Free Rad. Res. Comms., Vol. 3, No. 6, pp. 331-335
Photocopying permitted by license only

Printed in Great Britain

FREE RADICAL SCAVENGING DRUGS, ASSESSED BY ESR STUDIES: INFLUENCE OF HEMOGLOBIN

BERND LAMMEL,[†] SIGRID PILZ,[†]
HANS-JOACHIM FREISLEBEN,[†] ECKARD SCHRAVEN[‡] and
GUIDO ZIMMER[†]

[†]Gustav-Embden-Zentrum der Biologischen Chemie, Theodor-Stern-Kai 7, Universität Frankfurt and [‡]Medizinisch-Biologische Forschungsabteilung der Cassella AG, Hanauer Landstr. 526, D-6000 Frankfurt am Main, FRG

(Received October 10th 1986)

To monitor free radical scavenging properties of drugs, the 'stable' radical 2,2,6,6-tetramethylpiperidino-1-oxyl (TEMPO) was used. The sydnonimine molsidomine (SIN-1) effectively reduced the ESR signal whereas the nitrate isosorbidemononitrate (ISMN) did not. Thiol reagents like 2-mercaptopropionylglycine (MPG) or glutathione (GSH) only were effective in the presence of Fe^{2+} or Fe^{3+}. Protein-bound iron in hemoglobin proved about four times more effective in reducing ESR signal height by thiols. It is suggested that the decrease in thiol content adds to the lack in protein bound iron of hemoglobin to induce the burst of free radicals in hypoxia (ischemia) and reperfusion.

KEY WORDS: Free radicals, ESR signal reduction, molsidomine (SIN-1), isosorbidemononitrate (ISMN), 2-mercaptopropionylglycine (MPG), hemoglobin.

1. INTRODUCTION

In recent years, free radicals have become known to play a major role in hypoxic or ischemic diseases.[1] A particular interest is centered on cardiac hypoxia. Tissue damage becomes apparent during oxygen reperfusion and closer analysis revealed that besides Ca^{2+} overload, oxygen radicals were also responsible for cellular breakdown. The calcium and oxygen paradox seems to constitute facets of the same problem.[2]

Molecular oxygen has an unusual structure, in that it is a paramagnetic biradical containing two unpaired electrons with parallel spins. Oxygen radicals may be formed by uptake of 1 electron and further transformation into hydrogen peroxide may take place:

$$O_2 \xrightarrow{e^-} O_2^{\cdot-} \xrightarrow{e^- + 2H^+} H_2O_2.$$

The latter product can decompose into OH· radicals. These radicals may then cause membrane and cellular damage which results in permeability changes and exchange of calcium along the gradient from outside to inside.

The pharmaceutical issue of some drugs is thus such that they should be able to scavenge free radicals in order to protect the tissue against the sequence of damaging effects due to hypoxic or ischemic lesion.

Thus there is an obvious necessity to have a simple system to monitor the effectiveness of drugs in scavenging free radicals. Spin labels, introduced into biochemistry about 20 years ago, are free radicals, "stable" enough to become introduced into

diamagnetic species and yield signals from there. Stability, however is not absolute, so that we have introduced the label TEMPO as a simple means to measure the capacity of drugs to scavenge this organic radical.

2. MATERIALS AND METHODS

As a model substance for free radicals we used the stable radical 2,2,6,6-tetramethyl-piperidino-1-oxyl (TEMPO). Reactions were carried out in 0.5 M sodium phosphate buffer, pH 7.1 or at the indicated pH values (Fig. 1). Before use the buffer was passed through a chelex-100 column. Concentration of label was 100 μM, the concentrations of the investigated drugs were 100 mM. In this system, the final pH values were in the MPG series pH 6.7, with GSH 6.9 and with the active metabolite of molsidomine, SIN-1 7.0 (Table I). Consecutive measurements of line heights were carried out on the first line (low magnetic field line) of the ESR spectra, at 5 min intervals.

In all measurements the reaction rate/min from the beginning of the reaction was determined. The electron spin resonance spectrometer was a Bruker B-ER 420.

Substances were obtained from the following sources: 2-mercaptopropionylglycine (MPG) from Fresenius, Bad Homburg, SIN-1 from Cassella AG, Frankfurt, isosorbidemononitrate (ISMN) from Mack, Illertissen, FRG, glutathione and human hemoglobin (reduced form) from Sigma, Deisenhofen, FRG. Chelex 100 was obtained from Biorad labs, München, FRG, all buffer substances were from Merck. TEMPO was obtained from Aldrich, Steinheim, FRG. Fe (II)$Cl_2 \times 4H_2O$, Fe

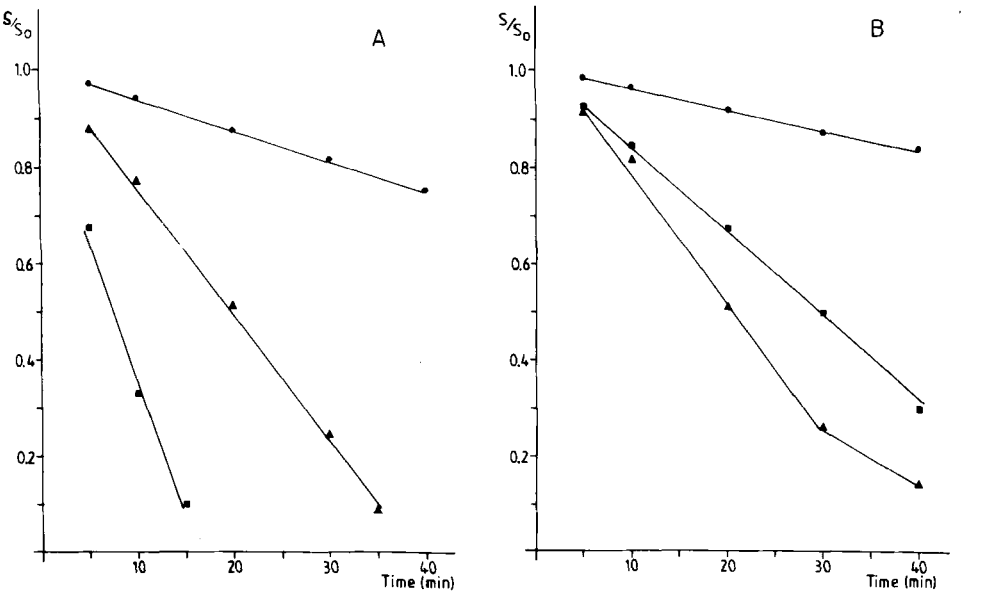

FIGURE 1 Reduction rate of TEMPO signal height by 100 mMol/l MPG in the presence of Fe^{2+} or Fe^{3+} at different pH values: A Fe^{2+} 40 μMol/l; pH 6.1 ■; pH 7.26 ▲; pH 8.0 ●; B Fe^{3+} 40 μMol/l; pH 6.1 ■; pH 7.28 ▲; pH 8.0 ●.

TABLE I
TEMPO signal height reduction by MPG or GSH in the presence of metal ions or hemoglobin, and by the drugs SIN-1 and ISMN

Substance at 100 mmol/l	Additional substance	μmol/l	TEMPo reduction rate of signal height μmol/min
MPG	0		0
MPG	Fe^{2+}	20	0.81
MPG	Fe^{2+}	40	1.785
MPG	Fe^{2+}	80	9.09
MPG	Fe^{3+}	20	0.735
MPG	Fe^{3+}	40	1.72
MPG	Fe^{3+}	80	6.0
MPG	Hb	5	0.97
MPG	Hb	10	2.22
MPG	Hb	20	11.1
MPG	Co^{2+}	40	0.23
GSH	0		0
GSH	Fe^{2+}	40	2.04
GSH	Hb	20	11.75
SIN-1	0		1.75
ISMN	0		0
ISMN	Fe^{2+} or Hb	40 or 20	0

All values are means of at least 3 experiments Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+} are ineffective.

(III)$Cl_3 \times 6H_2O$, $MgCl_2 \times 6H_2O$, $MnCl_2 \times 2H_2O$, $CuCl_2 \times 2H_2O$, $ZnCl_2$, $CoSo_4 \times 7H_2O$, $NiCl_2 \times 6H_2O$ and $CaCl_2 \times 2H_2O$ were from Merck, Darmstadt, FRG.

3. RESULTS

We used the TEMPO signal height reduction to monitor free radical scavenging properties of thiol compounds and different drugs.

The SH-compounds, 2-mercaptopropionylglycine (MPG) and reduced glutathion (GSH), in the absence of metal ions showed no activity at all (Table I). Addition of Fe^{2+} or Fe^{3+} ions proved to be highly effective in a dose-dependent manner. Cobalt revealed little activity, whereas the other tested metal ions were all ineffective (Table I). The activity of Fe^{2+} seems to be somewhat higher than that of Fe^{3+}.

We have then tested whether also protein-bound iron, like that in hemoglobin, exerts an effect similar to that observed with $FeCl_2$ or $FeCl_3$. Interestingly, the scavenging effect of either MPG or GSH, in the presence of hemoglobin, was, on a molar basis, about 4-fold compared to the metal ions. Activities of both tested thiol compounds MPG and GSH in the presence of hemoglobin and Fe^{2+} were nearly identical.

In contrast to the thiol compounds SIN-1 was active without addition of metal ions. The activity is comparable to that of MPG in the presence of 40 μmol/l iron. ISMN,

neither alone, in the presence of iron, nor of hemoglobin, had any free radical scavening properties.

We also tested the influence of pH on the effectiveness of iron (Fig. 1). Under alkaline conditions the activities of both forms of iron induce a considerable signal decrease (0.57 and 0.39 μmol/min respectively). At pH 7.28 Fe^{3+} shows the maximal activity (2.3 μmol/min) whereas Fe^{2+} was most effective at pH 6.1 (6.6 μmol/min).

4. DISCUSSION

The signal height reduction by TEMPO can be used to study the free radical scavenging properties of different compounds.

We showed that thiol compounds are only effective in the presence of metal ions, particularly, of iron, and to a much lower extent of cobalt confirming previous results.[3-5]

Furthermore, other metal ions were found to be inactive. Moreover, we demonstrated that also protein-bound iron, like that in hemoglobin, exerts a drastic decrease of the TEMPO signal in the presence of thiol reagents. These findings led us to consider possible pathophysiological consequences.

During hypoxia, overall protein-bound thiols of heart cells are decreased.[6] Similarly, mitochondrial membrane proteins become severely disarranged due to uncoupling or loose-coupling.[7,8]

In addition, as a consequence of a decreased blood supply during ischemia or in an infarcted area a reduction of available hemoglobin content should be taken into account. The combination of these two events (decrease in thiol content, decrease of available hemoglobin) may then result in the deleterious burst of free radical content in the tissue. This possibility is underlined by our present investigations.

These interrelations prompted us to study the effects of the antianginal drugs molsidomine and ISMN. The organic nitrate ISMN proved to be ineffective in our model system. By contrast, SIN-1 the active metabolite of molsidomine, showed radical scavenging activity also in the absence of iron. Therefore, the latter compound should be useful under conditions where enhanced radical formation may be the cause of cell damage.

Thiol compounds, like MPG, on the other hand, are potentially very efficient in restoring the reactivity of protein-bound SH-groups.[9,10]

Acknowledgement

Support of this work by the Deutsche Forschungsgemeinschaft (Zi 80/19-1 and 19-2) is gratefully acknowledged.

References

1. Hess, M.L. and Manson N.H., *J. Mol. Cell. Cardiol.*, **16**, 969, (1984).
2. Hearse, D.J., Humphrey, S.M. and Bullock, G.R., *J. Mol. Cell. Cardiol.*, **10**, 641 (1978).
3. Finkelstein, E., Rosen, G.M. and Rauckman, E.J. *Biochim. Biophys. Acta*, **802**, 90, (1984).
4. McConnell, H.M. and McFarland, B.G. *Quart. Rev. Biophys.*, **3**, 91, (1970).
5. Gaffney, B.J. in Spin Labeling-Theory and Application, ed. L.J. Berliner (Academic Press: New York, 1976) p. 183.
6. Ferrari, R., Ceconi, C., Curello, S., Guarnieri, C., Caldarera, C.M., Albertini, A. and Visioli, O. *J. Mol. Cell. Cardiol.*, **17**, 937, (1985).

7. Veit, P., Fuchs, J. and Zimmer, G. *Basic Res. Cardiol.*, **80** 107, (1985).
8. Fuchs, J., Mainka, L. and Zimmer, G. *Arzneim.-Forsch./Drug Res.*, **35**, 1394, (1985).
9. Fuchs, J., Veit, P. and Zimmer, G. *Basic Res. Cardiol.*, **80**, 231, (1985).
10. Zimmer, G., Beyersdorf, F. and Fuchs, J. *Mol. Physiol.*, **8**, 495 (1985).

Accepted by Dr. J.V. Bannister