewald formula for less than 400 mg/dl TG-containing samples (Friedewald et al., 1972). The Rat Insulin ELISA kit (Mercodia Corporation, Uppsala, Sweden) was used to measure serum insulin level.

HbA1c Measurement and AGE and FRAP Assay. HbA1c was measured by ion exchange chromatography method (Biosystems S.A., Barcelona, Spain). AGE determination was performed according to the method of Kalousová et al. (2002). Blood serum was diluted 1:50 with PBS, pH 7.4, and fluorescence intensity was recorded at the emission maximum (440 nm) upon excitation at 350 nm using the spectrofluorometer Shimadzu, model RF 5000 (Shimadzu, Kyoto, Japan). Fluorescence intensity was expressed as percentage of fluorescent emission.

The reducing ability of biological samples was determined by the FRAP assay of Benzie and Stain (1996). FRAP assay measures the change in the absorbance of FRAP reagent at 593 nm (Spectrophotometer Shimadzu, model 3101) due to the formation of a blue-colored Fe II-tripyridyltriazine complex from colorless oxidized Fe III formed by the action of electron-donating antioxidants in the serum.

Microalbuminuria Determination. Albumin content of urine samples was determined by ELISA kit (Orgentec, Mainz, Germany).

Determination of Serum Hsp70 Level. Serum Hsp70 was measured using a commercially available ELISA kit (StressGen Biotechnologies, Victoria, BC, Canada). The standard curve was plotted between 0.78 and 50 ng/ml of protein in the solution. The sensitivity of the assay was 0.2 ng/ml. The concentrations of the Hsp70 protein in the serum were determined using the standard curve.

Paraoxonase1 and LCAT Assay. Paraoxonase1 (PON1) activity was determined using paraoxon (*O,O*-diethyl-*O-p*-nitrophenylphosphate; Sigma Chemical Co.) as the substrate and measured by the increase in the absorbance at 412 nm due to the formation of 4-nitrophenol (Ferretti et al., 2001). In brief, the activity was measured at 25°C , by adding 50 μl of serum to 1 ml of Tris-HCl buffer (100 mM, pH 8.0) containing 2 mM CaCl_2 and 5.5 mM paraoxon. The rate of generation of 4-nitrophenol was determined at 412 nm by using a UV-visible spectrophotometer. Enzymatic activity was calculated using the molar extinction coefficient of substrate ($17,100 \text{ M}^{-1} \text{ cm}^{-1}$). LCAT activity was determined by a commercially available

LCAT kit (Roar Biomedical, Inc., New York, NY) based on the fluorescence decrement of the hydrolyzing substrate.

Statistical Analyses. All data were expressed as mean \pm S.D. Statistical comparisons were performed by one-way analysis of variance with least significant difference multiple comparison post-test analysis. Significance was set at $p < 0.05$.

In Vitro Studies

Glycation of BSA. The stock solution of BSA was prepared by dissolving it in PBS, pH 7.4. This solution was subsequently diluted with glucose solution made in the same buffer to form duplicate incubation mixtures of 10 mg/ml protein with 50 mM glucose in the absence and presence 20 mM ASA (Shaklai et al., 1984). After being sterilized by filtration (0.22- μ m filters; Millipore Corporation, Billerica, MA), the solutions were incubated at 37°C for 60 days in capped vials. Aliquots were collected every week, and unbound glucose and ASA were removed from BSA solution by extensive dialysis against PBS, pH 7.4, and stored at -80°C until they were analyzed by fluorometry and CD.

Fluorometry and CD Analyses. Production of fluorescent compounds or glycoporphs was monitored in samples with a concentration of 0.5 mg/ml BSA using a spectrofluorometer (Shimadzu, RF-5000) at excitation and emission wavelengths of 350 and 425 nm, respectively (Ogino and Okada, 1995). CD spectra were measured in a JASCO-810 spectropolarimeter (Jasco, Tokyo, Japan). The samples with a concentration of 0.1 mg/ml BSA were used for secondary structure analysis. The spectra have been smoothed and are presented in units of mean residue molar ellipticity. All measurements were performed at 25°C. The helicity of BSA estimated by using the simple assumption that θ_{222} for 100% helix is given by $40,000 \times [(n - 4)/n]$, where n is the number of residues (Jackson et al., 1991), which is 585 in this case.

Results

In Vivo Studies. Figure 1, A to C, shows the changes in the weight, glucose, and AGE in the serum of rats. The weights of two diabetic groups were significantly ($p < 0.01$) lower than those of the two normal groups, and ASA alone had no effects on this parameter (Fig. 1A). As seen in the Fig. 1B, the glucose level in the sera of both *N*-[methyl nitrosocarbamoyl]-D-glucosamine (streptozotocin; STZ)-treated groups is higher than that in the normal group. A slow decrease was observed in the glucose concentration of diabetic groups under treatment with ASA, but these are not significant. Average serum glucose levels during 5 months of experiment in groups 2 and 4 are 352.2 ± 27.4 and 329.5 ± 23.1 mg/dl, respectively. Figure 1C indicates that the AGE formation in the diabetic groups increased gradually; however, in the diabetic group under treatment with ASA, it is near the normal. The significant differences are observed ($p < 0.001$) between the diabetic group and other three groups at month 5.

Table 1 shows the HbA1c and serum insulin levels in rats during the course of the study. The percentage of HbA1c in diabetic rats treated with ASA decreased significantly in comparison with diabetic rats without treatment ($p < 0.05$). Serum insulin level also is seen in Table 1 and indicates a nonsignificant increase in the decreased insulin level due to the ASA treatment in diabetic rats.

In the study of lipid profile, our results (Fig. 2, A–D) show that the TG, TC, and LDL-c levels increases significantly in diabetic rats. However, these parameters significantly decreased under the effect of ASA administration during the course of the experiment. In addition, the HDL-c that de-

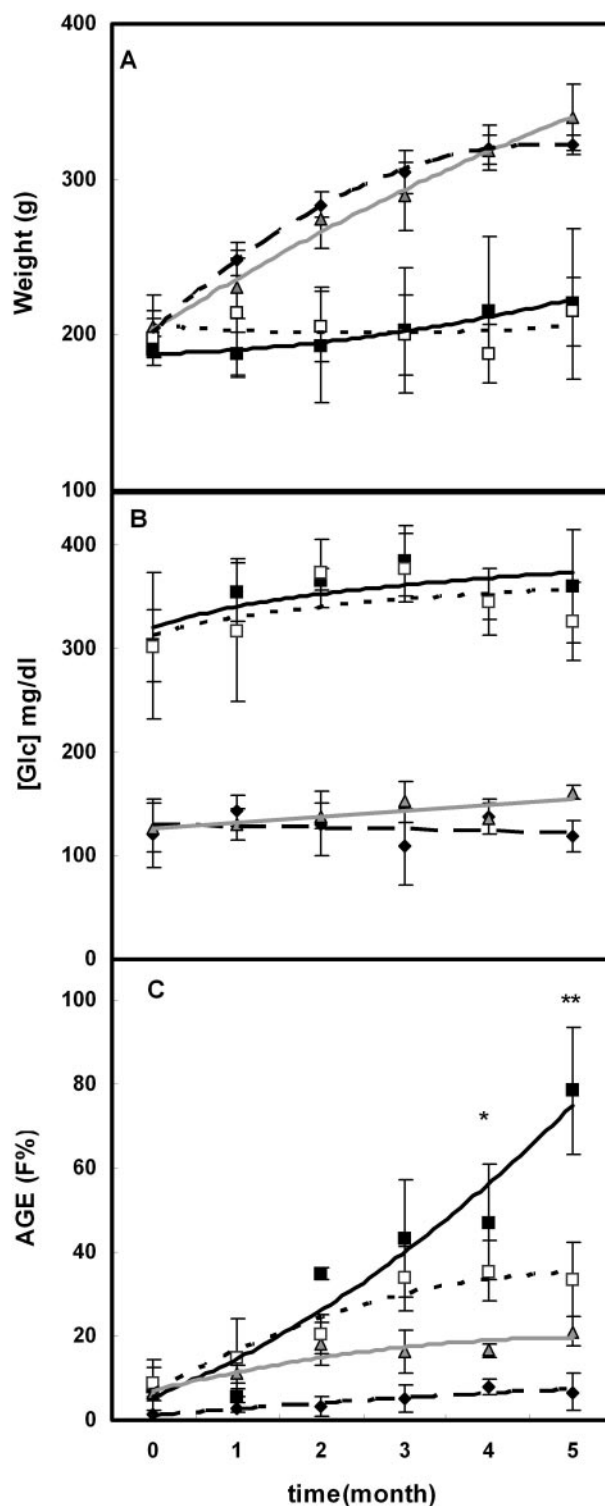


Fig. 1. Changes in the weight (A), glucose concentration (B), and fluorescence intensity (F%) of AGEs (C) during the time course of experiment in different groups of rats. In all figures: healthy rats, filled diamond (dashed line); healthy rats received acetyl salicylic acid, triangle (gray line); diabetic group, filled square (black line); and diabetic group received acetyl salicylic acid, open square (dotted line). Data are means \pm S.D. ($n = 5-7$). *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$, significant changes between two diabetic groups with or without acetyl salicylic acid administration.

creased significantly after diabetes induction significantly increased in the diabetic group treated with ASA.

Table 2 shows the activity of paraoxonase1 and LCAT

TABLE 1

Levels of glycated hemoglobin and serum insulin (%) at first (0) and after 2 and 4 months of STZ treatment

Group No.	Group Name	HbA1c			Serum Insulin*		
		0	2	4	0	2	4
			%			pM	
1	Healthy rats (mean \pm S.D.)	6.9 \pm 2.4	6.3 \pm 0.2	6.9 \pm 0.7	111.9 \pm 41.2	164.9 \pm 105.8	144.0 \pm 72.4
2	Diabetic rats (mean \pm S.D.)	6.7 \pm 1.6	7.5 \pm 2.2	7.8 \pm 0.9	37.6 \pm 20.2†	54.7 \pm 40.7†	54.8 \pm 52.8†
3	Healthy rats with ASA (mean \pm S.D.)	6.9 \pm 2.4	6.2 \pm 1.6	5.5 \pm 0.1	111.9 \pm 41.2	177.2 \pm 76.9	162.6 \pm 124.8
4	Diabetic rats with ASA (mean \pm S.D.)	6.7 \pm 1.6	7.9 \pm 1.5	6.6 \pm 0.1‡	37.6 \pm 20.2	87.4 \pm 38.0	84.0 \pm 39.3

* Significance of data comparing group 1 vs. all groups ($p < 0.05$).‡ Group 2 vs. groups of ASA-treated rats ($p < 0.05$).

* Picomolar.

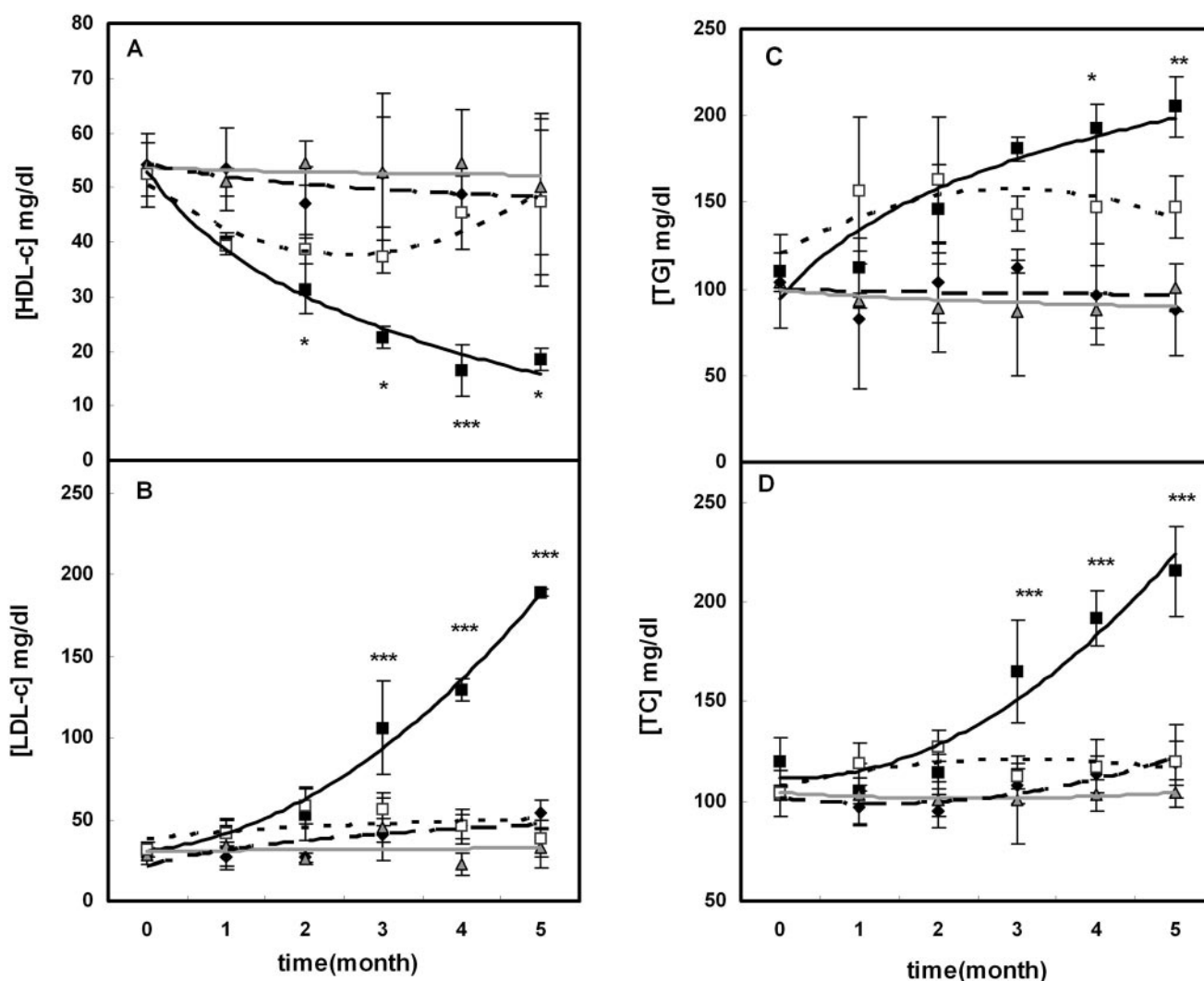


Fig. 2. Changes in the HDL-c (A), LDL-c (B), TG (C), and TC (D) during the time course of experiment in different groups of rats. In all figures: healthy rats, filled diamond (dashed line); healthy rats received acetyl salicylic acid, triangle (gray line); diabetic group, filled square (black line); and diabetic group received acetyl salicylic acid, open square (dotted line). Data are means \pm S.D. ($n = 5-7$). *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$, significant changes between two diabetic groups with or without acetyl salicylic acid administration.

during the experimental course. As illustrated in this table, the activity of these enzymes decreased due to the diabetes induction. The decreased paraoxonase1 activity was significantly ($p < 0.05$) compensated by ASA 2 months after the beginning of the experiment. Meanwhile, the improvement effect of ASA on LCAT activity was not significant.

The results in the Fig. 3 indicate that FRAP decreases gradually in the diabetic group in comparison with the control group and reaches the minimum at month 5 of experi-

ment ($p < 0.05$). Nevertheless, ASA suppresses the decreased amount in the diabetic group and retains the values near to the normal.

Table 3 shows that HSP70 levels gradually decreased in the serum of diabetic rats. The data show the significant increase of Hsp70 in the diabetic rat under treatment with ASA, after 4 months, in comparison with diabetic rats without treatment ($p < 0.05$).

Renal excretion of Alb (microalbuminuria), as an index of

TABLE 2

Paraoxonase1 and LCAT activity in different groups of rats at first (0) and after 2 and 4 months of STZ treatment

Group No.	Group Name	Paraoxonase1 Activity			LCAT Activity		
		0	2	4	0	2	4
		μM			$\mu\text{M}/\text{h}$		
1	Healthy rats (mean \pm S.D.)	165.3 \pm 33.5	189.3 \pm 13.2	188.5 \pm 20.5	81.6 \pm 10.8	84.0 \pm 7.2	85.2 \pm 3.0
2	Diabetic rats (mean \pm S.D.)	114.0 \pm 26.0	42.0 \pm 16.9*	56.5 \pm 14.4*	70.8 \pm 10.2*	61.2 \pm 18.6*	55.8 \pm 6.6*
3	Healthy rats with ASA (mean \pm S.D.)	165.3 \pm 33.5*†	181.0 \pm 46.1†	160.3 \pm 36.9	81.6 \pm 10.8†	85.0 \pm 11.4†	78.6 \pm 17.1†
4	Diabetic rats with ASA (mean \pm S.D.)	114.0 \pm 26.0	100.4 \pm 22.8*†	99.5 \pm 15.0*†	71.0 \pm 10.7*	71.4 \pm 10.8	64.9 \pm 8.2*

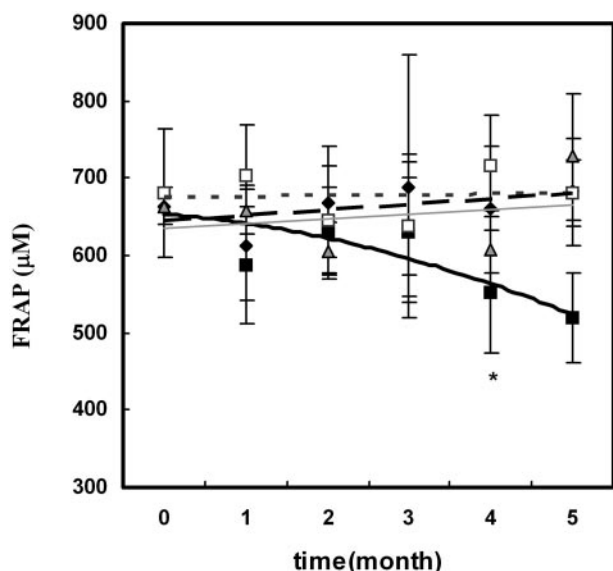
* Significances of data comparing group 1 vs. all groups ($p < 0.05$).† Group 2 vs. groups of ASA-treated rats ($p < 0.05$).

Fig. 3. Changes of the FRAP during the time course of experiment in different groups of rats. In this figure: healthy rats, filled diamond (dashed line); healthy rats received acetyl salicylic acid, triangle (gray line); diabetic group, filled square (black line); and diabetic group received acetyl salicylic acid, open square (dotted line). Data are means \pm S.D. ($n = 5-7$). *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$, significant changes between two diabetic groups with or without acetyl salicylic acid administration.

kidney function, was determined in the urine of rats. Table 4 shows a significant ($p < 0.001$) increase in this parameter after the 4th month of the experiment in the diabetic group, but no significant changes were observed in the diabetic group under treatment with ASA. No evidence of gross anatomical pathology, stomach hemorrhages, or ulceration was observed in both aspirin-treated groups, except one tumor, which was diagnosed as fibrosarcoma, in one of the animals in the control group receiving aspirin.

In Vitro Studies. The structural changes of BSA were studied as a model protein due to the high concentrations of glucose in the absence and presence of ASA. Figure 4A shows that incubation of BSA with 50 mM glucose results in a progressive increase in the fluorescence emission of glycoproteins from the 4th week of the beginning of the experiment, whereas in the presence of ASA, there is not a considerable change in fluorescence emission in comparison with the native BSA. The similar results were observed at 100 mM glucose.

The secondary structures of protein were also investigated by circular dichroism. As seen in the Fig. 4B, the molar ellipticity of BSA in the far-UV region (continuous line) changes due to the incubation with 50 mM glucose (dashed line), whereas the induced CD changes by glucose returned to the normal

value due to the presence of ASA in the solution (dotted line), and it seems similar to the unmodified protein. The helicity was 47% for BSA, 26% for BSA + Glc, and 48% for BSA + Glc + ASA in 0.1 M PBS, pH 7.4.

Discussion

There are clinical evidences that a reduction in the protein glycation and maintenance of protein structure can significantly lower the risk of diabetic complications (Harding and Ganea, 2006). Hence, attempts have been made to pharmacologically prevent or slow down the glycation of proteins and control the pathogenesis of the disease.

ASA, which was synthesized as a painkiller and antifever drug more than 100 years ago, has been applied for various therapeutic applications. This multifunctional drug affects different systems and metabolic pathways in the body with various mechanisms. The present study investigated these mechanisms in diabetic rats.

Figure 1B indicates a slight decrease in the serum glucose level of diabetic rats after ASA treatment. The hypoglycemic effect of ASA through the decrease in the intestinal absorption of glucose (Arvanitakis et al., 1977), reduction in the hepatic gluconeogenesis (Hundal et al., 2002), and the decrease in the insulin clearance (Hundal et al., 2002) has been reported previously in human subjects, normal or insulin resistant. However, it was pointed out that nonsteroidal anti-inflammatory drugs should not be considered as therapy for hyperglycemia (Mork and Robertson, 1983).

Our results also indicated (Table 1) a slight and nonsignificant increase in the serum insulin level due to the ASA administration to diabetic rats. As seen in Fig. 1B, there is not any increase in the weight of diabetic rats due to ASA treatment. It shows that a low increase in insulin level is not enough, not only for a significant reduction of glucose, but also for activation of the biosynthetic pathways.

ASA has been known as an inhibitor of glycation through acetylation of ϵ -NH₂ groups of lysine (Rao et al., 1985; Urios et al., 2007). Thus, the decreased levels of AGEs (Fig. 1C) and HbA1c formation (Table 1) in diabetic rats treated with ASA in comparison with the untreated group may be explained by the mentioned mechanism. In addition, a decrease in serum glucose level can be another reason for reduced AGEs and HbA1c. Similar changes in the AGEs (Urios et al., 2007) and HbA1c (Lapshina et al., 2006) have been reported elsewhere.

The in vitro data in Fig. 4 also confirm our in vivo results. The fluorescence intensity changes in the presence of glucose alone show a gradual increase up to the 2nd month of experiment, which indicate the formation of AGE compounds. However, this figure indicates that ASA prevents the forma-

TABLE 3

Serum HSP70 level at the beginning and after 2 and 4 months of the experiment

Group No.	Group Name	Serum HSP70		
		0	2	4
			<i>ng/ml</i>	
1	Healthy rats (mean \pm S.D.)	18.1 \pm 2.9	18.9 \pm 2.5	19.4 \pm 0.9
2	Diabetic rats (mean \pm S.D.)	22.6 \pm 8.8	15.0 \pm 6.0	15.2 \pm 5.5
3	Healthy rats with ASA (mean \pm S.D.)	18.1 \pm 2.9	19.1 \pm 5.6	20.9 \pm 4.8
4	Diabetic rats with ASA (mean \pm S.D.)	22.6 \pm 8.8	24.2 \pm 10.9	24.7 \pm 5.5*

* Significances of data comparing group 2 vs. groups of ASA-treated rats, all groups ($p < 0.05$).

TABLE 4

Levels of microalbuminuria after 2 and 4 months of experiment

Group No.	Group Name	Microalbuminuria		
		0	2	4
			<i>μg/ml</i>	
1	Healthy rats (mean \pm S.D.)	4.0 \pm 1.5	1.9 \pm 0.2	4.0 \pm 1.1
2	Diabetic rats (mean \pm S.D.)	12.5 \pm 5.4*	19.1 \pm 3.2*	32.0 \pm 5.6*
3	Healthy rats with acetyl salicylic acid (mean \pm S.D.)	4.0 \pm 1.5†	1.0 \pm 0.5†	4.0 \pm 1.1†
4	Diabetic rats with acetyl salicylic acid (mean \pm S.D.)	12.5 \pm 5.4*	25.3 \pm 6.8*	38.4 \pm 2.8*

* Significance of data comparing group 1 vs. all groups ($p < 0.05$).† Group 2 vs. groups of acetyl salicylic acid-treated rats ($p < 0.05$).

tion of these glycoproteins, and the fluorescence intensity of samples remains on the basal level. The data on Fig. 4 also show that the BSA conformation maintains, against structural changes due to the glycation, in the presence of ASA. The CD spectra of BSA in the presence of glucose indicated that the α -helical content of protein reduced significantly, but ASA conserved the secondary structure as the native one. It has been reported previously that serum albumin is acetylated in the presence of ASA (Hawkins et al., 1969). Our data confirm that ASA has a protective role on protein structure against nonenzymatic glycation and its subsequent events.

Hyperlipidemia independently increases the risk of cardiovascular mortality among patients with diabetes mellitus. Our results show a significant reduction in the levels of TC, TG, and LDL-c and a significant elevation in HDL-c in the diabetic group treated with ASA in comparison with diabetic rats without treatment (Fig. 2). These findings are in agreement with the results obtained in human subjects with type II diabetes (Hundal et al., 2002), in hypercholesterolemic rats (Tauseef et al., 2007), and in normal rats (Nakagawa et al., 1984). The direct antilipolytic effect on adipocytes, which reduced plasma free fatty acids, has been suggested as one of the mechanisms involved for these findings (Hundal et al., 2002).

As mentioned above, ASA can prevent protein glycation and hence conserve the protein structure and function. Thus, we investigated the activity of two important enzymes related to the HDL functionality. Our results show that the decreased activity of LCAT (Fournier et al., 1995) and paraoxonase1 (Mackness et al., 2002) due to diabetes induction improves significantly by ASA. An increase in the LCAT activity of serum due to the oral administration of ASA or salicylic acid, at a short-term study (3 days), has previously been reported (Nakagawa et al., 1984). It has also been shown that aspirin administration is associated with higher serum concentration and activity of PON1 in normal subjects (Blatter-Garin et al., 2003). Because PON1 is known as a powerful antioxidant system in plasma that delays the LDL oxidation, it prevents atherosclerosis, so the increase in its

functionality has a key role in cardioprotection. In addition, the antioxidant property of salicylic acid, as a main metabolite of ASA, has been reported (Sagone and Husney, 1987). It was also shown that PON1 can use ASA as a substrate to produce more salicylate ions (Santanam and Parthasarathy 2007). Thus, by using ASA, the antioxidant capacity, which decreased in diabetic patients, must be compensated. This assumption is confirmed not only by the observed increase in PON1 activity in diabetic rats but also by our data about FRAP assay. Figure 3 shows that the antioxidant capacity of plasma increased due to ASA administration to diabetic rats, in comparison with the untreated rats.

There are a few reports about the effect of ASA or SA on HSP70 level. Activation of the heat shock factor binding to heat shock elements in DNA by sodium salicylate has been reported in the HeLa cells (Jurivich et al., 1992). It has also been reported that short-term use (30 min) of aspirin has no significant effect on the HSP70 induction in the rat liver, kidney, and lung (Fawcett et al., 1997). However, the induction of HSP70 in the rat skin, due to the high-dose and short-term administration of ASA before heat treatment, has been shown (Ghavami et al., 2002). In a recent study, it has been demonstrated that ASA and some other nonsteroidal anti-inflammatory drugs (NSAIDs) cause the induction of HSP70 in mast cells (Mortaz et al., 2006a,b). Diabetes causes a reduction in serum HSP70 level (Table 3) due to glycation, misfolding, and inactivation of this protein (data not shown), which leads to removing it from the circulation or even the inability of monoclonal antibody to detect it. For the first time, we investigated the effect of high-dose and long-term use of ASA on serum HSP70 in diabetic and normal rats. Table 3 shows the increase in the serum HSP due to ASA administration in both groups. However, these increases are not significant in the normal group and significant in the diabetic group ($p < 0.05$) in comparison with the untreated groups. ASA induces the HSP70 in both groups receiving it. Thus, it can affect the protein stability and folding patterns.

In addition, ASA and some other NSAIDs inhibit the release of proinflammatory cytokines through inhibition of sig-

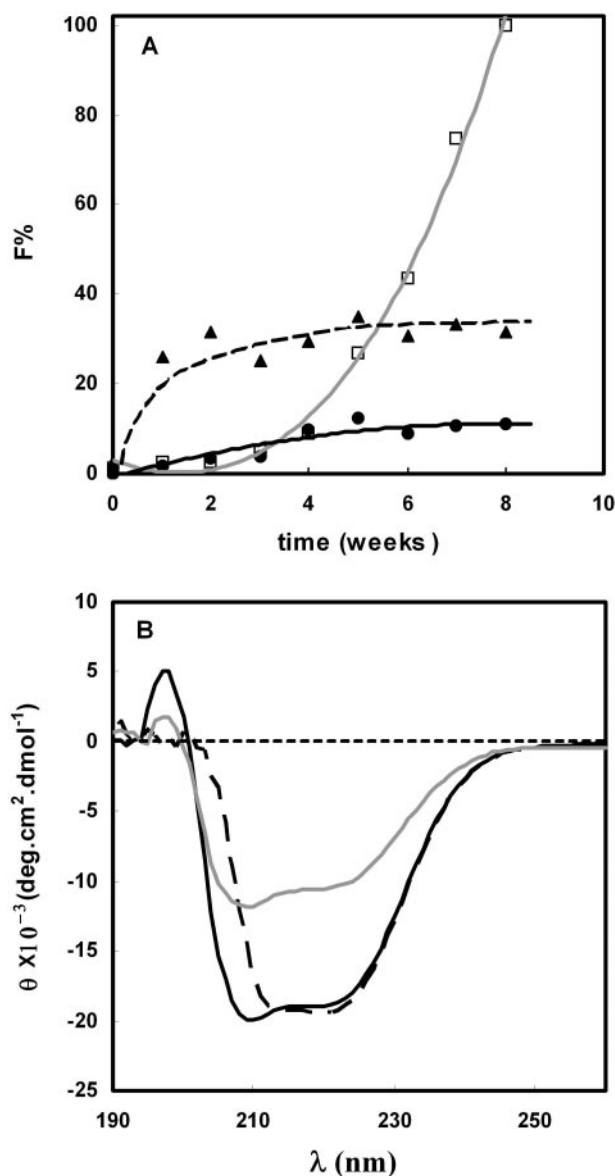


Fig. 4. A, fluorescence intensity of BSA alone (filled diamond, black line), BSA in the presence of 50 mM glucose with (open square, gray line) and without (filled triangle, dashed line) acetyl salicylic acid. B, CD spectra of BSA in far-UV region in 100 mM phosphate buffer, pH 7.4 (black line), in the presence of 50 mM glucose (gray line) and in the presence of both glucose and acetyl salicylic acid (dashed line) after 2 months of incubation at 37°C.

naling pathways, including the nuclear factor κ B and the mitogen-activated protein/p38 pathways (Mortaz et al., 2006a). This novel finding, which has been explained as a cyclooxygenase-independent anti-inflammatory effect of NSAIDs (Mortaz et al., 2006a), may be used as an affirmative reason for administration of ASA as an antidiabetic drug.

The accumulation of AGEs on collagen may cause the loss of glomerular function (Monnier et al., 1992). Aspirin has been inhibited by the formation of pentosidine, as an AGE product, in collagen incubated with glucose in an in vitro study (Urios et al., 2007). We assume that if the same mechanism happens in vivo, the improvement effect must be seen in the microalbuminuria. However, as Table 4 reveals, aspirin does not show such an effect. These findings are in agree-

ment with those obtained by others (Kern and Engerman, 2001).

Aspirin at 100 mg/kg is an anti-inflammatory and prostaglandin inhibitor in rodents, similar to a single 1-g dose in man. However, the cardioprotective doses of aspirin in human are 75 to 150 mg (approximately 1–10 mg/kg in rodents). In contrast to the adverse effects (gastrointestinal and kidney disorders) that have been reported for such high doses in human, in the present study, we do not see any side effects on rats. In addition, diabetic rats that received ASA in comparison with a group without it had a 40% reduction in mortality. This is an important finding that is the result of the beneficial effects of ASA such as prevention of glycated protein formation, lipid profile and HDL functionality, antioxidant capacity, HSP induction, and, in summary, protein structure conservation and protection. Would the same effects be obtained with a correspondingly lower dose in human? It is an important question that must be answered in the future.

Finally, the following reasons are suggested for the usefulness of ASA in diabetes: These explanations were confirmed by our in vitro results. ASA is one of the agents that can prevent in vitro and in vivo glycation of proteins by different mechanisms, including the following: 1) Hsp70 induction, 2) an increase in the antioxidant activity, 3) chemical competition with protein glycation through acetylation of free amino groups in proteins, and 4) chemical chaperone-like activity to protect the structure and function of the proteins, including that involved in the glucose and lipid metabolism.

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