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Original research paper

Nitrones: not only extraordinary spin traps, but also good nitric oxide sources *in vivo*

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Free radicals are involved in the development of reperfusion injuries. Using a spin trap, the intensity of such lesions can be reduced. Nitrones (effective *in vivo* spin traps) were tried in this work as *in vivo* nitric oxide donors. Nitrite and nitrate concentration values (rabbit blood) were used as biomarkers of nitric oxide production. Most nitrones did not increase plasma concentrations of nitrite and nitrate; on the contrary, reduced plasma concentrations of these indicators were noted. However, glyoxal isopropyldinitrone, in a dose of 50 mg kg^{-1}, was highly effective in increasing nitric oxide production. At the same time, nitrones do not react with hepatic homogenates, proving that the release of nitric oxide takes place in the tissues and is not related to hepatic metabolism. Before using nitrones *in vivo*, they were tested *in vitro* for the ability to release nitric oxide following a reaction with the hydroxyl radical.

Keywords: nitric oxide, nitrite, nitrate, nitrones, nitric oxide donor

When blood and nutrient flow is reduced in a tissue, ischemic diseases appear. During the ischemic period, tissue metabolism slows down, followed by over-active metabolic processes soon after the blood and nutrient flow is re-established, leading to generation of high quantities of free radicals. This excess of free radicals, both by apoptotic and necrotic pathways, has been proven to have a major impact on the development of reperfusion injuries (1, 2). The most common forms of free radicals are superoxide anion, hydroxyl radical, carbon-centered radicals, nitric oxide and nitrogen dioxide. Peroxynitrite and hydrogen peroxide, which are not free radicals themselves, can become important sources of free radicals (centered on oxygen and nitrogen) by electron accepting processes. The hydroxyl radical, considered to be the most damaging radical in cells, is extensively generated during ischemia and reperfusion by the Fenton reaction and decomposition of peroxynitrite at low pH, inducing cell membrane damage, a necrotic process, by peroxidation of unsaturated fatty acids. Superoxide anion causes mitochondrial membrane dysfunction and leads to cytochrome c release in the cytoplasm, activating pro-apoptotic signals (2, 3). Hydrogen

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peroxide increases the influx mode activity of the Na^+/Ca^{2+} exchanger, suggesting that oxidative stress might be involved in the Ca^{2+} overload, a key factor in the development of reperfusion injuries (4). The most affected organs by ischemia/reperfusion injuries are the myocardium and the brain.

There are several methods attempting to reduce the intensity of these lesions: (*i*) increasing the antioxidant defense systems and the amount of cell survival proteins by ischemic and remote ischemic preconditioning (5), (*ii*) inducing the reperfusion injury salvage kinase (RISK) pathway which, when activated specifically at the time of myocardial reperfusion, confers powerful cardioprotection (6), (*iii*) using naturally occurring spin traps, such as vitamin C, vitamin E, and polyunsaturated fatty acids for reduction of excessive superoxide anion production and protection of cardiac tissues from oxidative damage during heart surgery with extracorporeal circulation (7, 8).

These methods, proven effective in some studies, are designed for cardioprotection during heart surgery when the heart is stopped and restarted after an ischemic period. Unfortunately, reduced blood flow along with oxidative stress may also appear in other pathological conditions, such as coronary heart diseases, cerebrovascular disease, inflammation, hypertension, diabetes and atherosclerosis (9–12). These pathological conditions or their complications are usually associated with reduced nitric oxide activity (13–16).

Animal experiments have demonstrated that free radical traps are effective against ischemia/reperfusion injuries. Nitrones are a class of effective spin traps used in experimental biomedical research. These compounds have the ability to trap superoxide anion and hydroxyl radical, thus alleviating many of the toxic effects associated with radical formation, such as ischemia/reperfusion injuries (17), aging (18), LDL oxidation (19), CCl_4-induced liver injury (20), bacterial and viral infections (21), nerve degenerative conditions (22), death induced by buthylhydroxyperoxyde (23), drug induced diabetes and alcohol toxicity (24). Based on its radical trapping ability, one nitrone derivative (NXY-059) has been tested as a new drug in the anti-ischemic therapy in different phase-3 clinical trials (17).

We have previously demonstrated that nitrones are not only extraordinary spin traps, but they can also serve as nitric oxide sources *in vitro*. The formation of nitric oxide is self-limiting and takes place *via* interaction of a nitrone with a free radical. Increasing the nitrone concentration leads to an asymptotic increase in the amount of the produced nitric oxide (25). Finding an effective nitric oxide donor that can act as a spin trap, releasing the vasodilating agent after a spin trapping reaction, could bring extraordinary benefits to the current vasodilating therapy. The release of nitric oxide *in vitro* from nitrones has already been reported in the literature (26, 27). Our goal is to demonstrate that the release of nitric oxide takes place *in vivo* as well.

EXPERIMENTAL

All common chemicals and reagents (NaCl and KCl, Chimopar, Romania, Na_2HPO_4 and KH_2PO_4, Merck, Germany, DMSO, Sigma-Aldrich, USA; ethanol p. a. gradient-grade methanol for HPLC and gradient-grade acetonitrile for HPLC, Merck, were purchased from local providers and were used without further purification. Ultra-pure water was obtained using a Mili-Q purification system (Millipore, USA).

In vitro *release of nitric oxide from nitrones*

The chemical name and structures of the tested nitrones are given in Fig. 1 and Table I. Nitrones were prepared by methods described in detail elsewhere (25).

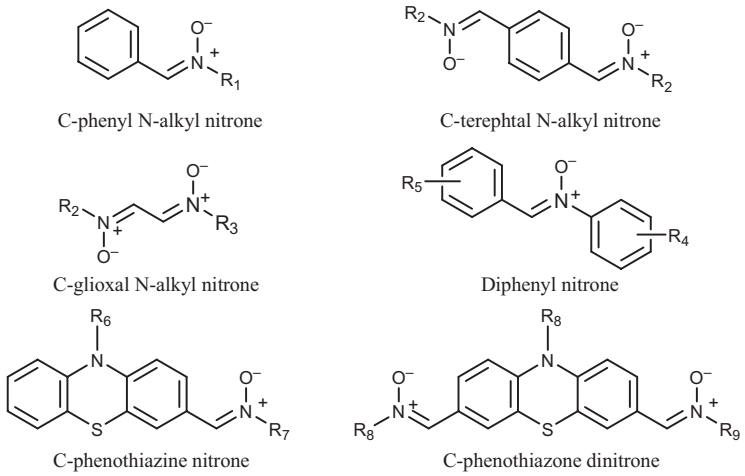

Fig. 1. Chemical structures of the tested nitrones (R_1 – R_9: substituents given in Table I).

Table I. Chemical names and substituents of the studied substances

Abbreviation	Chemical name	Substituent
C-phenyl N-alkyl nitrones		
PBN	phenyl-*N*-*tert*-butylnitrone	R_1 = *tert*-butyl
PEN	phenyl-*N*-ethylnitrone	R_1 = ethyl
C-terephtal N-alkyl dinitrones		
TIPN	(*N*,*N*′*Z*,*N*,*N*′*Z*)-*N*,*N*′-(1,4-phenylenebis(methanylylidene)) bis(propan-2-amine oxide)	R_2 = *i*-propyl
TTBN	(*N*,*N*′*Z*,*N*,*N*′*Z*)-*N*,*N*′-(1,4-phenylenebis(methan-1-yl-1-ylidene)) bis(2-methylpropan-2-amine oxide)	R_2 = *tert*-butyl
TSBN	(*N*,*N*′*Z*,*N*,*N*′*Z*)-*N*,*N*′-(1,4-phenylenebis(methanylylidene)) bis(butan-2-amine oxide)	R_2 = *sec*-butyl
C-glyoxal N-alkyl nitrones		
GIPN	(*N*,*N*′*Z*,*N*,*N*′*Z*)-*N*,*N*′-(ethane-1,2-diylidene)bis(propan-2-amine oxide)	R_3 = *i*-propyl
GTBN	(*N*,*N*′*Z*,*N*,*N*′*Z*)-*N*,*N*′-(ethane-1,2-diylidene)bis(2-methylpropan-2-amine oxide)	R_3 = *tert*-butyl
GSBN	(*N*,*N*′*Z*,*N*,*N*′*Z*)-*N*,*N*′-(ethane-1,2-diylidene)bis(butan-2-amine oxide)	R_3 = *sec*-butyl

Table I. continued

Abbreviation	Chemical name	Substituent
Diphenyl nitrone		
DPN1	(*Z*)-*N*-(4-hydroxybenzylidene)aniline oxide	R_5 = 4-OH
DPN2	(*Z*)-4-chloro-*N*-(4-methoxybenzylidene)aniline oxide	R_4 = 4-Cl, R_5 = 4-OMe
DPN3	(*Z*)-4-bromo-*N*-(4-methoxybenzylidene)aniline oxide	R_4 = 4-Br, R_5 = 4-OMe
DPN4	(*Z*)-4-chloro-*N*-(3,4-dimethoxybenzylidene)aniline oxide	R_4 = 4-Cl, R_5 = 3-OMe and 4-OMe
DPN5	(*Z*)-*N*-(4-methoxybenzylidene)aniline oxide	R_5 = 4-OMe
DPN6	(*Z*)-4-chloro-*N*-(3-chlorobenzylidene)aniline oxide	R_4 = 4-Cl, R_5 = 3-Cl
DPN7	(*Z*)-*N*-(3-chlorobenzylidene)aniline oxide	R_5 = 3-Cl
DPN8	(*Z*)-4-chloro-*N*-(4-hydroxybenzylidene)aniline oxide	R_4 = 4-Cl, R_5 = 4-OH
DPN9	(*Z*)-4-chloro-*N*-(3-hydroxybenzylidene)aniline oxide	R_4 = 4-Cl, R_5 = 3-OH
DPN10	(*Z*)-*N*-(4-chlorobenzylidene)aniline oxide	R_5 = 4-Cl
DPN11	(*Z*)-*N*-(2-nitrobenzylidene)aniline oxide	R_5 = 2-NO_2
DPN12	(*Z*)-4-chloro-*N*-(4-chlorobenzylidene)aniline oxide	R_4 = 4-Cl, R_5 = 4-Cl
Phenothiazine mono- and dinitrones		
PTZN1	(*Z*)-3-bromo-*N*-[(10-methyl-10*H*-phenothiazin-3-yl)methylene] aniline oxide	R_6 = methyl R_7 = 3-bromophenyl
PTZN2	(*Z*)-*N*-[(10-methyl-10*H*-phenothiazin-3-yl)methylene]aniline oxide	R_6 = methyl R_7 = phenyl
PTZN3	(*Z*)-3-acetyl-*N*-[(10-methyl-10*H*-phenothiazin-3-yl)methylene] aniline oxide	R_6 = methyl R_7 = 3-acetylphenyl
PTZN4	(*N*,*N*′*Z*,*N*,*N*′*Z*)-*N*,*N*′-(10-methyl-10*H*-phenothiazine-3,7-diyl) bis(methan-1-yl-1-ylidene)bis(aniline oxide)	R_8 = methyl R_9 = phenyl
PTZN5	(*Z*)-*N*-[(10-octadecyl-10*H*-phenothiazin-3-yl)methylene]aniline oxide	R_6 = octadecyl R_7 = phenyl
PTZN6	(*E*)-*N*-[(10-octedecyl-10*H*-phenothiazin-3-yl)methylene] ethanamine oxide	R_6 = octadecyl R_7 = ethyl
PTZN7	(*E*)-*N*-[(10-ethyl-10*H*-phenothiazin-3-yl)methylene]methanamine oxide	R_6 = ethyl R_7 = methyl
PTZN8	(*E*)-*N*-[(10-ethyl-10*H*-phenothiazin-3-yl)methylene]ethanamine oxide	R_6 = ethyl R_7 = ethyl

Reaction of nitrones with hydroxyl radical in an aqueous environment under UV radiation

The apparatus, reagents and methods used for testing the nitric oxide releasing ability of nitrones under UV irradiation are described in detail in ref. 25. The experiments were performed at 20 °C. The following modifications were made: (*i*) sampling time and rate were reduced (0, 20, 40, 60, 120, 180 and 300 minutes), (*ii*) a new method was applied for detection of oxidation products of nitric oxide (nitrite and nitrate ions) (28), (*iii*) the solvent used to dissolve the nitrones was acetonitrile/water 1:1 (*V*/*V*).

Interaction of nitrones with rat liver homogenate – preliminary testing

The following nitrones were tested during this experiment: PEN, DPN1, GIPN, TIPN, GTBN, TTBN, PBN, GSBN, TSBN, DPN11, DPN12, PTZN5, PTZN6, and PTZN7. The rat liver homogenate was prepared by homogenizing the liver of an adult male rat in 200 mmol L^{-1} PBS buffer solution (NaCl 137 mmol L^{-1}, KCl 2.7 mmol L^{-1}, Na_2HPO_4 10 mmol L^{-1} and KH_2PO_4 2 mmol L^{-1}). In different test tubes, 3.5 mg of each tested nitrone was weighed and dissolved in 0.5 mL of dimethylsulfoxide (DMSO), then 7 mL of rat liver homogenate was added. The test tubes were immersed in a water bath at 37 °C. Samples were centrifuged at 3000 rpm and were analyzed using the literature method described in ref. 28.

Nitric oxide releasing ability of nitrones in vivo

Eighteen rabbits (average body mass 4.62 ± 0.17 kg) were divided into two groups. Each group consisted of nine individuals, eight for blood sampling and one for preliminary acute toxicity testing, which was important because many of the tested substances were not administered to animals before and literature data about their toxicity is unavailable. All animal experiments were carried out with institutional approval received from our university's Committee on Ethics and in accordance with international regulations.

Nitroglycerin (NG) was not available as a pure substance. Thus nitroglycerin tablets containing 0.5 mg nitroglycerin per tablet (Zentiva S.A., Romania) were used. They were dissolved in the solvent, followed by filtration through a 0.5-μm filter.

The animals had food restriction for 16 hours before and during the experiment and free access to water. The first group of animals received PBN, GIPN, and TIPN. The second group received GSBN, GTBN, TTBN, DPN1 and sodium nitrite. Both groups were administered blank (solvent) and nitroglycerin. After the administration of a substance or blank, animals were left to recover for two weeks. Doses and route of administration of the tested nitrones are summarized in Table II. The solvent used for dissolving/suspending the sub-

Table II. Substances, dosages and routes of administration

Substance	Dose (mg kg^{-1})	Route of administration
Blank (solvent[a])	1.5 mL kg^{-1}	Intravenously
Blank (solvent[a])	1.5 mL kg^{-1}	Intraperitoneally
DPN1	100	Intraperitoneally
PBN	100	Intravenously
TIPN	70	Intraperitoneally
GIPN	100	Intraperitoneally
TTB	100	Intraperitoneally
GSB	50	Intraperitoneally
GTB	50	Intraperitoneally
NG (positive control)	1.0	Intraperitoneally

[a] Solvent: 20 % hydro-alcoholic solution

stances for the *in vivo* administrations was 20 % hydroalcoholic solution. The nitrones, except for PBN, were administered as suspensions because of their poor solubility.

Blood was sampled from veins in the hind legs of the animals in anticoagulant-free test tubes. The reasons for using anticoagulant-free test tubes, blood preparation steps and the HPLC UV-VIS method for sample analysis are described in detail elsewhere (28). The limits of quantification of the HPLC UV-VIS method were low and none of the samples contained lower concentrations of analyte than these limits (0.2 μg ml^{-1} for nitrate and 2 ng ml^{-1} for nitrite).

RESULTS AND DISCUSSION

Nitric oxide release from nitrones after in vitro *reaction with UV generated hydroxyl radical*

Most of the alkyl nitrones and some diphenyl nitrones were more active in releasing nitric oxide than PBN (Fig. 2). In the case of the diphenyl nitrones, it is difficult to estimate the release of nitric oxide based on the concentrations of nitrite and nitrate (NOx), since a nitrite consuming process is taking place as described in reference (25). In some cases, this process leads to lower concentrations of nitrite in the solution of the nitrone than are those recorded for the blank. The total concentrations of NOx products and the percent of the nitrones that released nitric oxide are presented in Table III.

During the UV generated hydroxyl radical attack on the nitrones, we observed that terephtal dinitrones and glyoxal dinitrones were the most active releasers of nitric oxide and, compared to the rest of the nitrones, nitrite was present in higher amounts in these solutions at the end of the experiment than nitrate. This suggests that, in the presence of

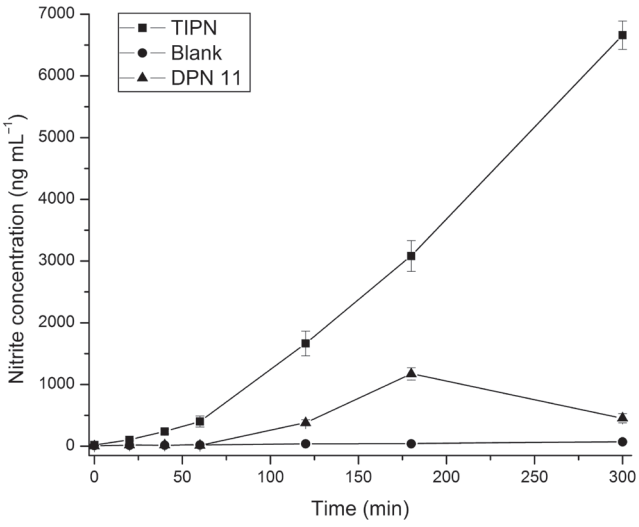

Fig. 2. Changes in nitrite concentrations in the solution of TIPN and DPN11 (mean ± SD, n = 3).

Table III. Concentration of NOx products and nitrone; percent of the nitrones that released NO

Nitrone	Nitrite (μmol L^{-1})[a]	Nitrate (μmol L^{-1})[a]	Nitrone (mmol L^{-1})	NO (%)[b]
Blank 1[c]	1.55	BLD	–	–
DPN1	0.31	7.25	2.347	0.26
DPN2	0.75	35.58	1.912	1.82
DPN3	1.06	10.17	1.634	0.59
DPN4	1.27	17.79	1.715	1.02
PBN	7.87	2.97	2.825	0.33
DPN5	0.58	9.68	2.203	0.40
DPN6	0.84	29.53	1.880	1.53
DPN7	1.80	15.05	2.160	0.71
DPN8	1.28	7.35	2.020	0.35
DPN9	1.08	22.82	2.020	1.11
Blank 2[c]	1.29	BLD	–	–
DPN10	2.18	15.16	2.160	0.73
DPN11	9.88	39.35	2.066	2.31
TIPN	144.79	24.82	2.128	7.90
TTBN	34.67	34.80	1.901	3.57
TSBN	44.39	52.17	1.901	5.00
DPN12	2.69	15.71	1.880	0.90
Blank 3[c]	0.79	BLD	–	–
PEN	27.84	2.03	3.030	0.96
GIPN	37.71	20.83	3.623	1.59
GTBN	38.20	44.83	3.012	2.73
GSBN	79.44	31.01	3.012	3.64
PTZN1	0.60	2.30	1.217	0.17
PTZN2	1.66	3.57	1.506	0.29
PTZN3	2.38	BLQ	1.337	0.12
PTZN4	11.19	11.67	1.109	2.06
PTZN5	11.99	4.02	0.876	1.74
PTZN6	5.45	2.24	0.956	0.72
PTZN7	9.37	8.01	1.761	0.94
PTZN8	0.25	BLQ	1.678	0.00

[a] Measured after 5 hours of UV irradiation.

[b] Percent of molecules that released nitric oxide during 5 hours of UV irradiation (the corresponding blank was subtracted from the calculation of these results); a different blank was used for each series of nitrones kept under UV radiation.

[c] Blank: acetonitrile/water (1:1).

BLD – below limit of detection; BLQ – below limit of quantitation.

these nitrones, nitric oxide may react directly with oxygen and water to form nitrite instead of being oxidized to $^{\bullet}NO_2$ (which in contact with water is expected to form equal amounts of nitrite and nitrate), or $^{\bullet}NO_2$ reacts with $^{\bullet}NO$ when N_2O_3 is formed; this compound forms only nitrite when reacted with water. We proved earlier that in the conditions and duration of this experiment only minor amounts of nitrite are oxidized to nitrate (25). Therefore, in the cases when high nitrate and low nitrite concentrations were recorded, the oxidation of nitric oxide to nitrogen(V) (N_2O_5: source of nitrate alone) either took place in steps before the formation of nitrite, or the nitrone or one of its decomposition products induced an oxidative process of nitrite. The presence of nitrite in the blanks (acetonitrile and water in 1:1 ratio) is due to the fact that the nitrogen oxides migrate from the air into the samples. The concentration changes on a daily basis, being influenced by atmospheric conditions. Thus, for every series of tested substances a blank is required and the concentration of nitrite in the blank should be subtracted from the results. However, concentrations of nitrite in blanks are insignificant compared to those yielded by many nitrones.

Since there are no available *in vivo* test results assessing the formation of nitric oxide from nitrones, we used our *in vitro* test results to decide which nitrone should be administered to the animals. Although diphenyl nitrones did not prove to be active nitric oxide releasers during the *in vitro* experiment, we decided to test DPN1 *in vivo*, too. The reason was the intriguing nitrite-consuming process which, at least theoretically, might be preceded by a nitric oxide release.

Reaction of nitrones with rat liver homogenate

If the metabolism of nitrones to nitrite and nitrate takes place in the liver, the value of nitrones as nitric oxide donors will be considerably reduced. Rat liver homogenate was used to verify if there were hepatic enzymes able to metabolize nitrones with release of nitric oxide. During the five-hour experiment, the concentration of nitrite from the blank sample (rat hepatic homogenate mixed with the solvent used in the case of nitrones) raised moderately in an asymptotic manner. Only four nitrones (PEN, GTBN, TSBN, and PTZN7) were able to raise nitrite concentrations in the preliminary rat hepatic homogenate test. The other nitrones did not affect or even reduced nitrite concentrations compared to the blank. Even those nitrones that proved to be very efficient in the hydroxyl radical test reduced significantly nitrite concentrations in the rat liver homogenate test (GIPN, TTBN, PBN, GSBN). Fig. 3 shows changes recorded for nitrite concentrations in the rat liver homogenate test in the case of PEN, PBN and blank. Nitrate concentrations were not changed by nitrones during this experiment.

Our results show that there was no interaction between the efficient nitric oxide donors (GIPN and GTBN) and the rat hepatic homogenate regarding the formation of nitric oxide decomposition products, proving that the increase of NOx products observed during the *in vivo* experiment took place in the tissues and was not related to hepatic metabolism.

Animal response to blank and »positive blank«

Administration of a blank was done in order to observe changes in the blood concentration of nitric oxide oxidation products brought by solvent administration. These changes, induced by blank administration, were used in the statistical evaluation of the modifications induced by the tested substances. Nitroglycerin was chosen as a »positive

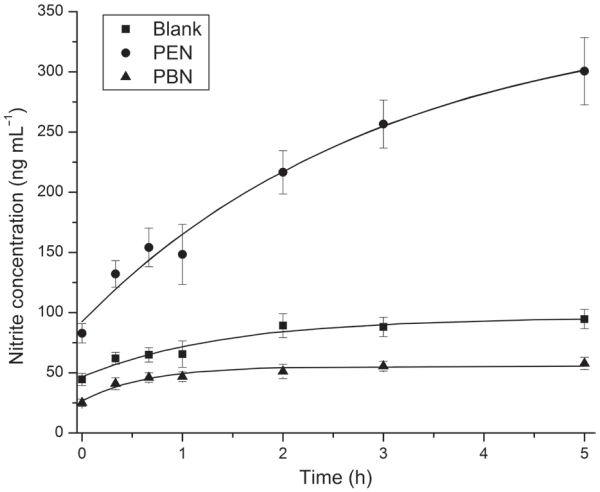

Fig. 3. Changes in the rat hepatic homogenate nitrite concentrations brought by PEN (highest increase) and PBN, compared to the blank (mean ± SD, n = 3).

blank«, a substance known to endogenously form nitric oxide. Nitroglycerin spectacularly increased the concentration of nitrite and nitrate in rabbit serum, showing that the animals reacted as expected to a nitric oxide donor.

Nitrogen oxide released by nitrones

Because of the need for repeated blood sampling, rabbits were chosen as experimental animals. The target dose of nitrones was 100 mg kg^{-1}, the dose at which PBN and its related compounds showed effective spin trapping abilities *in vivo* (29). However, when signs of intense vasodilatation (tachycardia, tachypnea, reduced motor activity) were obvious during acute toxicity testing, a dose reduction to 70 or even 50 mg kg^{-1} was applied.

Table IV shows the nitrite and nitrate concentration increase/decrease caused by nitrones administered to the tested animals, along with their statistical significance. Nitrite was used as preferred NO marker, for the reasons presented in ref. 30.

GIPN was the most active nitric oxide releasing nitrone and increased all NOx values at all time intervals in a manner similar to nitroglycerin. A second increase in nitrite concentrations was noticed after 5 hours. In previous studies, GIPN and other similar nitrones showed biological effects that were attributed to nitric oxide release *in vivo*, but the authors were not able to prove the release, since only spontaneous *in vitro* decomposition was followed (26). Our results prove that these previously observed effects were indeed results of nitric oxide release from nitrones.

GTBN increased nitrite concentrations statistically significantly only at the 7-h sampling time (Fig. 4).

PBN, DPN1, GSBN and TTBN acted as nitric oxide modulators, significantly reducing nitrite concentrations, while TIPN had no effect on NOx concentrations. It is interesting to

Table IV. Changes in nitrite and nitrate concentrations following nitrone administration to rabbits[a]

Nitrone administered	NOX[b,c]	Time (h)			
		0.666	2	5	7
PBN	Nitrate	4.97	6.19*	8.98*	ND
	Nitrite	–73.3*[e]	–38.1*	–33.4	
TIPN	Nitrate	–0.21	–0.63	0.30	ND
	Nitrite	11.8	–3.2	32.7	
GIPN	Nitrate	1.05*	1.20*	3.00*	ND
	Nitrite	16.9*	12.8*	49.9*	
TTBN	Nitrate	–0.74*	3.76*	0.87	–2.17
	Nitrite	–5.4	–26.7*	–7.0	–11.9*
GSBN	Nitrate	3.34*	3.40*	2.07	–0.55
	Nitrite	–28.7*	–41.1*	–21.1*	–12.7
GTBN	Nitrate	–1.37	3.08*	1.60*	–0.99
	Nitrite	3.9	–20.4	–1.8	42.8*
DPN1	Nitrate	11.18*	7.56*	1.71	1.01
	Nitrite	–81.0*	–114.2*	–83.9*	–67.5
NG	Nitrate	1.37*	1.97*	3.36*	ND
	Nitrite	83.2*	37.3*	40.9*	

[a] Changes were calculated using the formula: $[(T_{xN} - T_{0N}) - (T_{xB} - T_{0B})]$, where T_{xN} is concentration of nitrite or nitrate at a time point after nitrone administration; T_{0N} = concentration of nitrite or nitrate before nitrone administration (zero time concentration for nitrone); T_{xB} = concentration of nitrite or nitrate at a time point after blank administration; T_{0B} = concentration of nitrite or nitrate before blank administration (zero time concentration for blank)
ND – not determined
[b] Nitrate concentration is given in μg mL^{-1}.
[c] Nitrite concentration is given in ng mL^{-1}.
* Statistically significant differences compared to negative blank ($p < 0.05$).

observe that many promising nitrones, even if they acted as active *in vitro* nitric oxide releasers, proved to be the contrary when examined *in vivo*. An explanation might be their ability to interfere with NOS activity, so that an equilibrium is established between the ability to release nitric oxide and the ability to block the enzyme that normally produces it.

It is worth mentioning that, with the exception of DPN1, the administration of nitrones to animals was accompanied by signs of vasodilatation (tachycardia, dilated blood vessels in the ear, redness of the ear, easy blood sampling procedure). On the other hand, the nitrones that reduce significantly nitric oxide formation, such as DPN1, could also have therapeutic values since there are conditions (bacterial infection) in which nitric oxide is released in high amounts with unwanted effects.

In order to notice possible toxic effects of nitrones on the animals, food intake and body mass were monitored throughout the experiment. No signs of toxicity were observed during the administration of nitrones. The only observable effects were those related to the administration of substances with vasodilating activity but they were transient.

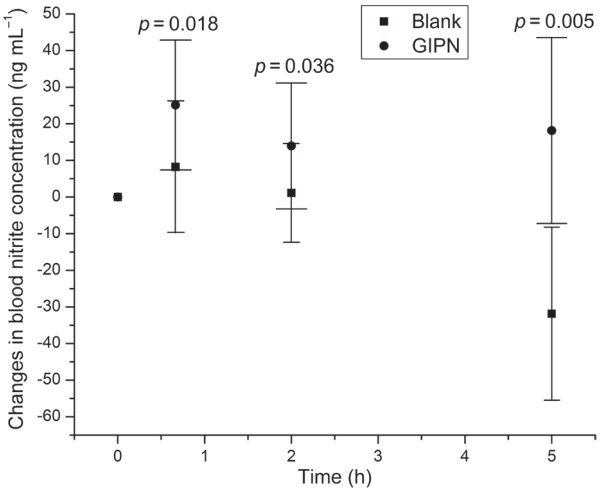

Fig. 4. Changes of nitrite concentrations compared to the blank after GIPN administration to animals (change in nitrite concentration was calculated by subtracting the concentration measured at time X from that measured at time 0).

Our research proved that nitrones can serve as important *in vivo* sources of nitric oxide. However, we have to emphasize that generalization cannot be applied to this statement since all nitrones acted to some extent as potential nitric oxide donors *in vitro* and only two were successfully proven as *in vivo* nitric oxide donors.

CONCLUSIONS

Our findings suggest that nitrones can be considered useful for therapeutic purposes since they can release nitric oxide and possess an extraordinary spin trapping ability. This can be a very useful characteristic of a new class of vasodilating agents. Such agents will not only provide vital vasodilatation in the ischemic tissue but will also prevent the damage brought about by the free radicals generated during reperfusion. The importance of hydroxyl radical in the formation of nitric oxide could even suggest that the targeted vasodilatation in the ischemic tissue might be provided. Further studies are needed to prove this claim.

Abbreviations, acronyms, symbols. – BLD – below limit of detection; BLQ – below limit of quantification; DPN1 – (*Z*)-*N*-(4-hydroxybenzylidene)anilineoxide; DPN10 – (*Z*)-*N*-(4-chlorobenzylidene)aniline oxide; DPN11 – (*Z*)-*N*-(2-nitrobenzylidene)aniline oxide; DPN12 – (*Z*)-4-chloro-*N*-(4-chlorobenzylidene)aniline oxide; DPN2 – (*Z*)-4-chloro-*N*-(4-methoxybenzylidene)aniline oxide; DPN3 – (*Z*)-4-bromo-*N*-(4-methoxybenzylidene)aniline oxide; DPN4 – (*Z*)-4-chloro-*N*-(3,4-dimethoxybenzylidene) aniline oxide; DPN5 – (*Z*)-*N*-(4-methoxybenzylidene)aniline oxide; DPN6 – (*Z*)-4-chloro-*N*-(3-chlorobenzylidene)aniline oxide; DPN7 – (*Z*)-*N*-(3-chlorobenzylidene)aniline oxide; DPN8 – (*Z*)-4-chloro-*N*-(4-hydroxybenzylidene)aniline oxide; DPN9 – (*Z*)-4-chloro-*N*-(3-hydroxybenzylidene)aniline oxide; GIPN – (*N*,*N*′*Z*,*N*,*N*′*Z*)-*N*,*N*′-(ethane-1,2-diylidene)bis(propan-2-amine oxide); GSBN – (*N*,*N*′*Z*,*N*,*N*′*Z*)-

N,*N*′-(ethane-1,2-diylidene)bis(butan-2-amine oxide); GTBN – (*N*,*N*′*Z*,*N*,*N*′*Z*)-*N*,*N*′-(ethane-1,2-diylidene)bis(2-methylpropan-2-amine oxide); HPLC – high pressure liquid chromatography; LDL – low density lipoproteins; NG – nitroglycerin; NOx – sum of nitrite and nitrate concentration; NXY-059 – disufenton sodium, a nitrone derivative; PBN – phenyltert-butylnitrone; PEN – phenyl-*N*-ethylnitrone; PTZN1 – (*Z*)-3-bromo-*N*-((10-methyl-10*H*-phenothiazin-3-yl)methylene)aniline oxide; PTZN2 – (*Z*)-*N*-((10-methyl-10*H*- -phenothiazin-3-yl)methylene)aniline oxide; PTZN3 – (*Z*)-3-acetyl-*N*-((10-methyl-10*H*-phenothiazin-3-yl)methylene)aniline oxide; PTZN4 – (*N*,*N*′*Z*,*N*,*N*′*Z*)-*N*,*N*′-(10-methyl-10*H*-phenothiazine-3,7-diyl)bis(methan-1-yl-1-ylidene)bis(aniline oxide); PTZN5 – (*Z*)-*N*-((10-octadecyl-10*H*-phenothiazin-3-yl)methylene)aniline oxide; PTZN6 – (*E*)-*N*-((10-octedecyl-10*H*-phenothiazin-3-yl) methylene)ethanamine oxide; PTZN7 – (*E*)-*N*-((10-ethyl-10*H*-phenothiazin-3-yl)methylene)methanamine oxide; PTZN8 – (*E*)-*N*-((10-ethyl-10*H*-phenothiazin-3-yl)methylene)ethanamine oxide; TIPN – (*N*,*N*′*Z*,*N*,*N*′*Z*)-*N*,*N*′-(1,4-phenylenebis(methanylylidene))bis(propan-2-amine oxide); TSBN – (*N*,*N*′*Z*,*N*,*N*′*Z*)-*N*,*N*′-(1,4-phenylenebis(methanylylidene))bis(butan-2-amine oxide); TTBN – (*N*,*N*′*Z*,*N*,*N*′*Z*)-*N*,*N*′-(1,4-phenylenebis(methan-1-yl-1-ylidene))bis(2-methylpropan-2-amine oxide).

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REFERENCES

1. J. Ahn and J. Kim, Mechanisms and consequences of inflammatory signaling in the myocardium, *Curr. Hypertens. Rep.* **14** (2012) 510–516; DOI: 10.1007/s11906-012-0309-0.
2. D. M. Ansley and B. Wang, Oxidative stress and myocardial injury in the diabetic heart, *J. Pathol.* **229** (2013) 232–241; DOI: 10.1002/path.4113.
3. A. M. Walters, G. A. Porter and P. S. Brookes, Mitochondria as a drug target in ischemic heart disease and cardiomyopathy, *Circ. Res.* **111** (2012) 1222–1236; DOI: 10.1161/CIRCRESAHA.112.265660.
4. C. Sai and L. Shuzhuang, The Na^+/Ca^{2+} exchanger in cardiac ischemia/reperfusion injury, *Med. Sci. Monit.* **18** (2012) 161–165; DOI: 10.12659/MSM.883533.
5. J. M. Pilcher, P. Young, M. Weatherall, I. Rahman, R. S. Bonser and R. W. Beasley, A systematic review and meta-analysis of the cardioprotective effects of remote ischaemic preconditioning in open cardiac surgery, *J. R. Soc. Med.* **105** (2012) 436–445; DOI: 10.1258/jrsm.2012.120049.
6. D. J. Hausenloy and D. M. Yellon, Reperfusion injury salvage kinase signalling: taking a RISK for cardioprotection, *Heart Fail. Rev.* **12** (2007) 217–234; DOI: 10.1007/s10741-007-9026-1.
7. R. Rodrigo, J. C. Prieto and R. Castillo, Cardioprotection against ischaemia/reperfusion by vitamins C and E plus n-3 fatty acids: molecular mechanisms and potential clinical applications, *Clin. Sci.* (Lond). **124** (2013) 1–15; DOI: 10.1042/CS20110663.
8. R. Rodrigo, J. Vinay, R. Castillo, M. Cereceda, R. Asenjo and J. Zamorano, Use of vitamins C and E as a prophylactic therapy to prevent postoperative atrial fibrillation, *Int. J. Cardiol.* **138** (2010) 221–228; DOI: 10.1016/j.ijcard.2009.04.043.
9. M. G. Andreassi, Coronary atherosclerosis and somatic mutations: an overview of the contributive factors for oxidative DNA damage, *Mutat. Res.* **543** (2003) 67–86; DOI: 10.1016/S1383-5742(02)00089-3.
10. H. A. Kim, A. A. Miller, G. R. Drummond, A. G. Thrift, T. V. Arumugam and T. G. Phan, Vascular cognitive impairment and Alzheimer's disease: role of cerebral hypoperfusion and oxidative stress, *Naunyn-Schmiedeberg. Arch. Pharmacol.* **385** (2012) 953–959; DOI: 10.1007/s00210-012-0790-7.
11. L. Kuo, N. Thengchaisri and T. W. Hein, Regulation of coronary vasomotor function by reactive oxygen species, *Mol. Med. Ther.* **1** (2012) 1–4; DOI: 10.4172/2324-8769.1000101.

12. A. S. Simon, V. Chithra, A. Vijayan, R. D. Dinesh and T. Vijayakumar, Altered DNA repair, oxidative stress and antioxidant status in coronary artery disease, *J. Biosci.* **38** (2013) 385–389; DOI: 10.1007/s12038-013-9313-z.
13. E. Bor-Seng-Shu, W. S. Kita, E. G. Figueiredo, W. S. Paiva, E. T. Fonoff, M. J. Teixeira and R. B. Panerai, Cerebral hemodynamics: concepts of clinical importance, *Arq. Neuropsiquiatr.* **70** (2012) 352–356.
14. F. Folli, D. Corradi, P. Fanti, A. Davalli, A. Paez and A. Giaccari, The role of oxidative stress in the pathogenesis of type 2 diabetes mellitus micro- and macrovascular complications: avenues for a mechanistic-based therapeutic approach, *Curr. Diabetes Rev.* **7** (2011) 313–324; DOI: 10.2174/157339911797415585.
15. N. A. Terpolilli, M. A. Moskowitz and N. Plesnila, Nitric oxide: considerations for the treatment of ischemic stroke, *J. Cereb. Blood Flow Metab.* **32** (2012) 1332–1346; DOI: 10.1038/jcbfm.2012.12.
16. N. Toda and H. Toda, Coronary hemodynamic regulation by nitric oxide in experimental animals: recent advances, *Eur. J. Pharmacol.* **667** (2011) 41–49; DOI: 10.1016/j.ejphar.2011.06.028.
17. M. R. Macleod, H. B. van der Worp, E. S. Sena, D. W. Howells, U. Dirnagl and G. A. Donnan, Evidence for the efficacy of NXY-059 in experimental focal cerebral ischaemia is confounded by study quality, *Stroke* **39** (2008) 2824–2829; DOI: 10.1161/STROKEAHA.108.515957.
18. R. A. Floyd, K. Hensley, M. J. Forster, J. A. Kelleher-Anderson and P. L. Wood, Nitrones as neuroprotectans and antiaging drugs, *Ann. N. Y. Acad. Sci.* **959** (2002) 321–329; DOI: 10.1111/j.1749-6632.2002.
19. B. Kalyanaraman, J. Joseph and S. Parthasarathy, The spin trap, alpha-phenyl N-tert-butylnitrone, inhibits the oxidative modification of low density lipoprotein, *FEBS. Lett.* **280** (1991) 17–20; DOI: 10.1016/0014-5793(91)80194-8.
20. E. G. Janzen, R. A. Towner and S. Yamashiro, The effect of phenyl tert-butyl nitrone (PBN) on CCl4-induced rat liver injury detected by proton magnetic resonance imaging (MRI) in vivo and electron microscopy (EM), *Free Radic. Res. Commun.* **9** (1990) 325–335; DOI: 10.3109/10715769009145691.
21. M. Thirumalaikumar, S. Sivakolunthu, S. Muthusubramanian, P. Mohan and S. Sivasubramanian, Synthesis, characterization and antimicrobial studies of metal (II) bis-chelates and mixed-ligand complexes of alpha-(2-hydroxyphenyl)-*N*-(1-phenyl-2-nitroethyl)nitrone, *Boll. Chim. Farm.* **35** (1999) 207–210.
22. K. T. Knecht and R. P. Mason, In vivo spin trapping of xenobiotic free radical metabolites, *Arch. Biochem. Biophys.* **303** (1993) 185–194; DOI: 10.1006/abbi.1993.1272.
23. S. Kuroda, R. Tsuchidate, M. L. Smith, K. R. Maples and B. K. Siesjo, Neuroprotective effects of a novel nitrone, NXY-059, after transient focal cerebral ischemia in the rat, *J. Cereb. Blood Flow Metab.* **9** (1999) 623–625; DOI: 10.1097/00004647-199907000-00008.
24. L. A. Reinke, D. R. Moore, C. M. Hague and P. B. McCay, Metabolism of ethanol to 1-hydroxyethyl radicals in rat liver microsomes: comparative studies with three spin trapping agents, *Free Radic. Res.* **21** (1994) 213–222; DOI: 10.3109/10715769409056573.
25. M. D. Croitoru, I. Fülöp, M. C. Pop, T. Dergez, B. Mitroi, M. T. Dogaru and B. Tőkés, Nitrones are able to release nitric oxide in aqueous environment under hydroxyl free radical attack, *Nitric oxide* **25** (2011) 309–315; DOI: 10.1016/j.niox.2011.05.007.
26. R. Camehn and K. Rehse, New NO donors with antithrombotic and vasodilating activities, N-(1-cyanocyclohexyl)-C-phenylnitrones and glyoxaldinitones, *Arch. Pharm. Pharm. Med. Chem.* **333** (2000) 130–134; DOI: 10.1002/(SICI)1521-4184(20005)333:53.0.CO;2-D.
27. W. Chamulitrat, S. J. Jordant, R. P. Mason, K. Saito and R. G. Cutler, Nitric oxide formation during light-induced decomposition of phenyl-N-tert-butylnitrone, *J. Biol. Chem.* **268** (1993) 11520–11527.
28. M. D. Croitoru, Nitrite and nitrate can be accurately measured in vegetal and animal samples using an HPLC-UV/VIS technique, *J. Chromatogr. B* **911** (2012) 154–161; DOI: 10.1016/j.jchromb.2012.11.006.

29. P. A. Lapchak, D. F. Chapman and J. A. Zivin, Pharmacological effects of the spin trap agents N-t-butyl-phenylnitrone (PBN) and 2,2,6, 6-tetramethylpiperidine-N-oxyl (TEMPO) in a rabbit thromboembolic stroke model: combination studies with the thrombolytic tissue plasminogen activator, *Stroke* **32** (2001) 147–153; DOI: 10.1161/01.STR.32.1.147.

30. M. D. Croitoru, I. Fülöp, E. Fogarasi and D. L. Muntean, Is nitrate a good biomarker of the nitric oxide status?, *Rev. Romana Med. Lab.* **23** (2015) 127–136; DOI: 10.1515/rrlm-2015-0001.