ELSEVIER

Biochemical Pharmacology 61 (2001) 547–554

Biochemical Pharmacology

Inhibitory effect of α-lipoic acid and its positively charged amide analogue on nitric oxide production in RAW 264.7 macrophages

Qiong Guo[1], Oren Tirosh[1], Lester Packer*,[2]

Department of Molecular and Cell Biology, University of California, 251 Life Sciences Addition, Berkeley, CA 94720-3200, USA

Received 9 June 2000; accepted 27 July 2000

Abstract

The aim of this study was to investigate the effect of the mitochondrial cofactor α-lipoic acid [*R* (+) LA] or its lipoamide analogue, 2-(*N*,*N*-dimethylamine) ethylamido lipoate [*R* (+) LA-plus], on nitric oxide (NO) production in RAW 264.7 macrophages. NO production from RAW 264.7 cells stimulated with 10 μg/mL of lipopolysaccharide and 50 U/mL of interferon-γ was measured directly by electron spin resonance using spin-trapping techniques. *R* (+) LA or *R* (+) LA-plus was found to inhibit NO production at pharmacologically relevant concentrations. However, in a cell-free chemical system, neither *R* (+) LA nor *R* (+) LA-plus was able to directly scavenge NO. Furthermore, in the presence of 2.5 or 25 mM glucose, the inhibitory effects of *R* (+) LA and *R* (+) LA-plus on NO production were decreased markedly, while they showed more potent inhibitory effects in the presence of 2 μM rotenone or 5 μg/mL of antimycin A, inhibitors of mitochondrial complex I and complex III, respectively. Glucose, rotenone, or antimycin A alone resulted in an increase of NO production. These results suggest that NO production in macrophages can be regulated by glucose and mitochondrial respiration, and that modulation of NO production by lipoic acid or lipoamide analogues in inflammatory situations is attributed not to their radical scavenging activity but to their redox properties.

Keywords: Nitric oxide; Electron spin resonance; Lipoic acid; Glucose; Mitochondrial respiration; Macrophages

1. Introduction

NO, a short-lived free radical, serves as a messenger molecule in diverse physiological and pathological conditions such as blood pressure regulation, inhibition of platelet aggregation, neurotransmission, inflammation, atherosclerosis, and septic shock [1]. NO production by NOS is divided into two major categories: (a) constitutive—low (nanomolar) amounts of NO are produced by a constitutive and calmodulin-dependent enzyme located predominantly in neuronal (nNOS) and endothelial (eNOS) tissues [2]; and (b) inducible—up to a 1000-fold greater amount of NO is produced by an inducible and calmodulin-independent NOS (iNOS) that can be induced by cytokines and endotoxins in immune-system cells [1,2]. The main role of constitutive NOS is to regulate physiological functions, e.g. blood vessel tone and synaptic transmission. iNOS has a pivotal role in the immune system [3,4].

R (+) LA is being used as a food supplement for its antioxidant and antidiabetic properties. It is an eight-carbon compound containing two sulfur atoms in a dithiolane ring structure (Fig. 1). Reduction of *R* (+) LA in living cells to DHLA (Fig. 1) is regulated by metabolic pathways and cellular enzymes [5–7]. Such a process can change the reducing equivalent homeostasis of a cell. Indeed, direct evidence describing the influence of *R* (+) LA on the levels of the cellular NAD(P)H/NAD(P) ratio in T-cells has been reported [8]. *R* (+) Lipoyllysine is a co-factor in several key enzymatic reactions located in the mitochondria. Lipoamide analogues more closely resemble the protein-bound lipoyl-

* Corresponding author. Tel.: +1-510-865-5461; fax: +1-510-865-5431.

E-mail address: packerresearch@aol.com (L. Packer).

[1] Q.G. and O.T. contributed equally to this work.

[2] Present address: Department of Molecular Pharmacology and Toxicology, School of Pharmacy, University of Southern California, 1985 Zonal Ave., Los Angeles, CA 90089-9121.

Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible NOS; *R* (+) LA, α-lipoic acid; DHLA, dihydrolipoic acid; *R* (+) LA-plus, 2-(*N*,*N*-dimethylamine) ethylamido lipoate; IFN-γ, interferon-γ; LPS, lipopolysaccharide; MGD, sodium *N*-methyl-D-glucamine dithiocarbamate; L-NMMA, N^G-monomethyl-L-arginine; SOD, superoxide dismutase; SIN-1, 3-morpholinosydnonimine-*N*-ethylcarbamide; and DPBS, Dulbecco's phosphate-buffered saline.

0006-2952/01/$ – see front matter
PII: S0006-2952(00)00569-4

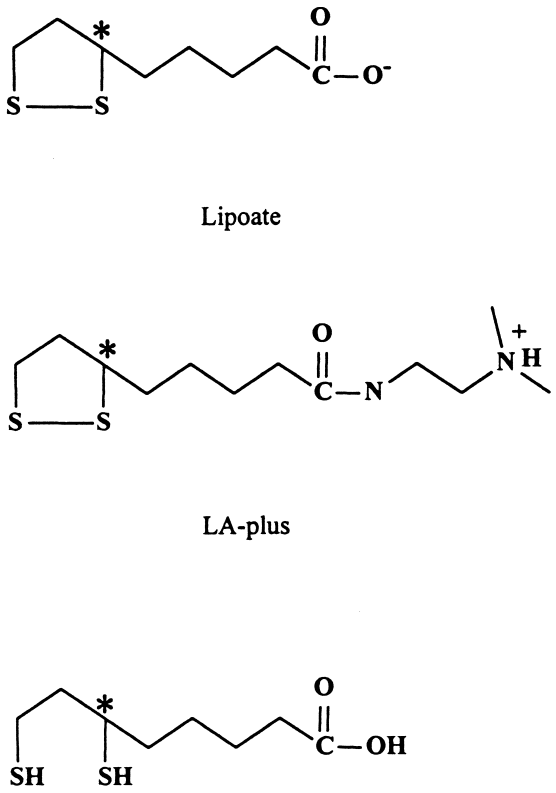

Fig. 1. Chemical structures. Top, the ionized form of lipoic acid. Middle, LA-plus (the protonated form of *N*,*N*-dimethyl,*N*′-2-ethylamido-lipoate at pH 7.4; the term plus indicates a positive ionic charge in neutral aqueous solutions). Bottom, the reduced form, dihydrolipoic acid. An asterisk (*) indicates chiral centers.

lysine than the free *R* (+) LA. Compared to the protein-bound lipoyllysine or to lipoamide analogues, free *R* (+) LA is considered to be an inferior substrate for dihydrolipoamide dehydrogenase, of the pyruvate dehydrogenase complex, in the mitochondria [9,10]. *R* (+) LA-plus, a water-soluble analogue of lipoamide (Fig. 1), has been shown to be more easily reduced in cells and to be a better substrate for the dihydrolipoamide dehydrogenase enzyme than *R* (+) LA [9,11].

At normal cellular calcium levels, NO production by iNOS was suggested to be limited only by substrate availability or cofactors present, such as L-arginine, oxygen, and NADPH [12]. This study investigated the effect of *R* (+) LA or its analogue, *R* (+) LA-plus, on NO production in RAW264.7 macrophages in the presence and absence of glucose or inhibitors of mitochondrial respiration. *R* (+) LA and *R* (+) LA-plus were used at relatively low concentrations and for short exposure times [13]. This treatment is consistent with the known pharmacokinetics of *R* (+) LA in plasma for up to 2 hr following oral supplementation [14].

2. Materials and methods

2.1. Materials

MGD was obtained from Polyscience Inc. SOD, L-lysine, L-NMMA, SIN-1, and L-arginine were from the Sigma Chemical Co. RPMI 1640 medium and DPBS were obtained from the Gibco BRL Co. IFN-γ and LPS were purchased from Genzyme. Fetal bovine serum was obtained from the cell culture facility of the University of California. An NO assay kit was purchased from the Calbiochem-Novabiochem Corp. R (+) LA-plus was prepared as described in the literature [11]. R (+) LA, DHLA, and S (−) LA were gifts from ASTA Medica.

The iron–dithiocarbamate complex spin-trap agent [$(MGD)_2$–Fe^{2+}] for trapping NO was prepared by reacting MGD (25 mM) with $FeSO_4$ (5 mM). The $FeSO_4$ solution was freshly prepared for each experiment [15]. Stock solutions (50 mM) of *R* (+) LA and *R* (+) LA-plus were prepared in DPBS, pH 7.4. Briefly, *R* (+) LA was first dissolved in an aqueous alkaline solution (1 N NaOH). Then DPBS was added to the solution, and the pH was neutralized with 1 N HCl. *R* (+) LA-plus was first dissolved in an acidic solution (1 N HCl). Then DPBS was added, and the pH was neutralized using 1 N NaOH [9].

2.2. Cell culture

The murine cell line of monocyte-macrophages RAW 264.7 (American Type Culture Collection) was used to produce $NO^{\cdot}$ in this study [13]. Cells were grown at 37°, 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% (w/v) penicillin-streptomycin, and 2 mM L-glutamine. Cells were used for experiments at about 90% confluence.

2.3. Activation and treatment of macrophages

RAW 264.7 cells were harvested in DPBS after a 6-hr stimulation with 10 μg/mL of LPS and 50 U/mL of IFN-γ. Cells were then incubated in the presence of the spin-trap complex [$(MGD)_2$–Fe^{2+}] with L-arginine, *R* (+) LA, *R* (+) LA-plus, glucose, and the mitochondrial respiratory inhibitor rotenone or antimycin A, alone or in various combinations, as indicated. The strategy was to avoid interference of *R* (+) LA, *R* (+) LA-plus, or glucose with iNOS gene expression during activation. The mixtures were incubated in a water bath at 37° for 90 min, and then all the samples were loaded into a quartz capillary and fitted into a quartz glass flat cell for ESR measurement. Each sample contained 2.4×10^6 cells in a final volume of 75 μL.

2.4. Chemical production of NO in a cell-free system

To test if R (+) LA and R (+) LA-plus can directly scavenge NO radical, SIN-1 was used to spontaneously

generate NO and $O_2^{\cdot-}$ during decomposition in an aqueous solution. The reaction medium containing 1.3 mM SIN-1, spin-trap complex [$(MGD)_2$–Fe^{2+}], and various concentrations of R (+) LA, R (+) LA-plus, DHLA, or SOD was incubated at 37° for 20 min, and then was transferred to a quartz capillary and fitted into a quartz glass flat cell for ESR measurement.

2.5. ESR spectrometer

ESR spectra were recorded using an IBM ER 200D-SRC ESR spectrometer. ESR spectrometer settings were: central field, 3420 G; modulation frequency, 100 kHz; modulation amplitude, 3.2 G; microwave power, 20 mW; scan width, 200 G; gain, 6.3×10^5; temperature, 298 K.

2.6. Griess reaction assay of nitrite formation from activated macrophages

The amount of nitrite production in the incubation medium was determined using the Griess reaction [16]. After a 6-hr stimulation with 10 μg/mL of LPS and 50 U/mL of IFN-γ, macrophages were harvested in DPBS and incubated with R (+) LA, R (+) LA-plus, or other compounds at 37° for 90 min. The Griess reaction was carried out to quantify the total nitrite production (nitrite plus nitrate) occurring during the incubation period, using an NO assay kit. Briefly, after 90 min of incubation, the medium was centrifuged, and supernatants were collected and mixed for 20 min with nitrate reductase and NADH, which were used to convert the nitrate to nitrite prior to quantitation using the Griess assay. Then the Griess Reagent (1% sulfanilamide/0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride in 3 M HCl) was added, and the samples were incubated for 10 min at room temperature. The total nitrite concentration was determined by measuring the absorbance at 540 nm in a Titertek Multiskan microplate spectrophotometer (Flow Laboratories). Total nitrite concentration was calculated using potassium nitrate as a standard.

2.7. HPLC measurement of uptake of R (+) LA and R (+) LA-plus

After a 6-hr stimulation with LPS and IFN-γ, macrophages were harvested and incubated for 90 min in the presence of 100 μM R (+) LA or R (+) LA-plus. Then R (+) LA, DHLA, R (+) LA-plus, and DHLA-plus from cell extracts and media were analyzed using HPLC as described before [9].

2.8. Statistical analysis

Statistical analysis was carried out by ANOVA using SPSS software. $P < 0.05$ was considered significant. Data are expressed as means ± SD of triplicate experiments.

3. Results

3.1. Detection of NO by ESR spin-trapping techniques

Introduction of a spin-trap agent, the [$(MGD)_2$–Fe^{2+}] complex, to macrophages activated with LPS and IFN-γ in the presence of 0.7 mM L-arginine produced a three-line ESR spectrum of the [$(MGD)_2$–Fe^{2+}–NO] complex (Fig. 2). The spectral parameters of this ESR signal ($g = 2.04$, $a^N = 12.5$ G) were identical to those reported in the literature when an aqueous solution of authentic NO was added to a solution containing a complex of iron and MGD [15]. Macrophages produced detectable amounts of NO after 4 hr of activation, as indicated by the relatively weak ESR signal (Fig. 2A). The level of NO production peaked at 6 hr and decreased thereafter. As a result, in subsequent experiments, macrophages were activated for 6 hr with LPS and IFN-γ before any treatment. In addition, L-arginine, the iNOS substrate, increased NO production in a concentration-dependent manner. Concentrations of L-arginine higher than 0.7 mM resulted in only an additional small increase in NO production (Fig. 2B).

L-NMMA, a competitive inhibitor of NOS, decreased NO production, in a concentration-dependent manner, in activated macrophages in the presence of exogenous L-arginine (Table 1). In the absence of exogenous L-arginine, L-NMMA at concentrations of 0.1 and 0.5 mM decreased NO signals to the basal noise level, indicating that NO production in cells was inhibited completely by L-NMMA. Similarly, L-lysine, a competitive inhibitor of L-arginine transport, also greatly decreased NO production in the presence of L-arginine. L-Lysine at 5 mM completely abolished NO production in the cells not treated with L-arginine. These results further confirmed that NO production was via the L-arginine–NOS pathway. The formation of NO in macrophages not treated with L-arginine suggests the existence of an endogenous pool of L-arginine that is capable of sustaining NO production. In contrast, SOD markedly increased NO production in the absence and presence of L-arginine, indicating that SOD scavenged superoxide radicals generated in activated cells, thereby reduced the formation of peroxynitrite.

3.2. Effect of lipoic acid and its analogues on NO production from macrophages

R (+) LA and its analogue, R (+) LA-plus, inhibited NO production, in a concentration-dependent fashion, in the presence of L-arginine (Fig. 3A). However, in the absence of L-arginine, their effect on NO production was negligible. One hundred micromolar R (+) LA or R (+) LA-plus showed no toxic effects on cell viability (data not shown). In addition, DHLA, the reduced form of lipoic acid, had a similar inhibitory effect on NO production, although HPLC measurement showed that DHLA was unstable and was

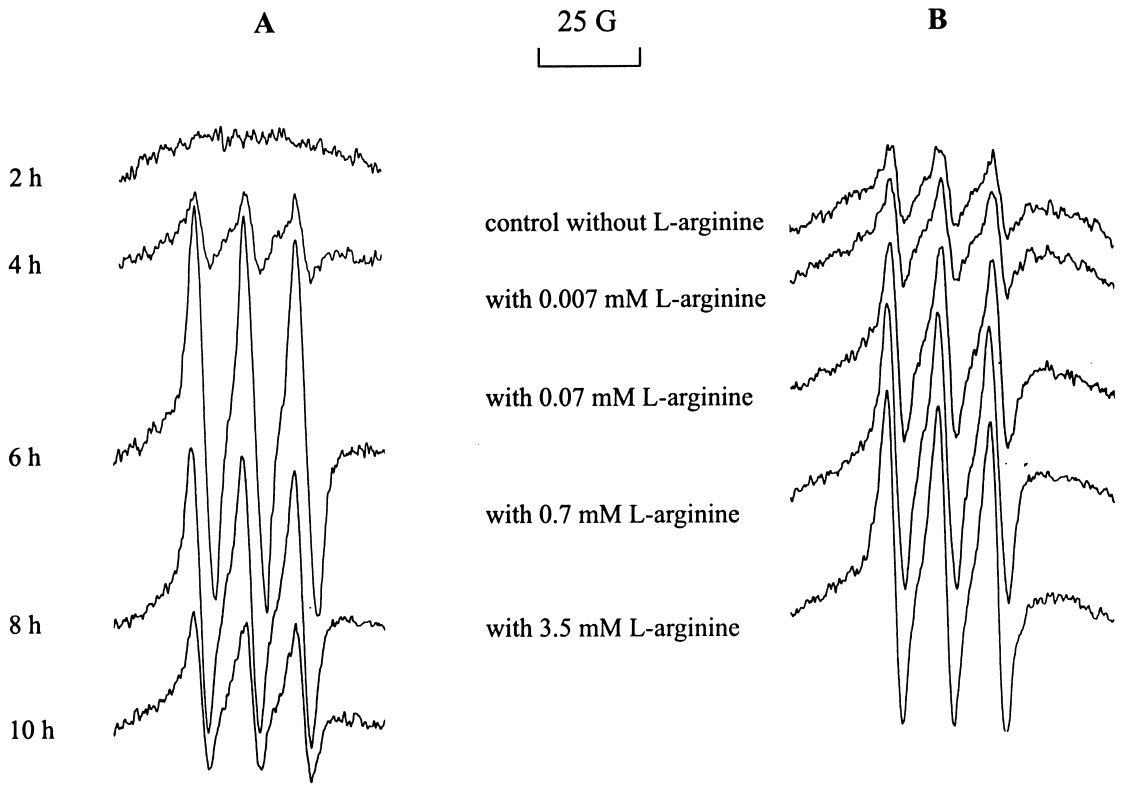

Fig. 2. iNOS-mediated NO production from RAW 264.7 macrophages measured by ESR spin-trapping techniques. (A) Effect of stimulation time on NO production. Macrophages were stimulated with 50 U/mL of IFN-γ and 10 μg/mL of LPS for various time intervals. Then stimulated cells were harvested in DPBS and incubated with 0.7 mM L-arginine in the presence of the spin-trap agent [$(MGD)_2$–Fe^{2+}] for 90 min. (B) Concentration-dependent effect of L-arginine on NO production. Cells were stimulated with 50 U/mL of IFN-γ and 10 μg/mL of LPS for 6 hr, harvested in DPBS, and incubated with increasing concentrations of L-arginine (0 to 3.5 mM) in the presence of the spin-trap agent [$(MGD)_2$–Fe^{2+}] for 90 min. The incubation mixtures were then loaded for ESR measurements.

oxidized completely to R (+) LA after 90 min of incubation (data not shown).

Table 1
NO production in activated macrophages via the L-arginine-iNOS pathway

Treatment	Concentration	NO production (% relative to control with L-arginine	
		Without 0.7 mM L-arginine	With 0.7 mM L-arginine
Control		36 ± 1.5	100 ± 5.6
L-NMMA	0.1 mM	NL[a]	44.7 ± 2.3**
L-NMMA	0.5 mM	NL	15.8 ± 2.5*
L-lysine	1.0 mM	13.6 ± 1.5**	54.3 ± 4.2*
L-lysine	5.0 mM	NL	8.9 ± 1.0*
SOD	100 U/mL	51.9 ± 4.2	129.8 ± 6.2
SOD	200 U/mL	62.2 ± 4.1	157.3 ± 2.1

Macrophages were harvested in DPBS and treated with L-NMMA, L-lysine, or SOD in the absence or presence of 0.7 mM L-arginine for 90 min after a 6-hr activation with 50 U/mL of IFN-γ and 10 μg/mL LPS. Activated cells were then loaded for ESR measurements (see "Materials and Methods"). Data are normalized to the control with L-arginine addition. values are mean ±SD, N = 3.

[a] NL, the signal intensity did not exceed the noise level.

* Lower compared with the control with L-arginine, $P < 0.05$.

** Lower compared with the control without L-arginine, $P < 0.05$.

In addition, the effects of naturally occurring *R* (+) LA and its isomer, *S* (−) LA, on NO production were compared in a similar experiment (Fig. 3B). It was found that *S* (−) LA was less efficient than *R* (+) LA at inhibiting NO production. Therefore, the inhibitory effect of those compounds on NO production decreased in the following order: *R* (+) LA-plus > *R* (+) LA > *S* (−) LA.

The effect of *R* (+) LA or *R* (+) LA-plus on NO release from macrophages was confirmed by measuring total nitrite accumulation in the incubation medium (Fig. 4). In the presence of L-arginine, total nitrite levels in media from cells treated with 0.1 mM L-NMMA, 5.0 mM L-lysine, 100 μM *R* (+) LA, or 100 μM *R* (+) LA-plus decreased compared with controls. Similar to the above results obtained using ESR spin-trapping techniques, *R* (+) LA-plus was more potent than *R* (+) LA in reducing total nitrite accumulation, although this effect was weaker in the absence of L-arginine.

3.3. Effect of lipoic acid and its analogues on NO production from macrophages in the presence of glucose

The addition of 2.5 mM glucose to the activated cell suspension in the presence of L-arginine increased NO pro-

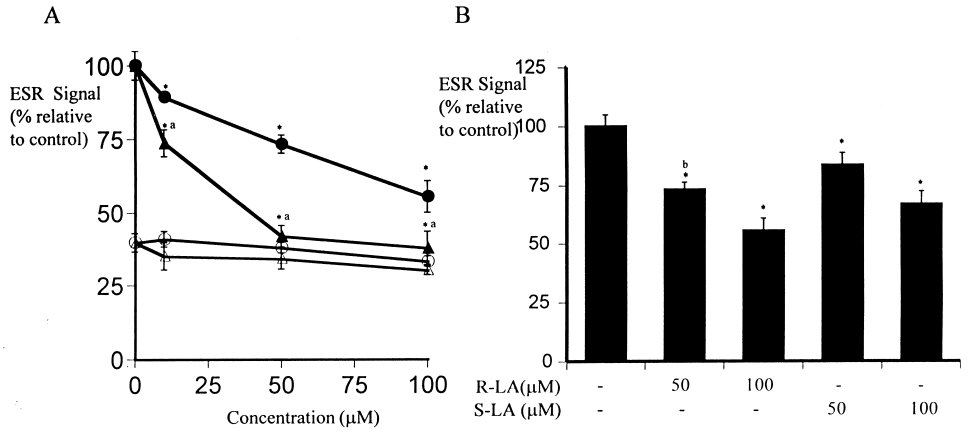

Fig. 3. Effects of *R* (+) LA and its analogues on NO production in RAW 264.7 macrophages measured by ESR spin-trapping techniques. (A) Effect of *R* (+) LA and its positively charged analogue, *R* (+) LA-plus, on NO production. Activated cells (2.4 x 10^6) in the absence (open symbols) or presence (closed symbols) of 0.7 mM L-arginine were treated with various concentrations of *R* (+) LA (circles) or *R* (+) LA-plus (triangles) for 90 min (see "Materials and methods"). (B) Effects of *R* (+) LA and its isomer, *S* (−) LA, on NO production. Activated cells (2.4 × 10^6) in the presence of 0.7 mM L-arginine were treated with the indicated concentrations of *R* (+) LA or *S* (−) LA for 90 min (see "Materials and methods"). NO production was then evaluated by ESR signal intensity. Data were normalized to the control. Key: (*) lower compared with the control, $P < 0.05$; (a) lower compared with the corresponding concentrations of *R* (+) LA-treated samples, $P < 0.05$; and (b) lower compared with the corresponding 50 μM *S* (−) LA-treated sample, $P < 0.05$. Values are means ± SD, N = 3.

duction more than 3-fold (Fig. 5). Supplementation with 25 mM glucose did not produce further augmentation. In the absence of glucose, treatment with 100 μM *R* (+) LA or *R* (+) LA-plus decreased NO production by 25 and 39%, respectively, compared with the untreated control. However, as compared to the corresponding concentrations of the glucose-treated control, the inhibitory effect of *R* (+) LA or *R* (+) LA-plus on NO production was decreased from 8 and 25% to 5 and 13% in the presence of 2.5 and 25 mM glucose, respectively.

3.4. Effect of lipoic acid and its analogues on NO production from macrophages in the presence of mitochondrial respiratory inhibitors

Treatment of activated macrophages with 2 μM rotenone, a mitochondrial complex I inhibitor, resulted in a considerable increase in NO production (Fig. 6). In the

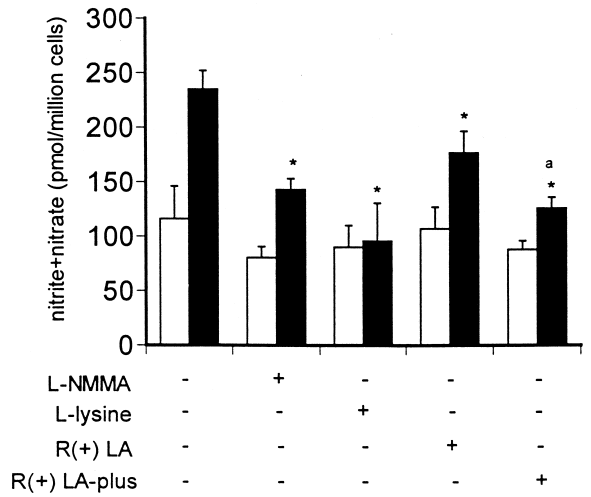

Fig. 4. Effects of *R* (+) LA and *R* (+) LA-plus on NO release from RAW 264.7 macrophages measured by the Griess reaction. Activated cells (2.4 × 10^6) in the absence (open bars) or presence (closed bars) of 0.7 mM L-arginine were treated with 0.1 mM L-NMMA, 5.0 mM L-lysine, 100 μM *R* (+) LA, or 100 μM *R* (+) LA-plus for 90 min (see "Materials and methods"). NO release was then evaluated spectrophotometrically at 540 nm. Key: (*) lower compared with the L-arginine-treated control, $P < 0.05$; and (a) lower compared with the corresponding *R* (+) LA-treated sample, $P < 0.05$. Values are means ± SD, N = 3.

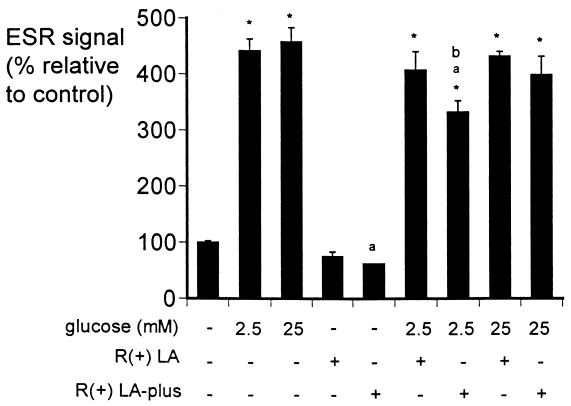

Fig. 5. Glucose-induced increase in NO production from RAW 264.7 macrophages and the effects of *R* (+) LA and *R* (+) LA-plus. Activated cells (2.4 × 10^6) in the presence of 0.7 mM L-arginine were treated with glucose, *R* (+) LA (100 μM), or *R* (+) LA-plus (100 μM) either alone or in combination for 90 min (see "Materials and methods"). NO production was then evaluated by ESR signal intensity. Data were normalized to the control. Key: (*) higher compared with the control, $P < 0.05$; (a) lower compared with the corresponding *R* (+) LA-treated samples, $P < 0.05$; and (b) lower compared with the 2.5 mM glucose-treated sample, $P < 0.05$. Values are means ± SD, N = 3.

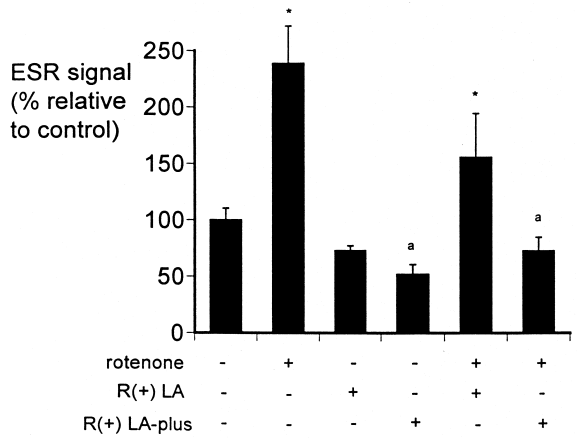

Fig. 6. Rotenone-induced increase in NO production from RAW 264.7 macrophages and the effects of *R* (+) LA and *R* (+) LA-plus. Activated cells (2.4×10^6) in the presence of 0.7 mM L-arginine were treated with rotenone (2 μM), *R* (+) LA (100 μM), or *R* (+) LA-plus (100 μM) either alone or in combination for 90 min (see "Materials and methods"). NO production was then evaluated by ESR signal intensity. Data were normalized to the control. Key: (*) higher compared with the control, $P < 0.05$; and (a) lower compared with the corresponding *R* (+) LA-treated samples, $P < 0.05$. Values are means ± SD, N = 3.

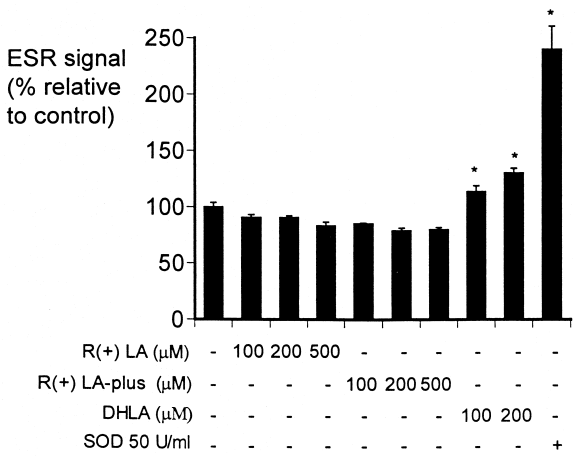

Fig. 7. Effects of *R* (+) LA and *R* (+) LA-plus on NO release from the NO donor SIN-1, measured by ESR spin-trapping techniques. SIN-1 (1.3 mM) was incubated with the indicated concentrations of *R* (+) LA, *R* (+) LA-plus, DHLA, or SOD in the presence of the spin-trapping agent [$(MGD)_2$–Fe^{2+}] for 20 min at 37°, and then NO release was evaluated by ESR signal intensity (see "Materials and methods"). Data were normalized to the control. Key: (*) higher compared with the control, $P < 0.05$. Values are means ± SD, N = 3.

absence of rotenone, treatment with 100 μM *R* (+) LA or *R* (+) LA-plus inhibited NO production 27 and 47%, respectively, as compared with the untreated control. In the presence of 2 μM rotenone, 100 μM *R* (+) LA or *R* (+) LA-plus decreased NO production by 35 and 70%, respectively, as compared with the rotenone-treated control. Similar results were obtained in the presence of 5 μg/mL of antimycin A (data not shown).

3.5. Radical scavenging properties

SIN-1 was used to chemically generate NO and superoxide radicals. SOD added to this system spared NO by scavenging superoxide radicals generated simultaneously in the chemical system (Fig. 7). *R* (+) LA and *R* (+) LA-plus showed only minor scavenging activity at a relatively high concentration of 0.5 mM, whereas DHLA, the reduced form of *R* (+) LA, spared NO in a SOD-mimicking manner, probably because DHLA scavenged superoxide radicals generated in the chemical system, as SOD did. The ability of DHLA to scavenge superoxide radicals has been demonstrated by Suzuki et al. [17].

3.6. Reduction of R (+) LA and R (+) LA-plus in macrophages

Incubation of activated macrophages with 100 μM *R* (+) LA or *R* (+) LA-plus for 90 min showed the formation of the corresponding reduced forms (Table 2). Levels of DHLA-plus in macrophages treated with *R* (+) LA-plus were more than 2-fold greater than those of DHLA in cells incubated with *R* (+) LA, indicating that *R* (+) LA-plus was reduced more efficiently than *R* (+) LA by macrophages.

4. Discussion

In the present study, using ESR and spin-trapping techniques, *R* (+) LA and its analogue, *R* (+) LA-plus, were found to inhibit NO production from activated macrophages. They had a smaller inhibitory effect on NO production in the absence of exogenous L-arginine than in the presence of L-arginine. As a consequence, such a selective inhibitory effect suggests that these compounds can regulate NO production under situations of pathological overproduction of NO but will not interfere with the delicate physiological balance of NO.

It has been reported that *R* (+) LA cannot directly scavenge superoxide radicals [17,18]. In our investigation, it was found that both *R* (+) LA and *R* (+) LA-plus had no direct NO-scavenging activity in a cell-free chemical system (Fig. 7). DHLA even spared NO in a SOD-mimicking manner. It was observed that incubation of *R* (+) LA and *R* (+) LA-plus with macrophages resulted in their reduction to DHLA and DHLA-plus, respectively, and *R* (+) LA-plus was reduced at a greater rate than *R* (+) LA (Table 2), in agreement with previous observations in human Wurzburg T cells [9]. In addition, our previous reports have shown that bio-reduction of *R* (+) LA and *R* (+) LA-plus was NAD(P)H dependent [6]. Taken together, these findings suggest that inhibition of NO production by *R* (+) LA or *R* (+) LA-plus probably is not attributed to the free radical-scavenging activities of *R* (+) LA or *R* (+) LA-plus, but to their metabolic properties, that is, *R* (+) LA and *R* (+)

Table 2
Bio-reduction of *R* (+) LA and *R* (+) LA-plus in activated macrophages

	R (+) LA	DHLA	Ratio reduced/ oxidized	*R* (+) LA-plus	DHLA-plus	Ratio reduced/ oxidized
Cellular (pmol/million cells)	245 ± 16	4.5 ± 0.3	0.018	158 ± 7.8	15.0 ± 3.2*	0.095
Extracellular (μM)	112 ± 5.6	1.06 ± 0.14	0.009	54.2 ± 2.4	3.7 ± 0.55*	0.68

Levels of *R* (+) LA, DHLA, *R* (+) LA-plus, and DHLA-plus in cells and medium were assayed using the HPLC-EC system after activated cells were treated with 100 μM *R* (+) LA or *R* (+) LA-plus for 90 min. Values are means ±SD, N = 3.

* $P < 0.05$, compared with the corresponding DHLA values.

LA-plus consumed cellular NAD(P)H during their bio-reduction and then indirectly regulated NO production from activated macrophages. NADPH supply also has been demonstrated to be important to sustain macrophage NO production [19].

The inhibitory effects on NO production by *R* (+) LA and *R* (+) LA-plus were investigated further in the presence of glucose or inhibitors of mitochondrial respiration. Addition of glucose alone to activated macrophages significantly increased NO production, indicating that the rate-limiting factor in NO production is indeed metabolic. This coincides with the fact that NADPH is one of the products of glucose metabolism through the pentose phosphate pathway. Additionally, the inhibitory effect of *R* (+) LA or *R* (+) LA-plus on NO production was much weaker in the presence of high glucose (25 mM) than that obtained in a 2.5 mM glucose-treated or a glucose-free system. These results suggest that the regulation of NO production directly correlated with the changes in the cellular levels of pyridine nucleotides.

Moreover, *R* (+) LA and *R* (+) LA-plus are substrates for the mitochondrial enzyme lipoamide dehydrogenase [9]. This enzyme, which supplies NADH to complex I of the mitochondrial respiratory chain, is dependent on NADH to reduce *R* (+) LA or *R* (+) LA-plus. Thus, the hypothesis that mitochondrial respiration could be a regulator of NO levels in cells was tested. Rotenone, an inhibitor of mitochondrial complex I, was found to enhance NO production. Antimycin A, a mitochondrial complex III inhibitor, showed a similar effect on NO production (unpublished data). These data support the idea that the regulation of NO production by mitochondrial respiratory inhibitors may not be mediated by superoxide radicals from a mitochondrial source since it has been reported that rotenone inhibits intramitochondrial superoxide production in macrophages, whereas antimycin A increases superoxide leakage from mitochondria [20,21]. In addition, both *R* (+) LA and *R* (+) LA-plus were more potent in inhibiting NO production in rotenone-treated macrophages than in glucose-treated cells. One possible explanation for this regulatory pattern is rotenone-induced acceleration of the rate of reduction of *R* (+) LA [6].

It is well known that NO acts as an inhibitor of mitochondrial respiration by inhibiting cytochrome oxidase or other respiratory chain complexes [2]. Interestingly, in this study, it was found for the first time that inhibition of mitochondrial respiration by rotenone or antimycin A regulated NO production. This may be due to changes in local oxygen and NAD(P)H concentrations in the vicinity of iNOS that were modulated by the status of mitochondrial respiration, an hypothesis supported by the finding that treatment of activated macrophages with rotenone increased cellular NAD(P)H levels (unpublished data). This involved mechanism was similar to that proposed in other reports indicating that mitochondria regulate cellular function by affecting local calcium concentrations in cells [22,23]. Therefore, this work provides evidence that there is a direct interaction between NO levels in macrophages and the status of the mitochondrial electron transport chain acting as a major route for cellular oxidation of pyridine nucleotides.

In conclusion, the inhibitory effects of *R* (+) LA and *R* (+) LA-plus on NO production from activated macrophages have been demonstrated for the first time. The inhibitory effects of *R* (+) LA and *R* (+) LA-plus were decreased in the presence of glucose supplementation, but were increased in the presence of inhibitors of mitochondrial respiration. Compounds such as *R* (+) LA and *R* (+) LA-plus, which decrease NO release from activated macrophages, might serve as potential anti-inflammatory agents.

Acknowledgments

This research was supported by BASF Aktiengesellschaft, Germany. The authors thank Drs. Hadi Moini and Sashwati Roy for helpful discussions.

References

[1] Schmidt HH, Walter U. NO at work. Cell 1994;78:919–25.
[2] Brown GC. Nitric oxide and mitochondrial respiration. Biochim Biophys Acta 1999;1411:351–69.
[3] Brown GC. Nitric oxide regulates mitochondrial respiration and cell functions by inhibiting cytochrome oxidase. FEBS Lett 1995;369:136–9.
[4] Murphy MP. Nitric oxide and cell death. Biochim Biophys Acta 1999;1411:401–14.
[5] Arner ES, Nordberg J, Holmgren A. Efficient reduction of lipoamide and lipoic acid by mammalian thioredoxin reductase. Biochem Biophys Res Commun 1996;225:268–74.

[6] Haramaki N, Han D, Handelman GJ, Tritschler HJ, Packer L. Cytosolic and mitochondrial systems for NADH- and NADPH-dependent reduction of α-lipoic acid. Free Radic Biol Med 1997;22:535–42.
[7] Pick U, Haramaki N, Constantinescu A, Handelman GJ, Tritschler HJ, Packer L. Glutathione reductase and lipoamide dehydrogenase have opposite stereospecificities for α-lipoic acid enantiomers. Biochem Biophys Res Commun 1995;206:724–30.
[8] Roy S, Sen CK, Tritschler HJ, Packer L. Modulation of cellular reducing equivalent homeostasis by α-lipoic acid. Mechanisms and implications for diabetes and ischemic injury. Biochem Pharmacol 1997;53:393–9.
[9] Tirosh O, Sen CK, Roy S, Kobayashi MS, Packer L. Neuroprotective effects of α-lipoic acid and its positively charged amide analogue. Free Radic Biol Med 1999;26:1418–26.
[10] Patel MS, Roche TE. Molecular biology and biochemistry of pyruvate dehydrogenase complexes. FASEB J 1990;4:3224–33.
[11] Sen CK, Tirosh O, Roy S, Kobayashi MS, Packer L. A positively charged α-lipoic acid analogue with increased cellular uptake and more potent immunomodulatory activity. Biochem Biophys Res Commun 1998;247:223–8.
[12] Abu-Soud HM, Stuehr DJ. Nitric oxide synthases reveal a role for calmodulin in controlling electron transfer. Proc Natl Acad Sci USA 1993;90:10769–72.
[13] Gao JJ, Zuvanich EG, Xue Q, Horn DL, Silverstein R, Morrison DC. Cutting edge: bacterial DNA and LPS act in synergy in inducing nitric oxide production in RAW 264.7 macrophages. J Immunol 1999;163:4095–9.
[14] Teichert J, Kern J, Tritschler HJ, Ulrich H, Preiss R. Investigations on the pharmacokinetics of alpha-lipoic acid in healthy volunteers. Int J Clin Pharmacol Ther 1998;36:625–8.
[15] Norby SW, Weyhenmeyer JA, Clarkson RB. Stimulation and inhibition of nitric oxide production in macrophages and neural cells as observed by spin trapping. Free Radic Biol Med 1997;22:1–9.
[16] Lancaster JR, Hibbs JB. EPR demonstration of iron-nitrosyl complex formation by cytotoxic activated macrophages. Proc Natl Acad Sci USA 1990;87:1223–7.
[17] Suzuki YJ, Tsuchiya M, Packer L. Thioctic acid and dihydrolipoic acid are novel antioxidants which interact with reactive oxygen species. [published erratum appears in Free Radic Res Commun 1992;17(2):155] Free Radic Res Commun 1991;15:255–63.
[18] Scott BC, Aruoma OI, Evans PJ, O'Neill C, Van der Vliet A, Cross CE, Tritschler H, Halliwell B. Lipoic and dihydrolipoic acids as antioxidants. A critical evaluation. Free Radic Res 1994;20:119–33.
[19] Hothersall JS, Gordge M, Noronha-Dutra AA. Inhibition of NADPH supply by 6-aminonicotinamide: effect on glutathione, nitric oxide and superoxide in J774 cells. FEBS Lett 1998;434:97–100.
[20] Boveris A, Chance B. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. Biochem J 1973;134:707–16.
[21] Chance B, Sies H, Boveris A. Hydroperoxide metabolism in mammalian organs. Physiol Rev 1979;59:527–605.
[22] Babcock DF, Herrington J, Goodwin PC, Park YB, Hille B. Mitochondrial participation in the intracellular Ca^{2+} network. J Cell Biol 1997;136:833–44.
[23] Hoth M, Fanger CM, Lewis RS. Mitochondrial regulation of store-operated calcium signaling in T lymphocytes. J Cell Biol 1997;137:633–48.