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Insulin Release from the Beta Cells in Acatalasemic Mice Is Highly Susceptible to Alloxan-Induced Oxidative Stress

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Abstract

Background: Catalase deficiency (acatalasemia) is sensitive to alloxan, and the administration to acatalasemic mice develops hyperglycemia under mild conditions. However, the mechanism is still poorly understood. Methods: Alloxan was used to induce the oxidative stress and intraperitoneally administered to acatalasemic and normal mice. The blood samples of these mice after 1, 3, 5 and 7 days were examined. The pancreatic islets 7 days after alloxan administration were isolated, and the insulin released under 3 mM and 20 mM glucose was examined. Results: After alloxan administration, increase of oxidative markers in blood and pancreatic apoptosis in acatalasemic mice were observed immediately. Insulin in blood was lowered after 3 days, and the insulin in acatalasemic mice was lower than that in normal mice. Hyperglycemia in the acatalasemic mice was observed after 3 days. The pancreatic islets after 7 days were isolated. A reduction of the insulin released from the islets under glucose stimulation was observed. The stimulation indexes of the normal and acatalasemic mice were 1.4 ± 0.6 and 0.7 ± 0.3, respectively. Conclusions: Alloxan induced a deterioration of glucose-dependent insulin secretion ability from the islets, and the deterioration mostly contributed to hyperglycemia, rather than apoptosis.

Keywords

Alloxan, Hyperglycemia, Acatalasemia, Insulin Release, Diabetes, Beta Cells

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1. Introduction

Catalase (EC1.11.1.6) plays a predominant role in the removal of hydrogen peroxide that is necessary to afford protection against the oxidative damage caused by high concentrations of hydrogen peroxide [1]. In 1948, Takahara and Miyamoto discovered deficiencies in erythrocyte catalase among some Japanese patients who had oral gangrene [2]. They named this congenital absence of erythrocyte catalase as acatalasemia, later called "Takahara's disease" [3]. Recently Goth *et al*. reported a high incidence of diabetes mellitus in Hungarian acatalasemia patients [4]. To make clear the reason why the high incidence of diabetes in acatalasemia patients was observed, we have examined acatalasemic mice established by Feinstein *et al*. [5] and normal mice by intrapenitoneal injection of diabetogenic alloxan [6]-[9]. Since it is known that alloxan generates reactive oxygen species by acting as a reducing agent in the body and selectively injures *β*-cells in the pancreas so as to cause insulin dependent diabetes mellitus [10] [11]. We indicated that alloxan generated hydrogen peroxide *in vitro*. Acatalasemic mice became hyperglycemic with a smaller amount of alloxan and the incidence of hyperglycemia was higher than normal mice. Insulin level in acatalasemic blood became lower than normal, and the insulin resistance was not observed. Increase of apoptosis in Langerhans islets was observed in acatalasemic mice but in normal mice. Finally, we deduced that acatalasemic mice promoted diabetes mellitus faster and more frequently than normal mice.

However, as the most part of these studies were carried out after one week from alloxan administrations, it is still unclear the exact point at which mice begin to suffer hyperglycemia after alloxan administration. At first, we examined glucose, insulin and oxidative stress markers in the blood and then apoptosis in the pancreas 1, 3, 5 and 7 days post alloxan administration. We also examined insulin release from the Langerhans islets isolated from alloxan-treated mice under glucose stimulation to investigate what kind of oxidative damage occurs in the pancreas, since it is widely accepted that diabetes is associated with reactive oxygen species, which contribute to pancreatic cell damage and dysfunction in both type 1 and type 2 diabetes [12].

2. Materials and Methods

2.1. Materials

Male mice of the C3H/AnL CS^aCS^a (normal) and C3H/AnL CS^bCS^b (acatalasemia) strains established by Feinstein *et al*. were maintained on a laboratory diet (CE-2 diet, Clea Japan, Tokyo, Japan) and water ad libitium. The experimental procedure was approved by the Ethics Review Committees for Animal Experimentation of Okayama University of Science. Catalase activity in the media was determined by our previously reported method [13].

2.2. Animal Experiments

Acatalasemic mice (23 ± 2 g) and normal mice (27 ± 2 g) (12 weeks old) were divided into two groups. One group was intraperitoneally administered alloxan (200 mg/kg of body weight) [6] [14], the other served as a control was treated with phosphate buffered saline (PBS). Each mouse group was starved for 20 hrs, and blood glucose of each mouse was determined. For examination of the blood and pancreas, the starved mice were sacrificed 1, 3, 5 and 7 days after the administration. The blood and pancreas were collected. Heparin was used as an anticoagulant, and the plasma was stored at −80˚C until it was used for analysis.

2.3. Determination of Glucose in the Blood

Glucose content in the blood obtained from the tail of 20 hr-starved mice was determined. As the volume for the determination of blood glucose was small (approximately 2 μL), the glucose content in the blood was measured with a "Glucose-Test-Ace R" apparatus (Sanwa Kagaku Kenkyusho Co., Nagoya, Japan) applying a glucose oxidase method.

2.4. Determination of Insulin and C-Peptide Levels in the Blood

Insulin and C-peptide levels in the plasma were determined using Mouse Insulin and C-peptide ELISA kits (Shibayagi, Gunma, Japan). Each determination was carried out according to the manufacturer's instructions. A

biotin-conjugated anti-insulin antibody (100 µL) was added to each well of an antibody-coated 96 well plate. To the well, 10 µL of sample or standard solutions were added and reacted for 2 hrs. Then, 100 µL of peroxidase-conjugated avidin solution were added and reacted for 30 min. Chromogenic substrate solution (100 µL) was added and reacted for 30 min. The reaction was terminated and the absorbance at 450 nm (sub-wave length, 620 nm) was recorded.

2.5. Measurement of Oxidative Stress Markers in the Blood

Lipid peroxide in the plasma was determined using a color reaction of malonyl dialdehyde and 4-hydroxyalkenals (OXIS Health Products Inc., CA, USA). 8-Hydroxydeoxy guanidine (8-OHdG) in the plasma was determined using an Amicon Ultra 10K device (Merck, Germany) and a highly sensitive ELISA kit for 8-OHdG (JaICA, Tokyo, Japan).

2.6. Microscopic Studies in the Pancreas

1, 3, 5 and 7 Days after alloxan administration, mice were sacrificed and each animal's pancreatic tissue was isolated, fixed in Bouin's fluid and embedded in paraffin. Serial sections (4 µm) were cut from each paraffin-embedded tissue block. After staining, the stained cells in each tissue section were recorded with an FX380 CCD Cameraunder microscopy (Olympus, Tokyo, Japan), and these cells were counted manually from the images. For the measurements in the photographs, the Image J program (NIH) was used.

2.6.1. Apoptosis Analysis

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was carried out using the In Situ Cell Death Detection Kit (Roche Diagnostics Japan) [15]. The apoptosis incidence was calculated from the TUNEL-positive cells per the total cells in the pancreatic islets.

2.6.2. Additional Staining Procedures

Sections were also stained with hematoxylin-eosin, Elastica van Gieson, 8-OHdG monoclonal antibody (MOG-020, Japan Institute for the Control of Aging) [16], protein-carbonyl immunohistochemical (Cosmo Bio. Co. Ltd, Tokyo, Japan) [17] and mouse anti-insulin antibody (H-86, sc-9168, Santa Cruz Biotechnology and Vectastain Elite ABC rabbit IgG, Vector Lab.) stains for visualization by light microscopy.

2.7. Isolation of Pancreatic Islets from the Mouse Pancreas

1, 3, 5 and 7 Days after alloxan administration, mice were sacrificed. Islets were isolated from the pancreas [18]. Five mL of collagenase solution (type IV, 160 U/ml, Worthington Biochem. Corp., NJ, USA) in Hank's balanced salt solution (pH 7.4, Sigma-Aldrich Co. LLC) was injected into the common bile duct of each mouse. The pancreas was taken and incubated with 1 mL of collagenase solution at 37˚C for 30 min. Digested pancreatic tissue was washed three-times with 5 mL of Hank's balanced salt solution. The suspension was centrifuged at 700 X *g* for 10 min. using a discontinuous gradient of Ficoll (Nacalai Tesque, Kyoto) at concentrations of 25%, 23%, 20% and 11% (2.0, 2.0, 2.0 and 1.0 mL, respectively) in Hank's solution. Islets were taken up with a micropipette from the interface between the 20% and 11% layers, and centrifuged. To the precipitate, 5 mL of RPIM 1640 medium (pH 7.4, Invitrogen Corp. CA, USA) were added twice and washed out. Purified islets were cultured in 5 mL of RPMI 1640 medium at 37˚C under air containing 5% CO_2 for 60 min and used for the experiments.

2.8. Assay of Insulin or C-Peptide Release under Glucose Stimulation

According to the manufacturer's instructions (Pancreatic islet culture kit, Cosmo Bio. Co. Ltd), 0.5 mL of RPIM 1640 medium containing 3 mM glucose (pH 7.4) was added to ten purified islets, and the suspension was incubated at 37˚C for 60 min under 5% CO_2. After centrifugation, the supernatant was removed. To the residue (the precipitated islets), 0.5 mL of RPIM 1640 medium containing 3 mM glucose was added, and the suspension was incubated at 37˚C for 60 min under 5% CO_2. The suspension was centrifuged again, and the supernatant was collected for analysis as the sample was stimulated with 3 mM glucose. To the residue, 0.5 mL of medium

containing 20 mM glucose was added and the mixture was incubated at 37˚C for a further 60 min under 5% CO_2. The suspension was centrifuged, and the supernatants were collected for analysis as the sample stimulated with 20 mM glucose. These supernatants were stored at −80˚C until analysis. The insulin and C-peptide levels in the supernatant were determined using Mouse Insulin and C-peptide ELISA kits (Shibayagi), and the total DNA content in each sample was measured with a CyQUANT assay (Invitrogen). In order to estimate the function of the islets for glucose stimulation, the stimulation indexes of insulin and C-peptide from the islets were calculated [19]. The stimulation index was dividing the insulin (or C-peptide) level stimulated with 20 mM glucose by the level stimulated with 3 mM glucose.

2.9. Statistics

Student's t-test was used to evaluate the statistical significance of difference. The difference was considered significant when $p < 0.05$.

3. Results

Catalase activity in acatalasemic mouse blood at 25˚C was 0.15 ± 0.09 μmol/s/g of Hb and the activity in normal mouse blood was 6.89 ± 0.57 μmol/s/g of Hb.

3.1. Blood Glucose in Normal and Acatalasemic Mice

Blood glucose level in both groups of mice became lower one day after alloxan administration (**Figure 1**). Both levels were significantly low ($p < 0.05$) compared to those of the control. Then, the level in the normal mice was slowly increased near the control level, and that in the acatalasemic mice was significantly increased after 3 days resulting in hyperglycemia ($p = 0.053$, $p < 0.05$). The incidence of hyperglycemia was indicated in **Table 1**.

3.2. Insulin in Normal and Acatalasemic Mouse Blood

The insulin concentration in normal and acatalasemic mouse blood transiently increased one day after alloxan administration and then decreased (**Figure 2**). After 3 days from alloxan administration the insulin level of both mice became low, and the insulin level of the acatalasemic mice was always lower than that of the normal mice.

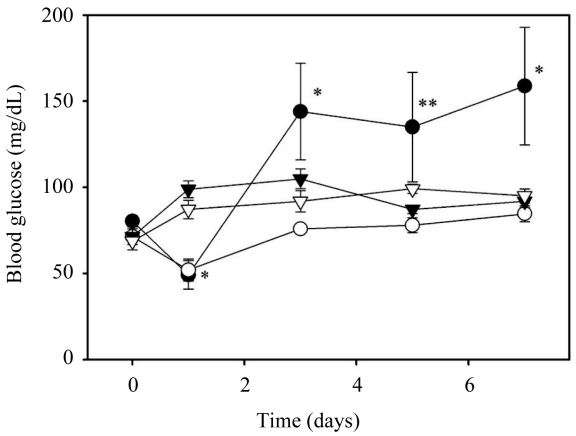

Figure 1. Blood glucose of fasting mice after alloxan administration. Alloxan was administered to each mouse on day 0, and mouse blood glucose on 0, 1st, 3rd, 5th and 7th days was determined. Closed circles indicate acatalasemic mice (n = 15), and open circles normal mice (n = 15) treated with alloxan. Closed triangles indicate acatalasemic mice treated with PBS (n = 6) as a control, and open triangles indicate the control of normal mice (n = 4). The bars of each symbol indicate the SE. * and ** indicate $p < 0.05$ and $p = 0.053$, respectively, compared to the control mice.

Table 1. Incidence of hyperglycemia in the mouse blood after alloxan administration.

Mice	Alloxan (mg/kg)	Incidence of hyperglycemia (%)				
		0 days	1 days	3 days	5 days	7 days
Normal* (3)	0	0	0	0	0	0
Normal (10)	200	0	0	0	0	10
Acatalasemia* (3)	0	0	0	0	0	0
Acatalasemia (15)	200	0	0	27	33	53

*Mice were treated with PBS as a control. Number in parenthesis indicates the number of mice. Hyperglycemia for mice is >126 mg/dL of blood glucose, which is determined from average fasting mouse blood glucose (77 ± 23 mg/dL).

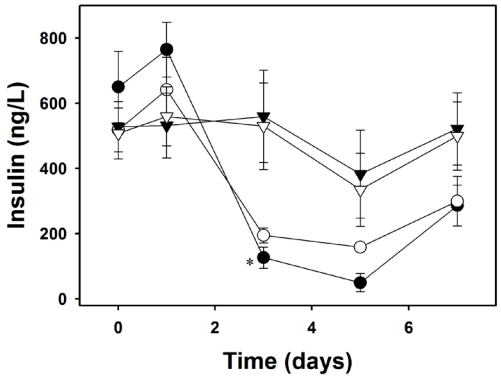

Figure 2. Insulin in mouse blood after alloxan administration. Alloxan was administered to normal and acatalacemic mice on day 0, and insulin in the blood was determined. Each open circle represents the normal mice (n = 6), and the closed circle is acatalasemic mice (n = 6) treated with alloxan. The open triangles indicate normal mice (n = 6) treated with PBS (n = 6) as a control, and the closed triangles are the control of acatalasemic mice (n = 4). The bars of each symbol indicate the SE. *indicates $p < 0.05$ compared to the control mice.

3.3. Oxidative Stress Markers in Normal and Acatalasemic Mouse Blood

Oxidative stress markers in normal and acatalasemic mouse bloods increased after alloxan administration (**Figure 3**). These oxidative stress markers were considerably higher than the control 7 days after alloxan administration as we previously reported [8]. As these marker levels in acatalasemic blood are similar to normal ones, it suggests that these markers are not directly generated by reactions of hydrogen peroxide.

3.4. Microscopic Studies of Mouse Pancreatic Tissues

3.4.1. Apoptosis in β Cells after Alloxan Administration

Apoptosis was calculated using TUNEL-positive cells per the total cells in the islets of Langerhans. After alloxan administration, pancreatic apoptosis in acatalasemic mice immediately increased (**Table 2**) though the increase was low (less than 6.2%). The increase was kept for more than 7 days. Increase of pancreatic apoptosis in normal mice was not observed.

3.4.2. Protein-Carbonyl and 8-OHdG Immunohistochemical Staining in the Mouse Pancreas

As the result of alloxan administration, mild increase of oxidized protein was observed on the inside of islets in the alloxan administrated group (**Figure 4**). It indicated that the increase in acatalasemic mice was larger than normal one.

In the case of 8-OHdG staining, the stained cells in normal and acatalasemic mice were hardly observed 1, 3, 5 and 7 days after alloxan administration (data not shown). It might be due to the low incidence of apoptosis in mouse pancreas.

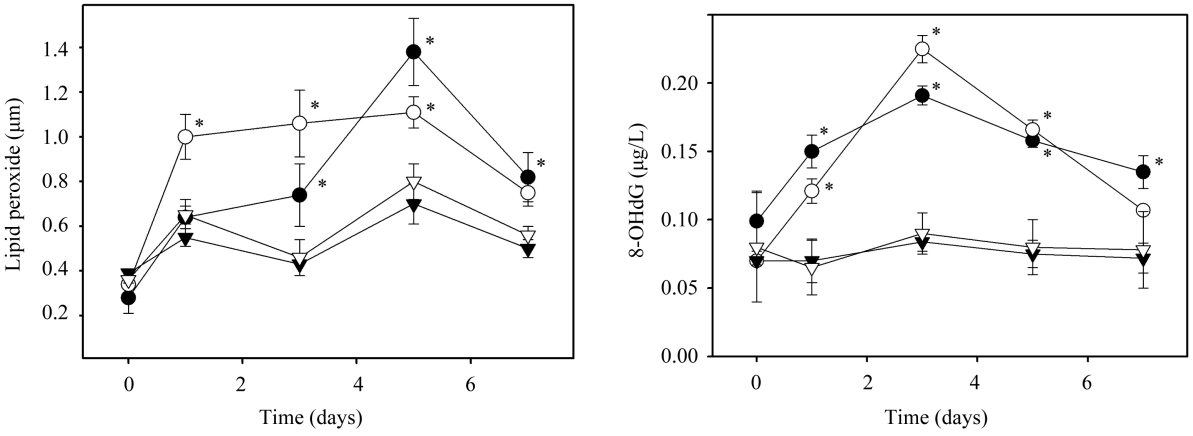

Figure 3. Lipid peroxide and 8-OHdG in the fasting mouse blood after alloxan administration. Alloxan was administered on day 0 to normal and acatalacemic mice. (a) indicates the lipid peroxide in the mouse blood and (b) 8-OHdG in the blood. Each open circle represents normal mice (n = 6) treated with alloxan, and the closed circle is the acatalasemic mice (n = 6). The open triangles indicate normal mice (n = 6) treated with PBS as a control, and the closed triangles are the control of acatalasemic mice (n = 4). The bars of each symbol indicate the SE. *indicates $p < 0.05$ compared to the control mice.

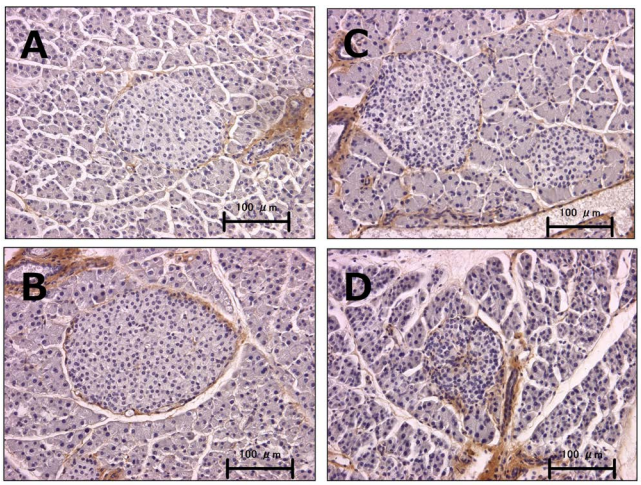

Figure 4. Protein-carbonyl immunohistochemical stains in the mouse pancreas 7 days after alloxan administration. (A) Normal mice treated with PBS as a control, (B) normal mice treated with alloxan, (C) control of acatalasemic mice and (D) acatalasemic mice treated with alloxan.

Table 2. Apoptosis in the pancreatic tissues after alloxan administration.

Mice (≥3)	Alloxan (mg/kg)	Incidence of apoptosis (%)			
		0 days	1 days	3 days	7 days
Normal*	0	0.9 ± 0.1	1.0 ± 0.5	0.9 ± 0.1	0.9 ± 0.3
Normal	200	1.0 ± 0.2	0.8 ± 0.4	0.8 ± 0.4	1.0 ± 0.4
Acatalasemia*	0	1.5 ± 0.7	2.3 ± 0.2	0.9 ± 0.2	1.3 ± 0.4
Acatalasemia	200	1.4 ± 0.6	7.4 ± 0.8**	7.1 ± 0.4**	4.5 ± 0.9**

*Mice were treated with PBS as a control. **$p < 0.05$ compared to the control mice.

3.4.3. Anti-Insulin Antibody Stain in Pancreas

After alloxan administration, numbers of β cells in normal and acatalasemic mice were decreased and the numbers in the acatalasemic mice were significantly decreased (**Figure 5**). The size of the β cells in the acatalasemic mice also significantly became small after alloxan administration.

3.5. Insulin and C-Peptide Release from Pancreatic Islets Stimulated with Glucose

We tried to isolate the islets of the acatalasemic mice 1, 3, 5 and 7 days after alloxan treatment, but were only able to isolate the islets 7 days after. The total DNA contents in the islet suspension of normal and acatalasemic mice were 226 ± 57 and 228 ± 64 ng/mL, respectively. Insulin and C-peptide releases from the ten isolated islets were summarized in **Table 3**. Both insulin and C-peptide releases were decreased by alloxan pretreatment, and the release from acatalasemic islets in response to 20 mM glucose stimulation dramatically became low compared to the control one.

The stimulation indexes were calculated and summarized in **Table 4**. Both of the indexes were decreased by alloxan pretreatment. The C-peptide index was similar to that of insulin, as supported by the insulin index. The indexes in the acatalasemic mice were substantially lower than the normal ones.

4. Discussion

To examine the time-point at which mice suffer hyperglycemia after alloxan administration, we first examined blood glucose and insulin as well as oxidative markers in the blood. As blood glucose decreased and insulin increased on the first day after alloxan administration, it might be that alloxan temporary induced insulin release

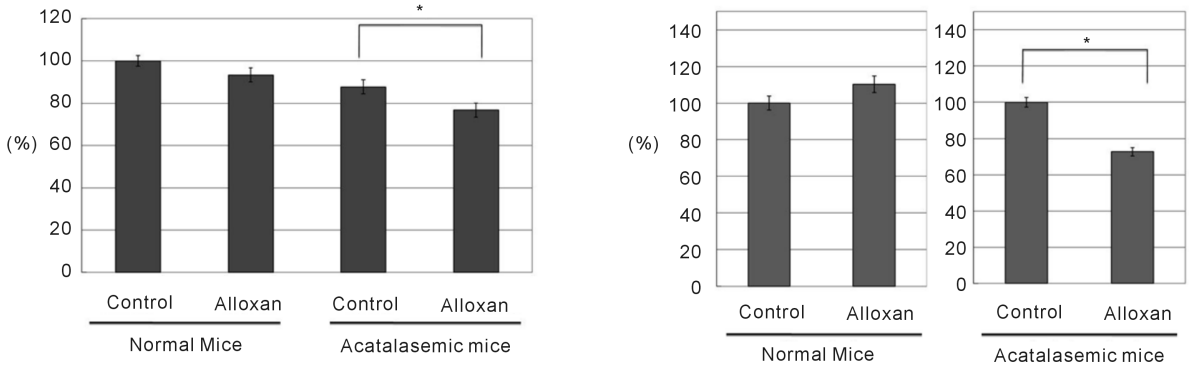

Figure 5. Relative number and the mean size of the β cells in Langerhans islets 7 days after alloxan administration. Relative number of β cells (a) and the mean size of the β cells; (b) were compared to these (100%) of control mice treated with PBS. The number of the control mice was 0.012/μm^2. The sizes of control normal and acatalasemic mice were 62.4 ± 2.4 and 81.5 ± 2.2 μm^2, respectively. The bars of each symbol indicate the SE. *indicates $p < 0.05$.

Table 3. Amounts of insulin and C-peptide release from Langerhans islets* stimulated with 3 and 20 mM glucose.

Compounds	Glucose (mM)	Concentration in supernatant (ng/mL)			
		Normal mice		Acatalasemic mice	
		Control (5)	Alloxan (3)	Control (6)	Alloxan (4)
Insulin	3	10.6 ± 3.3	5.6 ± 1.9	6.6 ± 1.8	5.7 ± 1.9
Insulin	20	27.0 ± 7.0	7.5 ± 2.5**	12.0 ± 0.2	4.0 ± 2.0**
C-peptide	3	15.3 ± 5.8	10.9 ± 3.7	19.0 ± 4.1	10.8 ± 6.3
C-peptide	20	37.0 ± 10.0	16.0 ± 8.0**	33.0 ± 0.02	8.0 ± 6.0**

*The islets were isolated 7 days from mice treated with alloxan (200 mg/kg of body weight) or PBS (as a control). Number in parenthesis indicated the number of mice. **$p < 0.05$ compared to each control group.

Table 4. Stimulation index (SI) of insulin and C-peptide from Langerhans islets* stimulated by glucose.

Compounds	Stimulation index**			
	Normal mice		Acatalasemic mice	
	Control (5)	Alloxan (3)	Control (6)	Alloxan (4)
Insulin	4.6 ± 2.6	1.4 ± 0.6***	2.2 ± 0.9	0.7 ± 0.3***
C-peptide	2.7 ± 1.6	1.6 ± 0.8***	1.7 ± 0.5	0.6 ± 0.3***

*The islets were isolated 7 days from mice treated with alloxan (200 mg/kg of body weight) or PBS (as a control). **Stimulation index = each insulin (or C-peptide) concentration at 20 mM glucose/the concentration at 3 mM glucose. ***$p < 0.05$ compared to the index of the control.

from islets of Langerhans in the pancreas [10] [11]. After 3 days, the insulin levels in the normal and acatalasemic bloods became lower than that of the controls and the level in the acatalasemic blood was lower than that in normal blood. The blood glucose level in the acatalasemic mice dramatically increased compared to that in normal mice 3 days after alloxan administration (**Figure 1**), and the incidence of hyperglycemia in acatalasemic mice was higher than normal mice. Oxidative markers in the blood of both mice increased after alloxan administration and gradually decreased but kept for more than 7 days. From these results, we confirmed that pancreas, especially the acatalasemic one, was sensitive to alloxan-induced oxidative stress thus resulting in hyperglycemia.

After alloxan administration, increase of apoptotic cells associated with decrease of number and the mean size of β-cells stained by insulin antibody (**Figure 5**) was observed in the acatalasemic mice but not in normal mice as previously reported [7]. Apoptosis cells in acatalasemic mice immediately increased, and the incidence was considerably small (less than 6.2%). However, hyperglycemia in acatalasemic mice was observed 3 days after alloxan administration (**Figure 1**), and the incidence was large (27%). To explain these phenomena, protein-carbonyl and 8-OHdG immunohistochemical stains of the pancreas were examined. A mild increase of the carbonyl-stained protein was observed in the islets of the alloxan-treated mice (**Figure 4**), and the staining of 8-OHdG from DNA degradation was hardly detected in pancreatic cells. These results suggested that the protein was oxidized rather than that the DNA was degraded by alloxan administration, suggesting that alloxan induced other damage than apoptosis in pancreas. As alloxan induced low insulin in blood (**Figure 2**), pancreatic islets from mice pretreated with alloxan were isolated. Insulin and C-peptide releases from the islets were examined under 3 mM and 20 mM glucose (**Table 3**) and calculated their insulin and C-peptide stimulation indexes [19] (**Table 4**). By alloxan pretreatment, insulin release from the islets was reduced, especially at 20 mM glucose stimulation, resulting low insulin indexes. It indicated that glucose-dependent insulin secretion ability of the islets had deteriorated. Insulin release from the islets of the acatalasemic mice was reduced compared to that of normal mice, suggesting that acatalasemic mice were not able to release enough insulin for the body even under hyperglycemic conditions. We deduced that insulin release ability from the islets is an important factor for the onset of hyperglycemia. This deterioration in pancreas may be in part similar to that of type 2 diabetes induced by chronic exposure to elevate levels of glucose and free fatty acid [12]. As the islets are impacted by the intracellular redox state [20], further study is currently underway on the insulin release from beta cells under glucose stimulation.

5. Conclusion

Pancreatic beta cells of acatalasemic mice are highly susceptible to alloxan-induced oxidative stress. The stress induced deterioration of glucose-dependent insulin secretion ability from the islets of Langerhans. The deterioration in the islets mostly contributed to hyperglycemia, rather than the apoptosis.

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