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The protective effect of *Amomum xanthoides* extract against alloxan-induced diabetes through the suppression of NFκB activation

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Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EMSA, electrophoretic mobility shift assay; iv, intravenous; ip, intraperitoneum; ROS, reactive oxygen species

Abstract

This study was undertaken to investigate the preventive mechanism of *Amomum xanthoides* extract against the development of alloxan-induced diabetics of mice. Pretreatment of mice with *A. xanthoides* extract *via* intraperitoneum prevented alloxan-induced hyperglycemia and hypoinsulinemia in a dose dependent manner. Histological examination of pancreatic tissue from *A. xanthoides* extract treated mice showed that the islet cells remain unaffected by alloxan treatment. NFκB activation in the pancreas 30 min after alloxan injection (60 mg/kg, *iv*), as assessed by an electrophoretic mobility shift assay, was not detected in the mice pretreated with *A. xanthoides* extract. These results suggest that NFκB activation may be one of the critical determinant in the progression of the disease. Considering the preventive effect of *A. xanthoides* extract from alloxan-induced diabetics development, these results may provide the possible therapeutic value of *A. xanthoides* extract for the prevention of diabetes mellitus progression.

Keywords: *Amomum xanthoides,* alloxan, NFκB, diabetes

Introduction

Alloxan is widely used for the development of experimental diabetes to induce selective dysfunctioning of pancreatic β-cells (Rerup, 1970; Lenzen and Panten, 1988). The mechanism of alloxan-induced diabetes has been the subject of many investigations, and it is generally accepted that reactive oxygen species (ROS) are involved in the initiation of the damage that ultimately leads to β-cell death (Yamamoto *et al*., 1981; Uchigata *et al*., 1982; Oberley, 1988; Rho *et al*., 2000). However the cellular mechanism responsible for β-cell death is still unclear. Our previous studies showed that increase of cytosolic free calcium in pancreatic β-cells played an important role in the alloxan-induced ROS generation (Kim *et al*., 1991; Kim *et al*., 1994).

Evidences suggest that ROS act as signal molecules in the regulation of gene expression, cell proliferation, and cell death (Palmer and Paulson, 1997; Rhee, 1999). Moreover, ROS are now known to play a critical role in the up-regulation of gene expression involved in the inflammatory and autoimmune responses.

During the course of oxidative cell death, the redox-sensitive transcription factor NFκB is activated (Schreck *et al*., 1991; Behl *et al*., 1994; Schmidt *et al*., 1995; Du *et al*., 1999). NFκB is predominantly consisted of the two subunits of p50 and p65. These proteins are members of the NFκB/Rel family of transcription factors that are known to control various genes involved in the inflammatory mechanisms. Typically, NFκB is sequestered in the cytoplasm by the specific inhibitory protein IκB. Activation and regulation of NFκB transition into the nucleus, where it can induce the transcription of NFκB-dependent target genes, is tightly controlled by IκB proteins (Baeuerle and Henkel, 1994; Baldwin, 1996).

Recently, Ho *et al.* (1999, 2000) suggested that inappropriate activation of NFκB by ROS might start a cascade of events that result in the inflammatory and autoimmune response in pancreas and inhibition of NFκB activation by antioxidants attenuated the severity of Type 1 diabetes. Once an autoimmune/inflammatory response is launched, the invading immune cells amplify ROS production, which ultimately destroys the β-cells. Thus, ROS are not simply cytotoxic agents that damage β-cells, but are key modulators of the cellular response pathways that initiate β-cell death and the development of Type 1 diabetes.

Diabetes mellitus is one of the most important health problems worldwide, showing high indices of prevalence and mortality. The inherent lack of antioxidant protection in the pancreatic islet may increase its sensitivity to diabetogenic agents that trigger ROS production (Lenzen *et al*., 1996; Tiedge *et al*., 1997). To date there have been different groups of hypoglycemic agents for clinical

use, having characteristic profiles of side effects. Management of diabetes without any side effects is still a challenge to the medical communities.

A. xanthoides comes from the fruit of *Amomum villosum* Lour and it belongs to the family of *Zingiberaceae*. It has been used to promote the well-being state of physiological system including gastroenteric organs (Ou, 1989). In the present study, we investigated the preventive effect of *A. xanthoides* extract on alloxan-induced diabetes. Pretreatment with *A. xanthoides* extract inhibited alloxan-induced NFκB activation, dysfunctioning of β-cells, and consequencial hyperglycemia and hypoinsulinemia.

Materials and Methods

Animals

Specific pathogen-free female ICR mice were purchased from the Korean Research Institute of Chemistry Technology (Daejon, Korea) and were housed throughout the experiments in a laminar flow cabinet, and maintained on standard laboratory chow *ad libitum*. All mice were 10-12 weeks-old of age.

Preparation of the extract

For water extraction, 200 g of *A. xanthoides* was ground and extracted with boiling water for 4 h. After the centrifugation at 3,000 *g* for 20 min, the supernatant was concentrated under reduced pressure and freeze-dried.

Effect of *A. xanthoides* extract on alloxan-induced diabetes

To induce diabetes, mice were injected with alloxan monohydrate *via* the tail vein in a dose of 60 mg/kg. Alloxan monohydrate was dissolved in saline just before use. To examine the effect of *A. xanthoides* extract on the development of alloxan-induced diabetes, *A. xanthoides* extract (2.5 mg/kg body weight) dissolved in saline was administered intraperitoneally 2 days before alloxan injection. Blood was obtained from the intra-orbital sinus. To minimize the effects of diurnal fluctuations, blood samples were collected at the same time every day.

Determination of blood glucose and insulin

Blood glucose was determined using glucose oxidase kit (Asan Pharm. Co., Korea), and serum insulin was assayed by standard radioimmunoassay technique (Amersham, USA).

Electrophoretic mobility shift assay

The nuclear extracts were prepared from the pancreases according to the method of Im *et al*. (1997). To inhibit endogenous protease activity, 1 mM phenylmethylsulfonyl fluoride was added. Protein contents in nuclear extracts were measured using the Bradford method (Bradford, 1976). As a probe for the gel retardation assay, NFκB specific oligonucleotide (κB, 5'-CCG GTT AAC AGA GGG GGC TTT CCG AG-3') was synthesized. The two complementary strands were annealed and labeled with [α-^{32}P]dCTP. Labeled oligonucleotides (10,000 cpm), 10 μg of nuclear extracts, and binding buffer (10 mM Tris-HCl, 500 mM KCl, 10 mM EDTA, 50% glycerol, 100 ng of poly(dI·dC), and 1 mM DTT, pH 7.6) were incubated for 30 min at room temperature in a final volume of 20 μl. The reaction mixture was analyzed by electrophoresis on a 4% polyacrylamide gel in 0.5X Tris-borate buffer. Specific binding was confirmed by competition with a 50-fold excess of cold κB oligonucleotide.

Histological examination

Following ablation 3 days after alloxan administration, pancreatic tissue was collected and the islet area was examined. The pancreas was fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin. At least 5 sections from each specimen, obtained at varying depths, were examined by light microscopy (Kim *et al*., 1991).

Statistical analysis

Statistical analysis of the data was performed with an unpaired Students *t*-test. Differences with $P < 0.05$ were considered statistically different.

Results and Discussion

Diabetes is possibly the world's fastest growing metabolic disease, so does the need for more appropriate therapies grow. Management of diabetes without any side effects is still a challenge to the medical community. Use of traditional plant medicines has been in practice for centuries at different regions of the world more so than others in order to alleviate diabetic symptoms. Despite insufficient evidences to support its therapeutic efficacy, the use of herbal medicine has increased considerably. A typical pharmacological action of *A. xanthoides* is considered to be a protective effect of gastrointestinal tract. Here we report another effect of *A. xanthoides* against alloxan-induced development of diabetics.

Mice that received 60 mg/kg of alloxan became hyperglycemic at 72 h (Figure 1A). Their blood glucose levels at 72 h were greater than 500 mg/dL, a value well within the acceptable diabetic range. In contrast, the mice pretreated with *A. xanthoides* extract (2.5 mg/kg, *ip*) and treated with alloxan remained to maintain blood glucose values in the normal range. This glucose lowering effect

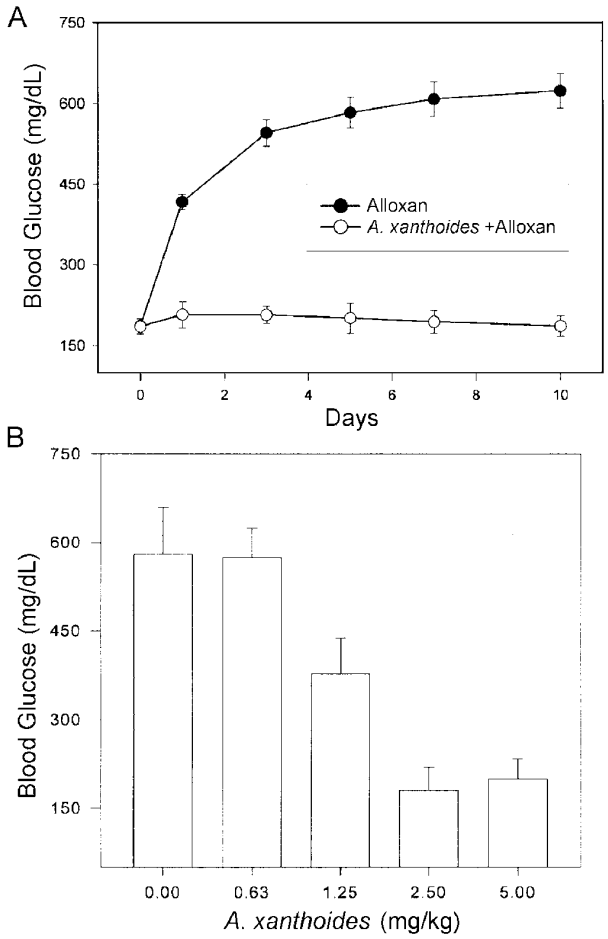

Figure 1. Effect of pretreated *A. xanthoides* extract on blood glucose levels in alloxan-treated mice. Alloxan (60 mg/kg) was injected *via* the tail vein of mice with or without pretreatment of *A. xanthoides* extract given intraperitoneally twice a day for 2 days prior to alloxan injection. The doses of pretreated *A. xanthoides* extract were either 2.5 mg/kg (A) or various ranges (B). Blood glucose was measured as described in "Materials and Methods". Each value denotes the mean ± S.D. of six separate experiments.

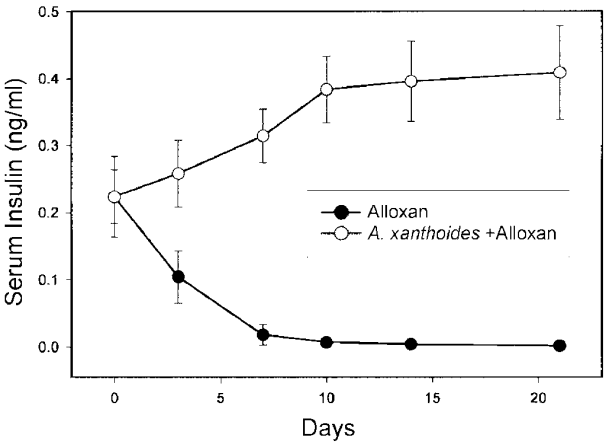

Figure 2. Effect of pretreated *A. xanthoides* extract on blood insulin levels in alloxan-treated mice. *A. xanthoides* extract (2.5 mg/kg) was given intraperitoneally twice a day for 2 days before alloxan injection. Blood insulin levels were determined by radioimmunoassay. Each value denotes the mean ± S.D. of six separate experiments.

was dose-dependent (Figure 1B). *A. xanthoides* extract treatment alone (no alloxan) did not affect blood glucose values in the mice (data not shown). Animals injected with alloxan alone showed a significant decrease in blood insulin levels in comparison with those of controls. Pretreatment of mice with *A. xanthoides* extract (2.5 mg/kg) attenuated the severity of alloxan-induced hypoinsulinemia (Figure 2). Thus, *A. xanthoides* extract at this dose are considered to be protective against the dose used to elicit alloxan-induced diabetes.

To elucidate the cellular effect of *A. xanthoides* extract, a histological examination on pancreatic islets was

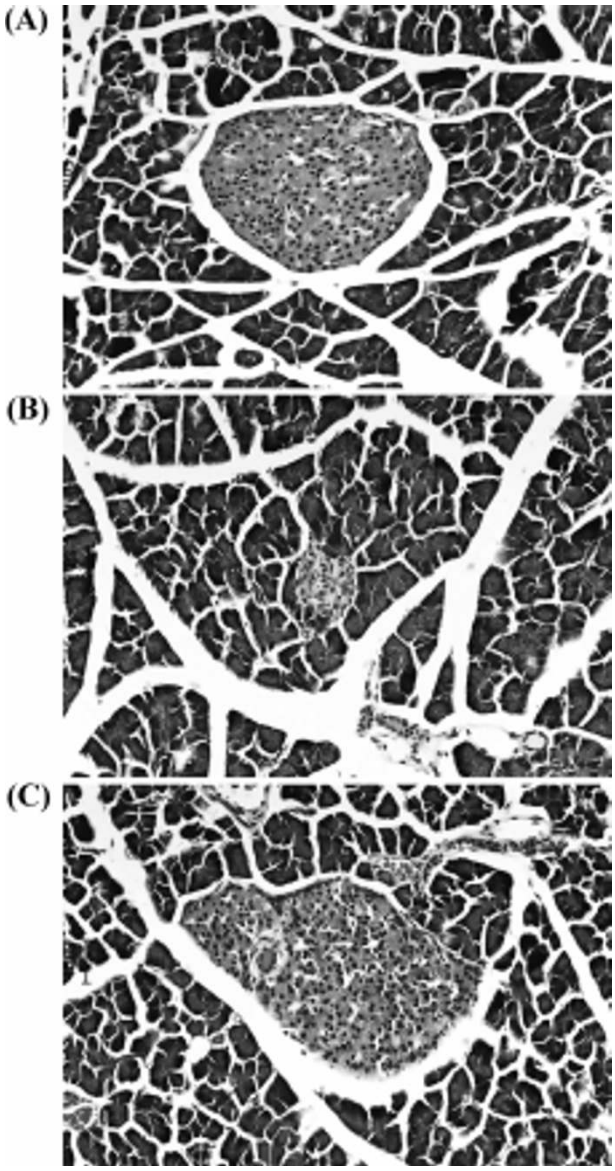

Figure 3. Histologic examination of pancreas. Pancreases were obtained 3 days after from normal control mouse (A), alloxan-induced diabetic mouse (B), and *A. xanthoides* extract (2.5 mg/kg)-pretreated mouse before alloxan-injection (C). The specimens were stained with hematoxylin and eosin and examined by light microscopy (× 200).

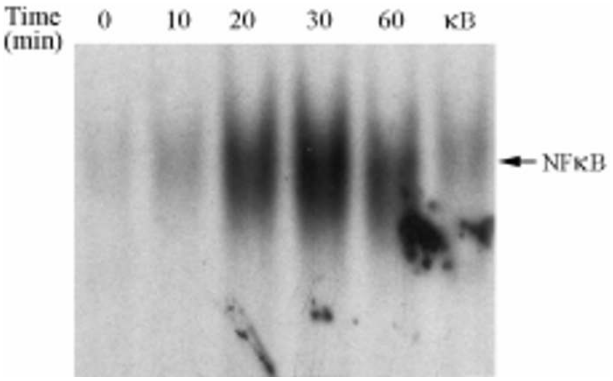

Figure 4. Time course of NFκB activation in the pancreas of mice injected with alloxan. Mice were injected with 60 mg/kg of alloxan or saline control. Mice were sacrificed at the indicated time, and pancreatic tissues were removed and immediately frozen. Nuclear extracts were incubated with ^{32}P-labeled κB oligonucleotide and electrophoresed on a 4% polyacrylamide gel. A 50-fold excess of unlabelled κB oligonucleotide was added to nuclear extract of 30 min as a competitor to confirm the specificity of NFκB binding. This experiment was repeated three times with similar results.

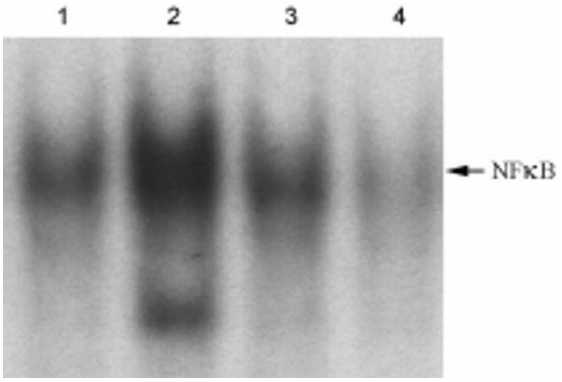

Figure 5. Inhibition of NFκB activation in pancreas by pretreatment of *A. xanthoides* extract. Mice were treated with alloxan in the presence or absence of *A. xanthoides* extract and NFκB activation was determined by electrophoretic mobility shift assay. Lane 1, control mouse; Lane 2, alloxan-treated mouse (for 30 min); Lane 3, Pretreatment with *A. xanthoides* extract before alloxan injection; Lane 4, A 50-fold excess of unlabelled κB oligonucleotide was added to nuclear extract of alloxan treated mouse. This experiment was repeated three times with similar results.

performed 3 days after alloxan administration with or without *A. xanthoides* extract pretreatment. Alloxan-treated mouse showed a marked change of pancreatic islets (Figure 3B): the islet decreased in its size and contained relatively small numbers of cells whereas numerous well-defined cells were seen in the pancreatic islets of the normal and *A. xanthoides* extract-pretreated mice (Figure 3B and C).

To elucidate the protective mechanism against alloxan-induced diabetes, we next examined the ability of alloxan to induce the activation of NFκB and determined if *A. xanthoides* extract could inhibit NFκB activation. Figure 4 shows a representative EMSA radiograph that depicts the ^{32}P-DNA/NFκB complex present in the nuclear extracts of the pancreas after injection of alloxan at various time points. The specificity of NFκB binding was confirmed by using an excess of unlabeled oligonucleotides, which comprise of NFκB binding sites as a specific competitor. NFκB activation was maximal at 30 min after alloxan injection in pancreatic nuclear extracts and still sustained until 60 min. Figure 5 illustrates that the pretreatment with *A. xanthoides* extract inhibits alloxan-induced NFκB activation. Similar to Figure 4, NFκB activation was observed at 30 min after alloxan administration. However, pretreatment of *A. xanthoides* extract (2 day before alloxan administration) prevented any activation of NFκB.

There is no doubt that alloxan induces NFκB activation *in vivo* and this is well correlated with the works of Ho *et al.* (1999, 2000). It is interesting to note that alloxan rapidly induces NFκB activation in pancreas within 30 min after an intravenous injection of alloxan. Pretreatment with *A. xanthoides* extract alone did not induced NFκB activation in the pancreas.

In conclusion, there appears to be a good correlation between the protective effect of *A. xanthioides* against alloxan-induced diabetics and its preventing effect of NFκB activation. This preventive effect against NFκB activation may explain the antidiabetic effect of *A. xanthoides* extract.

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