Cytotoxicity of spin trapping compounds

Reiner F. Haseloff[a,*], Katharina Mertsch[a], Elvira Rohde[a], Ingo Baeger[a], Igor A. Grigor'ev[b], Ingolf E. Blasig[a]

[a] *Research Institute of Molecular Pharmacology, Alfred-Kowalke-Str. 4, 10315 Berlin, Germany*
[b] *Institute of Organic Chemistry, Novosibirsk, Russia*

Received 3 October 1997; revised version received 20 October 1997

Abstract Spin trapping compounds are used frequently to detect free radicals released by cells. Their cytotoxicity has to be considered in order to prevent perturbations of normal cell growth and viability. Eleven spin traps (eight nitrones and three nitroso traps) have been tested for their effects on bovine aortic endothelial cells (toxicity range, 50% survival rate). The lowest cytotoxicity was found for 5,5-dimethylpyrroline-1-oxide and 2,2,4-trimethyl-2*H*-imidazole-1-oxide whereas nitrosobenzene and 2-methyl-2-nitrosopropane exerted the strongest cytotoxic effects. In addition, three nitronyl nitroxides were tested. Their cytotoxicity was found to be dependent on substitution, and the toxic concentration of a lipophilic derivative was found to be more than two orders lower as compared to a hydrophilic derivative. The results of this study indicate that most spin traps can be used in cell cultures at customary (i.e. millimolar) concentrations; caution is recommended when nitroso spin traps are applied to cells.

Key words: Cytotoxicity; Spin trap; Endothelial cell

1. Introduction

EPR spectroscopy of spin trapped radicals has become the method of choice for the detection and identification of free radicals formed in biological systems. The possibilities, advantages and disadvantages of the spin trapping technique have been discussed in various excellent reviews [1–3], and a systematic investigation of spin trapping methodology has been started recently [4–6]. There are several reports on protective effects exerted by spin trapping compounds in the heart [7] and, more recently, in the brain [8]. Cell cultures belong to the prominent targets of spin trapping investigations. Forced by relatively low rate constants for the reaction of spin traps with superoxide and the low amount of free radicals released by cells, it is necessary to apply rather high concentrations (usually ≥100 mM) of spin trap agents in order to detect intrinsic formation of free radicals by cells. However, extensive database search has revealed that, in contrast to nitroxide spin labels [9], there are no data available on the cytotoxicity of spin traps. In a recent in vivo study of nitrone toxicity, the most frequently used compounds 5,5-dimethylpyrroline *N*-oxide (DMPO) and *N-tert*-butyl-α-phenyl nitrone (PBN) have been found to exert a low toxicity [5]. These results support previous in vitro studies in isolated hearts [10] and rat pulmonary artery rings [11]. Moreover, there are data available on the mutagenicity of various spin trapping agents [12].

The objective of this work is the presentation of data on spin trap cytotoxicity using monolayers of bovine aortic endothelial cells (BAEC). In addition to commercially available nitrones and nitroso traps, this study includes 2*H*-imidazole-1-oxides [13] and a nitroxide spin label as well as 2-imidazoline nitroxides (nitronyl nitroxides) that have been applied recently for $^{\bullet}NO$ detection [14].

2. Materials and methods

2.1. Chemicals

Commercially available spin trapping compounds and nitroxides applied in this study were purchased from Sigma (St. Louis, MO) and were of the highest quality available. 2*H*-Imidazole-1-oxide spin traps and nitronyl nitroxides (Fig. 1) were synthesized according to [15] and [16], respectively.

2.2. Endothelial cell culture

Bovine aortic endothelial cells (passage 12–15) were cultivated in Corning flasks (25 cm^2) in Eagle minimum essential medium (MEM) (Sigma) supplemented with 10% fetal calf serum (Sigma), 2 mM glutamine without antibiotics at 37°C in 5% CO_2 and 95% air. Subcultivation was done twice a week with trypsin/EDTA (each 0.25% v/v) in PBS. Quality of cultivation was verified by the phase contrast microscopic cobblestone appearance at confluence, presence of factor VIII antigen (DAKO, LSAB, Denmark) and alkaline phosphatase (AP Kit 104, Sigma) as well as content of angiotensin converting enzyme (ACE, tested according to [17]).

2.3. Cytotoxicity determination

For studying cytotoxicity, the neutral red assay [18] was applied. Briefly, cells cultivated in 24-well plates (seeding density, 80 000/cm^2) were incubated for 24 h with the substance to be tested. Subsequently, incubation ensued for 2 h in neutral red (NR)-containing medium (50 μg/ml NR in Eagle MEM with 2% FCS). The dye absorbed by viable cells was extracted by shaking for 10 min in 50% ethanol/1% glacial acetic acid and spectrophotometrically determined at 540 nm using a microtiter plate reader (Cambridge Technology 7500, Cambridge, UK). Four to six independently cultivated plates were used for the evaluation of cytotoxicity. Control data were obtained from cells incubated under the same conditions without any test compounds added.

2.4. Statistics

The average absorbance at each concentration of a compound to be tested (six replicates) was expressed as a percentage of control. The

*Corresponding author. Fax: (49) (30) 51551-421.
E-mail: haseloff@fmp-berlin.de

Abbreviations: ACE, angiotensin converting enzyme; BAEC, bovine aortic endothelial cells; PBN, *N-tert*-butyl-α-phenyl nitrone; CMIO, 4-carboxy-2,2-dimethyl-2*H*-imidazole-1-oxide; CYIO, 4-cyano-2,2-dimethyl-2*H*-imidazole-1-oxide; DBNBS, 3,5-dibromo-4-nitroso-benzenesulfonic acid; NN 2, 4,4-dimethyl-2,5-diphenyl-5-methoxy-2-imidazoline-3-oxide-1-oxyl; MCIO, 2,2-dimethyl-4-methoxycarbonyl-2*H*-imidazole-1-oxide; DMPO, 5,5-dimethylpyrroline-1-oxide; NN 3, 4,4-dimethyl-2,5,5-triphenyl-2-imidazoline-3-oxide-1-oxyl; MNP, 2-methyl-2-nitrosopropane; NR, neutral red; NB, nitrosobenzene; POBN, α-(4-pyridyl-1-oxide)-*N-tert*-butyl nitrone; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; TMPO, 3,3,5,5-tetramethylpyrroline-1-oxide; NN 1, 4,4,5,5-tetramethyl-2-(4-trimethylammoniophenyl)-2-imidazoline-3-oxide-1-oxyl methyl sulfate; TMIO, 2,2,4-trimethyl-2*H*-imidazole-1-oxide

PII S0014-5793(97)01349-5

Table 1
Cytotoxicity of spin trapping compounds and nitroxides on endothelial cells

Compound	IC_{50} value (mM)	IC_{10} value (mM)
5,5-Dimethylpyrroline-1-oxide (DMPO)	138.34 ± 2.22	86.8
3,3,5,5-Tetramethylpyrroline-1-oxide (TMPO)	46.96 ± 11.88	4.5
N-tert-Butyl-α-phenyl nitrone (PBN)	9.37 ± 0.26	5.4
α-(4-Pyridyl-1-oxide)-*N-tert*-butyl nitrone (POBN)	5.41 ± 2.18	0.7
2,2,4-Trimethyl-2*H*-imidazole-1-oxide (TMIO)	104.56 ± 2.26	72.0
4-Carboxy-2,2-dimethyl-2*H*-imidazole-1-oxide (CMIO)	12.96 ± 0.79	9.4
2,2-Dimethyl-4-methoxycarbonyl-2*H*-imidazole-1-oxide (MCIO)	8.98 ± 0.06	6.8
4-Cyano-2,2-dimethyl-2*H*-imidazole-1-oxide (CYIO)	1.62 ± 0.03	1.1
2-Methyl-2-nitrosopropane (MNP)	0.10 ± 0.03	0.007
Nitrosobenzene (NB)	0.06 ± 0.007	0.006
3,5-Dibromo-4-nitroso-benzenesulfonic acid (DBNBS)	0.64 ± 0.07	0.07
4,4,5,5-Tetramethyl-2-(4-trimethylammoniophenyl)-2-imidazoline-3-oxide-1-oxyl methyl sulfate (NN 1)	3.72 ± 0.64	0.62
4,4-Dimethyl-2,5-diphenyl-5-methoxy-2-imidazoline-3-oxide-1-oxyl (NN 2)	5.27 ± 0.24	2.8
4,4-Dimethyl-2,5,5-triphenyl-2-imidazoline-3-oxide-1-oxyl (NN 3)	0.15 ± 0.01	0.11
2,2,6,6-Tetramethylpiperidine-1-oxyl (TEMPO)	0.72 ± 0.05	0.31

IC_{50} and IC_{10} values represent the concentrations that correspond to 50% and 90% cell viability.

inhibitory concentration of the compound reducing the viability of the cells by 50% (IC_{50} value) was determined from the dose-response curve using a non-linear curve fitting procedure (model, Hill function with two parameters).

3. Results

BAEC applied in this study were characterized by specific markers of endothelial cells such as von Willebrandt factor and ACE (content of ACE, 362 ± 70 pmol/min/mg protein).

Table 1 presents the data obtained for the cytotoxicity of spin trapping compounds and nitroxides. The most toxic compounds found were the nitroso spin traps nitrosobenzole and 2-methyl-2-nitrosopropane. Their IC_{50} values were more than one order lower as compared to all other spin traps tested in this study. With an IC_{50} value of 640 μM, the water soluble nitroso trap DBNBS exerted a much lower toxicity.

The toxicity of the commercially available nitrone spin traps varied with the general structure of the compounds. The lowest toxicity was observed for pyrroline-1-oxides. The IC_{50} values of PBN and POBN were found to be more than one order lower as compared to DMPO. The toxic concentrations of 2*H*-imidazole-1-oxides differed by approximately two orders of magnitude, with TMIO giving the highest and CYIO the lowest IC_{50} values. The evaluation of the nitroxides' toxicity revealed IC_{50} values of 0.72 mM for TEMPO and of 3.72, 5.27 and 0.15 mM for NN 1, NN 2 and NN 3, respectively.

4. Discussion

Presenting data on the cytotoxicity of spin trapping compounds, this study attempts to fill a gap which has been left so far in the literature on the application of spin trapping compounds. Several reasons justify the selection of endothelial cells for this cytotoxicity study. Endothelial cells attracted (and attract) strong interest particularly due to their ability to produce and release nitric oxide and to contribute to the regulation of the vascular tone [19]. Moreover, there is a growing body of evidence from spin trapping investigations that significant amounts of other radical species, especially superoxide anions, are produced by endothelial cells under certain conditions [14,20,21].

Regarding the most frequently used spin trap DMPO it can be stated that the data obtained are in agreement with those reported for in vivo toxicity [5]: its cytotoxicity was found to be the lowest of all traps tested. On the other hand, TMPO (additionally -CH_3-substituted in the 3-position only) also exerted a relatively low cytotoxicity as compared to PBN and 2H-imidazole derivatives (except TMIO). This result contradicts the high in vivo toxicity of TMPO and indicates that its toxic effects result from specific interactions which obviously do not occur in endothelial cells.

Using 2*H*-imidazole spin traps it was possible to investigate the influence of different substituents in the 4-position on the cytotoxicity. It is not surprising that the highest toxicity was found for the 4-cyano derivative; on the other hand, it may be a reasonable assumption that the structural similarity of TMIO with DMPO results in a comparably low toxicity.

The toxicity (and mutagenicity) of nitroso compounds is a well-known phenomenon [22,23]; most of the spin trapping applications of nitroso traps are devoted to cell-free model systems. DBNBS has been applied in several biological studies; advantages of this compound are its water solubility (partition coefficient 0.04 [6]) and, as shown here, its lower cytotoxicity (6 times higher IC_{50} value as compared to MNP).

Independently of the spin trap family, there was a tendency for higher toxicity with increasing lipophilicity. This reflects the key role of cell membranes for cytotoxic effects. Disturbances of membrane integrity (which must considered to occur with a higher probability in the presence of lipophilic xenobiotics) seem to be a main reason for cell death.

Derivatives of 2-imidazoline nitroxides have been applied for 'spin trapping' of nitric oxide[1]; thus, three of these compounds have been included into this study. As already observed for 'genuine' spin traps, a considerable difference of more than one order of magnitude was found in the toxicity of the hydrophilic and amphiphilic nitroxides NN 1 and NN 2 and the very lipophilic (octanol/water partition coefficient >20) nitroxide NN 3. This again confirms the tendency

[1] The term 'spin trap' seems to be incorrect in this context: usually, spin traps are considered to be diamagnetic compounds reacting with short-lived free radicals forming a longer-lived radical adduct. Nitronyl nitroxides are paramagnetic molecules, their reaction with •NO results in the formation of imino nitroxides which have an ESR spectrum that differs considerably from the spectrum of nitronyl nitroxides.

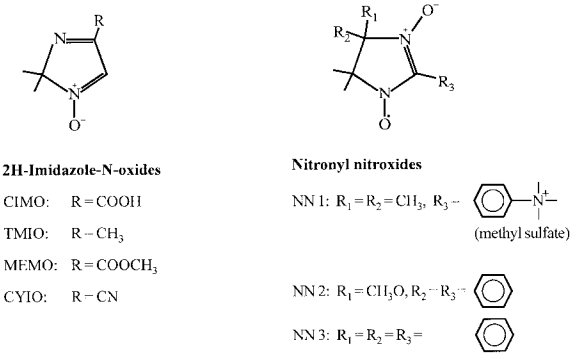

Fig. 1. Structural formulas of 2*H*-imidazole-1-oxides and nitronyl nitroxides.

that higher lipophilicity may be accompanied by a higher toxicity in this cell culture model.

A similar dependence on the lipophilicity has been observed in a cytotoxicity study of nitroxide radical spin probes [9]. These data were obtained in Chinese hamster ovary cells using a different experimental approach. Thus, in order to acquire information on the compatibility of the data obtained, one of these spin probes was included into the experiments presented here. The divergence in the toxicity of TEMPO (IC_{50} value not given, toxicity starting at concentrations >1 mM [9] vs. $IC_{50} = 0.8$ mM, this study) is sufficiently low to be attributed to the specificity of cells and the differences in the experimental approach. This observation justifies the assumption that the toxicity values of spin trapping compounds found in endothelial cells can be used as a clue for selecting concentrations in studies devoted to other types of cells.

Acknowledgements: This work was supported by the Bundesministerium für Bildung und Forschung (Grants BEO 021/0310015A/B and X224.5).

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