Allopurinol Protects Pancreatic β Cells from the Cytotoxic Effect of Streptozotocin: In Vitro Study

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Isolated rat pancreatic β cells in monolayer culture were shown to be protected from the cytotoxic effect of streptozotocin (STZ) by allopurinol. Pretreatment with allopurinol for 2 h caused dose-dependent inhibition of the decreased secretion of insulin by the cells induced by STZ (2 mM, for 1 h), 500 μM allopurinol causing complete inhibition of this effect of STZ. Pretreatment with allopurinol (250 μM) also prevented the rapid decrease in intracellular adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide concentrations in β cells induced by treatment with STZ. High performance liquid chromatography revealed that the intracellular concentration of uric acid in STZ-treated cells was about 3 fold that of control cells. This finding suggests that the reaction of xanthine oxidase is facilitated in the cells exposed to STZ probably due to an increased supply of substrate resulting from decrease in intracellular ATP. Based on these results, a possible mechanism of the effect of allopurinol on the cytotoxic effect of STZ via xanthine oxidase is discussed.

Keywords — allopurinol; ATP; pancreatic β cell; streptozotocin; xanthine oxidase

Introduction

Superoxide dismutase (SOD) and dimethylurea protect pancreatic β cells from the cytotoxicity of streptozotocin (STZ) suggesting that free radicals derived from oxygen may be related to the toxic effect of STZ. However, the mechanism of generation of free radicals by STZ is unknown. Recently, we reported that the activity of xanthine oxidase (XOD) in a homogenate of rat pancreatic β cells was high, but that of SOD was extremely low. In general, cellular superoxide anion (O₂⁻) is thought to be generated by an XOD system. Therefore, assuming that XOD is present intrinsically in β cells, the cells might be exposed to a high concentration of O₂⁻ radical when sufficient substrate of XOD is present.

As shown in this paper and reported by others the cellular adenosine triphosphate (ATP) level of pancreatic β cells was markedly decreased by STZ. Therefore, in this study we examined whether STZ increases the level of substrate for XOD by decreasing the intracellular level of ATP. We also examined the influence of allopurinol, an inhibitor of XOD, on the cytotoxic effect of STZ in β cells.

Materials and Methods

Allopurinol, hypoxanthine, xanthine, uric acid, luciferin-luciferase mixture (FLE-40), thiazolyl blue, penicillin G and streptomycin sulfate were purchased from Sigma. Nicotinamide adenine dinucleotide (NAD) and alcohol dehydrogenase were from Oriental Yeast (Tokyo). Phenazine methosulfate and an insulin assay kit (Insulin B-Test) were from Wako (Osaka). Medium 199 was from Nissui (Tokyo). Fetal bovine serum (FBS) was from Flow Laboratories (Sydney). All other reagents used were of analytical grade.

Islet cells were isolated from the pancreas of neonatal Wistar rats as described previously and were plated into 100 mm plastic culture dishes (Falcon). The cells were cultured in medium 199 supplemented with 10% FBS, 100 U/ml penicillin G, 100 mg/l streptomycin sulfate and 16.7 mM glucose. After incubation for 16 h, non-adherent cells were collected and resuspended in the same medium, and 0.5 ml volumes of cell suspension (5 x 10⁶ - 10⁶ cells) were plated into 16 mm culture wells (24 wells/plate, Falcon) and maintained in a CO₂-incubator (37 °C, 100% humidity, 5% CO₂-95% air). To eliminate contaminating fibroblasts, we
added 3 \( \mu g/ml \) of monoiodoacetic acid for 4 h.\(^{83} \) The cultured cells were incubated with the indicated concentrations of allopurinol for 2 h, washed thoroughly with medium 199 and then exposed to 2 mM STZ for 1 h.\(^{93} \) The cells were treated with STZ only as described above and then incubated for the indicated periods.

Insulin released into the medium was determined by enzyme immunoassay with an Insulin B-Test Kit. Homogenates of the cells were used for measurements of intracellular NAD and ATP and XOD activity. NAD was determined by the method of Nisselbaum and Green\(^{10} \) and ATP was measured with luciferin-luciferase\(^{11} \) in a Lumac/3M Biocounter. The activity of XOD was determined as described by Hashimoto\(^{12} \) using 2 mM xanthine as substrate.

Metabolites of the XOD reaction in \( \beta \) cells were examined by high performance liquid chromatograph (HPLC) equipped with a Shimadzu LC-6A. The stationary phase was Shimpack CLC-ODS in a stainless-steel column (150 \( \times \) 6 mm). Phosphate buffer (50 mM, pH 6.5 containing 20% methanol) was used as the mobile phase at a flow rate of 0.8 ml/min. The cultured \( \beta \) cells were exposed to distilled water and heat treatment in boiling water for 10 min. The preparation was then centrifuged and 20 \( \mu l \) of the supernatant was applied to an HPLC column.

**Results**

Figure 1 shows the release of insulin from cultured \( \beta \) cells into the medium measured after 6 h incubation. In the absence of allopurinol, STZ decreased the secretion of insulin to about 30% of that of control cells. But pretreatment of allopurinol for 2 h suppressed this decrease dose-dependently and allopurinol at a concentration of 500 \( \mu M \) or more restored insulin secretion to the control level.

The effects of STZ and allopurinol on the intracellular NAD level are shown in Table I. Addition of 2 mM STZ decreased the level to less than 10% of that of control cells, while 250 \( \mu M \) allopurinol prevented this decrease and maintained the NAD concentration at the control level. Allopurinol has similar effects at 250 and 375 \( \mu M \), indicating that its effect leveled off at a concentration of 250 \( \mu M \) or less.

STZ is reported to decrease intracellular ATP as well as NAD in mouse islet-cells.\(^{63} \) In isolated rat \( \beta \) cells, treatment with STZ resulted in rapid decrease in intracellular ATP as shown in Fig. 2. Allopurinol caused dose-dependent inhibition of this decrease in ATP concentration observed 6 h after treatment with STZ; 250 \( \mu M \) allopurinol maintaining the control level of ATP (Table II).

**Table I. NAD Concentration of Cultured Pancreatic \( \beta \) Cells**

<table>
<thead>
<tr>
<th>Allopurinol conc. (( \mu M ))</th>
<th>( n )</th>
<th>NAD (ng/10(^5 ) cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>243 ± 11</td>
</tr>
<tr>
<td>0</td>
<td>4</td>
<td>15.0 ± 0.1</td>
</tr>
<tr>
<td>250</td>
<td>4</td>
<td>257 ± 17</td>
</tr>
<tr>
<td>375</td>
<td>3</td>
<td>277 ± 18</td>
</tr>
</tbody>
</table>

The cells were pretreated with the indicated concentrations of allopurinol for 2 h, exposure to 2 mM streptozotocin for 1 h and then washed and incubated for 6 h. They were then collected and homogenized for NAD determination. Control cells were incubated in the same conditions without test chemicals. Values are means ±S.E.
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Fig. 2. Effect of Streptozotocin Treatment of Intracellular ATP Level of Cultured Pancreatic β Cells

The cells were exposed to 2 mM streptozotocin for 1 h and then washed thoroughly. C, ATP level in control cells incubated without streptozotocin (n = 8). Points represent means for numbers of samples shown in parentheses. At times SEs were less than 5% of the means.

Next we examined the inhibition of XOD activity in β cells by allopurinol. The activity was 14.4 ± 1.0 mU/sample in control cells (n = 3), 10.0 ± 0.5 mU/sample with 250 μM allopurinol (n = 3), and undetectable with 500 μM allopurinol (n = 3). With 250 μM allopurinol, which maintained the intracellular NAD and ATP concentration at control levels, the inhibition of XOD was 30%.

We determined intracellular uric acid in control and STZ-treated β cells by HPLC, and typical HPLC patterns are shown in Fig. 3. Quantitative analysis revealed that the concentration of uric acid in STZ-treated cells was about 300% of that in control cells; 0.67 ± 0.13 nmol/10^6 in control cells (n = 4) and 2.10 ± 0.24 nmol/10^6 in STZ-treated cells (n = 5). This finding suggests that the reaction of the XOD system was facilitated in cells exposed to STZ, probably because of an increased supply of the enzyme substrate, hypoxanthine, by degradation of ADP as the level of ATP was much lowered by STZ.

Fig. 3. High Performance Liquid Chromatograms of Metabolites of Xanthine Oxidase in Control and Streptozotocin-treated Cultured Pancreatic β Cells

The stationary phase was Shimpack CLC-ODS (Shimadzu). Phosphate buffer (50 mM, pH 6.5) containing 20% methanol was used as the mobile phase at a flow rate of 0.8 ml/min. Std., Authentic samples of hypoxanthine (Hypox), xanthine (X) and uric acid (UA); control, control cells; STZ, streptozotocin-treated cells.

TABLE II. ATP Concentration in Cultured Pancreatic β Cells

<table>
<thead>
<tr>
<th>Allopurinol conc. (μM)</th>
<th>n</th>
<th>ATP (ng/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>83.0 ± 1.7</td>
</tr>
<tr>
<td>0</td>
<td>4</td>
<td>29.8 ± 1.6</td>
</tr>
<tr>
<td>125</td>
<td>4</td>
<td>60.7 ± 5.7</td>
</tr>
<tr>
<td>250</td>
<td>4</td>
<td>94.0 ± 6.5</td>
</tr>
<tr>
<td>375</td>
<td>4</td>
<td>79.2 ± 4.6</td>
</tr>
</tbody>
</table>

Experimental conditions were as for Table I. Values are means ±S.E.

Discussion

In this study we showed that allopurinol protects pancreatic β cells from the cytotoxic effect of STZ. XOD is known to be transformed from xanthine dehydrogenase (XDH) in vivo in a variety of conditions and it is hard to determine whether the activity of XOD observed in certain tissue is present intrinsically or is an artifact formed during experimental procedures such as homogenization of the tissues. Thus the actual
XOD/XDH ratios in intact tissues are unknown. In particular we know of no report on the relation between XOD and XDH in pancreatic β cells. However, assuming that β cells intrinsically have XOD activity, decrease in their intracellular ATP concentration caused by STZ would result in increased degradation of ADP and provide endogenous hypoxanthine as a substrate of XOD. XOD in β cells would oxidize hypoxanthine to uric acid as a terminal metabolite, since the activity of uricase in pancreas is reported to be very low or undetectable. Our finding showing the accumulation of intracellular uric acid in β cells supports these possibilities. When XOD operates, an O$_2^-$ radical is generated stoichiometrically. As the activity of SOD in β cells is extremely low as previously reported, the β cells would be exposed to a high concentration of O$_2^-$ or hydroxyl radical produced by the Haber-Weiss reaction from O$_2$. The injury of β cells by this oxygen-derived radical might be amplified by STZ, because STZ itself activates XOD and enhances the generation of O$_2^-$ radical as we reported previously.

In our experimental conditions, the possibility that allopurinol acts as a radical scavenger is excluded, by the fact that the effective concentration of allopurinol was less than 500 μM. The reason for the decreased level of ATP in β cells by STZ is unknown. This effect of STZ in decreasing the ATP level may be explained by our finding in separate experiments that STZ or its decomposition product(s) impaired the functions of rat hepatic mitochondria (unpublished).

Although many problems still require elucidation, our new finding that allopurinol protects pancreatic β cells from the cytotoxic effect of STZ indicates an alternative explanation for the mechanism of the action of STZ in pancreatic β cells.

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References