Scavenging Effect of Nicorandil on Free Radicals and Lipid Peroxide in Streptozotocin-Induced Diabetic Rats

Toshiki Mano, Rikio Shinohara, Akio Nagasaka, Hifumi Nakagawa, Keiko Uchimura, Ritsuko Hayashi, Itsuko Nakano, Toru Tsugawa, Fumiko Watanabe, Takako Kobayashi, Kentaro Fujiwara, Akira Nakai, and Mitsuyasu Itoh

Free radicals and lipid peroxide (LPO), easily formed in the diabetic state, play an important role in the development of diabetic complications. Potentially, nicorandil may reduce the production of free radicals and LPO in various organs. In fact, increased LPO levels in the serum, kidney, and cardiac muscle of diabetic (DM) rats were reduced by nicorandil treatment (N treatment). Xanthine oxidase (XOD), which produces free radicals, was decreased in the liver and increased in the kidney of DM rats compared with control rats, and these changes were prevented by N treatment. The concentration of Cu, Zn-superoxide dismutase (SOD) decreased in the cardiac muscle and increased in the kidney of DM rats, and these changes returned to normal after N treatment. The decreased concentration of Mn-SOD in the liver, kidney, and cardiac muscle from DM rats was also reversed by N treatment. The changes in catalase and glutathione peroxidase (GSH-PX) activities in DM rats were not improved effectively by N treatment. Another K-adenosine triphosphate (K-ATP) channel opener, tilisolol hydrochloride, had an effect similar to that of nicorandil. The effects of nicorandil and tilisolol were studied only in DM rats. These data imply that N treatment, as an antioxidative therapy, may be beneficial in preventing diabetic complications due to lipoperoxidation and free radicals in DM rats.

GLYCATED PROTEINS produce free radicals and hydrogen peroxide in diabetes mellitus (DM).[1] The increased glycation of proteins may induce an increase in free radical production and affect several enzyme activities,[2-4] and then accelerate atherogenesis as a result of oxidative modifications to the vascular membrane lipids.[5] Thus, free radicals and lipid peroxides (LPOs), easily formed during this process, are thought to play an important role in the development of diabetic complications, because free radicals and hydrogen peroxide damage cell components.[6]

Nicorandil (*N*-(2-hydroxyethyl)-nicotinamide nitrate), a vasodilator that acts as a potassium channel opener, is thought to inhibit superoxide anion production by canine neutrophils activated with either phorbol myristate acetate (PMA) or opsonized zymogen.[7] Naito et al[8] verified that nicorandil has an antioxidative action using a liver perfusion experiment.

In this study, we hypothesized that nicorandil reduces the production of free radicals and LPO in various organs of DM rats. Therefore, we examined the changes in the LPO concentrations and xanthine oxidase (XOD) activity in various organs from DM rats treated with or without nicorandil, comparing them with DM rats treated with tilisolol hydrochloride, which also acts as a potassium channel opener. In addition, changes in the activity of free-radical–scavenging enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSH-PX) were also evaluated.

MATERIALS AND METHODS

Experimental Animals

Male 6-week-old Wistar rats weighing about 200 g were used in this study. The animals were housed in stainless steel cages under conditions of controlled temperature, humidity, and light. The rats were fed with a standard diet and water. All procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Animal Treatment

DM was induced with a single intraperitoneal injection of streptozotocin (50 mg/kg body weight; Sigma Chemical, St Louis, MO) dissolved in 10-mmol/L citrate buffer, pH 4.5. The control rats received an equivalent volume of citrate buffer. DM rats with a fasting blood glucose concentration less than 200 mg/dL were excluded from the study on day 3 after injection. The blood glucose level was measured using a GlucoBoy kit (Eiken, Tokyo, Japan) after overnight fasting. N-treated DM rats and N-treated nondiabetic rats were prepared by administration of nicorandil 15 mg/kg body weight/d (generously supplied by Chugai Pharmaceutical, Tokyo, Japan) via intramuscular injection every day for 4 weeks from day 4 after the streptozotocin injection. N-treated nondiabetic rats were prepared by the same dose of nicorandil as the nondiabetic rats every day for 4 weeks. Another K-ATP channel opener,[9] tilisolol hydrochloride, which also has a nonselective β-blocking action[10] (generously supplied by Toyama Chemical, Tokyo, Japan), was administered to the DM rats in the same manner used for the N-treated rats (tilisolol (T)-treated). Control rats were treated with a saline injection. The kidneys, liver, and heart were quickly removed from anesthetized rats and immediately frozen at −80°C until use.

Assays

LPO. LPO was determined as the concentration of malondialdehyde (MDA). The level of MDA, a secondary product of lipid peroxidation, was measured by adding thiobarbituric acid[11] to the homogenates and determining the LPO reaction according to the method of Ohkawa et al.[12] This method is the optimum assay for LPO, when compared with other assay systems.[13]

XOD. XOD activity expressed as the concentration was measured according to the method described by Hashimoto.[14]

SOD. SOD concentrations were measured as described previously.[15,16] One hundred microliters of tissue homogenate was incubated

From the Department of Internal Medicine and Pharmacy and Department of Biochemistry, Fujita Health University School of Medicine and Hygiene, Toyoake, Aichi, Japan.

Submitted June 6, 1998; accepted September 21, 1999.

Supported by the Nakagawa Science Foundation and Chugai Pharmaceutical Co, Ltd (Tokyo, Japan).

Address reprint requests to Akio Nagasaka, MD, Department of Internal Medicine, Fujita Health University School of Medicine, Toyoake, Aichi 470-1192, Japan.

0026-0495/00/4904-0005$10.00/0

in a mixture (0.8 mL) containing 0.5 mL distilled water, 0.1 mL boric acid buffer (0.156 mmol/L, pH 8.0), 0.1 mL hypoxanthine (2 mmol/L), and 0.1 mL $NH_2OH \cdot HCl$ (10 mmol/L). The reaction was started by the addition of XOD 20 U/mL diluted with 1 mmol/L disodium EDTA (pH 7.0) in a water bath at 37°C for 30 minutes. To determine nitrite formation as the product of hydroxylammonium chloride oxidation catalyzed by XOD, 2 mL reaction mixture (5 mg *N*-1-naphthylethylene diamine, 250 mg sulfanilic acid, and 66.8 mL 100% acetic acid solution, to yield a final vol of 400 mL with distilled water) was added and the mixture was incubated at 37°C for 45 minutes. Absorbance of the nitrite-produced reaction mixture at 540 nm was measured with a spectrophotometer. To discriminate between Cu,Zn-SOD and Mn-SOD, 1 mmol/L cyanide, which inactivates Cu,Zn-SOD, was added to the mixture. SOD activity is expressed as the enzyme concentration using bovine erythrocyte SOD (Sigma Chemical) as a standard.

Catalase and GSH-PX. Catalase and GSH-PX activities were measured using a method previously described.[17,18]

Protein. Protein concentrations were determined with a Bio-Rad (Richmond, CA) protein assay kit using bovine serum albumin as the standard.[19]

Statistical Analysis

Data are expressed as the mean ± SEM and were analyzed by ANOVA and a post hoc test (Scheffé F test) to determine differences among the mean values of the groups. A P value less than 5% was considered significant.

RESULTS

Animal Parameters

The body weight was monitored during the 4-week experimental period. Plasma glucose in DM and N-treated or T-treated DM rats significantly increased compared with the control (saline-treated) and N-treated rats. The body weight of DM and N-treated or T-treated DM rats significantly decreased compared with the control rats, but the body weight of DM and T-treated DM rats was not different from that of N-treated rats. The urinary protein concentration tended to increase in DM rats and decrease in N-treated DM rats (Table 1).

Changes in LPO Concentration in the Serum, Liver, Kidneys, and Cardiac Muscle

The LPO concentration increased in the serum, cardiac muscle, and kidneys from DM rats compared with control rats.

Table 1. Comparison of Plasma Glucose Level, Body Weight, and Urinary Protein Concentration Among Control (saline-treated), N-Treated, DM, N-Treated DM, and T-Treated DM Rats

Group	Plasma Glucose (mg/dL)	Body Weight (g)	Urinary Protein (score)
Control	92 ± 6	382 ± 14	1.6 ± 0.4
N-treated	89 ± 5	339 ± 24	1.4 ± 0.5
DM	417 ± 59*	298 ± 52*	2.3 ± 1.5
N-treated + DM	399 ± 56*	300 ± 16*	1.0 ± 1.8
T-treated + DM	387 ± 75*	339 ± 30*	1.8 ± 1.5

NOTE. Values are the mean ± SD for n = 10 rats per group. Urinary protein concentrations were measured using urinary protein test paper (Bayer Medical, New York, NY) and were scored as follows: 0 mg/dL = 0, 30 mg/dL = 1, 100 mg/dL = 2, 300 mg/dL = 3, and 1,000 mg/dL = 4.

*$P < .05$ *v* control.

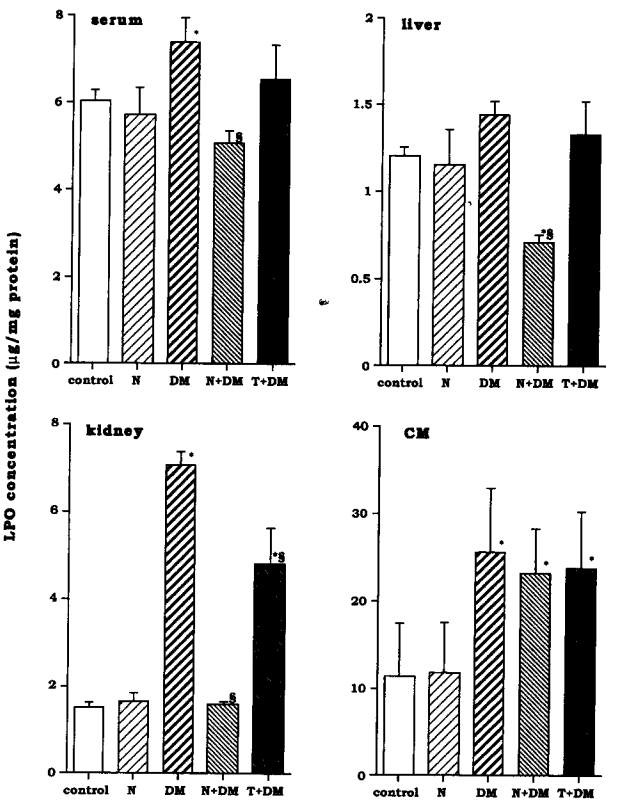

Fig 1. Comparison of LPO concentrations in the serum, liver, kidneys, and cardiac muscle (CM) among control, N-treated (N), DM, N-treated DM (N + DM), and t-treated DM (T + DM) rats. Each value (μg/mg protein) is the mean ± SEM for data from 10 rats. *$P < .05$ *v* control rats; §$P < .05$ *v* DM rats.

In N-treated DM rats, the increased LPO levels in the serum and kidneys were reduced to the values in the control rats by N treatment. Nicorandil did not prevent the DM-induced increase in LPO in the cardiac muscle. In the liver, nicorandil induced a decrease in LPO to a subnormal level. The elevated LPO concentration in DM rats was reduced only in the kidneys by T treatment (Fig 1).

Changes in XOD Concentration in the Liver, Kidneys, and Cardiac Muscle

XOD concentrations decreased in the liver, increased in the kidneys, and were unchanged in the cardiac muscle from DM rats, and these changes were restored to the normal levels by N or T treatment (Fig 2).

Changes in Free-Radical–Scavenging Enzyme Concentrations

Liver. The decreased concentration of Mn-SOD in DM rats was restored to a supranormal level by N treatment and to the normal level by T treatment, but Cu,Zn-SOD concentrations were unchanged by either treatment. The decreased catalase activity in DM rats was not restored by N or T treatment. The increased activity of GSH-PX in DM rats was not restored by N or T treatment (Fig 3).

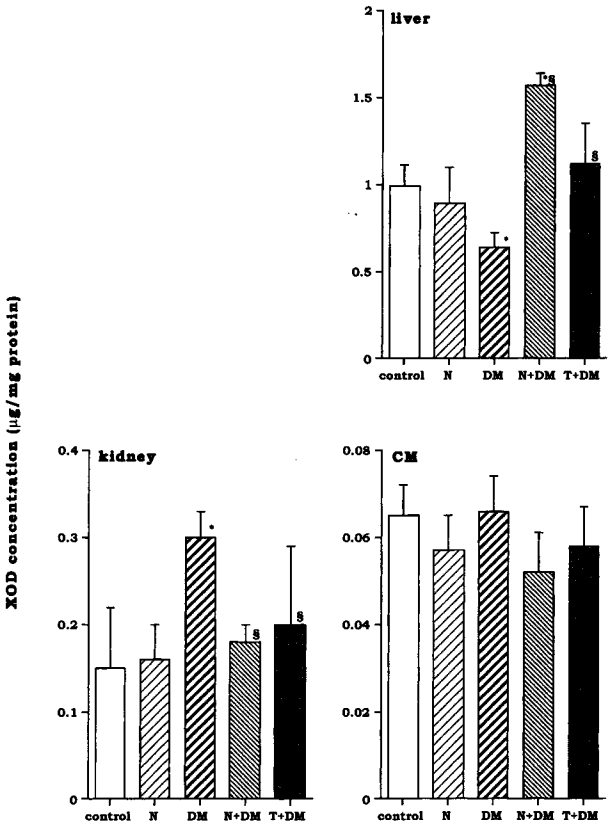

Fig 2. Comparison of XOD concentrations in the liver, kidneys, and cardiac muscle (CM) among control, N-treated (N), DM, N-treated DM (N + DM), and T-treated DM (T + DM) rats. Each value (μg/mg protein) is the mean ± SEM for data from 10 rats. *$P < .05$ *v* control rats; §$P < .05$ *v* DM rats.

Kidneys. The decreased concentration of Mn-SOD in DM rats was restored to the normal level by N treatment but not by T treatment, while the increased concentration of Cu,Zn-SOD in DM rats was reduced to the normal level by N or T treatment. The reduced activity of catalase in DM rats was reduced further by N treatment, but it was restored to a subnormal level by T treatment. The markedly increased activity of GSH-PX in DM rats decreased to a subnormal level after N or T treatment (Fig 4).

Cardiac muscle. The decreased concentrations of both Mn-SOD and Cu,Zn-SOD in DM rats were restored to the normal levels by N treatment, but T treatment restored only the Mn-SOD concentration. The increased catalase activity in DM rats was unchanged by N or T treatment. The increased activity of GSH-PX in DM rats was decreased by both N and T treatment (Fig 5).

All N or T treatment data were the same for nondiabetic rats and control rats.

DISCUSSION

Treatment with nicorandil or tilisolol was effective only in DM rats. The increased lipid peroxidation in the serum, kidneys, and cardiac muscle of DM rats was restored to the same level as the control rats by N treatment. Thus, nicorandil may protect various organs against LPO invasion under diabetic conditions.

In our experiment, the changes (increases and occasional decreases) in free-radical–scavenging enzyme levels in the diabetic state were improved by N treatment. Probably, the high levels of LPO induced by hyperglycemia resulted in the induction of scavenging enzymes, and these distorted metabolic changes were recovered by N treatment.

Nicorandil was reported to act as both a nitrovasodilator and a K^+ channel opener.[20,21] A pure K^+ channel opener did not inhibit lipid peroxidation, suggesting that the antioxidative action of nicorandil is not mediated via a K^+ channel.[8] Since nicorandil may act as a direct scavenger of hydroxy radicals[7] and nicotinamide is known as a hydroxyl scavenger,[7,22] the putative beneficial effects of nicorandil due to its radical-scavenging action or its inhibition of free radical production in both human[23] and canine[7] leukocytes are proposed. It may also act as an antioxidant via the formation of nitric oxide,[24] which can interfere with free radical production,[25,26] or via other mechanisms.[8]

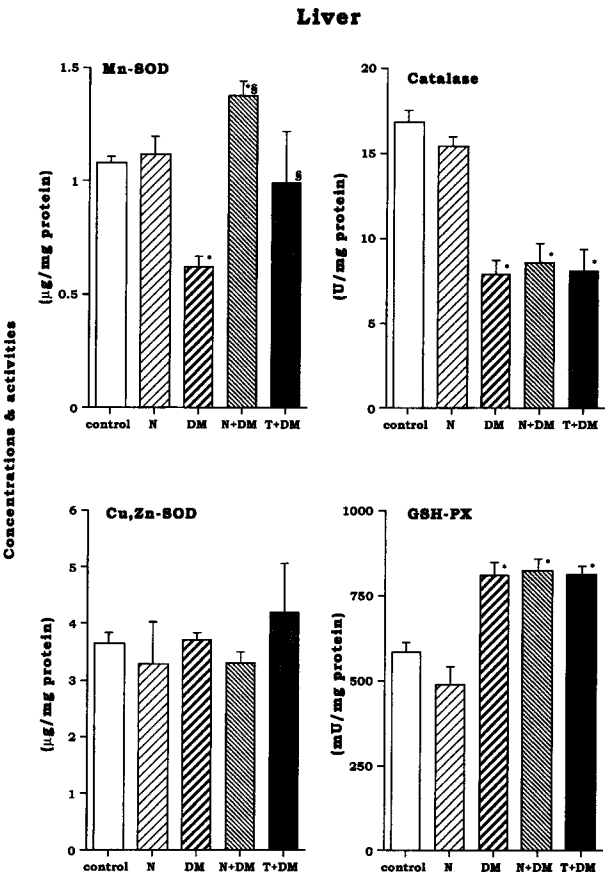

Fig 3. Comparison of scavenging enzyme concentrations and activities in the liver from control, N-treated (N), DM, N-treated DM (N + DM), and T-treated DM (T + DM) rats. Each value is the mean ± SEM for data from 10 rats. *$P < .05$ *v* control rats; §$P < .05$ *v* DM rats.

The concentration of XOD, which produces free radicals, was high in the kidneys and low in the liver from DM rats, and these changes were restored by N treatment. Free radicals produced by activated XOD may be easily induced in the kidneys and then cause diabetic complications, although the mechanism of the changes in XOD concentrations under diabetic conditions and with N treatment was not clarified.

There is a controversy concerning the activity and concentration of SOD in DM.[27-32] In our study, the activity of Cu,Zn-SOD, expressed as a concentration, decreased in cardiac muscle but increased in the kidneys from DM rats. Kakkar et al[32] reported that the increase in free radicals in DM may increase GSH-PX activity, which in turn may prevent SOD inactivation due to H_2O_2 and thus increase SOD activity. In contrast, glycation and inactivation of Cu,Zn-SOD in erythrocytes has been reported.[33,34] Since blood glucose levels were unchanged by nicorandil, it seems unlikely that nicorandil affected the glycation of SOD.

Mn-SOD activity expressed as a concentration decreased in the liver, kidneys, and cardiac muscle from DM rats. Mn-SOD plays a key role in the protection against mitochondrial oxidative stress.[31] The reduced induction of Mn-SOD in DM may contribute to the damage of diabetic organs. To our knowledge, there are no reports concerning the glycation of Mn-SOD, implying that other factors such as cytokines induce these changes in Mn-SOD activity.

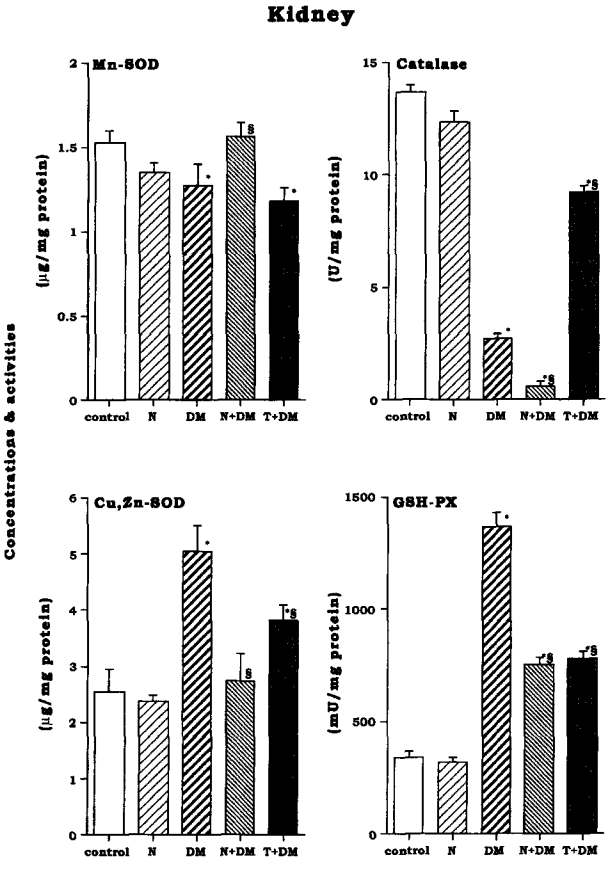

Fig 4. Comparison of scavenging enzyme concentrations and activities in the kidneys from control, N-treated (N), DM, N-treated DM (N + DM), and T-treated DM (T + DM) rats. Each value is the mean ± SEM for data from 10 rats. *$P < .05$ *v* control rats; §$P < .05$ *v* DM rats.

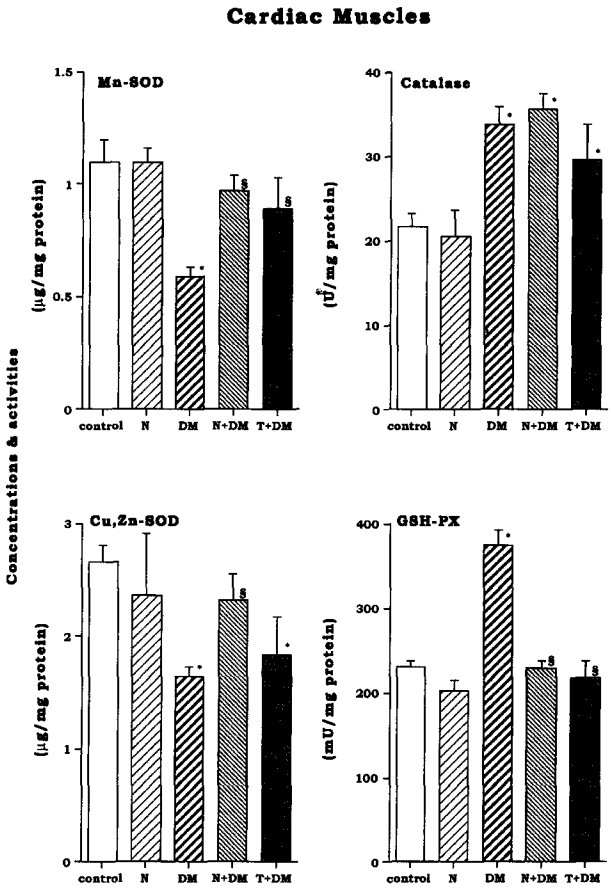

Fig 5. Comparison of scavenging enzyme concentrations and activities in cardiac muscle from control, N-treated (N), DM, N-treated DM (N + DM), and T-treated DM (T + DM) rats. Each value is the mean ± SEM for data from 10 rats. *$P < .05$ *v* control rats; §$P < .05$ *v* DM rats.

The results of this study imply that lipid peroxidation and free radicals play a role in diabetic complications, and nicorandil treatment as an antioxidative therapy may be beneficial in preventing diabetic complications in rats rendered diabetic with streptozotocin.

ACKNOWLEDGMENT

We thank Chugai Pharmaceutical (Tokyo, Japan) for providing nicorandil and N. Takekawa for preparing the manuscript.

REFERENCES

1. Wolff SP, Bascal ZA, Hunt JV: Autooxidative glycosylation: Free radicals and glycation theory, in Baynes JW, Monnier VM (eds): The Maillard Reaction in Aging, Diabetes and Nutrition. New York, NY, Liss, 1989, pp 259-275
2. Heath MM, Rixon KC, Harding JJ: Glycation-induced inactivation of malate dehydrogenase protection by aspirin and a lens molecular chaperone, α-crystallin. Biochim Biophys Acta 1315:176-184, 1996
3. Nerlich AG, Sauer U, Kolm-Litty V, et al: Expression of glutamine: fructose-6-phosphate amidotransferase in human tissues: Evidence for high variability and distinct regulation in diabetes. Diabetes 47:170-178, 1998
4. Zhao W, Devamanoharan PS, Varma SD: Fructose induced deactivation of glucose-6-phosphate dehydrogenase activity and its prevention by pyruvate: Implications in cataract prevention. Free Radic Res 29:315-320, 1998
5. Mullarkey CJ, Edelstein D, Browlee M: Free radical generation by early glycation products: A mechanism for accelerated atherogenesis in diabetes. Biochem Biophys Res Commun 173:932-939, 1990
6. Tappel AI: Lipid peroxidation damage to cell components. Fed Proc 32:1870-1874, 1973
7. Pieper GM, Gross GJ: Anti–free-radical and neutrophil-modulating properties of the nitrovasodilator, nicorandil. Cardiovasc Drug Ther 6:225-232, 1992
8. Naito A, Aniya Y, Sakanashi M: Antioxidative action of the nitrovasodilator nicorandil: Inhibition of oxidative activation of liver microsomal glutathione S-transferase and lipid peroxidation. Jpn J Pharmacol 65:209-213, 1994
9. Sugai T, Kojima K, Iwakami N, et al: Vasorelaxant and hypotensive effects of tilisolol hydrochloride (N-696) in isolated rat thoracic aorta and pithed rats. Jpn J Pharmacol 57:367-375, 1991
10. Nakagawa Y, Sugai T, Chin WP, et al: Pharmacological profile of a new β-adrenoceptor blocker, 4-[3-(*tert*-butylamino)-2-hydroxypropoxy]-*N*- methylisocarbostyril hydrochloride (N-696). Arzneimittelforschung 34:194-199, 1984
11. Patton S, Kurtz GW: 2-Thiobarbituric acid as a reagent for detecting milk fat oxidation. J Dairy Sci 34:669-674, 1951
12. Ohkawa H, Ohnishi N, Yagi K: Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95:351-358, 1979
13. Shinohara R, Miura N, Kanno K, et al: Comparison of lipid peroxide values determined by various methods during heating of unsaturated fatty acids. J Anal Biosci 19:194-198, 1996
14. Hashimoto S: A new spectrophotometric assay method of xanthine oxidase in crude tissue homogenate. Anal Biochem 62:426-435, 1974
15. Mano T, Sinohara R, Sawai Y, et al: Effects of thyroid hormone on coenzyme Q and other free radical scavengers in rat heart muscle. J Endocrinol 145:131-136, 1995
16. Elstner EF, Heupel A: Inhibition of nitrite formation from hydroxylammonium chloride: A simple assay for superoxide dismutase. Anal Biochem 70:616-620, 1976
17. Hochstein P, Utley H: Hydrogen peroxide detoxication by glutathione peroxidase and catalase in rat liver homogenate. Mol Pharmacol 4:574-579, 1968
18. Aebi H: Catalase in vitro. Methods Enzymol 105:121-126, 1984
19. Bradford M: A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254, 1976
20. Edwards G, Weston AH: Structure-activity relationships of K^+-channel openers. Trends Pharmacol Sci 11:417-422, 1990
21. Ohta H, Jinno Y, Harada K, et al: Cardioprotective effect of KRN 2391 and nicorandil on ischemic dysfunction in perfused rat heart. Eur J Pharmacol 204:171-177, 1991
22. Ledoux SP, Hall CR, Forbes PM, et al: Mechanisms of nicotinamide and thymidine protection from alloxan and streptozotocin toxicity. Diabetes 37:1015-1019, 1988
23. Gross GJ, Auchampach JA, Maruyama M, et al: Cardioprotective effects of nicorandil. Cardiovasc Pharmacol 20:S22-S28, 1992 (suppl 3)
24. Kanner J, Harel S, Grant R: Nitric oxide as an antioxidant. Arch Biochem Biophys 189:130-136, 1991
25. Pieper GM, Clarke GA, Gross GJ: Stimulatory and inhibitory action of nitric oxide donor agents vs. nitrovasodilators on reactive oxygen production by isolated polymorphonuclear leukocytes. J Pharmacol Exp Ther 269:451-456, 1994
26. Demiryurek AT, Kennedy S, Wainwright CL, et al: Influence of nitric oxide on luminol-enhanced chemiluminescence measured from porcine-stimulated leukocytes. J Cardiovasc Pharmacol 30:332-337, 1997
27. Matkovics B, Varga SI, Szabo L, et al: The effect of diabetes on the activities of the peroxide metabolism enzymes. Horm Metab Res 14:77-79, 1982
28. Wohaieb SA, Godin DV: Alterations in free radical tissue-defence mechanism in streptozotocin-induced diabetes in rat. Diabetes 36:1014-1018, 1987
29. Loven D, Schedl H, Wilson H, et al: Effect of insulin and oral glutathione levels and superoxide dismutase activities in organs of rats with streptozotocin-induced diabetes. Diabetes 35:503-507, 1986
30. Lammi-Keefe CJ, Swan PB, Hegarty PVJ: Evidence for increased peroxidative activity in muscles from streptozotocin-diabetic rats. Proc Soc Exp Biol Med 176:27-31, 1984
31. Asayama K, Hayashibe H, Dobashi K, et al: Antioxidant enzyme status and lipid peroxidation in various tissues of diabetic and starved rats. Diabetes Res 12:85-91, 1989
32. Kakkar R, Kalra J, Mantha SV, et al: Lipid peroxidation and activity of antioxidant enzyme in diabetic rats. Mol Cell Biochem 151:113-119, 1995
33. Kawamura N, Ookawara T, Suzukui K, et al: Increased glycated Cu, Zn-superoxide dismutase levels in erythrocytes of patients with insulin-dependent diabetes mellitus. J Clin Endocrinol Metab 74:1352-1354, 1992
34. Katsura A, Maguchi S, Fujii S, et al: Glycation and inactivation of human Cu-Zn-superoxide dismutase. J Biol Chem 262:16969-16972, 1987
35. Yan H, Harding JJ: Glycation-induced inactivation and loss of antigenicity of catalase and superoxide dismutase. Biochem J 328:599-605, 1997
36. Stadler J, Curran JB, Ocoha JB, et al: Effect of endogenous nitric oxide on mitochondrial respiration of rat hepatocytes in vitro and in vivo. Arch Surg 126:186-191, 1991
37. James SL, Glaven J: Macrophage cytotoxicity against schistosomula of *Schistosoma mansoni* involves arginine-dependent production of reactive nitrogen intermediates. J Immunol 143:4208-4212, 1989