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Antioxidative activity of 4-oxy- and 4-hydroxy-nitroxides in tissues and erythrocytes from rats[1]

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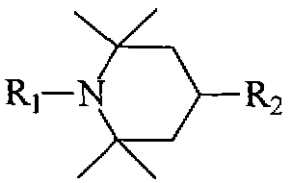
KEY WORDS nitroxides; free radicals; antioxidants; malondialdehyde; erythrocytes; neutrophils; free radical scavengers

AIM: To compare the activities of antioxidation of 4-oxy- and 4-hydroxy-nitroxides in tissues and RBC from rats. **METHODS**: The homogenates of liver, heart, and kidneys of rats were used to determine malondialdehyde (MDA) formation using TBA colorimetric method. H_2O_2-caused hemolysis was measured spectrometrically. Superoxide anion from zymosan-stimulated neutrophils of rats was assayed by NBT reduction method. **RESULTS**: Nitroxide free radicals OTMPO and HTMPO inhibited MDA generation caused by ·OH generation system (MIC 10.5 and 21 $\mu mol \cdot L^{-1}$, respectively), antagonized hemolysis induced by H_2O_2 (MIC: 338 and 168 $\mu mol \cdot L^{-1}$, respectively), but did not affect O_2^- formation from activated neutrophils. 1-Hydroxyl compounds OTMPOH and HTMPOH possessed similarly potent antilipoperoxidative activities. But nonfree radical OTMP and HTMP had no effect on peroxidation of tissues. **CONCLUSION**: Nitroxides exert their antilipoperoxidative effect by specifically scavenging ·OH free radicals in biological system. Trapping of ·OH free radicals by nitroxides is not by reduction of NO· group in nitroxides. Both NO· group and NOH group are essential active groups.

The nitroxides belong to stable free radicals which are widely used for spin labeling in electron spinning resonance technique[1] and possess some antitumor activities[2]. Such compounds trap hydroxyl radical in nonbiological system like aqueous solution[3]. ·OH radicals attack them by abstraction H atoms from 4-hydroxy group as well as bound to carbon on nitroxides[3]. The formed intermediates react further producing stable free radicals and biradicals[3]. But it is unknown whether they have similar actions in biological system. This paper was to study the antioxidative activities of nitroxides in tissues and erythrocytes (RBC) of rats.



2,2,6,6-Tetramethylpiperidine compounds

Compounds	R_1	R_2
OTMP	H－	=O
OTMPO	·O－	=O
OTMPOH	HO－	=O
HTMP	H－	－OH
HTMPO	·O－	－OH
HTMPOH	HO－	－OH

MATERIALS AND METHODS

4-Oxy-2,2,6,6-tetramethylpiperidine (OTMP), 4-oxy-2, 2, 6, 6-tetramethylpiperidinooxyl (OTMPO), 4-oxy-2,2,6,6-tetramethyl-1-hydroxy-piperidine (OTMPOH), 4-hydroxy-2,2,6,6-tetramethyl-piperidine (HTMP), 4-hydroxy-2,2,6,6-tetramethylpiperidinooxyl (HTMPO), and 4-hydroxy-2, 2, 6, 6-tetramethyl-1-hydroxy piperidine (HTMPOH) were synthesized by Laboratory of Applied Organic Chemistry, Lanzhou University, the purity >98 %, and were dissolved in 5 % Me_2SO. Zymosan A (Sigma) was opsonized with rat serum[4] and suspended in phosphate buffer 0.15 $mol \cdot L^{-1}$ (pH 7.4). Bovine superoxide dismutase (SOD) was purchased from Xiahe Biological Preparation Factory, Gannan, China. The other test agents were AR.

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Wistar rats ($n = 50$, ♀♂, 8 wk) weighing 176 ± s 14 g were provided by Animal Breeding Center, Lanzhou Medical College

Determination of malondialdehyde (MDA) The heart, liver, and kidneys from rats were prepared as 5 % tissue homogenate in Tris-KCl buffer (containing trihydroxymethyl-aminomethane 0.1 mol·L^{-1} and KCl 1 mmol·L^{-1}, pH 7.4). The tissue homogenate 1 mL was incubated with test compounds or vehicle (Me_2SO 2.5 mL·L^{-1}) at 37 ℃ for 10 min. Fe^{2+}-ascorbic acid solution ($FeSO_4$/ascorbic acid = 50/50 μmol·L^{-1}) was added. After 30-min incubation, MDA was determined[5]. The spontaneous MDA generation from rat liver was measured after 2-h incubation with test compounds and without Fe^{2+}-ascorbic acid.

Superoxide anion formation analysis Leukocytes from rat abdominal cavity were prepared[4] and suspended in Hanks' solution ($CaCl_2$ 1.3, $MgSO_4$ 0.8, and glucose 10 mmol·L^{-1}, pH 7.4). The leukocyte viability assessed by trypan blue exclusion was >98 %. The neutrophils were 97 % in leukocytes. All the procedures were carried out below 4 ℃. Superoxide anion formation was determined by NBT (nitroblue tetrazolium) reduction assay[6]: 0.1 mL of KCN 10 mmol·L^{-1}, 0.4 mL of 0.1 % NBT-normal saline, 0.4 mL of neutrophil suspension (1×10^{10} cells·L^{-1}), 0.1 mL of zymosan A 2.5 g·L^{-1} and 50 μL of test compound solution or vehicle were added in plastic tubes. The mixture was incubated at 37 ℃ for 35 min and reaction was stopped by adding 5 mL of cold HCl 0.5 mol·L^{-1}. The tubes were then spinned at 4 ℃ for 10 min. The purple pellet was extracted with 3 mL of pyridine in boiling water (>95 ℃) for 20 min. The absorbance (A) of reduced NBT (formazan) was determined by spectrometer at 515 nm against a pyridine blank. The specificity of assay for superoxide anion was determined by adding superoxide dismutase (SOD) into assay system.

Hemolysis test Rat RBC was washed 3 times with normal saline and made into 0.5 % suspension. RBC suspension 1 mL was incubated with H_2O_2 (100 mmol·L^{-1}) at 37 ℃ for 1 h after adding test compounds or vehicle. After diluted 5 times with normal saline, the mixture was spinned at 1000 × g for 10 min. The absorbance (A) of supernatant was measured at 415 nm under 0.5 cm optical path. The A of control tube was defined as 100 %. The hemolysis extent was calculated by referring to control tubes[7].

Statistics Data ($\bar{x} \pm s$) were compared using group t-test. The inhibitory concentration which caused 50 % reduction relative to control tubes was defined as IC_{50}. The IC_{50} and its 95 % confidence limits were determined from linear regression of effect vs lg dose[8].

RESULTS

MDA generation from tissues MDA was spontaneously formed in homogenate of rat liver after 2-h incubation (Tab 1). OTMPO, OTMPOH, HTMPO, or HTMPOH inhibited spontaneous MDA generation from liver, but OTMP and HTMP did not (Tab 1).

The homogenates of liver, heart, and kidneys, stimulated by hydroxyl free radical generation system (Fe^{2+}-ascorbic acid) for 30 min, produced MDA 4 – 8 times over unstimulated homogenates did. The effects of test compounds on MDA generation induced by Fe^{2+}-ascorbic acid were quite similar on spontaneous MDA generation: OTMPO, HTMPO, OTMPOH, and HTMPOH strongly inhibited MDA generation from homogenates of liver, heart, and kidneys of rats stimulated by Fe^{2+}-ascorbic acid. But OTMP and HTMP did not show any antioxidative activity (Tab 1).

Hemolysis of rat RBC stimulated by H_2O_2 Free radicals OTMPO and HTMPO alleviated hemolysis caused by H_2O_2 but their inhibitory effects were weaker than those of 1-hydroxyl compounds OTMPOH and HTMPOH. However, OTMP and HTMP did not antagonize hemolysis induced by H_2O_2 (Tab 2).

O_2^- generation from rat neutrophils NBT was reduced to formazan with purple color by O_2^- from neutrophils stimulated by zymosan. The specificity of assay for O_2^- was demonstrated by the fact that SOD 600 kU·L^{-1} inhibited formazan formation by 84 ± s 5 % (n = 5). OTMP, HTMP, OTMPO, HTMPO, OTMPOH, and HTMPOH all did not affect O_2^- generation from activated neutrophils (Tab 3).

IC_{50} values The potencies of stable free radicals (OTMPO and HTMPO) and 1-hydroxyl compounds (OTMPOH and HTMPOH) were not all the same in different test systems. In hydroxyl free radical generation system, the potency of 1-hydroxyl compounds was stronger than that of stable free radicals except in kidneys. In H_2O_2-caused hemolysis test, 1-hydroxyl compounds were also more potent than stable free radicals: the ratio of potency of antihemolysis of OTMPO/OTMOH

Tab 1. Effect of nitroxides on malondialdehyde generation from homogenates of rat liver, heart, and kidneys. $n = 5$ homogenates (each was pooled from 6 − 7 rats). $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Drug /μmol·L^{-1}	OTMP	OTMPO	OTMPOH	HTMP	HTMPO	HTMPOH
	Malondialdehyde content, nmol/g tissues					
	Induced by Fe^{2+}-ascorbic acid in liver					
Basal	27.6 ± 1.5^c	46.1 ± 1.5^c	52.3 ± 2.3^c	30 ± 5^c	77 ± 8^c	43.9 ± 2.3^c
Control	232 ± 16	266 ± 3	250 ± 12	243 ± 13	274 ± 12	217 ± 6
168	241 ± 8^a	36.6 ± 2.1^c	30 ± 7^c	239 ± 11^a	52 ± 8^c	23.5 ± 1.2^c
84	239 ± 8^a	27 ± 3^c	31 ± 6^c	252 ± 8^a	129 ± 7^c	33.5 ± 1.2^c
42	232 ± 10^a	56 ± 10^c	47 ± 6^c	241 ± 15^a	203 ± 14^c	68 ± 11^c
21	234 ± 7^a	151.7 ± 2.8^c	109 ± 13^c	244 ± 7^a	231 ± 10^c	116 ± 4^c
10.5	235 ± 8^a	258 ± 7^a	136 ± 8^c	245 ± 8^a	257 ± 18^a	136 ± 6^c
5.25	233 ± 6^a	262 ± 4^a	175 ± 10^c	246 ± 10^a	265 ± 14^a	160 ± 7^c
	Induced by Fe^{2+}-ascorbic acid in heart					
Basal	44 ± 7^c	71 ± 6^c	76 ± 5^c	42 ± 6^c	45 ± 3^c	67.0 ± 2.1^c
Control	239 ± 39	232 ± 8	232 ± 12	197 ± 9	240 ± 15	188 ± 10
168	225 ± 19^a	23.1 ± 2.4^c	22 ± 3^c	192 ± 9^a	31.8 ± 1.8^c	30 ± 3^c
84	236 ± 19^a	29.1 ± 1.2^c	38 ± 4^c	202 ± 8^a	129 ± 16^c	67 ± 12^c
42	230 ± 20^a	177 ± 11^c	129 ± 13^c	198 ± 6^a	203 ± 8^c	171 ± 9^b
21	231 ± 12^a	215 ± 7^c	206 ± 15^c	198 ± 10^a	206 ± 14^b	183 ± 12^a
10.5	234 ± 8^a	224 ± 6^b	229 ± 12^a	195 ± 5^a	236 ± 10^a	187 ± 8^a
5.25	237 ± 9^a	232 ± 8^a	230 ± 8^a	199 ± 9^a	232 ± 9^a	189 ± 7^a
	Induced by Fe^{2+}-ascorbic acid in kidneys					
Basal	65 ± 5^c	114 ± 8^c	104 ± 5^c	62 ± 8^c	128 ± 8^c	102 ± 9^c
Control	242 ± 20	276 ± 15	224 ± 4	214 ± 9	276 ± 9	243 ± 8
168	236 ± 13^a	74 ± 8^c	90 ± 7^c	217 ± 8^a	89 ± 4^c	75 ± 9^c
84	232 ± 25^a	76 ± 3^c	107 ± 9^c	220 ± 10^a	108 ± 6^c	175 ± 5^c
42	254 ± 18^a	126 ± 11^c	195 ± 8^c	214 ± 8^a	116 ± 7^c	204 ± 6^c
21	243 ± 8^a	178 ± 14^c	238 ± 6^a	210 ± 10^a	133 ± 11^c	210 ± 20^c
10.5	243 ± 10^a	248 ± 16^b	225 ± 8^a	212 ± 9^a	159 ± 9^c	216 ± 15^c
5.25	246 ± 8^a	261 ± 18^a	226 ± 9^a	217 ± 6^a	257 ± 13^b	226 ± 15^a
	Spontaneously formed in liver					
Control	140 ± 7	180 ± 10	119.1 ± 2.5	133 ± 6	141 ± 9	137 ± 9
168	139 ± 8^a	16 ± 7^c	21 ± 6^c	135 ± 6^a	33 ± 4^c	28.4 ± 2.9^c
84	142 ± 9^a	20 ± 4^c	29 ± 4^c	137 ± 10^a	74 ± 4^a	32 ± 5^c
42	138 ± 5^a	43 ± 6^c	47.8 ± 2.7^c	132 ± 6^a	106 ± 9^c	74 ± 5^c
21	147 ± 10^a	77 ± 8^c	87.9 ± 2.3^c	134 ± 6^a	122 ± 3^c	97.6 ± 1.1^c
10.5	137 ± 9^a	114 ± 10^c	107.0 ± 2.6^c	135 ± 6^a	137 ± 9^a	120 ± 6^c
5.25	139 ± 8^a	130 ± 7^c	123 ± 6^a	132 ± 9^a	136 ± 3^a	121 ± 6^c

Tab 2. Effect of nitroxides on the hemolysis of rat RBC induced by H_2O_2. $n = 5$ suspensions (each was pooled from 1 − 2 rats). $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Drug /μmol·L^{-1}	OTMP	OTMPO	OTMPOH	HTMP	HTMPO	HTMPOH
	Hemolysis extent/%					
Basal	2.1 ± 0.5^c	9 ± 4^c	6.8 ± 1.7^c	2.6 ± 1.5^c	5.3 ± 2.3^c	5.3 ± 1.2^c
Control	100	100	100	100	100	100
336	98 ± 6^a	49 ± 6^c	11 ± 3^c	101 ± 4^a	18.7 ± 1.4^c	14 ± 4^c
168	102 ± 6^a	99 ± 8^a	11.3 ± 2.8^c	102.6 ± 1.5^a	70 ± 11^c	16 ± 4^c
84	102.1 ± 1.4^a	102 ± 5^a	13.8 ± 1.6^c	103 ± 4^a	102 ± 9^a	57.5 ± 2.1^c
42	102 ± 4^a	101 ± 7^a	75 ± 10^c	102.6 ± 2.9^a	100 ± 4^a	90 ± 9^b
21	98 ± 8^a	104 ± 6^a	100 ± 6^a	98 ± 6^a	102 ± 6^a	102 ± 5^a

Tab 3. Effect of nitroxides on the superoxide anion production from zymosan-activated neutrophils of rats. $n = 5$ Neu suspensions (each was pooled from 3 - 4 rats). $\bar{x} \pm s$.

Drug /μmol·L^{-1}	Reduced NBT ($A_{515\ nm}$) OTMP	OTMPO	OTMPOH	HTMP	HTMPO	HTMPOH
Basal	0.089 ± 0.018	0.092 ± 0.023	0.098 ± 0.005	0.093 ± 0.023	0.09 ± 0.03	0.099 ± 0.005
336	0.255 ± 0.028	0.243 ± 0.024	0.265 ± 0.016	0.26 ± 0.03	0.25 ± 0.03	0.246 ± 0.023
168	0.24 ± 0.03	0.246 ± 0.019	0.257 ± 0.024	0.25 ± 0.04	0.26 ± 0.03	0.242 ± 0.025
84	0.256 ± 0.022	0.256 ± 0.023	0.255 ± 0.018	0.247 ± 0.022	0.253 ± 0.021	0.25 ± 0.03

was 16.3/100 and HTMPO/HTMPOH was 43.1/100, demonstrating that reduction of NO· group to NOH group on nitroxides by H atoms enhances activity of nitroxides to scavenge hydroxyl radicals and to antagonize lipoperoxidation. However, 1-hydroxyl compounds were weaker than stable free radicals in inhibiting MDA generation from kidneys stimulated by Fe^{2+}-ascorbic acid (Tab 4).

DISCUSSION

In our experiment, MDA generation was used as an indicator of ·OH-caused lipid peroxidation and indirectly reflected the amount of ·OH formation[9] in tissue homogenate stimulated by Fe^{2+}-ascorbic acid. H_2O_2-caused hemolysis mainly reflects the extent of lipoperoxidation of RBC membrane[7]. The formazan, the NBT-reduced product by superoxide anion during neutrophil activity, represents amount of O_2^- generation from activated neutrophils[10]. Our experiment demonstrated that nitroxide free radicals inhibited MDA generation caused by hydroxy free radical generation system in tissue homogenate, antagonized hemolysis induced by H_2O_2, but failed to affect formation of superoxide anion from activated neutrophils; suggesting that nitroxide free radicals exert their antioxidative actions by specifically scavenging ·OH free radicals in biological systems or by directly antagonizing lipoperoxidation as demonstrated in H_2O_2-caused hemolysis test. 1-Hydroxyl compounds OTMPOH and HTMPOH also possessed potent antioxidative activity, implying that trapping of hydroxy free radicals by nitroxides is not by reduction of NO· group. This result is justly concordant with the test *in nonbiological systems*[3]. Because non-free radicals OTMP and HTMP had no effect on peroxidation of tissues, it is rational to conclude that either NO· group or NOH group on nitroxides is essential active group.

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Tab 4. IC_{50} and 95 % confidence limits (μmol·L^{-1}) of nitroxides.

	Inhibition of malondialdehyde formation Induced by Fe^{2+}-ascorbic acid in Liver	Heart	Kidneys	Spontaneously in liver	Anti-hemolysis
OTMP	No effect	No effect	No effect	No effect	No effect
OTMPO	22.2 (20.5 - 23.9)	38.5 (35.2 - 41.8)	18.8 (17.6 - 20.0)	15.0 (13.7 - 16.3)	329.0 (327.9 - 330.1)
OTMPOH	8.3 (6.6 - 10.0)	28.6 (26.8 - 30.4)	47.8 (45.8 - 49.8)	36.5 (35.0 - 38.0)	53.7 (52.6 - 54.8)
HTMP	No effect	No effect	No effect	No effect	No effect
HTMPO	41.3 (39.7 - 42.9)	59.3 (57.0 - 61.6)	11.8 (9.5 - 14.1)	81.1 (79.5 - 82.7)	201.2 (200.1 - 202.3)
HTMPOH	11.6 (10.2 - 13.0)	49.7 (47.6 - 51.8)	53.8 (50.7 - 56.9)	39.8 (38.3 - 41.3)	86.8 (85.6 - 88.0)

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4-氧和4-羟氮氧自由基对大鼠组织和红细胞的抗脂质过氧化活性[1]

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关键词 氮氧自由基；自由基；抗氧化剂；丙二醛；红细胞；中性白细胞；自由基清除剂

目的：研究氮氧自由基的抗脂质过氧化作用. **方法**：用·OH生成系统 Fe^{2+} + 抗坏血酸刺激大鼠心、肝、肾组织匀浆，用TBA比色法测MDA生成；用分光光度法测 H_2O_2 诱导的大鼠RBC溶血度；用NBT还原法测酵母多糖诱导大鼠中性白细胞生成的 O_2^-. **结果**：氮氧自由基OTMPO和HTMPO抑制MDA生成，对抗溶血反应，但不影响 O_2^- 生成. 1-羟基化合物OTMPOH和HTMPOH也有类似作用. 但非自由基OTMP和HTMP对组织脂质过氧化无任何影响. **结论**：氮氧自由基通过清除·OH而对抗脂质过氧化，其捕捉·OH不是通过NO·的还原作用，NO·基和NOH基都是必需活性基团.

APS 1997 年全国生化药理与生物工程新药研制与开发学术研讨会

中国药理学会中国药理学报(APS)编辑委员会将于1997年11月5－8日在广东省珠海市召开"全国生化药理与生物工程新药研制与开发学术研讨会". 会议将由珠海恒通生物工程制药公司协办. 会议将邀请著名专家作有关专题报告. 会议主题为总结和交流：1) 生物化学、生物物理学、生化药理学研究进展及在新药研制与开发中的应用；2) 生物工程的研究进展，基因工程、细胞工程、蛋白质工程、酶工程、发酵工程等生物技术在新药研制与开发中的应用；3) 参观考察恒通生物工程制药公司并组织座谈. 会议征文要求：来稿必须是未公开发表过的学术性论文. 综述性文章请提前联系. 请寄800字以内(中文或英文)论文结构式摘要，包括 AIM (目的)，METHODS (方法)，RESULTS (结果)，CONCLUSION (结论)四部分. 请加盖公章或附单位介绍信，注明工作单位、地址、邮编、联系电话与传真. 务必在信封或传真上注明"药理学报会议征文". 征文截止日期为1997年9月5日. 无论文者也可索取报名单. 报名截止日期为1997年9月20日. 本次会议注册费500元/人，车旅费及食宿费自理. 具体安排见报到通知. 在收到您的报名之后将适时寄去报到通知. 征文或报名请寄：200031 上海市太原路294号《中国药理学报》编辑部 朱倩蓉收，联系电话：(021) 6431-1833 转200，传真：(021) 6437-0269. E-mail: aps@iris3.shmm.ac.cn.