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Synthetic Water-Soluble Phenolic Antioxidant Regulates L-Arginine Metabolism in Macrophages: a Possible Role of Nrf2/ARE

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Abstract—Synthetic water-soluble phenolic antioxidant TS-13 exhibits pronounced anti-inflammatory properties *in vivo* and induces intracellular signal system Nrf2/ARE. At concentrations 150-1000 μM it inhibits nitric oxide (NO) production in mouse peritoneal macrophages. However, this compound at low concentrations (1-100 μM) paradoxically increases NO production and decreases activity of arginase. These results are indicative of an ambiguous role of NO and its metabolites in the mechanism of development of inflammatory reaction.

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Key words: nitric oxide, arginase, macrophages, inflammation, antioxidant responsive element, Nrf2, TS-13

L-Arginine metabolism in immune-competent cells involves two opposite enzyme systems with key participants type I-III NO synthases, catalyzing L-arginine oxidation to L-citrulline and nitric oxide (NO), and type I and II arginases converting the amino acid to L-ornithine and urea [1]. Peculiarities of L-arginine metabolism play an important role in the development of inflammation and immune response. According to traditional concepts, NO and its metabolites provide for the phagocyte effector functions (their bactericidal and cytotoxic activities) due to oxidative damage to macromolecules and subcellular and cellular structures accompanied by changes in the organism's own tissues and enhancement of inflammation [2]. On the other side, NO and its metabolites are involved in negative regulation of inflammatory reaction and stimulate its resolution due to lowering the blood vessel wall permeability and restriction of leucocyte entry into the inflammation focus [3] along with induction of apoptosis in activated leucocytes [4]. The increase in arginase activity in macrophages is specific for some types of inflammation caused by allergens or extracellular parasites [5] and is also noticed upon transition of inflammatory reaction to chronic form [6]. High arginase activity and, correspondingly, decreased NO production stimulate cell proliferation and synthesis of intercellular matrix, thus stimulating tissue repair that under some conditions can result in cell fibrosis [7].

The intracellular signal system Nrf2/ARE plays a decisive role in maintenance of intracellular homeostasis by ensuring protection against reactive oxygen and nitrogen species as well as against some xenobiotics and carcinogens [8, 9] due to regulation of expression of genes of some enzymic antioxidants and enzymes of the second phase of detoxification [8, 10]. Nrf2/ARE inducers exhibit pronounced anti-inflammatory properties [11, 12] by lowering production of anti-inflammatory cytokines and NO, which is in many respects due to inhibition of transcription factor NF-κB activation [13].

Abbreviations: ARE, antioxidant responsive element; DCF, 2,7-dichlorofluorescein; IC_{50}, concentration required for 50% inhibition; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NO, nitric oxide; PMA, phorbol 12-myristate-13-acetate; TS-13, sodium 3-(3′-*tert*-butyl-4′-hydroxyphenyl)propyl thiosulfonate.

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However, there are certain contradictions. It was shown that NO itself is an Nrf2/ARE activator inducing modification of Nrf2/Keap1, Nrf2 translocation into the cell nucleus, and enhancement of expression of ARE-dependent genes [14, 15]. Besides, to date the involvement of Nrf2/ARE in the regulation of L-arginine metabolism in macrophages, in particular of arginase activity, is poorly studied.

We have synthesized a structurally related series of hydrophilic monophenols differing in the number of *tert*-butyl substituents in *ortho*-position relative to the OH group and in the presence of sulfonate or thiosulfonate group in the *para*-propyl substituent. The compounds exhibited high antioxidant activity in model systems *in vitro* and high anti-inflammatory activity *in vivo* due to their ability to induce antioxidant responsive element [11]. The partially hindered thiosulfonate TS-13 (sodium 3-(3′-*tert*-butyl-4′-hydroxyphenyl)propyl thiosulfonate) exhibited the most pronounced efficiency.

The goal of this work was to investigate the effect of the phenolic antioxidant TS-13 on peculiarities of L-arginine metabolism in resident mouse peritoneal macrophages (as shown previously, this compound exhibited pronounced anti-inflammatory activity).

MATERIALS AND METHODS

Experimental animals. Cells from intact two month old hybrid female mice (C57Bl/6×DBA/2)F1 obtained from the Experimental Biological Clinic for Laboratory Animals (Siberian Branch of the Russian Academy of Medical Sciences) were used in this work.

Isolation and cultivation of resident peritoneal macrophages. Peritoneal macrophages were isolated by washing the mouse abdominal cavity with cold culture medium RPMI-1640 (Bio-West, France) containing 1% fetal bovine serum (Biolot, Russia) and following washing off non-adherent cell fraction 2 h after beginning of cultivation in 96-well plates (Orange Scientific, Belgium) in complete culture medium (RPMI-1640 without phenol red, 10% fetal bovine serum, 15 mM Hepes (Sigma, USA), and 0.3% L-glutamine (Vector, Russia)). To activate NO synthase, at the beginning of cultivation lipopolysaccharide (LPS) was added to final concentration 1 μg/ml (LPS *E. coli* B5:055; Sigma).

Isolation of leucocytes. After decapitation, mouse blood was collected in plastic tubes with heparin (final concentration 40 units/ml), and then tenfold excess of lysing buffer (0.8% NH_4Cl, 0.08% EDTA, 0.08% $NaHCO_3$) was added. Lysed erythrocytes were washed out by centrifugation (10 min, 300*g*), and leucocytes were resuspended in Hanks' balanced salt solution without phenol red.

Antioxidant activity of the studied compounds was determined by inhibition of the 2,7-dichlorofluorescein (DCF)-dependent fluorescence of peripheral blood neutrophils. To do this, leucocytes were incubated for 15 min at 37°C with 2,7-dichlorodihydrofluorescein diacetate (Sigma). TS-13 obtained by the reaction of 3-(3′-*tert*-butyl-4′-hydroxyphenyl)propylbromide with sodium thiosulfate in water–alcohol solution [11] was added in different concentrations to cell suspensions 10 min before introduction of 2,7-dichlorofluorescin diacetate. The known antioxidant, α-tocopherol analog Trolox (Sigma), 6-hydroxy-2,5,7,8-tetramethylchroman-carboxylic acid was used as a reference preparation due to its hydrophilicity and hindering of hydroxyl group joined to a benzene ring. To activate reactive oxygen species, phorbol 12-myristate 13-acetate (PMA; Sigma) was added to cells to final concentration 100 ng/ml. Intensity of DCF-dependent fluorescence (λ_{em} = 488 nm, λ_{ex} = 520 nm) was measured using a FACSCalibur flow cytofluorimeter (Becton-Dickinson, USA). The neutrophil containing region was distinguished on the basis of indexes of the side and forward light scattering.

Nitric oxide production was evaluated by nitrite content in the cell culture supernatant 48 h after beginning of cultivation using Griess reagent (Fluka, Switzerland) by measuring optical density at 540 nm using a TriStar LB 941 spectrophotometer (Berthold Technologies, Germany) [16].

Arginase activity was determined by a micromethod measuring the rate of urea formation from exogenous L-arginine [17] 24 h after beginning of cell cultivation. Macrophages were lysed in wells with 0.1% Triton X-100 for 1 h. Then 50 μl 50 mM Tris-HCl, pH 7.4, was added to 50 μl lysate. Arginase was activated by introduction of 10 μl 50 mM $MnCl_2$ solution and heating in a water bath at 57°C for 10 min, then 100 μl 0.5 M L-arginine solution was added to each well and samples were incubated for 1 h at 37°C. The reaction was stopped by addition of solutions of sulfuric and phosphoric acids in water in volume ratios 1 : 3 : 7, respectively. Urea concentration was colorimetrically determined using the TriStar LB 941 spectrophotometer at 540 nm after addition of 9% alcohol solution of α-isonitrosopropiophenone and heating in a boiling water bath for 45 min. The result was expressed in enzyme activity units. The enzyme amount that synthesized 1 mmol urea in 1 min was taken as 1 activity unit.

Statistical analysis. From four to six measurements were carried out for each designated concentration of tested compounds and all parameters under study. Results are expressed as ratios to corresponding control (cell incubation without compounds) and are shown in figures as median values. The significance of differences was estimated using the non-parametric Wilcoxon criterion and considered as reliable at $p < 0.05$. The concentration at which 50% inhibition was achieved (IC_{50}) was calculated by interpolation from curves of non-linear regression (decreasing function by the first order exponent $y = y_0 + A \cdot e^{-x/t}$) of the "dose–response" dependences.

RESULTS

Comparative investigation of the effects of TS-13 and Trolox on generation of reactive oxygen species by neutrophils showed that both studied compounds dose-dependently decrease the DCF-mediated fluorescence of neutrophils (Fig. 1). In this case TS-13 is far inferior to Trolox in its antioxidant properties. Thus, 50% decrease in intensity of spontaneous DCF-dependent neutrophile fluorescence ($IC_{50spont}$) was registered at TS-13 concentration 485.8 μM and after cell stimulation (IC_{50stim}) it was observed at concentration 161.5 μM, whereas $IC_{50spont}$ and IC_{50stim} calculated for Trolox are 31.6 and 39.8 μM, respectively. The difference in IC_{50stim} and $IC_{50spont}$ of phenolic antioxidant TS-13 can be explained by different rate of interaction of this compound with different reactive oxygen species, in particular with more efficient neutralization of hypochlorites that are practically not synthesized by non-activated neutrophils, whereas in the case of respiratory burst over 28% of consumed oxygen is spent for hypochlorite generation [18]. Thus we showed earlier in *in vitro* systems that TS-13 more efficiently inhibited the hypochlorite-dependent component of the neutrophile respiratory burst comparing to that defined by superoxide anion [11]. Another possible explanation for enhancement of TS-13 antioxidant properties is the inhibition of the process by PMA-induced assembly of NADPH-oxidase complex due to protein kinase C inactivation. It is known that some phenol and selenium-containing antioxidants inhibit it via modification of cysteine residues in the catalytic subunit [19]. The presence of a thiosulfonate group within TS-13 structure probably makes easier its interaction with sulfhydryl groups of the protein amino acid residues and causes its ability to inactivate the enzyme. Trolox has no effect on protein kinase C activity [20].

Studying the ability of TS-13 to inhibit NO synthesis by inducible NO synthase (iNOS) and its comparison with the effect of Trolox showed that a single addition of the tested compounds at different concentrations (1-100 μM) to peritoneal macrophages at the beginning of cell cultivation does not alter spontaneous production of nitric oxide (data not shown in figures). Effects of the studied preparations on the activity of macrophagic NOS were revealed after cell stimulation by LPS (Fig. 2a). Trolox added simultaneously with LPS at concentrations 1-500 μM has no effect on stimulated NO production by peritoneal macrophages, whereas at the dose of 1 mM it more than twice decreases this production. In turn, TS-13 at low concentrations (0.1-10 μM) increases the LPS-stimulated production of NO in a dose-dependent manner, whereas at concentrations above 10 μM it dose-dependently decreases the nitrite content in the culture medium. In this case it is necessary to note that TS-13 more efficiently inhibits NO production than Trolox at analogous concentrations.

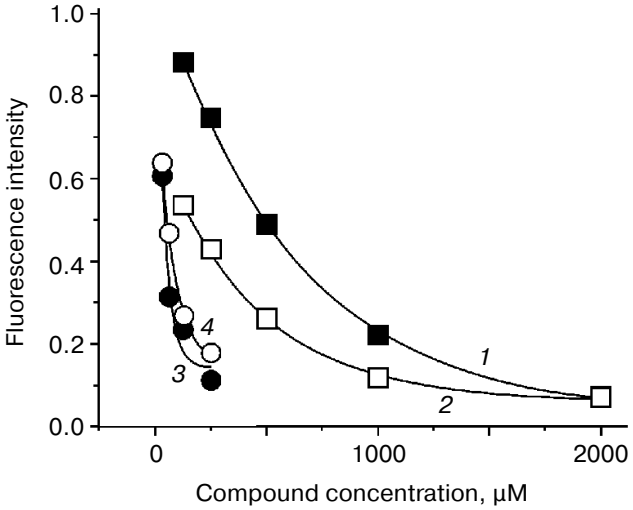

Fig. 1. Effect of phenolic antioxidant TS-13 (*1*, *2*) and Trolox (*3*, *4*) on intensity of DCF-dependent fluorescence of mouse blood neutrophils. Here and in Fig. 2 ratios of measured parameter values to corresponding control are shown. Lines *1* and *3* show spontaneous fluorescence, lines *2* and *4* show PMA-stimulated fluorescence.

To study the effects of TS-13 and Trolox on induction of macrophagic NOS, these compounds were added at concentrations 1-100 μM to cells simultaneously with LPS, 6 h later macrophages were washed off, culture medium was changed, and cells were grown without the studied compounds for 48 h. At these conditions both TS-13 and Trolox increase nitrite concentration at the end of cell growth, which is indicative of their ability to enhance the LPS-stimulated activation of iNOS of macrophages (Fig. 2b). In this case the phenolic antioxidant TS-13 in lower concentrations (1 μM) exhibits this effect, whereas Trolox enhances induction of macrophagic NOS at doses more than 10 μM.

Under conditions *in vitro* single addition of Trolox at concentrations 1-100 μM 24 h before measurement had no effect on arginase activity in mouse peritoneal macrophages, whereas TS-13 at doses of 10 and 100 μM decreases this activity by 38 and 25% compared to control level, respectively (Fig. 2c).

DISCUSSION

The novel water-soluble phenolic antioxidant TS-13 described in this work is an activator of Nrf2/ARE-dependent gene expression, and its high anti-inflammatory activity is probably due to this [11]. Unlike partially hindered phenol TS-13, water-soluble hindered phenol Trolox, widely used as a reference antioxidant, has no effect on the heme oxygenase 1 gene expression and intracellular reduced glutathione level in different cell

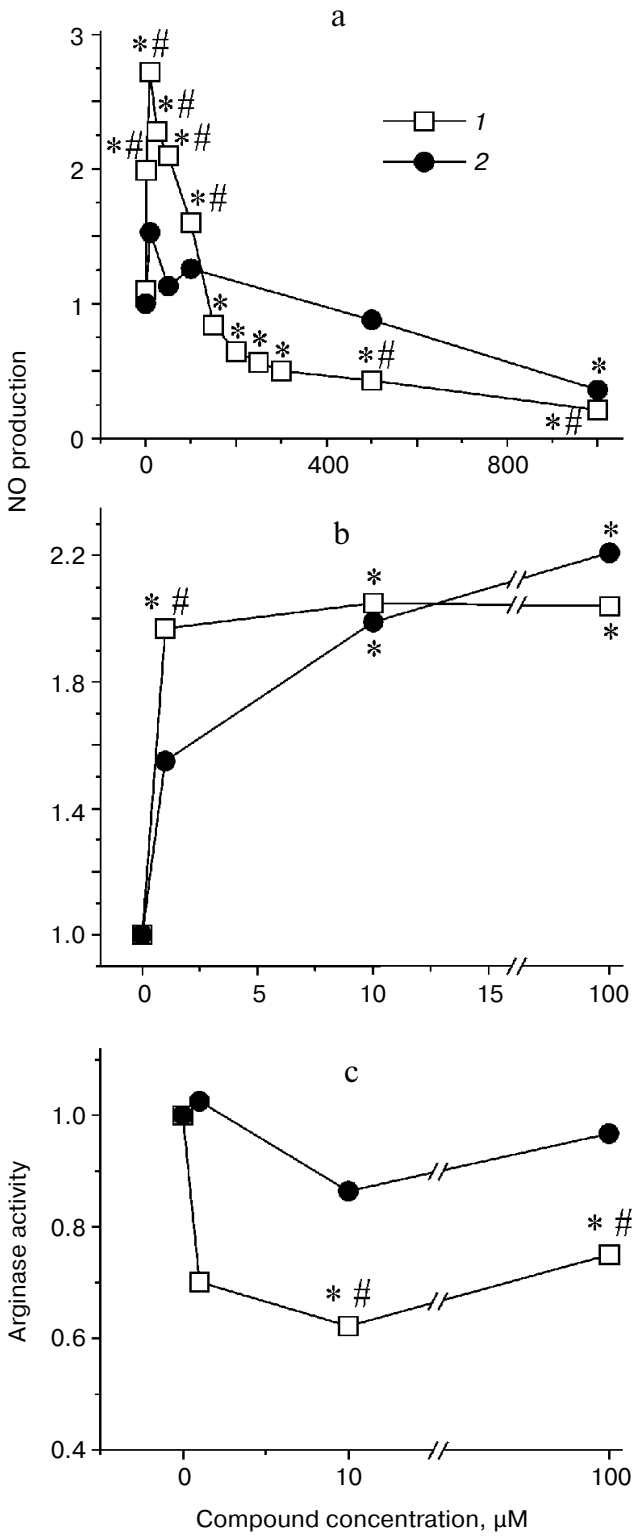

Fig. 2. Effect of TS-13 (*1*) and Trolox (*2*) on LPS-stimulated NO production by mouse peritoneal macrophages *in vitro* (a), on LPS-induced activation of macrophagic NOS estimated by NO production (b), and on arginase activity in macrophages (c). * Significant difference from corresponding control ($p < 0.05$); # significant difference from the value obtained upon Trolox addition at the same concentration.

lines [21], which points to its probable inability to activate transcription factor Nrf2. Considering also the fact that TS-13 is far inferior to Trolox in its direct antioxidant activity, it is reasonable to suppose that the effect of the studied compounds on L-arginine metabolism in macrophages is due, at least in part, to activation of the Nrf2/ARE signal system.

Inhibition of NOS activity caused by TS-13 at high doses (over 100 μM) cannot be explained by its direct interaction with NO or nitrite ion [11] and, most likely, it is due to activation of the Nrf2/ARE signal system, which agrees well with data from the literature [12, 22]. The classical Nrf2 activator sulforaphan exhibits pronounced anti-inflammatory effect by inhibition of iNOS expression [12]. It was found in an extensive investigation of a group of authors that inducers of Nrf2/ARE-dependent signal pathway belonging to different classes of chemical substances decrease in dose-dependent manner the LPS-stimulated NO production by mouse peritoneal macrophages and cells of the RAW264.7 line [22]. In this case there is linear relationship between the ability of studied compounds to induce expression of the Nrf2/ARE-dependent genes and to inhibit NO production. Among mechanisms of negative effect of Nrf2 inducers on NOS activity is inhibition of activation of transcription factor NF-κB [13, 23] regulating expression of iNOS.

Nevertheless, the fact that sulforaphan at doses above 20 μM already does not act as an inhibitor of iNOS expression attracts attention, although in this case there still exists its negative effect on expression of other anti-inflammatory genes [12]. Thus, at certain concentrations inversion of effect of the Nrf2/ARE-signal pathway inducers is possible. Besides, it was shown in some works using NO donors that NO efficiently activates the Nrf2/Keap1/ARE signal system [14, 15]. We found in this work that TS-13 at low concentrations (<100 μM) increases NO production. In the same interval of concentrations the phenolic antioxidant induced expression of the Nrf2/ARE-dependent genes [11] owing to which this property can be explained by its ability to enhance inducible NO generation.

Our data are indicative of arginase activity inhibition in non-stimulated macrophages in response to TS-13, which evidently cannot be due to iNOS activation, because without additional stimulation by lipopolysaccharide the phenolic antioxidant was not able to enhance NO production. It can be supposed that arginase inhibition is the result of increased catalase activity, because expression of a corresponding gene in macrophage is under Nrf2 control [24]. It was found that exogenous catalase decreases activity of cellular arginase in the dose-dependent manner [25], but exogenous hydrogen peroxide at high concentrations, on the contrary, increase this parameter [26]. It is important that reliable enhancement of LPS-stimulated NO production is observed upon action on cells of 1 μM

TS-13, whereas the phenolic antioxidant at this concentration has no effect on arginase. This indirectly confirms the ability of the studied compound to exhibit direct activating effect on iNOS, possibly due to enhancement of the corresponding gene expression.

It can be supposed in accordance with this that the effect of TS-13 on expression of enzymes providing for L-arginine metabolism is concentration dependent. At low concentrations TS-13 stimulates expression of the *NOSII* gene and inhibits arginase activity, possibly due to increased catalase activity, and when TS-13 concentration is increased it decreases NO production due to blocking the NF-κB activation. TS-13, the inducer of Nrf2/ARE-dependent signal pathway, exhibits pronounced anti-inflammatory properties, and data obtained on its ability to activate iNOS confirm the opinion concerning the ambiguous role of NO in development of inflammatory reaction. There are data indicative of the participation of NO in resolution of inflammatory process associated with NO-dependent apoptosis of immunocompetent cells [27, 28]. However, taking into account anti-apoptotic functions traditionally attributed to Nrf2/ARE inducers, the problem of the relationship between the iNOS-activating effect of TS-13 and its ability to decrease manifestation of acute inflammation requires further investigation.

The effect of phenolic antioxidant TS-13 on enzyme systems of L-arginine metabolism in macrophages is closely linked with its ability to activate the Nrf2/ARE signal system. In this case induction of Nrf2-dependent signal pathway in response to the studied compound at low doses can be due to enhanced NO production. Activation of transcription factor Nrf2 is accompanied by iNOS inhibition, causing reversion of TS-13 effect on NOS activity in response to higher dose. The decrease in nitrite concentration in the culture medium in response to Trolox at high dose is due to direct antioxidant activity of the latter. Nevertheless, the physiological role of the described alterations in L-arginine metabolism caused by Nrf2 activation is still not clear. Our data point to the ambiguous role of iNOS and NO in inflammation.

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