# Gliclazide Scavenges Hydroxyl and Superoxide Radicals: An Electron Spin Resonance Study

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**The role of reactive oxygen species in diabetes and its complications are well known. Two therapeutic agents commonly used in the treatment of diabetes are the sulfonylureas gliclazide and glibenclamide. These drugs effectively reduce blood sugar in non–insulin-dependent diabetes mellitus, by augmenting insulin release. Gliclazide is known to be a general free radical scavenger as shown by its inhibition of o-dianisidine photo-oxidation. In this study, the effects of gliclazide and glibenclamide on free radicals were examined in vitro, using electron spin resonance spectroscopy. Superoxide radical ($O_2^-$) generated from the hypoxanthine-xanthine oxidase system or hydroxyl radical ($OH^\cdot$) generated via the Fenton reaction were analyzed as spin adducts of 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). Gliclazide scavenged $O_2^-$ and $OH^\cdot$ in a dose-dependent manner whereas glibenclamide was without effect. These findings suggest that gliclazide is not only effective in reducing blood sugar, but may also be beneficial as a result of inhibition of lipid and protein denaturation, which is believed to lead to the development of diabetic complications.**



DIABETES REPRESENTS A state of increased oxidative stress based on increased peroxidation[1-3] and reduced antioxidant reserves.[4,5] Furthermore, in vitro experiments with diabetic animal models show that the nonenzymatic glycation of protein yielded Schiff base intermediates, which may arrange to form an Amadori compound, which can generate the superoxide anion by auto-oxidation.[6-9] Superoxide dismutase (SOD) loses activity when exposed to glucose in vitro, and high levels of glycated erythrocyte SOD are found in erythrocytes in diabetes.[10] In these states of increased peroxidation and reduced antioxidant reserve in diabetes, oxidative stress may play an important role in the pathogenesis of diabetic vascular complications, such as neurovascular dysfunction,[11,12] β-cell damage in the pancreas,[13] cataract,[14] atherosclerotic peripheral arterial disease,[15] and teratogenesis.[16] Therefore, the generation of reactive oxygen species may contribute to the overall complications of diabetes.[17] Moreover, antioxidants have been shown to attenuate diabetic complications such as muscular and neurovascular deficits in experimental diabetes.[18-20,21]

Gliclazide and glibenclamide are sulfonylureas commonly used in the treatment of diabetes, as these drugs effectively reduce blood sugar in non–insulin-dependent diabetes mellitus (NIDDM) by augmenting insulin release. A general free radical scavenging activity of gliclazide was shown by the inhibition of photo-oxidation of o-dianisidine, though glibenclamide has no effect.[22] Furthermore, significant improvement of oxidative status in patients with NIDDM after treatment with gliclazide, but not glibenclamide, was shown by examining lipid peroxides and erythrocyte SOD.[23] In this study, the effects of gliclazide and glibenclamide on hydroxyl and superoxide radicals were examined in vitro, using an electron spin resonance (ESR) technique.

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## MATERIALS AND METHODS

### *Reagents*

Xanthine oxidase ([XOD], 1 U/mg, from cow's milk) was obtained from Boehringer Mannheim Corp (Indianapolis, IN). Hypoxanthine (HPX) and the spin trap 5,5-dimethyl-1-pyrroline N-oxide (DMPO) were obtained from Labotec (Tokyo, Japan). All other chemicals were from Sigma Chemical Co (St Louis, MO) and were of the highest grade.

### *Standards*

L-Ascorbic acid, 2-[3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-2H-1-benzopyran-6yl-hydrogen phosphate] potassium salt (EPC-$K_1$) was a gift from Senju Pharmaceutical Co, Ltd (Osaka, Japan). SOD standard solution kit was purchased from Labotec.

### *Samples*

Gliclazide and glibenclamide were gifts from Dainippon Pharmaceutical Co, Ltd (Osaka, Japan).

### *ESR Spectrometer*

For the measurements of superoxide and hydroxyl radical scavenging, the Free Radical Monitor (JES-FR 30; JEOL, Tokyo, Japan) was used.[24] This compact, sensitive, ESR spectrometer has the function of normalizing all spectra for accurate calculation using manganese oxide (MnO) as an internal standard. MnO provided a constant signal to which all peak heights were compared. Sample peak height was divided by the MnO peak height to give the relative peak height.

ESR measurements were made as follows: magnetic field: 335.5 ± 5 mT; power: 4 mT; modulation frequency: 9.41 GHz; modulation amplitude: 1 × 0.1 mT; response time: 0.1 seconds; amplitude: 1 × 200; and the sweep time: 2 minutes. ESR spectra were measured at 23°C. Data analyses were performed using a computerized program (version 5.2 for JES-FR 30) connected to the Free Radical Monitor.

Methods for spin trapping of hydroxyl and superoxide radicals were based on earlier reports.[25-27]

### *Hydroxyl Radical Scavenging Activity*

Hydroxyl radical scavenging activity was estimated essentially according to the method of Liu et al.[28] All solutions except $FeSO_4$ were dissolved in 0.1 mol/L potassium phosphate buffer, pH 7.4; $FeSO_4$ was dissolved in distilled water. Fifty microliters of sample solution, 50 μL of 0.18 DMPO, 50 μL of 2 mmol/L $H_2O_2$, and 50 μL of 0.2 mmol/L $FeSO_4$ were mixed and put into a flat cell (200-μL capacity, quartz from JEOL). Exactly 30 seconds after mixing, ESR spectra of the DMPO-OH

spin adduct were recorded. As a standard for hydroxyl radical scavenging, EPC-$K_1$ was used. EPC-$K_1$ is composed of ascorbate and vitamin E joined by a phosphate linkage, and has potent hydroxyl radical scavenging activity, especially for hydroxyl radicals generated by iron-catalyzed reactions.[24,29,30]

*Superoxide Scavenging Activity*

Superoxide scavenging activity was estimated according to the method of Liu et al.[28] Superoxide was generated by a HPX-XOD system. All solutions except dimethylsulphoxide (DMSO) were in 0.1 mol/L potassium phosphate buffer, pH 7.4. Fifty microliters of 4 mmol/L HPX, 30 μL of DMSO, 50 μL of sample solution, 20 μL of 4.5 mol/L DMPO, and 50 μL of XOD (0.4 U/mL) were mixed and transferred into a 200-μL capacity flat cell. ESR spectra of the DMPO-$OO^-$ spin adduct were analyzed. SOD-like activity was expressed as SOD-equivalent U/mg.

*Statistics*

The mean values of hydroxyl and superoxide scavenging activities were calculated from several data points, which fit the linear relationship between the concentration and the scavenging activity. These data were confirmed by the results of more than 3 separate experiments. All data are expressed as means ± SEM. Statistical analysis was performed using the Student *t* test.

## RESULTS

*Hydroxyl Radical Scavenging Activity*

The ESR signal of the hydroxyl radical decreased markedly in the presence of 25 mmol/L gliclazide (Fig 1). Gliclazide itself did not show any ESR signal. The specific quartet signal of the DMPO-OH spin adduct disappeared in the presence of DMSO. The hydroxyl radical scavenging activity of gliclazide was dose-dependent up to 25 mmol/L and was calculated as 0.18 ± 0.06 EPC-$K_1$ equivalent (μmoL/mg) (n = 5). Glibenclamide did not show any scavenging effect on hydroxyl radicals (data not shown).

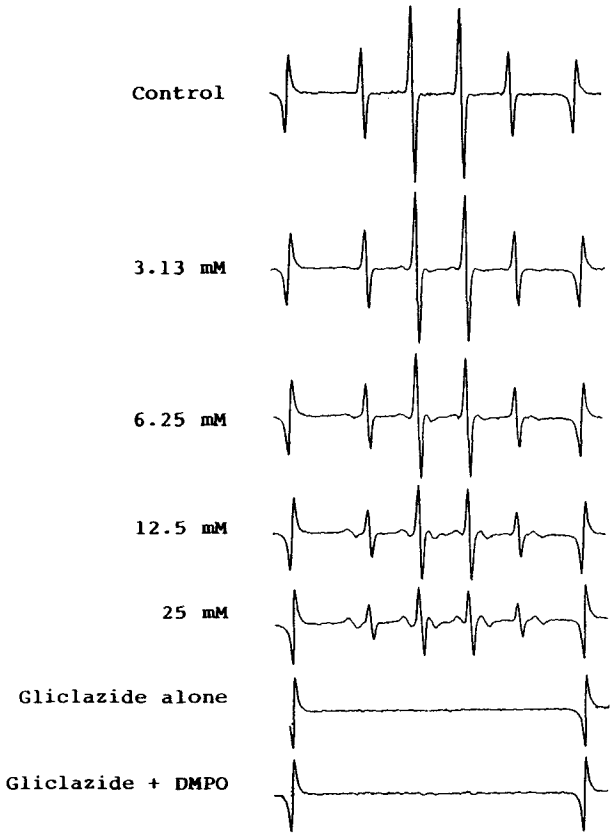


**Fig 1. Hydroxyl radical scavenging activity of gliclazide.**

*Superoxide Scavenging Activity*

The ESR signal of superoxide decreased slightly in the presence of 25 mmol/L gliclazide (Fig 2). This effect of gliclazide was also dose-dependent up to 25 mmol/L, and the superoxide scavenging activity was calculated as 0.18 ± 0.08 SOD equivalent U/mg (n = 4). As for hydroxyl radical scavenging, glibenclamide showed no superoxide scavenging activity (data not shown).

## DISCUSSION

In this study the free radical scavenging activities of gliclazide for hydroxyl and superoxide anion radicals were clearly shown. No such activities were observed with the other sulfonylurea glibenclamide. The superoxide scavenging activity of gliclazide was estimated as 0.18 ± 0.08 SOD equivalent U/mg, which is much less when compared to ascorbic acid (approximately 500 SOD equivalent U/mg). On the other hand, hydroxyl radical scavenging activity was estimated at 0.18 ± 0.06 EPC-$K_1$ equivalent (μmoL/mg). This is about 20% of the potency of EPC-$K_1$, 8-fold greater than that of Trolox, a water soluble vitamin E analogue, and 6-fold greater than that of mannitol. Therefore, the antioxidant activity of gliclazide could principally depend on hydroxyl radical scavenging activity.

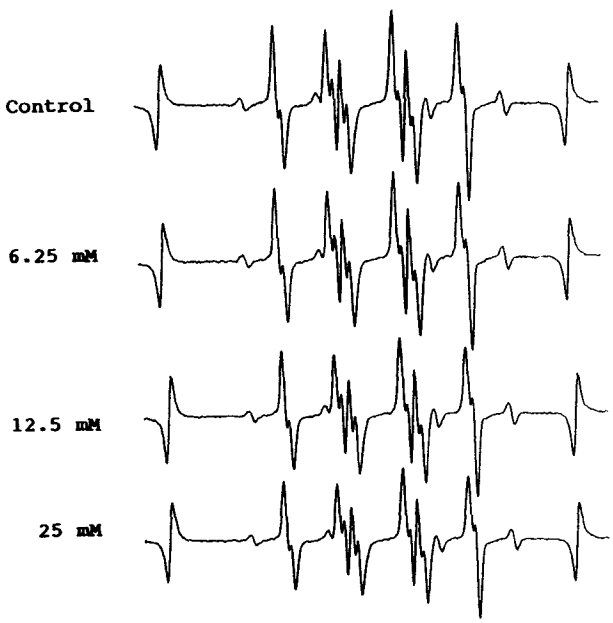


**Fig 2. Superoxide scavenging activity of gliclazide.**

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