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Interaction Between Nitric Oxide and Respiratory Burst Responses of Mouse Peritoneal Macrophages against Salmonella typhimurium

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SUMMARY

Nitric oxide (NO) and respiratory burst (RB) responses are major elements of the "oxygen dependent" defence mechanisms of macrophages, and since their roles have been also explained in human, many studies focused on NO and RB. In this study, NO and RB responses of mouse macrophages against live and dead forms of Salmonella typhimurium as an intracellular pathogen, and a possible relationship between these responses were evaluated. In live bacteria groups NO and RB responses were significantly higher while in dead bacteria groups responses were low. Inhibition of the NO response by an inhibitor (N-nitro-L-arginine methyl ester (L-NAME)), was not affected the RB responses. L-arginine addition was not effective on NO production if the response was not triggered. In conclusion, this study suggests that NO and RB relations, and effects of inhibitors and stimulators of them deserve further studies in order to explain their possible therapeutic effects on human diseases caused by intracellular pathogens.

Key Words: Nitric oxide, respiratory burst, macrophage, Salmonella typhimurium

ÖZET

Salmonella typhimurium'a Karşı Fare Peritoneal Makrofajlarının Solunum Patlaması ve Nitrik Oksit Yanıtlarının Etkileşimi

Nitrik oksit (NO) ve solunum patlaması (SP) yanıtları makrofajların "oksijene bağımlı" savunma mekanizmalarının temel ögelerini oluşturur. NO ve SP yanıtlarının insanlardaki rolleri açıklandığından beri de birçok çalışma bu konulara odaklanmıştır. Bu çalışmada, fakültatif hücre içi bir patojen olan Salmonella typhimurium'un ölü ve canlı biçimlerine fare peritoneal makrofajlarının yanıtları ve bu yanıtların biribirileri ile ilişkileri değerlendirilmiştir. Canlı bakteri grubunda NO ve SP yanıtları, ölü bakteri grubunda oluşan yanıtlara göre belirgin olarak daha yüksek bulunmuştur. NO yanıtlarının bir baskılayıcı ile baskılanması (N-nitro-L-arginine-methyl ester (L-NAME)), SP yanıtlarını etkilememiştir. L-arjininin (NO yolağı ara maddesi) eklenmesi, eğer yanıt tetiklenmemişse, NO üretimine etkili olmamıştır. Sonuç olarak, bu çalışma NO ve SP arasındaki etkileşim ve ilişkilerin infeksiyon patogenezine etkileri, hücre içi patojenlerce oluşan infeksiyonlarda bu iki yolağın baskılayıcı ve ara maddelerinin olası sağaltıcı etkileri gibi nedenlerle ileri çalışmalar yapılmasını desteklemektedir.

Anahtar Kelimeler: Nitrik oksit, solunum patlaması, makrofaj, Salmonella typhimurium

INTRODUCTION

Natural immune system is the first responding system against intracellular pathogens and macrophages are the main cells of this system (1). After phagacoytosis period, mainly two defence systems are activated in macrophages: oxygen dependent sytem (ODS) and oxygen independent system (OIS). Two different parts are involved in ODS: reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs) (2). In RNI, a free gaseous molecule molecule, nitric oxide (NO) is produced by a number of cell types from molecular oxygen. ın activated macrophages, NO is exerted through induction of expression of the inucible nitric oxide synthase (iNOS) gene (3, 4). In ROI, after macrophages activation nicotine adenine dinucleotide phosphate (NADPH) carries the molecular oxygen bound to respiratory burst (RB) and as a result oxygen beco-

mes superoxide anion, hydrogen peroxide, singlet oxygen and hydroxyl radicals which have strong microbicidal effects (2, 5, 6).

Salmonella typhimurium is an facultative intracellular pathogen for human (7) and mice (8), and can cause entrocolitis in human (9, 10, 11).

In this study, NO and RB responses of macrophages were evaluated when macrophages were incubated with live or dead intracellular bacteria for 24 hours, and the RB responses were evaluated when the NO responses were inhibited for determining an outlet of any possible relationship between these ODSs.

MATERIAL AND METHODS

Inbred, healthy, female BALB/c mice were obtained from Dokuz Eylül University, School of Medicine. Mice were used at an age of 8-12 weeks.

RPMI-1640 without phenol red and sodium bicarbonate and L-glutamine was obtained from Sigma. Foetal calf serum (FCS) with a low endotoxin level was from Seromed-Biochrom.

N-nitro-L-arginine methyl ester (L-NAME) (Sigma), as an inhibitor of NO pathway, and L-arginine (Sigma) as a metobolite in NO pathway, were suspended in RPMI-1640 medium, filtered through 0.22 µm filter before use.

The Griess Reagent for measuring nitrite levels were consisted of equal volumes of 0.1 % N-(1-napthyl) ethylene diamine dihydrochloride and 1% p-aminobenzene-sulfanilamide diluted in 2.5% phosphoric acid (both from Sigma).

For respiratory burst (RB) determination nitro blue tetrazolium (NBT) dye was used (Sigma). NBT was diluted in autoclaved (PBS (1 mg / 1 mL) and filtered through 0.22 µm filter before use.

Experiments were performed by resting mouse peritoneal cells. Cells were harvested from mice by lavage of the peritoneum with cold PBS supplementd with 2% FCS and 20 IU/mL of heparin. Cells were kept on ice, washed once in RPMI-1640 in medium and seeded in cultures of 2x106 cells in volume of 0.-0.9 mL of RPMI-1640 medium (10% FCS + 1% Penicillin/Streptomycin + 1% HEPES + 1% L-glutamine) in 24-well plates (multidish wells; 16 mm diameter; NUNC).

Human isolated, non mutant, non 37°C resistant Salmonella typhimurium strain was prepared in RPMI-1640 medium in 10:1 multiplicity of infection (M.O.I) for final concentrations. The same amount of bacteria were prepared by keeping bacteria for 30 minutes in 56°C in water bath.

Cultures were incubated overnight in a humidified atmosphere with 5% CO_2, at 37°C after establishment. Infection and stimulation were done initially by adding 100 µL of live and dead bacteria with cells in same concentrations for 30 minutes at 37°C with 5% CO_2. At the end of the period cells were washed three times with RPMI-1640 (with 200 mg/mL) gentamycin) in order to eliminate extracellular bacteria (7).

After washing step, by adding 100 µL - 200 µL of L-NAME and L-arginine combinations (1 mM/mL, 0,4 mM/mL, respectively) were added to macrophages which were then kept in culture with RPMI-1640 (with 100 mg/mL gentamycin) for 24 hours at 37°C with 5% CO_2.

After incubation step, triplicate samples were harvested for determination of nitrite concentrations (L-NAME and L-arginine concentrations were in highest limit concentrations) (Data not shown)

Nitrite is generated oxidation of NO and stable in culture medium at least for 3 days. It reflects the amount of NO produced. ın nitrite assay, a modification of previously published method was used (12). Aliquots of 100 µL culture supernatans were mixed with equal volumes of Griess Reagent in 96-well microtitre plate (Maxisorb Immunoplate, NUNC). After 10 minutes of incubation at room temperature the absorbance at a wavelength of 550 nm was measured. A range of 2-fold dilutions of sodium nitrite (0-100 µM) in RPMI-medium was run in each assay to generate a standard curve.

Nitro blue tetrazolium (NBT) is a redox dye, and reduction of NBT shows the presence of superoxide and oxygen radicals, mainly the RB. In NBT test two known methods were modified (13,14). NBT was prepared by a concentration of 1 mg/mL in PBS and filtered through 0.22 µm filter before use. After nitrite de-

termination, culture medium were poured off and 100 µL of PBS-NBT mixtures were dispensed for incubation 30 minutes at 37°C. At the end of incubation mixtures were poured off and cells with blue precipitations were counted. Spontaneous formation limit was kept at 50% cells per well (positives were higher than 50%, negatives were lower than 50%). Results were either (+) or (-) in at least more than the half of the total cells. Counts were done either visually or by microscope.

The NO response against live Salmonella typhimurium alone was tested by the sign test on mean values from all experiments. Statistical testing of live/dead bacteria on NO production was performed by the paired t-test.

RESULTS

In a series of experiments we examined the NO production of resting macrophages when stimulated for 24 hours in cultures with live and dead S. typhimurium. Figure 1 and Table 1 showed that NO responses of live bacteria groups were higher than that of dead bacteria groups and controls (without any chemical or bacterial stimulation). No phagocytosis inhibitors were used in experiments for showing phagocytosis because of confusing effects on results. Lipopolysaccharide (LPS) content of bacteria shows a low NO response in dead bacteria group, we may state that, elevated NO response depends on the invasion of bacteria because of sufficient incubation period. Decreasing of NO response with L-NAME (1 mM/mL) and a little increase with L-arginine (0.4 mM/mL) shows us that produced NO is the specific product of nitrogen metobolism in these experiments.

No spontaneous NO and RB productions were examined when L-NAME and/or L-arginine combinations were applied to macrophage cultures. Table 2 demonstrated that NO responses were very low and RB responses were negative providing that all the NO/RB responses in all experiments were without any additional effects of any chemicals and their combinations. And note that L-arginine addition alone is not effective on NO production without any stimulation on macrophages.

In live bacteria groups RB responses were positive whereas in dead bacteria groups responses were negative (Table 3). Note that there was no inhibition on RB responses in at least the half of the total cells when NO response was inhibited in live bacteria groups.

DISCUSSION

Uptil 1987 in explaining the cytotoxicity of activated

Table 1. Nitric oxide responses of live and dead S. typhimurium groups, including L-NAME and L-arginine addings (µM±SEM)

	Control	Live bacteria	Dead bacteria	Live bacteria+L-NAME	Dead bacteria+L-NAME	Live bacteria+L-NAME+L-arginine	Dead bacteria+L-NAME+L-arginine
Experiment 1	1±0.3	25±1.4	4.5±0.2	13±1.0	2.8±0.1	18±0.6	3.4±0.1
Experiment 2	1±0.1	27±1.0	4.7±0.2	13±0.5	2.7±0.1	19±0.2	3.2±0.1
Experiment 3	1±0.2	26±1.2	4.6±0.2	16±0	2.9±0.1	20±0.4	3.0±0.1
Mean	1±0.2	26±1.2	4.5±0.2	14±0.5	2.8±0.1	19±0.4	3.2±0.1

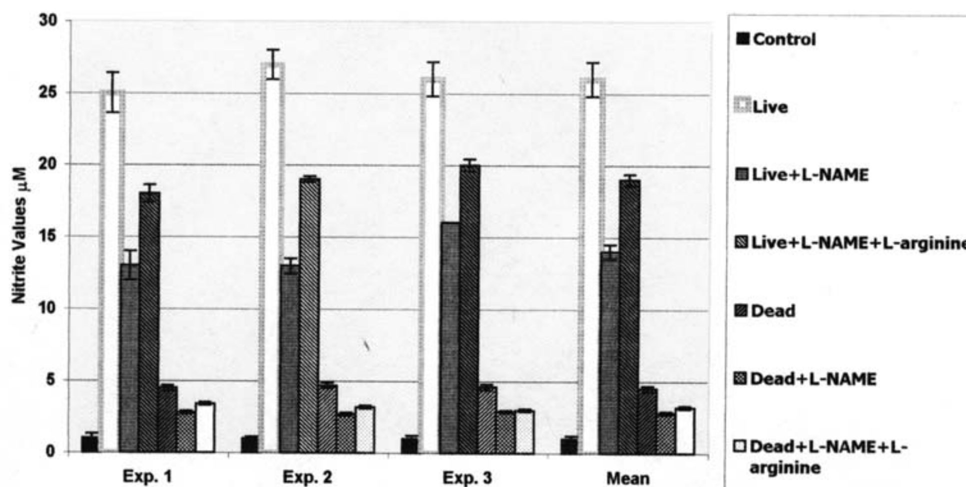
Table 2. Nitric oxide (NO) and respiratory burst (RB) responses of macrophages as were incubated by L-NAME and L-arginine alone (NO responses were as µM±SEM).

	Nitric oxide control	Respiratory burst control	L-NAME (NO)	L-NAME (RB)	L-arginine (NO)	L-arginine (RB)	Live bacteria+L-NAME+L-arginine	Live bacteria+L-NAME+L-arginine
Experiment 1	1±0.3	Negative	0.6±0.2	Negative	0.8±0.1	Negative	1.0±0.6	Negative
Experiment 2	1±0.1	Negative	0.5±0.2	Negative	0.7±0.1	Negative	0.8±0.1	Negative
Experiment 3	1±0.2	Negative	0.7±0.2	Negative	0.8±0.1	Negative	0.9±0.1	Negative
Mean	1±0.2	Negative	0.7±0.2	Negative	0.8±0.1	Negative	0.9±0.1	Negative

Table 3. Respiratory burst (RB) responses of live and dead bacteria.

	Control	Live bacteria	Dead bacteria	Live bacteria+ L-NAME	Dead bacteria+ L-NAME	Live bacteria+L-NAME+L-arginine	Dead bacteria+L-NAME+L-arginine
Experiment 1	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Experiment 2	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Experiment 3	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Result	Negative	Positive	Negative	Positive	Negative	Positive	Negative

Figure 1: Nitric oxide levels (µM±SEM) of different groups in three separate expriments in three wells for 24 hours.



macrophages only NADPH oxidase synthesis with reactive oxygen intermediates mechanism has been explained. But this mechanism was not clear enough to explain inducible cytotoxicity (15). In 1987 it was shown that cytokine inducible cytotoxicity in activated macrophages was related to L-arginine (16). Today it is known that NO has a cytotoxic effect against a broad spectrum of intracellular pathogens such as Mycobacterium tuberculosis (17), Chlamydia trachomatis (18),... etc., and it is shown that human macrophages also produce NO response (19, 20), like RB response (21).

On the other hand, loss or excess of oyxgen redicals may cause many diseases. For instance in chronic granulomatous disease (CGD), RB can not be produced. In CGD cases persistant bacterial infections can be seen. Also an excess of NADPH oxidase can cause tissue damages in inflammation (21).

These results were suggested us to question a possible relationship between NO and RB, and behaviours of macrophages against live and dead S. typhimurium as an intracellular pathogen. First of all, macrophages showed strong NO and RB responses against live S. typhimurium, but showed very low and weak responses against dead S. typhimurium. It seems that endotoxin content of the bacteria is not enough to trigger the NO and RB responses in macrophages, they need to be stimulated by an intracellular message evoked by live bacteria.

Secondly, NO inhibitors (L-NAME) did not affect RB responses. On the contrary, in a previous study it was shown that catalase enzyme (RB inhibitor) additions decreased NO responses (22). NO and RB are the members of ODS, but RB response may act more freely from ODS from NO response.

As a third result, L-arginine addition did not effect the low NO responsiveness of macrophages against dead S. typhimurium, but in contrast increases the NO responses against live S. typhimurium when inhibited by L-NAME. This suggests that if NO response is not

triggered, L-arginine addition is not effective on NO production.

In conclusion, we may state that NO and RB relationships together with the inhibitors and stimulators's effects deserve further studies because of their possible therapeutic effects on human diseases when caused by alive intracellular pathogens. And in explaining these relationships, in animal models involving susceptibility and resistance to intracellular pathogens may be of help.

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