BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **245,** 11–16 (1998)
ARTICLE NO. RC988368

Generation of Nitric Oxide from Streptozotocin (STZ) in the Presence of Copper(II) plus Ascorbate: Implication for the Development of STZ-Induced Diabetes

Akihiro Tsuji and Hiromu Sakurai[1]

Department of Analytical and Bioinorganic Chemistry, Kyoto Pharmaceutical University, Nakauchi-cho 5, Misasagi, Yamashina-ku, Kyoto 607-0825, Japan

Received February 10, 1998

Streptozotocin (STZ) is widely used as a strong inducer of insulin-dependent diabetes in experimental animals. Although nitric oxide (NO) generation from STZ has been proposed to be responsible for the toxicity of pancreatic B-cells, the mechanism is yet unknown. We found that STZ generates NO in the presence of Cu(II) plus ascorbate. In addition, nicotinamide, which is an antidiabetic agent against STZ, has been found to inhibit NO generation from STZ during the reaction with Cu(II) plus ascorbate. Since rat pancreatic islets contain both ascorbate and Cu at the concentrations of 3.5 mM and 1.0 nmol/mg protein, respectively, our present results indicate that (1) NO generation is responsible for the development of STZ-induced diabetes and (2) mechanism for the protection of diabetes by nicotinamide is due to the inhibition of NO generation from STZ through complex formation between nicotinamide and Cu (I), which is reduced by ascorbate.

Key Words: **streptozotocin; nitric oxide; copper; ascorbate; nicotinamide.**

Streptozotocin (STZ, 2-deoxy-D-glucose derivative of N-methyl-N-nitrosourea) (1, 2), shows selective toxicity to pancreatic B-cells (3, 4). Therefore, STZ is widely used as a strong inducer of diabetes in experimental animals (5) as well as the clinical treatment of the pancreatic neoplasm (6, 7). STZ is uptaken through glucose transporter into pancreatic B-cells (8) and causes impairment of mitochondrial oxidative processes in terms of glucose oxidation, inhibition of protein syntheses and suppression of insulin release (9-12).

Recently, nitric oxide (NO) generation from STZ has been proposed to be responsible for the toxicity of pancreatic B-cells (12-14), the factors affecting NO generation from STZ in solution being ultraviolet and fluorescence lights, temperature, and pH (15-18). STZ has also been reported to generate NO in incubation with the intact islet cells or hepatocytes (14). But the metabolic pathway of NO generation from STZ within pancreatic B-cells is yet unclear.

On the other hand, the toxic actions of STZ for pancreatic B-cells is known to be inhibited by nicotinamide (19-22). When nicotinamide is given to rats before or after the administration of STZ, this compound prevents the destruction of pancreatic B-cells (19, 20). However, the mechanism for the protective effect of nicotinamide has not yet been understood.

During the investigations on the chemical induction of diabetes in experimental animals (23, 24), we noticed that the pancreatic islets of rats contain both ascorbate and Cu in relatively high amounts rather than other organs (25). Then, we studied the action mechanism of STZ in terms of these two important factors, and found that STZ generates NO in the presence of Cu(II) plus ascorbate. In addition, nicotinamide was found to inhibit NO generation from STZ during the reaction with Cu(II) plus ascorbate. These results strongly suggested that induction of diabetes by STZ and its protection by nicotinamide are responsible for NO generation and inhibition of NO generation, respectively.

MATERIALS AND METHODS

Materials. Streptozotocin (STZ), hemin, superoxide dismutase (SOD), and catalase were purchased from Sigma Chemical Co. (St. Louis, USA). Bathocuproine disulfonic acid and NOC7 were obtained

[1] To whom correspondence should be addressed at Department of Analytical and Bioinorganic Chemistry, Kyoto Pharmaceutical University, Nakauchi-cho 5, Misasagi, Yamashina-ku, Kyoto 607, Japan. Fax: 81-75-595-4753. E-mail: sakurai@mb.kyoto-phu.ac.jp.

Abbreviations: NO: nitric oxide, NOC7: 3-(2-hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-propanamine, NOx: nitrite and nitrate, SOD: superoxide dismutase, STZ: streptozotocin, Tris: tris(hydroxymethyl)aminomethane.

0006-291X/98 $25.00

from Dojin Chemical Co. (Kumamoto, Japan). All other reagents were of analytical reagent quality. Stock solutions of STZ (0.02 M) and ascorbate (0.04 M) were prepared in 1.0 mM HCl bubbled with Argon gas for 20 min, and were kept in dark. Since we have found that citrate acts as a Cu chelator, STZ was dissolved in 1.0 mM HCl. Stock solution of 1,10 - phenanthroline (100 mM) was dissolved in 50% (V/V) EtOH. Hemin (1 mM) and NOC7 (2 mM) were dissolved in 10 mM NaOH.

Reaction systems. A mixture of STZ, $CuSO_4$, and ascorbate was dissolved in 20 mM sodium phosphate buffer (pH 7.4), and voltexed in a light-protected 1.5-ml polypropylene tube. The reaction mixture (0.5 ml) was incubated in shaking water bath at 60 cycle / min and at 25°C or 37°C. After 1hr, nitrite and nitrate (NOx) concentration was determined for an aliquot (0.1 ml) of the sample solution.

Determination of nitrite and nitrate (NOx) concentration. Total nitrite and nitrate (NOx) concentrations in the reaction solution were determined by the Griess method after conversion of nitrate to nitrite with a TCI-NOX 1000 automated NOx analyser (Tokyo Kasei Co., Tokyo, Japan) (26, 27). NOx concentrations were quantified by the absorbance at 540 nm for the known amounts of KNO_3 as standard.

NO determination by difference spectra using hemin. Real time detection of NO was required to show the fact that STZ generates NO but not NOx in the presence of Cu(II) plus ascorbate. Thus, the difference absorption spectra due to the nitrosyl-heme complex were measured using hemin (28). The mixture containing 20 μM hemin and 2 mM ascorbate in 20 mM sodium phosphate buffer (pH 7.4) (total volume of 8 ml) was preincubated for 10 min at 37°C under the dark, and followed by addition of 0.1 mM $CuSO_4$. Aliquots (3.5 ml) were placed in both sample and reference cuvettes. After recording the base-line, NOC7 (0.02 mM) or STZ (0.2 mM) was added to the sample cuvette and the vehicle was to the reference. The difference absorption spectra at 37°C were recorded from 700 to 500 nm at every 2 min with an MPS-2000 spectrophotometer (Shimadzu, Kyoto, Japan).

ESR spectral measurement. A mixture of 0.1 mM $CuSO_4$ and 2 mM ascorbate in the presence of bathocuproine disulfonate (2.5 mM) or nicotinamide (2.5 mM) in 20 mM sodium phosphate buffer (pH 7.4) was incubated for 10 min at 25°C. ESR spectra were measured at liquid nitrogen temperature (77 K) with a JES-RE1X X-band ESR spectrometer (JEOL, Tokyo, Japan). Conditions for ESR measurement were as follows: microwave power 5 mW, modulation amplitude width 0.63 mT, scanning field 300 ± 100 mT, response time 0.1 s, sweep time 4 min, and receiver gain 400.

RESULTS

We examined first whether STZ releases NO in the presence of Cu(II) and ascorbate in terms of NOx. Since commercially available STZ (purity 98 %) contaminates both NO_2^- (0.12 ± 0.01 %) and NO_3^- (1.5 ± 0.1 %) (n = 21), the results are represented by subtracting 1.62% from observed values in NOx measurements. NOx generation due to spontaneous degradation of 1 mM STZ was not observed for 60 min at pH 7.4. However, incubation of STZ in the presence of Cu(II) plus ascorbate was found to induce dose-dependent formations of NOx, as shown in Fig. 1(a). Neither Cu(II) nor ascorbate alone exhibited significant effects on NOx generation from STZ. Fe(III) instead of Cu(II) had no effect on NOx generation from STZ with ascorbate (data not shown).

Fig. 1(b) shows the reaction time- and temperature-dependent NOx generations from 1 mM STZ caused by 0.1 mM Cu(II) and 2 mM ascorbate at pH 7.4 and 25°C

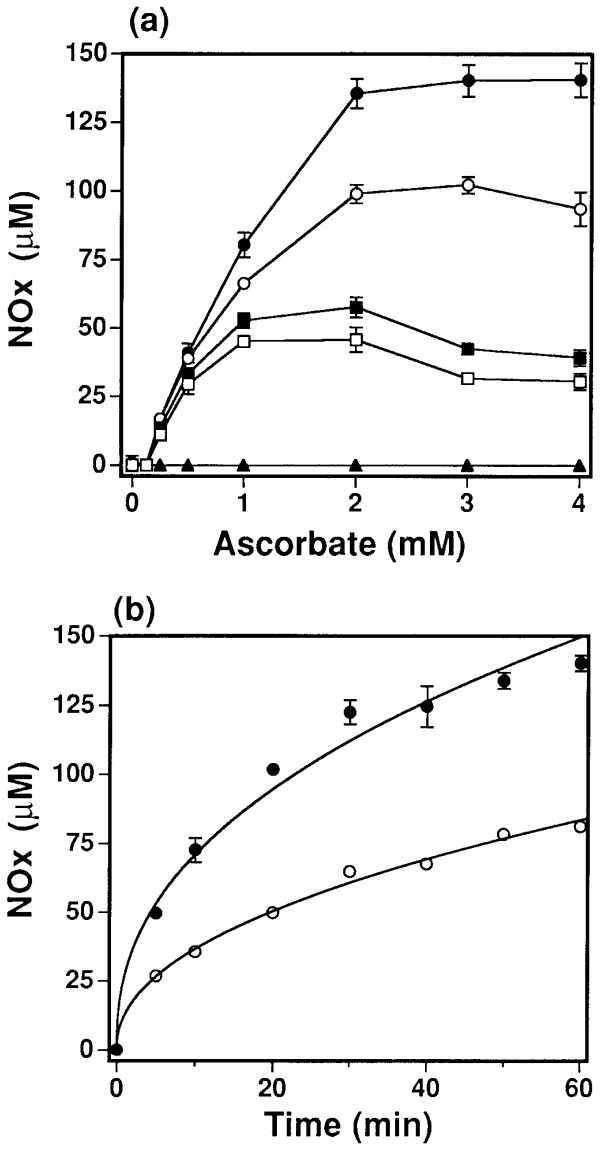

FIG. 1. NOx generation from STZ in the presence of Cu(II) plus ascorbate. (a) The mixtures consisting of 1 mM STZ, 5 ~ 100 μM Cu(II) and 0.25 ~ 4 mM ascorbate were incubated at 37°C for 60 min. Cu(II) concentration was 0 μM (▲), 5 μM (□), 10 μM (■), 50 μM (○) and 100 μM (●). (b) The mixture containing 1 mM STZ, 0.1 mM Cu(II) and 2 mM ascorbate was incubated at 25°C (○) or 37°C (●). Each symbol is expressed as the mean ± the standard deviation of 4 determinations.

or 37°C. NOx generation from STZ was enhanced from 5 min to 60 min depending on the incubation period, and at 37°C than at 25°C.

Then we examined whether addition of nicotinamide or EDTA was able to prevent NOx generation from STZ in the presence of Cu(II) plus ascorbate. When 1 mM STZ was incubated for 60 min in the presence of 0.1mM Cu(II) plus 2 mM ascorbate by addition of increasing amounts of either nicotinamide or EDTA, both nicotin-

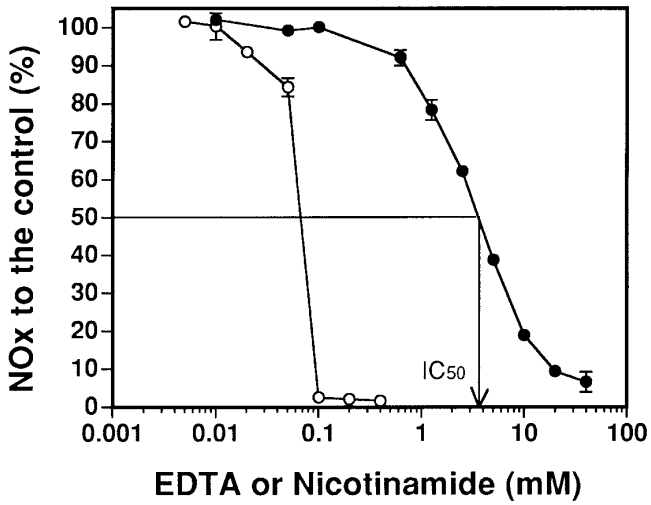

FIG. 2. Dose-dependent inhibitory effects of nicotinamide or EDTA on NOx generation from STZ in the presence of Cu(II) plus ascorbate. The mixture of 1 mM STZ, 0.1 mM Cu(II) and 2 mM ascorbate was incubated at 37°C for 60 min in the presence of nicotinamide (●) or EDTA (○). Each symbol is expressed as the mean ± the standard deviation of 4 determinations.

amide and EDTA suppressed NOx generation in dose-dependent manners (Fig. 2). The inhibitory effect of the compound was evaluated by IC_{50} values, 50% inhibition concentration of a compound to the generated NOx levels, being 3.5 mM for nicotinamide and 0.05 ~ 0.1 mM for EDTA. EDTA inhibited completely the NOx generation at 0.1mM, which corresponds to the equivalent mole concentration of Cu(II).

Since both nicotinamide and EDTA inhibited NOx generation derived from STZ in the presence of Cu(II) and ascorbate, we examined the effect of other metal chelators and active oxygen scavengers (Table I). EDTA and bathocuproine disulfonate, which is a Cu(I) chelator, exhibited the strongest inhibition, followed by the effects of 1, 10-phenanthroline, histidine, citrate and Tris, suggesting the participation of Cu(I) in the reaction. ·OH scavengers, such as mannitol and ethanol, had weak effects on the inhibition of NOx generation. In addition, SOD and catalase exhibited essentially no effects on NOx generation, indicating that active oxygen species involving O_2^-, H_2O_2, and ·OH were less important for the NOx generation from STZ caused by Cu(II) plus ascorbate.

Since NOx generation from STZ in the presence of Cu(II) plus ascorbate has been confirmed, we examined further whether NOx detected by the Griess method is due to NO radicals generated in the reaction system. As a reliable method for NO determination, we used the difference spectral method based on the formation of nitrosyl-heme complex. Authentic nitrosyl-heme complex was obtained in the reaction of hemin and NOC7, which is a spontaneous NO donor generating 2 moles of NO per mole of NOC7 (29). As shown in Fig. 3a, a mixture of hemin, NOC7, Cu(II) and ascorbate developed the absorption maximum at 575 nm and minimum at 630 nm due to nitrosyl-heme complex formation (28), giving an isosbestic point at 610 nm. The spectrum was not obtained for the solution containing 0.02 mM nitrite instead of 0.02 mM NOC7, indicating the generation of NO from NOC7. Therefore, the method was confirmed to be a specific one to detect NO radicals, but not NOx. A reaction mixture consisting of hemin, STZ, Cu(II) and ascorbate developed the absorption maximum at 575 nm and the minimum at 630 nm, with an isosbestic point at 610 nm (Fig. 3(b)). STZ itself showed no absorption above 500 nm, and the nitrosyl-heme formation was not observed without Cu(II) or ascorbate. Thus the reaction of STZ with Cu(II) plus ascorbate was found to form an identical spectrum to that of NOC7 with Cu(II) plus ascorbate. These results indicated that STZ generates NO radicals during the reaction with Cu(II) plus ascorbate.

Aqueous Cu(II) shows ESR spectrum ($g_\perp = 2.076$, $g_\parallel = 2.399$, $A_\parallel = 138.6 \times 10^{-4}\ cm^{-1}$) at 77K (Fig. 4(a)). A mixture of Cu(II) and ascorbate also developed ESR spectrum due to Cu(II) ($g_\perp = 2.077$, $g_\parallel = 2.356$, $A_\parallel = 143.5 \times 10^{-4}\ cm^{-1}$) (Fig. 4(b)). However, when bathocuproine disulfonate or nicotinamide was added to the solution, no ESR spectra due to Cu(II) were observed (Fig. 4(c) and (d)).

DISCUSSION

NO generation in pancreatic B-cells treated with STZ has been proposed and discussed by several research-

TABLE I

Inhibitory Effects of Metal Chelators and Active Oxygen Scavengers on NOx Generation from STZ in the Presence of Cu(II) and Ascorbate

Compound	Concentration (mM)	Inhibition (%)
Chelators		
EDTA	0.1	100
Bathocuproine disulfonate	1	100
1,10-Phenanthroline	1	88
Histidine	2	89
Citrate	2	66
Tris	2	54
Nicotinamide	10	73
	2	36
Active oxygen scavengers		
Mannitol	100	26
Ethanol	100	0
Sodium azide	50	49
SOD	400 unit/ml	17
	80 unit/ml	0
Catalase	400 unit/ml	31
	80 unit/ml	3

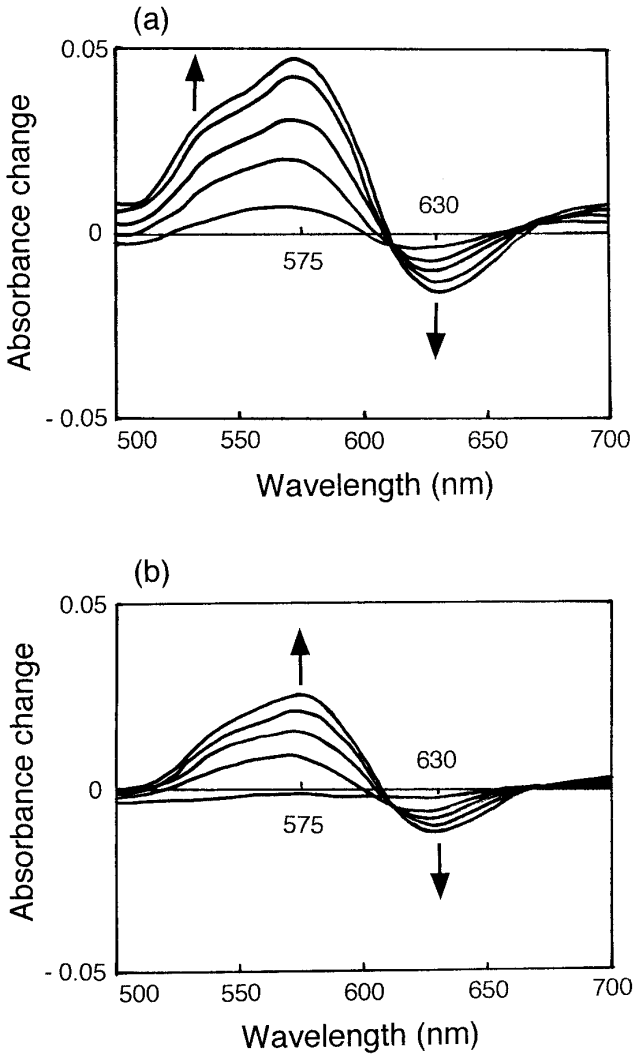

FIG. 3. Difference spectra of nitrosyl-heme formation by the reaction of hemin and NOC7 or STZ in the reaction with Cu(II) plus ascorbate. (a) The spectra were recorded for the reaction mixture containing 20μM hemin, 0.02 mM NOC7, 0.1 mM Cu(II) and 2 mM ascorbate at 37°C. (b) The spectra were obtained for the reaction mixture of 20μM hemin, 0.2 mM STZ, 0.1 mM Cu(II) and 2 mM ascorbate at 37°C. The spectra were recorded at every 2 min.

ers (12, 14, 30). However, the mechanism for the NO generation from STZ has not been understood. In the present investigation, we examined the factors affecting NO generation from STZ under physiological conditions and found that both Cu(II) and ascorbate are indispensable for enhancing NO generation from STZ at pH 7.4 and 37°C (Fig. 1(a) and (b)). Furthermore, NO generation from STZ was found to be suppressed by an anti-diabetic agent against STZ, nicotinamide (Fig. 2).

On the basis of the results, we propose a possible mechanism for the development of STZ-induced diabetes and its protection by nicotinamide in terms of NO generation, as follows.

[1] NO generation from STZ required 250 μM or more of ascorbate and 5 μM or more of Cu(II) (Fig.1(a)). Ascorbate and Cu in plasma are normally present at 50 $\pm$ 20 μM (31) and 17 $\pm$ 3 μM (32), respectively. While, rat pancreatic islets have been shown to contain ascorbate at 3.53 $\pm$ 0.1 mM and total Cu at 1.04 nmol/mg protein, and notably both mitochondria and secretion granule fraction accumulate both Cu and ascorbate (25). Therefore, STZ is assumed to react with Cu(II) and ascorbate for generating NO radicals within islet mitochondria and secretion granule, which in turn damages pancreatic B-cells following the development of diabetes.

[2] Reaction of Cu(II) and ascorbate in the presence of molecular dioxygen has been reported to produce both H_2O_2 (33, 34) and $\cdot$OH (35-37) at physiological pH and temperature. Ascorbate reduces Cu(II) to Cu(I), which reacts with molecular dioxygen to form superoxide anion radicals ($\cdot O_2^-$), following the formation of H_2O_2 (33, 34). Thus, resulting Cu(I) and H_2O_2 react to form $\cdot$OH (35-37), as shown in the equations 1) $\sim$ 4).

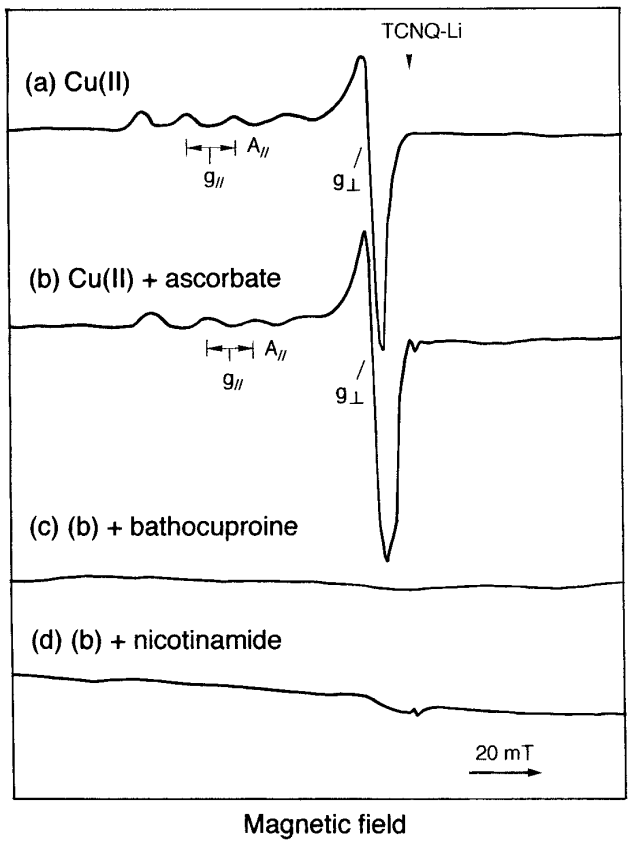

FIG. 4. ESR spectra at 77K for a mixture of Cu(II) and ascorbate in the presence of bathocuproine disulfonate or nicotinamide. (a) 0.1 mM Cu(II). (b) 0.1 mM Cu(II) and 2 mM ascorbate. (c) (b) + 2.5 mM bathocuproine disulfonate. (d) (b) + 2.5 mM nicotinamide.

$$2Cu(II) + \text{ascorbate} \rightarrow 2Cu(I) + \text{dehydroascorbate} + 2H^+ \quad 1)$$

$$Cu(I) + O_2 \rightarrow Cu(II) + \cdot O_2^- \quad 2)$$

$$Cu(I) + \cdot O_2^- + 2H^+ \rightarrow H_2O_2 \quad 3)$$

$$Cu(I) + H_2O_2 \rightarrow Cu(II) + OH^- + \cdot OH \quad 4)$$

However, O_2^-, H_2O_2 and $\cdot OH$ have been found to play no major roles in NO generation from STZ (Table I). Recently, an alternative Cu(I)-OOH complex, which has a similar reactivity to that of $\cdot OH$ but not inhibited by $\cdot OH$ scavengers such as mannitol and ethanol, was proposed to be formed in the reaction between Cu ion and H_2O_2 (39-41). Such Cu(I)-OOH complex might be proposed to be formed in our present system. In a preliminary spectrophotometric analysis, we observed the disappearance of STZ with an absorbance maximum at 390 nm due to —N—N═O moiety, indicating the cleavage of —N—N═O bond followed by the generation of NO radicals.

[3] The complete inhibitory effect of bathocuproine disulfonate and 1, 10-phenanthroline on NO generation from STZ catalyzed by Cu(II) plus ascorbate (Table I) suggests that Cu(I) plays a central role in NO generation from STZ. Since auto-oxidation of Cu(I), which was formed during the reaction of Cu(II) and ascorbate, is quite rapid (42), Cu(II) state was observed in ESR spectrum (Fig. 4(b)). However, in the presence of nicotinamide or bathocuproine disulfonate, ESR spectrum due to Cu(II) was not detected, indicating rapid and stable complex formation between the ligand and Cu(I). In fact, ligands with a pyridyl ring such as 2-picolylamine and 3-picolylamine have been shown to form stable Cu(I) complexes rather then the corresponding Cu(II) complexes (43). Thus, Cu(I) complex formation is important to prevent NO generation from STZ catalyzed by Cu(II) and ascorbate. Nicotinamide is known to prevent the pancreatic B-cells destruction caused by STZ *in vivo and in vitro* (19-22). Therefore, the protection of pancreatic B-cells by nicotinamide may be due to the inhibition of NO generation from STZ in terms of complex formation between Cu(I) and nicotinamide in the cells.

In conclusion, Cu(II) and ascorbate, that are present in pancreatic islet B-cells, were found to enhance remarkable NO generation from STZ at pH 7.4 and 37°C. In addition, nicotinamide, an antidiabetic agent against STZ, was found to inhibit NO generation from STZ in the presence of Cu(II) plus ascorbate. The present results indicate that both development and protection of STZ-induced diabetes are responsible for NO radicals generated from STZ in the presence of Cu(II) and ascorbate in pancreatic islet B-cells. Further investigation is required to reveal the detailed mechanism of NO generation *in vivo*.

ACKNOWLEDGMENTS

This study was supported in part by the grants from the Ministry of Education, Science, Sports and Culture of Japan to H.S.

REFERENCES

1. Herr, R. R., Jahnke, H. K., and Argoudelis, A. D. (1967) *J. Am. Chem. Soc.* **89,** 4808–4809.
2. Wiley, P. F., Herr, R. R., Jahnke, H. K., Chidester, C. G., Mizsak, S. A., Spaulding, L. B., and Argoudelis, A. D. (1979) *J. Org. Chem.* **44,** 9–16.
3. Tjalve, H., Wilander, E., and Johansson, E. B. (1976) *J. Endocr.* **69,** 455–456.
4. Morgan, N. G., Cable, H. C., Newcombe, N. R., and Williams, G. T. (1994) *Biosci. Reports* **14,** 243–249.
5. Rerup, C. G. (1970) *Pharmacol. Rev.* **22,** 485–518.
6. Broder, L. E., and Carter, S. K. (1973) *Ann. Internal. Med.* **7,** 108–118.
7. Murray-Lyon, I. M., Eddleston, A. L., Williams, R., Brown, M., Hogbin, B. M., Bennett, A., Edwards, J. C., and Taylor, K. W. (1968) *Lancet* **2,** 895–898.
8. Kawada, J., Okita, M., Nishida, M., Yoshimura, Y., Toyooka, K., and Kubota, S. (1987) *J. Endocr.* **112,** 375–378.
9. Ledoux, S. P., and Wilson, G. L. (1984) *Biochim. Biophys. Acta* **804,** 387–392.
10. Eizirik, D. L., Sandler, S., Ahnstrom, G., and Welsh, M. (1991) *Biochem. Pharmacol.* **42,** 2275–2282.
11. Janjic, D., and Wollheim, C. B. (1992) *Diabetologia* **35,** 482–485.
12. Turk, J., Corbett, J. A., Rammanadham, S., Bohrer, A., and McDaniel, M. L. (1993) *Biochem. Biophys. Res. Commun.* **197,** 1458–1464.
13. Kaneto, H., Fujii, J., Seo, H. G., Suzuki, K., Matsuoka, T., Nakamura, M., Tatsumi, H., Yamasaki, Y., Kamada, T., and Tanigichi, N. (1995) *Diabetes* **44,** 733–738.
14. Kroncke, K. D., Fehsel, K., Sommer, A., Rodriguez, M. L., and Kolb-Bachofen, V. (1995) *Biol. Chem. Hoppe-Seyler* **376,** 179–185.
15. Kroncke, K. D., and Kolb-Bachofen, V. (1996) *Free Rad. Res.* **24,** 77–80.
16. Forist, A. A. (1964) *Anal. Chem.* **36,** 1338–1339.
17. Chang, K. C., Chong, W. S., Park, B. W., Seung, B. W., Chun, G. W., Lee, I. J., and Park, P. S. (1993) *Biochem. Biophys. Res. Commun.* **191,** 509–514.
18. Kwon, N. S., Lee, S. H., Choi, C. S., Kho, T., and Lee, H. S. (1994) *FASEB J.* **8,** 529–533.
19. Schein, P. S., Cooney, D. A., and Vernon, M. L. (1967) *Cancer Res.* **27,** 2324–2332.
20. Dulin, W. E., and Wyse, B. M. (1969) *Diabetes* **18,** 459–466.
21. Gunnarsson, R., Berne, C., and Hellerstrom, C. (1974) *Biochem. J.* **140,** 487–494.
22. Bedoya, F. J., Solano, F., and Lucas, M. (1996) *Experientia* **52,** 344–347.
23. Nukatsuka, M., Sakurai, H., Yoshimura, Y., Nishida, M., and Kawada, J. (1988) *FEBS Lett.* **239,** 295–298.
24. Nukatsuka, M., Sakurai, H., and Kawada, J. (1989) *Biochem. Biophys. Res. Commun.* **165,** 278–283.
25. Zhou, A., Nielsen, J. H., Farver, O., and Thorn, N. A. (1991) *Biochem. J.* **274,** 739–744.

26. Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., and Tannenbaum, S. R. (1982) *Anal. Biochem.* **126,** 131–138.
27. Habu, H., Yokoi, I., Kabuto, H., and Mori, A. (1994) *Neurochem.* **5,** 1571–1573.
28. Wayland, B. B., and Olson, L. W. (1974) *J. Am. Chem. Soc.* **96,** 6037–6041.
29. Hrabie, J. A., Klose, J. R., Wink, D. A., and Keefer, L. K. (1993) *J. Org. Chem.* **58,** 1472–1476.
30. Delaney, C. A., Dunger, A., Matteo, M. D., Cunningham, J. M., Green, M. H. L., and Green, I. C. (1995) *Biochem. Pharmacol.* **50,** 2015–2020.
31. Menditto, A., Menotti, A., Morisi, G., Patriarca, M., and Spagnolo, A. (1992) *Arch. Gerontol. Geriatr.* **15,** 257–266.
32. Gutteridge, J. M., Rowry, D. A., Halliwell, B., and Westermarck, T. (1982) *Lancet* **1,** 459–460.
33. Taqui-Khan, M. M., and Martel, A. E. (1967) *J. Am. Chem. Soc.* **89,** 4176–4185.
34. Weissberger, A., and Lu Valle, J. E. (1944) *J. Am. Chem. Soc.* **66,** 700–705.
35. Biaglow, J. E., Manevich, Y., Uckun, F., and Held, K. D. (1997) *Free Rad. Biol. Med.* **22,** 1129–1138.
36. Kadiiska, M. B., Hanna, P. M., Hernandez, L., and Mason, R. P. (1992) *Mol. Pharmacol.* **42,** 723–729.
37. Gutteridge, J. M. C., and Wilkins, S. (1982) *FEBS Lett.* **137,** 327–330.
38. Marx, G., and Chevion, M. (1985) *Biochem. J.* **236,** 397–400.
39. Drouin, R., Rodriguez, H., Gao, S. W., Gebreyes, Z., Connor, T. R., Holmquist, G. P., and Akman, S. A. (1996) *Free Rad. Biol. Med.* **21,** 261–273.
40. Yamamoto, K., and Kawanishi, S. (1989) *J. Biol. Chem.* **264,** 15435–15440.
41. Pecci, L., Montefoschi, G., and Cavallini, D. (1997) *Biochem. Biophys. Res. Commun.* **235,** 264–267.
42. Zuberbuhler, A. (1970) *Helv. Chim. Acta* **53,** 473–485.
43. Couturier, Y., and Petitfaux, C. (1978) *Bull. Soc. Chim. France* 435–441.