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Protective Effect of Copper against Pancreatic Insult in Streptozotocin-induced Diabetogenic Rat

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Abstract□The present study examined the alterations in endogenous oxy-radical scavenging system of pancreatic tissue associated with the dose of 45 mg/kg streptozotocin (STZ) alone or with various combinations. The activities of pancreatic Mn-superoxide dismutase (SOD) and catalase were no apparent changes in the other groups except for the Cu(II) 4 mg/kg pretreated group. The presence of 4 mg/kg of Cu(II) with or without 125 mg/kg of diethylenetriaminepentaacetic acid (DTPA) markedly attenuated the fall in activity of Cu, Zn-SOD by STZ stress. In particular, STZ-induced superoxide generation was dramatically abolished by prior administration of Cu(II) 4 mg/kg. Conclusively, We suggest the possible involvement that copper may enhance the defense mechanism of pancreatic oxidative damage by STZ challenge.

Keywords□Oxy-radical scavenging system, streptozotocin, Cu(II).

Streptozotocin (STZ) is widely used in studies of experimental diabetes because this agent destroys the pancreatic β cells with relative selectivity[1,2]. Recent evidence indicates the damage of STZ on the pancreatic tissue results from oxygen free radical in the presence of transition metals[3]. During the course of normal metabolism, 98% of molecular O_2 undergoes complete reduction to water via oxidase pathway. The reminder is converted by an oxygenase pathway to potentially toxic reactive species including superoxide and hydroxyl radicals as well as H_2O_2, which are capable of causing oxidative damageto alteration in subcellular organelle structural and functional integrity[4]. The levels of these intermediate reduction products of O_2 metabolism are controlled by free radical scavenger i.e. superoxide dismutase, catalase, and glutathione peroxidase. Alteration in these mechanism may be important in various pathological states, including diabetes[5,6]. Superoxide anions dismute to form H_2O_2, and catalase also antagonized H_2O_2, peroxide appears to be involved. Most attention has been lately focused on antioxidant effect by the mechanism as a quencher in toxic metabolism of oxygen.

Based on the foregoing finding, we measured the activity of several oxygen radical scavenging system by pretreatment of various chemicals in exocrine plus endocrine pancreatic tissue in rat with STZ-induced hyperglycemic state.

EXPERIMENTAL METHODS

Materials

Streptozotocin, diethylenetriaminepentaacetic acid, cupric acetate , ferric chloride, xanthine oxidase, superoxide dismutase, cytochrome C, bovine serum albumin, xanthine, sodium deoxycholate, and collagenase were purchased from Sigma. Co. USA. Glibenclamide was obtained from Boehringer Mannheim Co. All other chemicals were used special grade.

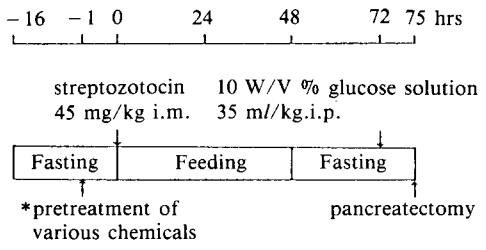
Male Spraque-Dawley rats (200 ± 20g) were used. Experimental animals were adapted for 3 weeks under the contant conditions.

Experimental protocol

According to the method of Nakhoda *et al*[7], diabetogenic state was induced. Like Scheme 1, the pretreatments of various chemicals were carried out prior to STZ dosing.

Dispensing of chemicals

STZ was dissolved in 100 mM citrate buffer (pH 4.5) and kept in ice bath. STZ solution was prepared immediately prior to injection. 625 mg of



*Group 1: Normal control.
2: STZ 45 mg/kg.i.m.
3: DTPA 250 mg/kg.i.p. + STZ 45 mg/kg.i.m.
4: DTPA 125 mg/kg.i.p. + STZ 45 mg/kg.i.m.
5: Glibenclamide 1 mg/kg.p.o. + STZ 45 mg/kg.i.m.
6: Cu(II) 4 mg/kg.i.p. + STZ 45 mg/kg.i.m.
7: Fe(III) 10 mg/kg.i.p. + STZ 45 mg/kg.i.m.
8: [Cu(II) 4 mg/kg.i.p. + DTPA 125 mg/kg.i.p.] + STZ 45 mg/kg.i.m.
9: [Fe(III) 10 mg/kg.i.p. + DTPA 125 mg/kg.i.p.] + STZ 45 mg/kg.i.m.

Scheme 1. Experimental protocol.

diethylenetriaminepentaacetic acid (DTPA) which was evaporated and condensed in 0.1 N-NaOH solution (pH 7.0) was dissolved in 10 m*l* of physiologic saline solution. Cupric acetate, ferric chloride and glibenclamide were dissolved in physiologic saline solution, respectively.

Tissue preparation

According to the method of Beppu *et al*[8], the pancreatic tissue was cut into small species and homogenated by slight collagenase digestion for 5 min at 37°C [4 U/5 m*l* phosphate buffered saline, pH 7.5 (PBS)]. The resulting pancreatic homogenate was washed three times with PBS for 3 min at 200×g. This homogenate was immediately used for experimental assay.

Detection of superoxide radical formation

Superoxide anion production in pancreatic tissue was determined by the rate of superoxide dismutase (SOD) inhibitable acetylated cytochrome C reduction[9]. The initial rate of acetylated cytochrome C reduction in the presence and absence of SOD was monitored spectrometrically at 550 nm and 37°C in a Beckman model M-35 recording spectrophotometer equipped with a circulatng water bath. Superoxide generation was calculated from the rate of acetylated cytochrome C reduction that was inhibited by SOD using extinction coefficiednt for cytochrome C (reuced minus oxidized) of 19.6 $mM^{-1}cm^{-1}$.

Enzymatic assay

Approximately half of the homogenate was removed for determination of catalase activity. The reminder was centrifuged at 10000×g for 30 minutes. The supernatant was mixd with 0.25 times the volume of ethanol and 0.15 times the volume of chloroform to remove Mn-SOD.

After recentrifuging at 5000×g for 20 minutes, the supernatant was stored at 4°C until assayed for SOD activity and protein. Cu, Zn SOD activity was measured spectophotometrically by its ability to inhibit the superoxide mediated reduction of ferric cytochrome C by xanthine oxidase plus xanthine[9-11]. The amount of xanthine oxidase was adjusted to obtain an initial reaction rate of 0.025 absorbance unit per minutes. The amount of total SOD was determined by the above procedure, whereas the absence of Mn SOD was verified by its insensitivity to cyanide inhibition[12].

For mitochondrial fractions, sonication is necessary to measure full Mn SOD activity.

We sonicated at the maximum output of a Bronson cell disruptor for 5 min, while on ice.

Cu. Zn SOD was calculated from the difference between the two assays. Under these conditions, one unit of activity was defined as that amount of enzyme which caused by 50% inhibition of the reaction.

Catalase was assayed by the method of Aebi[13] at 240 nm and expressed as the rate of the reaction with H_2O_2. Protein was determined by the method of Lowry[14] with bovine serum albumin as standard.

RESULTS

As shown in Table I, the several oxy-radical scanvenging effects of various injection 1 hr prior to STZ were determined.

Influence of various pretreatment on the generations of superoxide radical in STZ-induced cytotoxic pancreas in rats

Solely treated STZ tended to increase the rate of superoxide generation in rat although the differcene was not significant. When several chemicals were pretreated before STZ, the rate of superoxide generation was the highest in the group of DPTA 250 mg/kg, and the lowerest in the group of Cu(II) 4 mg/kg. On the other hand, the pretreatments except for Cu(II) 4 mg/kg or DPTA 250 mg/kg did not influence on the rate of superoxide generation induced by STZ.

Influence of various pretreatment on the specific activities of mitochondrial Mn-SOD in STZ-induced cytotoxic pancreas in rat

Table I. The effects of various combinations on streptozotocin (STZ) toxicity in isolated pancreas.

Group	No of rat	Superoxide mediated cytochrome C reduction (u mole/ /min/tissue g)	Mitochondrial SOD (U/protein mg)	Cytosolic SOD (U/ protein mg)	Catalase (n mole/min/ protein mg)
Normal control	10	28.37 ± 5.65	0.86 ± 0.12	0.84 ± 0.10	54.87 ± 5.61
STZ 45 mg/kg.i.m.	10	48.30 ± 7.44	0.94 ± 0.11	0.41 ± 0.05[a]	35.87 ± 4.13
DTPA 250 mg/kg.i.p. + STZ 45 mg/kg.i.m.	6	107.50 ± 5.80[c]	1.22 ± 0.20	0.62 ± 0.15	26.00 ± 2.56
DTPA 125 mg/kg.i.p. + STZ 45 mg/kg.i.m.	5	62.78 ± 12.01	1.08 ± 0.15	0.70 ± 0.26	38.70 ± 4.78
Glibenclamide 1 mg/kg.p.o. + STZ 45 mg/kg. i.m.	6	59.05 ± 5.25	0.97 ± 0.04	0.56 ± 0.11	46.30 ± 7.99
Cu (II) 4 mg/kg.i.p. + STZ 45 mg/kg.i.m.	6	0.50 ± 0.05[d]	2.45 ± 0.39[c]	2.02 ± 0.25[d]	59.15 ± 6.52[b]
Fe (III) 10 mg/kg.i.p. + STZ 45 mg/kg.i.m.	6	42.72 ± 1.91	0.97 ± 0.11	0.27 ± 0.04	30.08 ± 3.48
[Cu (II) 4 mg/kg.i.p. + DTZ 125 mg/ kg.i.p.] + STZ 45 mg/kg.i.m.	6	49.30 ± 12.96	2.01 ± 0.35[b]	0.98 ± 0.13[b]	37.04 ± 8.25
[Fe (III) 10 mg/kg.i.p. + DTPA 125 mg/kg.i.p.] + STZ 45 mg/kg.i.m.	6	59.03 ± 7.26	1.67 ± 0.35	0.81 ± 0.27[f]	45.75 ± 5.29[e]

Each value shows the mean ± S.E.

SOD one unit was defined as the quantity of SOD required to produce 50% inhibition of the rate of reduction of cytochrome C under the specified conditions.

Catalase was expressed as the rate of the reaction with H_2O_2 at 240 nm.

a: $p<0.02$, V.S. Normal control

b: $p<0.05$, V.S. STZ 45 mg/kg

c: $p<0.01$, V.S. STZ 45 mg/kg

d: $p<0.001$, V.S. STZ 45 mg/kg

e: $p<0.05$, V.S. DTPA 250 mg/kg + STZ 45 mg

f: $p<0.05$, V.S. Fe (III) 10 mg/kg.i.p. + STZ 45 mg/kg.i.m.

The activities of the mitochondrial enzymes of rat receiving Cu(II) 45 mg/kg alone or with DPTA 125 mg/kg were significantly increased by 2.6 ($p<0.01$) or 2.1 ($p<0.05$) fold as compared with STZ alone. On the other hand, DPTA 125 mg/kg with Fe(III) 10 mg/kg tended to activate the level of the Mn SOD, but the effects was not found in the group of Fe(III) 10 mg/kg alone.

Influence of various pretreatment on the specific activities of cytosolic Cu, Zn SOD in STZ-induced cytotoxic pancreas in rat

The STZ showed significantly lower activity of Cu, Zn SOD compared with normal group ($p<0.02$). Remarkably, those animals received Cu(II) 4 mg/kg with or without DPTA 125 mg/kg prior to STZ did differ significantly from solely treated STZ ($p<0.05$, $p<0.001$). The lowered activity of Cu, Zn SOD in Fe(III) 10 mg/kg group was restored by additional DPTA 125 mg/kg ($p<0.05$). However, the administrations with DPTA 125 mg/kg, DPTA 250 mg/kg, Glienclamide, or Fe(III) before STZ were not effective.

Influence of various pretreatment on the specific activities of catalase in STZ-induced cytotoxic pancreas in rat

The STZ group exhiniting a slight decrease in catalase activity as compared with normal group. The lowest activity of catalase was found in group receiving DPTA 250 mg/kg before STZ in all groups. Although the catalase activity induced by STZ treatment was returned to the in the presence of Cu(II) 4 mg/kg, Glibenclamide, or Fe(III) 10 mg/kg plus DPTA 125 mg/kg in rats, the effect was only significant in Cu(II) 4 mg/kg pretreated rat.

DISCUSSION

The major source of superoxide in the massive damaged tissue appears to be the enzyme xanthine oxidase. This enzyme was the first documented biologic source of the superoxide radical and has been the subject of intensive study[15]. When most tissue are homogenized without special precautions, the xanthine dehydrogenase converts to the xanthine oxidase as a result of sulfhydryl oxidation[16].The xanthine oxidase

can use molecular oxygen instead of NAD^+, producing superoxide, as follows:

$$\text{Xanthine} + H_2O + 2O_2 \xrightarrow{\text{xanthine oxidase}} \text{uric acid} + 2O^-_2 + 2H^+$$

Recently, Kawada[17] observd that the xanthine oxidase reaction was facillitated by STZ. This observation was evidenced by the elevation in intracellular uric acid and the increase in xanthine oxidase activity from cultured pancreatic beta cells in the conditions induced by STZ. Similarly, our *in vivo* experiments indirectly confirm the finding of Kawada that the state of rise in xanthine oxidase, major source of superoxide, may play a pathogenic role in diabetes. Our speculation is supported by the result that the rate of superoxide generation in STZ group is increased by 1.7 fold as compared with the control group.

Especially, the STZ group in this study manifested markedly low activity of Cu, Zn SOD compared with the control group, agreed with the findings of Gandy *et al*[18]. It is known that the DPTA binds and inactivates iron with respect to the Harber-Weiss reaction may protect pancreatic islet efficiently against diabetogenics[19]. In the present study, unexpectedly, we observed the dosage 250 mg/kg of DPTA-induced unfavorable effect that was evidenced by the activation in superoxide generation, However, when the dosage of 125 mg/kg DPTA was administered, the unfavorable effect was inclined to inactivate.

Particularly, the rats of superoxide formartion and the alterations of mitochondrial and cytosolic SOD from STZ stress were markedly attenuated by addition of Cu(II) 4 mg/kg in the present assay system. Several studies showed the possibility that copper ions and copper complexes have SOD-like activity. In contrast, SOD activity is also inhibited in animal treated with copper chelator, diethyldithiocarbamate[20,21]. The Cu, Zn-SOD is present in relatively high level in the beta cells of pancreatic islets[22,23]. Thus, Cu, Zn-SOD may play an important role in pancreatic homeostsis. It seems that the rise in fallen the cytozolic Cu Zn-SOD level may result in some amelioration of the defense mechanism in cytotoxic effects of STZ.

Although the detail experiment was not performed in this study, it is suggesting the probability that catalase may be the one of the imporant determinants of commercial antidiabetics, glibenclamide, in antioxidant status. Catalase has been implicated in the detoxification of high H_2O_2 concentratons[24]. The results obtained in the prior administration of copper suggest that marked elevation in catalase activity may be an important adaptive response against the conditions of increased H_2O_2 production by dismutation. However, the reasons for the lowered catalase activities in the presence of DPTA 250 mg/kg and Cu(II) 4 mg/kg plus DPTA 125 mg/kg are not ready to reply. Therefore, further research in peroxidase system is highly required. Conclusively, our data do permit evalution of the possibility that the Cu(II) administration prior to STZ stress may enhance the antioxidant capacity. However, the present observations only provide a basis for further exploration in antioxidant effect of suitable transition metal ion against cytotoxicity of STZ. Continuously, we will focus on the effects of trace elements such as, copper and selenium affecting tissue antioxidant status as an approach to device rational therapeutic measures.

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