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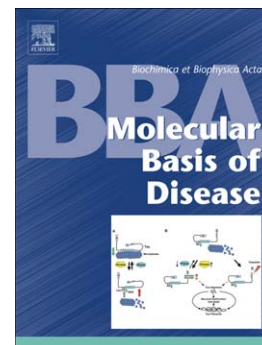
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## Sensitization to alloxan-induced diabetes and pancreatic cell apoptosis in acatalasemic mice

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### Keywords

Acatalasemia, Diabetes mellitus, Oxidative stress, Catalase, Apoptosis, Angiotensin  $\square$  receptor antagonist

**Abstract**

Human acatalasemia may be a risk factor for the development of diabetes mellitus. However, the mechanism by which diabetes is induced is still poorly understood. The impact of catalase deficiency on the onset of diabetes has been studied in homozygous acatalasemic mutant mice or control wild-type mice by intraperitoneal injection of diabetogenic alloxan. The incidence of diabetes was higher in acatalasemic mice treated with a high dose (180 mg/kg body weight) of alloxan. A higher dose of alloxan accelerated severe atrophy of pancreatic islets and induced pancreatic  $\beta$  cell apoptosis in acatalasemic mice in comparison to wild-type mice. Catalase activity remained low in the acatalasemic pancreas without the significant compensatory up-regulation of glutathione peroxidase or superoxide dismutase. Furthermore, daily intraperitoneal injection of angiotensin II type 1 (AT1) receptor antagonist telmisartan (0.1mg/kg body weight) prevented the development of alloxan-induced hyperglycemia in acatalasemic mice. This study suggests that catalase plays a crucial role in the defense against oxidative-stress mediated pancreatic  $\beta$  cell death in an alloxan-induced diabetes mouse model. Treatment with telmisartan may prevent the onset of alloxan-induced diabetes even under acatalasemic conditions.

## 1. Introduction

Diabetes mellitus is a world-wide disease and one of the major causes of death, thus it is essential to clarify the pathogenesis and effective preventive or therapeutic measures of the disease. Diabetes is characterized by progressive  $\beta$  cell loss and, it is widely accepted that reactive oxygen species (ROS) contribute to pancreatic cell or tissue damage and dysfunction both in type 1 and 2 diabetes, even though the underlying mechanisms differ [1].

The degree of oxidative stress and the severity of subsequent tissue injury may depend on an imbalance between the excessive production of ROS and antioxidant defense within the pancreatic islet. These antioxidants include the enzymes superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX), which detoxify ROS. Catalase(E.C.1.11.1.6) is a major enzyme that catalyzes the decomposition of hydrogen peroxide ( $H_2O_2$ ) and plays a role in cellular antioxidant defense mechanisms [2]. The main reaction of catalase is the catalytic reaction ( $2H_2O_2 \rightarrow O_2 + 2H_2O$ ) and it is essential for the removal of excessive  $H_2O_2$  and for regulation of the  $H_2O_2$  concentration in signaling pathways [3]. Catalase limits the accumulation of  $H_2O_2$  generated by various oxidases in tissue and serves as a substrate for the Fenton reaction to produce the highly injurious hydroxyl radicals.

Genetic defects of catalase were first documented by Takahara [4] in Japanese patients who exhibited a deficiency of catalase enzyme activity in their blood (acatalasemia). The short-time clinical manifestations of human acatalasemia after exposure to  $H_2O_2$  or infection with peroxide-generating bacteria such as streptococci appear predominantly in the mouse. Oral ulcerations, alveolar gangrene and atrophy resulting in a loss of teeth have been reported. A high frequency (12.7%) of diabetes mellitus and deleterious changes in lipid and carbohydrate metabolism is observed in Hungarian acatalasemia, thus suggesting that this inherited disorder may be a risk factor

for the development of diabetes or atherosclerosis, and catalase deficiency may not be the benign disorder [5].

ROS are involved in many of the angiotensin  $\square$  (Ang  $\square$ ) signaling pathways. Ang  $\square$  stimulates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity via the AT1 receptor to produce the superoxide anion,  $H_2O_2$  and hydroxyl radicals [6]. The existence of a local renin-angiotensin system (RAS) is now recognized in pancreas, the activities of which are subjected to regulation by physiological and pathophysiological stimuli such as hypoxia, pancreatitis, islet transplantation, hyperglycemia and diabetes mellitus [7]. Thus, the increased RAS in pancreas may aggravate pancreatic cell damage induced by ROS, and the blockade of RAS may be useful for preventing diabetes.

The present study hypothesized that a functional catalase deficiency would render the pancreas more susceptible to oxidant tissue injury, and the effect of dysregulation of the antioxidant system on the onset of diabetes was investigated using an acatalasemic mouse strain induced by the diabetogenic compound alloxan. The acatalasemic mice differentially express their catalase activity in a tissue specific manner. The catalase activities in these mice range from 1-2% of the normal mice in red blood cells, to approximately 20% in kidney, or to nearly normal levels in liver [8, 9]. Alloxan is a mild oxidant which shows the selective toxicity to pancreatic  $\beta$  cells. The diabetologic action of alloxan is initiated by the generation of ROS [10]. In addition, since ROS are associated with many Ang  $\square$  signaling pathways, we investigated whether the AT1 receptor antagonist, telmisartan, could inhibit the alloxan-induced hyperglycemia in acatalasemic mice.

## 2. Materials and methods

### 2.1. Animals and experimental protocol

Male wild-type mice (C3H/AnLCs<sup>a</sup>Cs<sup>a</sup>) and male homozygous acatalasemic mutant mice (C3H/AnLCs<sup>b</sup>Cs<sup>b</sup>) were used at the age of 8 to 10 weeks old. All animals were

housed in a group of five and fed standard laboratory chow and water. Diabetes mellitus was induced by the intraperitoneal injection with 120 or 180 mg/kg body weight (BW) of alloxan (2,4,5,6-Tetraoxypyrimidine) (Sigma-Aldrich Co., St. Louis, MO) dissolved in phosphate buffered saline (PBS) at the first two consecutive days of experimental protocol. In the control group of mice, the same volume of PBS was injected intraperitoneally. Each group consisted of 15 to 20 mice. The BW was measured at day 0 and 7 of protocol. The blood glucose concentration was determined by a portable glucose meter using Glutest Sensor (Sanwa Kagaku Kenkyusho Co., Nagoya, Japan) at day 0, 2 and 7, using tail tip blood. Plasma insulin at day 7 was measured using a rat insulin radioimmunoassay kit (Linco Research Inc., St. Charles, MO). The pancreases were dissected out at day 7 under pentobarbital anesthesia. The development of diabetes was defined as over 200 mg/dl of blood glucose concentration [11]. Telmisartan (BIBR 277) was dissolved in PBS, adjusted to pH 8.0, and injected intraperitoneally daily from one day before administration of 180mg/kg BW of alloxan to day 7, at a dose of 0.1mg/kg BW [12]. A vehicle-treated group received the intraperitoneal injection of PBS alone. In this treatment experiment, mice were divided into 8 subgroups (N=10 to 15/group). The BW and blood glucose concentration was checked as described above. Streptozotocin (STZ) is another prominent diabetogenic compounds. In a pilot study, the intraperitoneal injection of different concentrations of STZ (120 to 160 mg/kg of BW) did not sensitize acatalasemic mice to diabetes (Supplementary Figure 1). Therefore, we utilized alloxan-induced diabetes model in this study. The experimental protocol was approved by the Ethics Review Committees for Animal Experimentation of Okayama University Graduate School.

## 2.2. Light microscopic studies

Formalin-fixed, paraffin-embedded 3- $\mu$ m sections were assessed using periodic acid-Schiff (PAS) stain. Each tissue section was observed using an Olympus BX51 light microscope (Olympus, Tokyo, Japan) with a high-resolution digital camera system

(Penguin 600CL; Pixera Co., CA). The measurement of the pancreatic islets size was performed using a Microanalyzer program (version 1.1; Nippon Poladigital Co., Tokyo, Japan).

### 2.3. Apoptosis detection

DNA fragmentation associated with apoptosis was detected in situ by the addition of nucleotides to free 3' hydroxyl groups in DNA as described previously [13, 14]. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was performed using a MEBSTAIN Apoptosis Kit Direct (Medical and Biological Laboratories, Nagoya, Japan).

### 2.4. Catalase, GPX and SOD activity

After harvesting pancreatic tissue, the samples were immediately snap frozen in liquid nitrogen and stored at -80°C until assayed. The catalase activity was determined by measuring the removal rate of 70 µM H<sub>2</sub>O<sub>2</sub> based on a method described previously [15, 16]. The activity of GPX or SOD was measured as described previously [15, 17].

### 2.5 RNA extraction and quantitative real-time PCR analysis of catalase in isolated pancreatic islets

Before harvesting pancreatic tissue, mice were perfused with saline and the samples were fixed in PAX gene<sup>TM</sup> Tissue Containers (Qiagen, Valencia, CA). Total RNA was extracted from pancreatic islets dissected by the laser-capture microdissection (LCM) technique as described previously [18, 19], using LCM Staining Kit (Applied Biosystems, Foster City, CA), PAX gene<sup>TM</sup> Tissue miRNA Kit (Qiagen) and PALM MicroBeam System (Carl Zeiss Inc., Bernried, Germany). Real-time PCR was carried out as described previously [12, 15, 19, 36]. TaqMan PCR primers and labeled probes were purchased from Applied Biosystems and the oligonucleotide primers used for PCR were custom-ordered from Nihon Gene Research Lab's Inc. (Sendai, Miyagi, Japan)[20]. The amount of PCR product was normalized with β-actin to determine the relative expression ratios for each



mRNA in relation to  $\beta$ -actin mRNA.

### 2.6. Statistical analyses

The data, presented as the mean  $\pm$  SEM, were analyzed by the Mann-Whitney U test using the Stat View statistical software package (Hulkins, Tokyo, Japan). *P* values  $<0.05$  were considered to be statistically significant.

## 3. Results

### 3.1. Acatalasemia promotes alloxan-induced diabetes in mice

Alloxan is an oxidative stress agent that is relatively specific to, and destroys the insulin-producing pancreatic  $\beta$  cells, so alloxan-induced diabetes is considered to be a model of type 1 diabetes mellitus [21]. Hyperglycemia was not observed in the control mice in both groups (N=15 to 20 animals in each group). The incidence of diabetes at 7 days after intraperitoneal injection with 120 mg/kg BW of alloxan (ALX 120) was 0% in the wild-type mice group, 7.7% in the acatalasemic mice group, 31.6% in the wild-type mice treated with ALX 180, and 72.2% in the acatalasemic mice treated with ALX 180. There were no significant changes in body weight throughout the experiment in the wild-type mice, while a significant decrease in body weight was observed in the acatalasemic mice at 7 days after injection with ALX 180 (Table 1). Blood glucose significantly increased in both groups at 7 days after the administration of ALX 180. Moreover, the elevation of blood glucose in the acatalasemic mice was remarkable at 2 and 7 days after injection with ALX 180 in comparison to that in the wild-type mice (Fig. 1). The concentrations of plasma insulin at day 7 were  $0.84 \pm 0.21$ ng/ml in wild-type control mice,  $0.26 \pm 0.07$ ng/ml in the wild-type ALX180 group,  $0.40 \pm 0.05$ ng/ml in the acatalasemic control, and  $0.14 \pm 0.02$ ng/ml in the acatalasemic ALX180 group. No statistically significant differences in concentrations of plasma insulin were observed between wild-type and acatalasemic same groups. While, plasma insulin significantly decreased in both

ALX 180 groups in comparison to the appropriate control group ( $P=0.02$  in wild-type and  $P=0.005$  in acatalasemic mice).

### *3.2. A higher dose of alloxan accelerates severe atrophy of pancreatic islet and induces apoptosis in pancreatic islet cells in acatalasemic mice*

The atrophy of pancreatic islets can frequently be observed from the early phase of the disease in other animal models of type 1 diabetes [22]. A histological and morphometric analysis of pancreatic islets of wild-type and acatalasemic mice was performed to evaluate the effect of acatalasemia on the administration of alloxan. No histological abnormalities of the pancreatic islets were observed at the light microscopic level in wild-type and acatalasemic control mice (Fig. 2A, C). Severe atrophy of pancreatic islets developed 7 days after intraperitoneal ALX 180 injection in acatalasemic mice, (Fig. 2D) in comparison to that observed in wild-type treated with ALX 180 (Fig. 2B). The mean sizes of pancreatic islets in the wild-type and acatalasemic mice at 7 days after injection with alloxan are shown in Figure 2E. The average size of pancreatic islets was significantly decreased only in acatalasemic mice treated with ALX 180.

Pancreatic  $\beta$ -cell death is fundamental in the pathogenesis of type 1 diabetes [23]. Oxidative stress is one of the most important causes of pancreatic cell death, and many studies have showed relationships between  $\beta$ -cell loss and apoptosis [24-26]. Oxidative stress enhanced by acatalasemia may sensitize pancreatic islet cells to apoptosis. TUNEL-positive pancreatic islet cells were hardly found in the wild-type and acatalasemic control mice, while they were observed in the wild-type and acatalasemic mice at 7 days after injection with ALX 180 (Fig. 2F and G). The number of TUNEL positive cells in atrophic pancreatic islets significantly increased only in acatalasemic mice treated with ALX 180 in comparison to wild-type ALX 180 mice at the same time point (Fig. 2H).

### 3.3. No significant compensation of GPX or SOD for catalase in acatalasemic pancreas

The maintenance of tissue homeostasis requires an appropriate balance between oxidants and antioxidants. Catalase and GPX are physiologically involved in the detoxification of H<sub>2</sub>O<sub>2</sub> and protect cells or tissues from oxidant-mediated injury. The ability of other antioxidant enzymes to compensate for catalase in acatalasemic pancreatic tissue was investigated. Pancreatic catalase activity from the acatalasemic mice exhibited a 1.4 fold ( $P<0.01$ ) decrease in comparison to wild-type mice and remained low 7 days after ALX injection (Fig. 3A). The activity of pancreatic catalase significantly increased in both the wild-type and acatalasemic mice treated with ALX 180. To examine the effect of acatalasemia on other pancreatic antioxidant enzymes in alloxan-induced diabetes, the activities of GPX and SOD were measured in wild-type and acatalasemic mice. There was not any compensatory up-regulation of GPX in the acatalasemic pancreas or a significant difference in GPX activities between the two mice groups (Fig. 3B). The activity of SOD in acatalasemic mice treated with ALX 180 tended to be higher than those in the wild-type ALX 180-treated group, but no statistically significant differences were observed among the groups. There were no significant changes in the activities of SOD between the two groups (Fig. 3C). The expression of catalase mRNA in acatalasemic and wild-type control pancreatic islets dissected by LCM was measured by real-time PCR. They were not significantly different (Supplementary figure 2).

### 3.4. Telmisartan inhibits alloxan-induced hyperglycemia in acatalasemic mice

We then investigated the effect of telmisartan on the onset of diabetes mellitus in wild-type or acatalasemic alloxan-induced diabetes model. Hyperglycemia was not observed in the control mice in both groups whether they were treated with telmisartan or not (N=10 to 15 animals in each group). The incidence of diabetes at 7 days after intraperitoneal injection with ALX 180 was 36.4% in the wild-type mice, 65.2% in the

acatalasemic mice. While, in the 0.1mg/kg BW telmisartan-treated groups, diabetes did not develop in the wild-type mice and was observed only 20% in the acatalasemic mice. Significant BW decreasing was seen only in the acatalasemic ALX 180 mice without telmisartan administration. Treatment with telmisartan resulted in a significant prevention of the development of hyperglycemia in acatalasemic mice treated with ALX 180 in comparison to wild-type ALX 180 treated-mice (Fig. 4).

#### 4. Discussion

The present study examined the effects of functional catalase deficiency on the development of diabetes mellitus utilizing an acatalasemic mouse strain. The results showed that acatalasemic mice developed more severe atrophy of pancreatic islets and apoptosis of islet cells, promoted diabetes in comparison to the wild-type mice in an alloxan-induced experimental diabetes model. Plasma insulin decreased in both the acatalasemic and wild-type mice treated with a higher dose of alloxan, but the decrease was more significant in the acatalasemic mice. Pancreatic catalase activity remained low without significant compensatory up-regulation of GPX and SOD. Daily intraperitoneal injection of AT1 receptor antagonist telmisartan prevented the development of alloxan-induced hyperglycemia in acatalasemic mice. Therefore, the results suggest that catalase may play a crucial role in protecting pancreatic islet cells from atrophy and apoptosis, and that ROS, particularly hydroxyl radicals associated with the reduction of catalase activity, might be involved in the acceleration of the onset of diabetes in acatalasemic disease conditions. In addition, telmisartan may have beneficial effects on prevention of the onset and progression of alloxan-induced diabetes in acatalasemia.

An acatalasemic mouse strain (Cs<sup>b</sup>) was established by Feinstein et al. [8] from the progeny of X-ray-irradiated mice. The residual catalase activities of acatalasemic mice range from 1-2% of wild-type mice in red blood cells, to approximately 20% in kidney, or

to nearly normal levels in liver [8, 9]. Acatlasemic mice develop and grow normally. The mice are as fertile as wild-type mice, and no apparent abnormalities are seen in the morphology of the major organs, including the liver, heart, lung, kidney, and pancreas. However, after exposure to certain toxic agents, particularly oxidative stressors such as nitrogen monoxide, carbon tetrachloride and so on, caused severe tissue or cell damage in comparison to that observed in wild-type mice [27]. These mice are more susceptible to diethylnitrosamine, leading to enhanced hepatocarcinogenesis in comparison to normal mice [28]. Furthermore, the catalase deficiency enhances renal tubulointerstitial injury and fibrosis in a model of unilateral ureteral obstruction (UUO) [14], sensitizes remnant kidneys to albuminuria and tubulointerstitial fibrosis in a 5/6 nephrectomized model [15], and develop more severe tissue injury and peritoneal fibrosis in a chlorhexidine gluconate-induced experimental peritoneal fibrosis model [29]. These studies suggest that the increased oxidative stress caused by catalase deficiency may play important roles in the defense against injuries in different organs. The current study showed for the first time that acatalasemic mice promoted diabetes mellitus faster and more frequently than wild-type mice. Similar results with catalase gene mutations and type 2 diabetes were reported in humans, and this risk might be due to peroxide damage of normally catalase-poor pancreatic  $\beta$  cells [30]. Goth reported a proband of type D Hungarian acatalasemia develop type 2 diabetes at a relatively early age [31] and there is higher incidence of diabetes (type 1 and 2) in a catalase deficient family than a normocatalasemic family [5]. The acatalasemic diabetes mice model may reveal the exact mechanisms by which hydroxyl radical affects pancreatic dysfunction and it may lead to the development of new treatments for human diabetes.

Pancreatic islet cells contain low levels of the antioxidant enzymes SOD, GPX or catalase relative to other organs [32], so the antioxidative defense mechanisms are weak and can be overwhelmed by redox imbalance resulting from overproduction of ROS [33].

Their function as glucose sensors and insulin producers requires an intracellular environment rich in oxygen and glucose in order to generate the signal for insulin secretion and to supply adequately the target tissues with insulin. This special internal milieu makes the pancreatic  $\beta$  cell particularly susceptible to oxidative stress [33] and this may be involved in the pathogenesis of diabetes. Xu et al. produced transgenic mice that have an increase in  $\beta$  cell catalase activity and showed that the increase in catalase activity protected islets against hydrogen peroxide and STZ [34]. Tabatabaie et al. used electron paramagnetic resonance spectroscopy in conjunction with spin-trapping methodology to demonstrate that the *in vivo* administration of proinflammatory cytokines, tumor necrosis factor- $\alpha$ , interleukin-1 $\beta$  (IL-1 $\beta$ ) and interferon- $\gamma$  into the rat pancreas leads to the formation of free radicals in the  $\beta$  cells [35]. In addition lower antioxidant enzyme activities in islets from diabetes-prone BB/S rats is a factor in the development of type 1 diabetes and in susceptibility to DNA damage *in vitro* [36]. These studies have defined the apparent relationships between the failure of antioxidant systems and the pathogenesis of diabetes, and they are consistent with the findings of the current study.

The expression of catalase mRNA in pancreatic islets was not significantly different between acatalasemic and wild-type mice. The tissues of acatalasemic mice express normal catalase mRNA levels in comparison to those of wild-type mice, suggesting that the mutation does not act at the level of gene transcription or mRNA stability, but rather during mRNA translation and/or protein turnover. The mutation is mapped to the mouse catalase structural gene on chromosome 2 and is expressed by modification of the enzyme active site but not of the antigenic site. This mutation may render the catalase molecule unstable in acatalasemic mice [9] and may lead to a significant difference in catalase activities. Blood catalase could contribute to the defense of organs with low catalase such as the pancreas or brain. Acatalasemic blood catalase activity accounts for about 1-2% of the normal catalase activity [16], and therefore a low blood catalase activity could be

associated with the onset of diabetes in this animal model.

There are many differences between the mechanism of islet cell death in type 1 and 2 diabetes mellitus [1].  $\beta$  cell death in the insulinitis in type 1 diabetes is caused by contact with activated macrophages and T cells, and/or exposure to soluble mediators secreted by these cells, including cytokines, nitric oxide (NO), and oxygen free radicals [37]. Apoptosis is thought to be the main cause of  $\beta$  cell death at the onset of type 1 diabetes and it is a highly regulated process activated or modified by a lot of signals and expression of apoptosis-related genes [37]. INS-1 cells, a rat pancreatic  $\beta$  cell line, treated with alloxan show decreasing viability, intracellular ATP levels, and glucose-stimulated insulin release and the appearance of a DNA ladder, thus suggesting that alloxan induces apoptosis in these cells [24]. Alloxan selectively inhibits glucose-induced insulin secretion through specific inhibition of glucokinase and also causes a state of insulin-dependent diabetes through its ability to induce ROS formation in a cyclic reaction with its reduction product, dialuric acid [38]. Elsner et al. showed that catalase protected insulin-producing cells against the cytotoxic action of alloxan and dialuric acid due to the breakdown of  $H_2O_2$  and the prevention of hydroxyl radical formation [39]. This indicates that the hydroxyl radical is the ultimate toxic ROS and optimal protection against the cytotoxicity of alloxan is provided by a combination of SOD and catalase, which completely prevents redox cycling between alloxan and dialuric acid, and therefore the generation of all ROS in this pathway [38]. The current results also reflected and supported this fact. Catalase and GPX are two major enzymes involved in the degradation of  $H_2O_2$ . GPX provides a high affinity but low capacity degradative system, whereas catalase represents a relatively low affinity and high capacity system. Therefore, the main regulator at high concentrations of  $H_2O_2$  is enzyme catalase [40-42]. GPX or SOD might compensate for the catalase deficiency in acatalasemic pancreas, though, no significant compensatory up-regulation of GPX and SOD were found in alloxan-induced diabetes in acatalasemic pancreas. The pancreatic

catalase activities in this study increased in alloxan-treated diabetes. A hyperglycemic state might lead to an increased concentration of H<sub>2</sub>O<sub>2</sub> and cause the induction of catalase activity as a defense mechanism against free radicals, but it might not be sufficient to prevent the onset of diabetes. On the other hand, Tanaka et al. showed that overexpression of GPX in islets isolated from the model rats of type 2 diabetes provided enhanced protection against oxidative stress [43]. GPX protects cells from both excessive levels of H<sub>2</sub>O<sub>2</sub> and intracellular lipid peroxides, while catalase catabolizes only H<sub>2</sub>O<sub>2</sub>. The contradiction of these results may, in part, be explained by the difference in the involvement of lipid peroxides catabolism between type 1 diabetes under excessive oxidative stress in the acatalasemia and type 2 diabetes.

We found that treatment with 0.1mg/kg BW of telmisartan significantly inhibited the alloxan-induced hyperglycemia in acatalasemic mice. A number of clinical studies have suggested that treatment with AT1 receptor antagonist prevented the new-onset or the development of type 2 diabetes mellitus. Recently, local RAS in pancreas has been recognized [7] and, has been regarded as playing important roles in the pathogenesis of diabetes in animal models [44-47]. These studies suggest that islet RAS activation may be involved in oxidative-stress mediated islet apoptosis and fibrosis and that AT1 receptor antagonist may attenuate NADPH oxidase-induced oxidative stress in pancreas. As for type 1 diabetes mellitus, Chipitsyna et al. demonstrated that Ang II elicited an inflammatory response in the islets and  $\beta$ -cells by stimulation of Monocyte Chemoattractant Protein-1 (MCP-1) production and that hyperglycemia and progression to diabetes correlated with up-regulation of anigiotensin-converting enzyme, the enzyme responsible for production of Ang II [48]. The mechanism of beneficial effect of telmisartan on the onset of diabetes in acatalasemic mice should be elucidated. Telmisartan ameliorated renal fibrosis by inhibition of oxidative stress, but did not change the concentrations of renal antioxidant enzymes in acatalasemic UUO model [12]. This finding suggests that telmisartan inhibits



the generation of ROS rather than effecting ROS detoxification.

Finally, this study demonstrated that a higher dose of alloxan accelerated the severe atrophy of the pancreatic islets and induced apoptosis in catalase deficient conditions using acatalasemic mice, and promoted a high incidence of diabetes. In addition, telmisartan prevented alloxan-induced hyperglycemia under acatalasemic conditions. However, the mechanisms of induction of pancreatic islet cell death in this model were not apparent, and maybe not only apoptosis but necrosis also could be involved in the islet cell death, since alloxan can act as a diabetogenic agent and usually cause  $\beta$  cell death by necrosis [38]. Further studies of apoptosis-related cytokines or molecules and elucidation of the mechanisms of the efficacy of AT1 receptor antagonists in this model, including whether the inhibition of NADPH oxidase could be involved, would provide crucial information about the relationship between the oxidative stress and the onset of diabetes in the presence of a catalase deficiency. Regardless of the type of diabetes, its development and complications are life-threatening and this disease remains one of the most important social and health problems. Future studies on the development of novel therapeutic strategies to inhibit the development of diabetes mellitus, including the specific detoxification of hydroxyl radicals should be carried out in acatalasemic mice.

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ACCEPTED MANUSCRIPT

**Figure legends**

**Fig. 1.** Changes in the blood glucose concentration in wild-type and acatalasemic mice. The blood glucose concentrations significantly increased in both groups at 7 days after the intraperitoneal injection with 180 mg/kg body weight (BW) of alloxan (ALX 180). The elevation of blood glucose in acatalasemic mice is remarkable at 2 and 7 days after the injection with ALX 180 in comparison to that in wild-type mice. The data are expressed as the mean  $\pm$  SEM; N=15 to 20 in each group. \*\*  $P<0.01$  vs. control in the same mouse group; ##  $P<0.01$  vs. wild-type ALX 180 mice at the same time point.

**Fig. 2.** The mean size of pancreatic islet and apoptosis in pancreatic islet cells in wild-type and acatalasemic mice at 7 days after injection with alloxan. Histological and morphometric analyses of pancreatic islets. Light micrographs of wild-type (A) or acatalasemic (C) control pancreatic islets and wild-type (B) and acatalasemic (D) pancreatic islet tissue 7 days after injection with ALX 180. PAS stain. Note that the atrophy of the pancreatic islet is significant only in the acatalasemic mice treated with ALX 180 (D, E). Panels F and G are fluorescent micrographs of TUNEL-positive pancreatic islet cells (arrowheads) in wild-type (F) and acatalasemic (G) mice at 7 days after the injection with ALX 180. A significant increase in the number of TUNEL positive cells in pancreatic islet in acatalasemic mice treated with ALX 180 (H). Scale bars represent 50  $\mu$ m in panels A to D, F and G. In panels E and H, each column consists of the means  $\pm$  SEM. N=7 to 10 (E), 5 to 9 (G) in each group. \*\*  $P<0.01$  vs. control in the same mouse group; ##  $P<0.01$  vs. wild-type ALX 180 mice at the same time point.

**Fig. 3.** Pancreatic content of antioxidant enzymes in alloxan-induced diabetes model of wild-type and acatalasemic mice. Catalase (A), glutathione peroxidase (GPX) (B), and



superoxide dismutase (SOD) (C) activities in pancreatic tissue. Each enzyme activity is expressed as nmol/sec/mg protein. Mean  $\pm$  SEM, N=6 to 7 in each group. \* $P$ <0.05; \*\* $P$ <0.01 vs. control in the same mouse group. #  $P$ <0.05; ##  $P$ <0.01 vs. wild-type mice at the same time point.

**Fig. 4.** Changes in blood glucose concentration in wild-type and acatalasemic mice treated with telmisartan. Telmisartan (0.1mg/kg/BW) treatment significantly prevented the development of hyperglycemia in acatalasemic mice treated with ALX 180 in comparison to that in wild-type ALX 180 mice. The data are means  $\pm$  SEM; N=10 to 15 in each group. \*\* $P$ <0.01 vs. control in the same mouse group; ##  $P$ <0.01 vs. wild-type ALX 180 mice at the same time point; †  $P$ <0.05, ‡  $P$ <0.01 vs. telmisartan-treated acatalasemic mice at the same time point. TS, telmisartan.

**Supplementary Fig.1.** Changes in the blood glucose concentration in wild-type and acatalasemic mice after the intraperitoneal injection of streptozotocin (STZ). STZ did not sensitize acatalasemic mice to diabetes.

**Supplementary Fig.2.** Laser-capture microdissection (LCM) of pancreatic islets of wild-type (A, C) and acatalasemic (B, D) control mice. Cresyl Violet stain. Scale bars represent 150  $\mu$ m in panels A to D. Panel E shows the relative expression ratio of catalase mRNA to  $\beta$ -actin mRNA in pancreatic islets and kidneys of wild-type or acatalasemic control mice. There was no significant difference between acatalasemic and wild-type mice. The mRNA expression of the islets was lower than that of the kidneys.

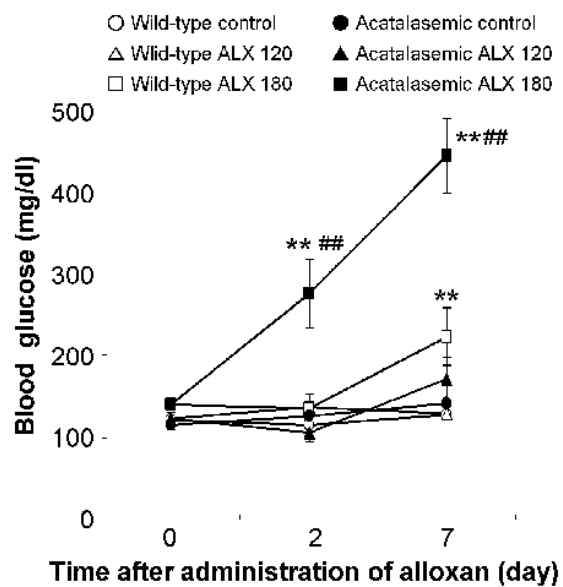
**Table 1. Body weight of mice with alloxan-induced diabetes**

Group		Day 0	Day 7
Wild-type	Control	28.5 ± 0.4	29.5 ± 0.4
	ALX 120 (mg/kg bw)	29.5 ± 0.6	30.0 ± 0.5
	ALX 180 (mg/kg bw)	30.1 ± 0.4	30.0 ± 0.4
Acatalasemic	Control	27.0 ± 0.4	28.2 ± 0.6
	ALX 120 (mg/kg bw)	27.2 ± 0.3	27.1 ± 0.3
	ALX 180 (mg/kg bw)	26.5 ± 0.4	25.3 ± 0.3 <sup>a,b,c</sup>

Values are means ± SEM (g) of 15 to 20 animals in each group.

<sup>a</sup> $P < 0.005$  vs. day 0 in the same group; <sup>b</sup> $P < 0.001$  vs. acatalasemic control day 7;

<sup>c</sup> $P < 0.0001$  vs. wild-type ALX180 group at day 7.

**Figure 1**

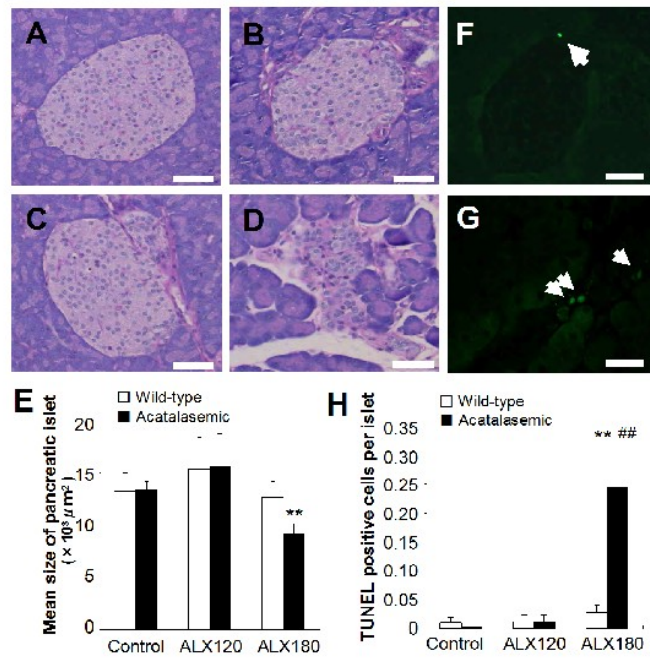


Figure 2

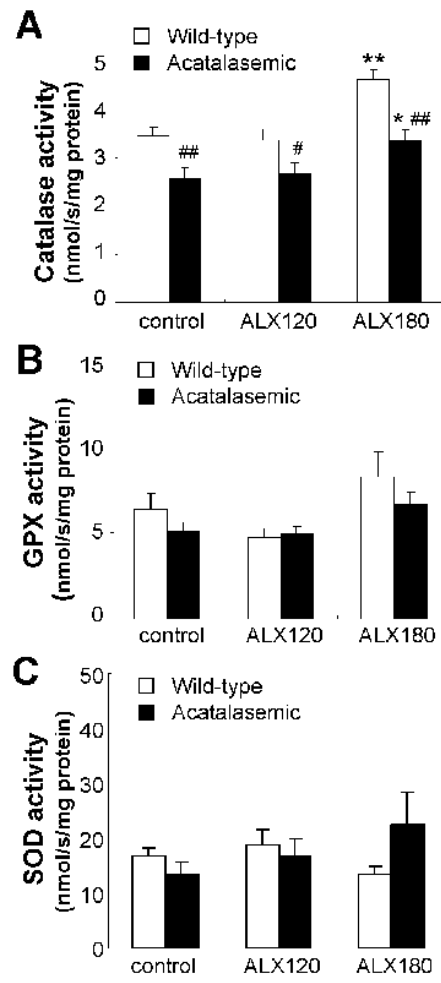


Figure 3

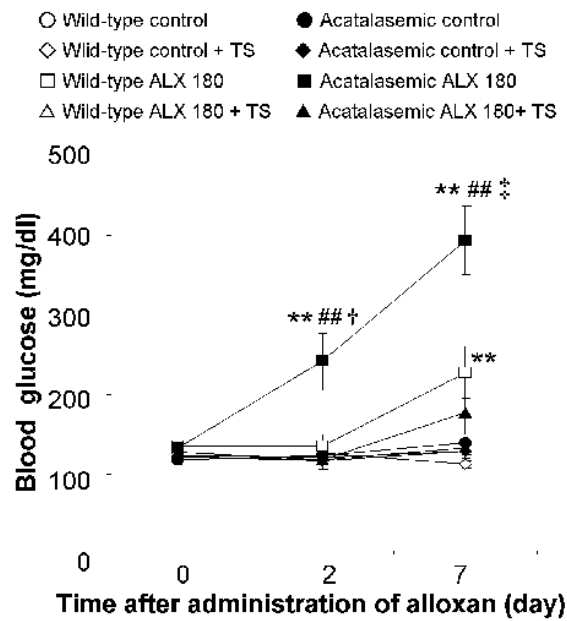


Figure 4