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Streptozotocin-Induced Pro-Inflammatory Response in Rat Blood and its Attenuation by External Albumin Administration

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Abstract : Previous report has suggested that the albumin levels were reduced in the peripheral blood mononuclear cells (PBMCs) and consequently oxidative stress was elevated in streptozotocin (STZ)-induced diabetic rats as albumin is the predominant antioxidant in plasma. In this study, we suggest that the levels of pro-inflammatory cytokine such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were increased by approximately 3.4- and 2.9-fold, respectively, in the serum of STZ-diabetic rats, compared to those of normal rat. In addition to the cytokines, the levels of C-reactive protein (CRP) were also about 3.6-fold higher, indicating that STZ induced a pro-inflammatory response in rat blood. However, when purified rat albumin was externally co-administrated with STZ through the tail vein, the serum levels of IL-6, TNF-α, and CRP were markedly reduced, although the values were still higher than those of normal (non-diabetic) rats. Albumin administration also decreased STZ-induced oxidative stress in serum and PBMCs. Moreover, the decrease in cytokine and CRP levels was dependent on the dose of injected albumin. These results suggest that STZ-induced pro-inflammation and oxidative stress in rat blood might be attenuated by treatment with exogenous albumin.

Key words **:** albumin, streptozotocin, diabetes, reactive oxygen species, pro-inflammation, PBMC.

Introduction

Streptozotocin (STZ) has been used to induce type 1 diabetes mellitus (DM) in animal models. It has been suggested that hyperglycemia, auto-oxidation of glycated proteins, and increased production of reactive oxygen species (ROS) lead to increased oxidative stress In STZ treated rats, that is accompanied by pancreatic β-cell damage (5). Moreover, STZ has been shown to deplete antioxidant pools in cells, making them more susceptible to oxidative damage (9).

Inflammation is an exceedingly a complex protective response against noxious stimuli such as infection and cell injury, and is considered as a mechanism of innate immunity (21). Inflammatory response also comprises of various molecular mediators, endo- and exogenous inducers, and tissues and cellular effectors that coordinately participate in the inflammatory pathway (10). The mediators can be classified into several groups according to their biochemical properties: vasoactive amines, complements, lipid mediators, cytokines and chemokines. Reactive oxygen species (ROS) and reactive nitrogen species commonly function in the inflammation process to successfully eliminate infectious agents (11). However, host cells are also inevitably damaged by these species and therefore repair responses (or healing stage) and anti-inflammatory phase follow the pro-inflammatory reaction.

C-reactive protein (CRP) is an inflammatory plasma protein that is synthesized primarily in liver hepatocytes and also in smooth muscle cells, macrophages, endothelial cells, and lymphocytes (20). CRP levels in serum dramatically increase in response to injury, infection, and inflammatory conditions. Thus CRP is classified as an acute marker of inflammation (6). CRP plays important roles in inflammatory process that include the complement pathway, phagocytosis, nitric oxide release, and the cytokine production, to eliminate infection agents. Increased level of CRP links with pathophysiology of many diseases such as cardiovascular disease, appendicitis, pancreatitis, meningitis, and type 2 diabetes (1).

Albumin is the most abundant circulating protein present in plasma, accounting for approximately 60% of total plasma proteins. Albumin has several important physiological and pharmacological functions including the transport of metals, fatty acids, cholesterol, bile pigments, and drugs. In general, albumin is also a predominant antioxidant in plasma, a body compartment that is continuously exposed to oxidative stress (17). Previous studies have shown that more than 70% of the free radical-trapping activity of serum attributed to serum albumin (3). The implication of this function is that hypoalbuminemia patients have reduced potential for scavenging of oxygen free radicals (12).

In this study, we investigated STZ-induced pathological changes in rat blood, particularly pro-inflammatory response. We further studied the effect of exogenously administrated albumin in alleviating the symptoms of the STZ-induced pathology.

Experimental Animals

Six-week-old male Sprague Dawley rats weighting 200-

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250 grams were obtained from Samtako Bio Korea (Osan, South Korea) and used for inducing type I DM, after one week of quarantine and acclimation. The rats were housed individually in stainless steel cages in a room with a controlled temperature of 23 ± 2°C and lighting (alternating 12 h period of light and dark). They were fed a commercial rodent chow (Samyang Feed, Wonju, South Korea). The experimental design was approved by the committee for the care and use of laboratory animals at the Chonnam National University (CNU IACUC-YB-2014-7).

Induction of DM and albumin administration

Diabetes was induced by a single intraperitoneal injection of STZ (100 mg/kg body weight in 0.1 M citrate buffer). Rats in the non-diabetic group (control) were injected with equivalent volume of 0.1 M citrate buffer only. One week after the injection, severity of the induced diabetic state was assessed by monitoring blood glucose levels using reagent strips (ACCUTREND, Roche Diagnostics GmbH, Germany). Rats with blood glucose levels exceeding 300 mg/dl were classified as diabetic rats. Rat bloods were collected from the caudal vena cava. Purified rat serum albumin (Abcam, Cambridge, UK) was dissolved in sterile phosphate buffered saline (PBS) and co-administrated with STZ to rats through the tail vein.

Blood biochemistry

Blood samples from the caudal vena cava were centrifuged to obtain hemolysis-free clear serum. The amounts of total protein (TP) and albumin (ALB) in the serum were assayed using an autoanalyzer (Dir-chem 4000i, Fujifilm, Japan) by standard methods. The levels of IL-6 and TNF-α in serum were measured using dedicated ELISA assay kits (Sigma-Aldrich, St. Louis, MO, USA). The levels of serum CRP and albumin bound to peripheral blood mononuclear cell (PBMC-albumin) were analyzed using dedicated ELISA kits (Abcam).

Isolation of PBMCs

PBMCs were prepared from whole blood using Ficoll-Paque PLUS (GE Healthcare Biosciences), as described previously (15). Briefly, EDTA-treated blood was diluted 1:1 with PBS containing 2% fetal bovine serum, and layered onto Ficoll-Paque PLUS with a ratio of blood plus PBS:Ficoll (2:1 ratio of blood + PBS:Ficoll). The blood was centrifuged at 400 ×*g* for 40 min at 25°C. The buffy coat layer was removed and followed by washing steps. The cells were suspended in a balanced salt solution (provided by the manufacturer) and then the sample was centrifuged at 100 ×*g* for 10 min at room temperature. The pellet was collected after removal of supernatant and this washing step was repeated twice. The isolation of PBMC from rat blood was verified by flow cytometry analysis using anti-pan B-cells antibody (clone number of 68-IB3, Santa Cruz, Dallas, TX, USA).

Fractionation of cells and measurement of oxidative stress

The cytosolic and microsomal fractions were obtained using a previously described method (22). The amount of hydrogen peroxide (H_2O_2) in serum was measured spectrofluorimetrically using the Amplex® Red (AR) assay kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Emission fluorescence for the AR assay was recorded at 585 nm with an excitation wavelength of 571 nm. Lipid peroxidation (LPO) in the membranes of PBMCs was estimated by measuring thiobarbituric acid-reactive substances, using malondialdehyde as a standard, as described previously (14).

Other methods

The concentration of live PBMCs was assayed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (Sigma-Aldrich) in accordance with the manufacturer's instructions. Briefly, after isolation of PBMC from 1 mL blood, MTT solution was added to the cells in 96-well plates. After the incubation of the plates at 37°C for 3 h, the required volume of solubilizing solution was added and the absorbance at 590 nm was measured. Statistical data were expressed as the mean ± standard deviations (S.D.) of at least five samples. One-way ANOVA computed by SPSS version 19.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis and a p-value < 0.05 was considered significant.

Results and Discussion

It has been shown previously that STZ causes a decrease in PBMC-albumin (cell surface-bound albumin) levels, and subsequently stimulated the generatin of oxidative stress in PBMCs (15). In this study, we further explored the effect of STZ treatment alone and in combination with external albumin on the physiology of rat blood, particularly concentrating inflammatory response.

We first confirmed the previous studies by analyzing the biochemical parameters of blood, including oxidative stress in the serum of STZ-induced diabetic rats. As shown in Table

Table 1. Biochemical parameters of serum and PBMCs obtained from normal (Control) and STZ-induced diabetic (STZ) rats

	TP (g/dL)	ALB (g/dL)	H_2O_2 (mM)
Control	4.33 ± 0.45	2.82 ± 0.21	1.64 ± 0.54
STZ	4.04 ± 0.38	2.40 ± 0.25	2.96 ± 0.71
	ALB/P	H_2O_2/P (pmol/mg protein)	LPO/P (nmol/mg protein)
Control	1	2.92 ± 0.54	5.44 ± 1.08
STZ	0.72 ± 0.08	6.43 ± 1.13	8.75 ± 1.46

ALB/P, H_2O_2/P, and LPO/P represent the levels of albumin, hydrogen peroxide (H_2O_2), and lipid peroxidation (LPO) in PBMCs, respectively.
ALB/P (cell surface-bound albumin) was expressed as relative degree compared to control, which was set to one.

1, the levels of total protein (TP) and albumin in serum, and PBMC-albumin were reduced by approximately 6%, 15%, and 28%, respectively, in STZ-diabetic rats, compared to non-diabetic control rats. In contrast, the amounts of H_2O_2 in serum increased by approximately 1.8-fold compared to those of the control. Especially, these results paralleled the previous suggestions that serum albumin was vulnerable to ROS generated during type 1 DM (16). STZ treatment also stimulated the production of H_2O_2 and LPO in PBMCs by approximately 2.2- and 1.6-fold, respectively (Table 1). These results confirmed the previous report and implied that STZ administration was implicated in enhanced generation of oxidative stress and decreased albumin levels in both serum and PBMCs. This deduction is also supported by the results that while serum albumin acted as an anti-oxidant, such as radical-trapping, STZ depleted the anti-oxidant pool in cells, making them more susceptible to oxidative damage and (9,17). However, the direct correlation between the albumin concentration in serum or PBMCs and the degree of oxidative stress is still unclear. Furthermore, the molecular mechanisms for STZ-induced ROS production in blood and reduction in albumin are also unknown.

Oxidative stress is well-known to induce cell death (18). To correlate STZ-induced oxidative stress in serum and PBMC, and cell viability, viable PBMCs upon STZ treatment were counted by MTT assay and flow cytometry, as previously described (4). As a result, the number of viable PBMCs in blood was decreased by approximately 45% upon STZ treatment compared to that of control group which was set to 100% (Table 1). Although the molecular mechanism(s) for STZ-induced reduction of PBMC concentration are unknown at present, these results suggest that STZ-mediated ROS production and/or reduction of anti-oxidant pool may contribute to the death of PBMCs. However, we could not exclude the possibility that STZ itself may cause the decreased viability of PBMC, as STZ also functions as a DNA synthesis inhibitor in both bacterial and mammalian cells (8). The viabilities of other cell types in the blood should be also examined to understand the relationship between STZ or STZ-mediated oxidative stress and viability of blood cells.

STZ-induced type 2 DM rats showed enhanced plasma IL-6 and TNF-α levels, indicative of a pro-inflammatory response, and oxidative stress (2). Further, STZ-induced hyperglycemic mice showed a systemic pro-inflammatory state, characterized by broad infiltration of macrophages (13). Hence, we investigated the status of pro-inflammatory response in STZ-induced type 1 DM rats. The levels of IL-6 and TNF-α in the serum of STZ-diabetic rats increased by approximately 3.4- and 2.9-fold, respectively, compared to the untreated rats (control) (Fig 1). Moreover, the levels of CRP in the serum, a pro-inflammatory protein produced in liver, were also approximately 3.6-fold higher than control (Fig 1). These results confirmed the previous suggestions and collectively implied that STZ induced pro-inflammatory response as well as oxidative stress in type 1 DM rats, although changes in other parameters of inflammation were not systematically investigated in this study.

To examine the effect of exogenous administration of albumin on ROS production and pro-inflammatory response in

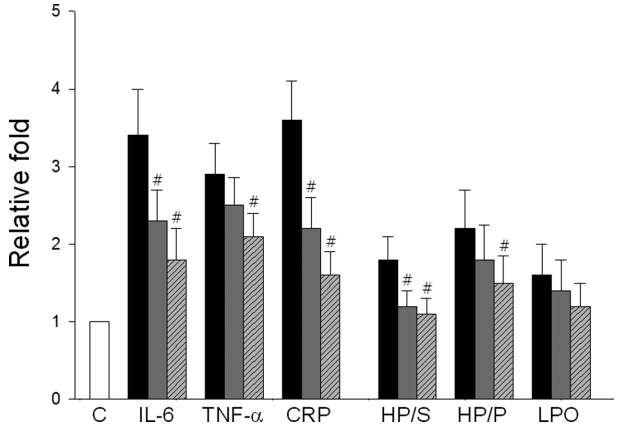

Fig 1. STZ-induced changes in the levels of IL-6, TNF-α, and CRP in serum and the effect of external albumin administration on the inflammatory parameters and ROS production in PBMCs. The relative concentrations of IL-6, TNF-α, and CRP in serum were measured from 1 mL blood of sample 1 week after treating rats with STZ (black bars). The parameters for ROS were also measured after the co-administration of STZ and albumin. The values of cytokines, CRP, and ROS for untreated rat group (C, control) were set to one (1). The amounts of injected albumin were 7.5 mg/100 g body weight (gray bars) or 12 mg/100 g (hatched bars). HP/S and HP/P represent the relative levels of H_2O_2 in serum and PBMCs, respectively. Values represent the mean ± S.D. of five independent samples. #: $p < 0.05$, compared to the STZ-diabetic rats.

blood, purified rat albumin (7.5 mg/100 g body weight) was co-administered with STZ to rats and the parameters for ROS and inflammation were measured at time intervals after the injection. As a result of the co-administration, the levels of H_2O_2 and inflammatory cytokines including CRP in serum were significantly reduced compared to those of diabetic rats treated with STZ alone (Fig 1). However, the different parameters showed variable degrees of decrease. The concentration of H_2O_2 was particularly comparable to that of the normal group, confirming that albumin scavenged ROS (19). However, the blood glucose levels upon the co-administration were similar to those of diabetic rats (results not shown). These results suggested that albumin injection potentially alleviated ROS production and pro-inflammatory response induced by STZ treatment in rats. In addition to the reduction of H_2O_2 in serum, the external albumin decreased ROS generation in PBMCs, although the reduced degrees were not statistically significant. These results collectively suggested that exogenous albumin may exerted function similar to endogenous albumin alleviating STZ-induced damages in blood. Although the origin of cell-bound albumin including PBMC-albumin is still unclear, it has been suggested that lymphocyte- and macrophage-bound albumin originate from the serum *in vivo* and is not produced by the cells themselves (7). Based on this report, we assume that serum albumin and PBMC-albumin could function interchangeably, to attenuate the ROS generation in PBMCs.

To confirm the above result and investigate external albumin-dose dependent changes in blood biochemistry, the measurements were repeated after increasing the amount of co-

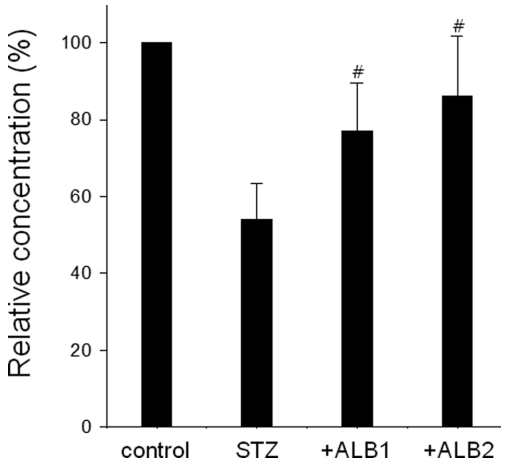

Fig 2. Effect of external albumin administration on the concentration of PBMC in blood. The concentrations of live PBMCs were determined from 1 mL blood 1 week after the co-administration of STZ and albumin and expressed as relative concentration (%) in y-axis. The symbols +ALB1 and +ALB2 represent the injected amount of albumin, 7.5 mg/100 g body weight and 12 mg/100 g body weight, respectively. Values represent the mean ± S.D. of five independent samples. #: $p < 0.05$, compared to the STZ-diabetic rats.

injected albumin to 12 mg albumin/100 g body weight. At this dose of external albumin, the parameters showed further decrease including remarkable reduction in ROS levels in serum and PBMCs, although the overall levels of these parameters were still higher than the control. However, we have not tested the effect of even higher doses of albumin. Furthermore, as inflammation is an exceedingly complicated reaction in animals, the possible influences of albumin on other indices of inflammation should be also investigated.

In addition to blood biochemistry, the effect of external albumin on the concentration of PBMCs in blood was also examined, as STZ treatment led to a decrease in cell viability of PBMCs, as described (Table 1). Fig 2 shows that cell viability of PBMCs was remarkably recovered when purified rat albumin was co-injected with STZ and the treatment with higher dose of albumin resulted in further increase in live PBMC counts in blood. As control experiments, the blood glucose levels, however, exceeded 300 mg/dL and were similar to those of STZ-diabetic rats, even when the exogenous albumin was co-administrated (results not shown). Therefore, it could be expected that external albumin had no effect on STZ-induced injury of pancreatic cells and resultant diabetes.

Conclusion

All the current results collectively suggested that externally administered albumin alleviated ROS production, proinflammatory response, and reduction of PBMC concentration in blood, which were caused by STZ treatment to rats. The present study also presents the possibility that external albumin could be used as a remedy to attenuate detrimental effects of type 1 diabetes on blood. In future, the relationship between albumin and STZ-mediated lesions should be investigated at the molecular level.

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