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Short communication

Single gavage with *Porphyromonas gingivalis* reduces acute systemic nitric oxide response in mice

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Nemec A, Pavlica Z, Šentjurc M, Crossley DA, Jerin A, Eržen D, Zdovc I, Petelin M, Skalerič U. Single gavage with Porphyromonas gingivalis *reduces acute systemic nitric oxide response in mice.*
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Introduction: *Porphyromonas gingivalis*, an important periodontal pathogen, can also induce host responses in distant tissues. *P. gingivalis* induces nitric oxide (NO) production in immune system cells and non-immune system cells, therefore NO might be involved in an acute systemic host response.
Methods: Eighteen female BALB/c mice were perorally inoculated with 10^8 colony-forming units live *P. gingivalis* ATCC 33277. Plasma nitrite and nitrate (NOx) and NO production in lungs, aorta, heart, liver, spleen, kidneys, and brain were measured at intervals after inoculation and compared with levels in 11 control animals.
Results: NOx levels were significantly ($P = 0.017$) lower at 7, 13, and 25 h after *P. gingivalis* inoculation. A similar trend in NO production occurred in most tested organs, but never reached statistical significance. The correlation between NOx in plasma and NO in liver was positive (Spearman correlation coefficient = 0.81, $P = 0.0025$) and marginal for kidney (0.58, $P = 0.059$).
Conclusion: Single peroral inoculation of mice with *P. gingivalis* reduces the acute systemic NO response. As NO is important for host defense, the reduction of NO levels after exposure is likely to delay the host response, increasing the chances that infection with *P. gingivalis* will become established.

Key words: acute host response; mice; *Porphyromonas gingivalis;* systemic nitroxidative stress

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Porphyromonas gingivalis is important in the pathogenesis of periodontal disease (15). Modulation of host immune responses by bacteria permits their long-term survival, increasing their pathogenic potential (15). Bacteremia commonly occurs in periodontal disease and the bacteria are also swallowed or aspirated from the oral cavity, affecting other sites (23). Remote sites are also affected by the bacterial antigens and cytokines absorbed at sites of infection and distributed throughout the body (15, 20, 23). *P. gingivalis* lipopolysaccharide (LPS) alone has the potential to modulate the cytokine network (15). *In vitro*, it induces nitric oxide (NO) production in both immune system cells and non-immune system cells (4, 13, 24, 26) by binding to host cell receptors CD14 and Toll-like receptor 2 (12). However, *in vivo*, the inflammatory environment influences cellular activity.

Systemic cytokine modulation occurs after a single intratracheal exposure of mice to sonicated *P. gingivalis* (20) and chronic *in vivo* stimulation with *P. gingivalis* upregulates NO production in murine macrophages (7); therefore NO is likely to be involved in a systemic acute host response to *P. gingivalis* exposure. This hypothesis was tested in this study by measuring nitrite and nitrate (NOx) plasma and NO production in different organs of mice perorally inoculated with live *P. gingivalis*.

Materials and methods

Experimental design

Twenty-nine female BALB/c mice between 2 and 3 months of age were randomly divided into three groups (Table 1), housed

Table 1. Experimental groups

Experimental group	Procedure	Number of mice
1	Untreated control group Immediate sacrifice	3
2	Spin-trap-treated control group Sacrificed 60 min after spin trap injection	8 (5 NOx)[1]
3	*P. gingivalis* broth inoculation (0.1 ml per os) at the start with spin trap administered 60 min before sacrifice Sacrifice of three mice at each of 2.5, 7, and 13 h; and of nine mice at 25 h.	18 (6 NOx at 25 h)[1]

[1]NOx assays only performed on five or six mice because of difficulties obtaining plasma samples (hemolysis).

separately and given standard laboratory chow (Teklad Global 16% Protein Rodent Diet, Harlan Italy, San Pietro al Natisone, Italy) and water *ad libitum* following the Veterinary Administration of the Republic of Slovenia approved animal protocol No. 323–02–187/2004/2.

The *P. gingivalis* ATCC 33277 culture was prepared as described previously (10) and the cell concentration was adjusted to approximately 10^9 colony-forming units (CFU)/ml.

Based on our previous findings that 10^8 CFU *Escherichia coli* in 0.1 ml induces systemic NO production without clinical signs of illness (19), each mouse was orally inoculated with 10^8 CFU (0.1 ml) *P. gingivalis* while the nose was occluded to stimulate aspiration. No sham-inoculated group was used in this study because single inoculation with sterile broth had previously been found not to influence the systemic NO response (19).

Spin trap, required for electron paramagnetic resonance (EPR) NO detection, was administered in two parts (2): diethyldithiocarbamate (DETC; Alexis Biochemicals, Alexis Corporation, Lausen, Switzerland) 500 mg/kg body weight in 0.1 ml of water for injection was given intraperitoneally, followed by a mixture of ferrous sulfate (Johnson, Matthey & Co. Limited, London, UK) 100 mg/kg and sodium citrate (Alkaloid, Skopje, Macedonia) 500 mg/kg in 0.1 ml of water for injection subcutaneously. These components combine in the tissues creating $Fe(DETC)_2$, which binds NO, forming a stable $FeNO(DETC)_2$ adduct that gives EPR signals with a typical *g*-factor ($g = 2.04$) (2). Spin trap was administered 1 h before sacrifice because a preliminary study had demonstrated that the highest signals were obtained with this interval (Table 1). Blood was collected immediately postmortem by cardiac puncture into cold ethylenediaminetetraacetic acid tubes and immediately centrifuged (1500 ***g***, 4°C, 15 min); the plasma was stored at −70°C until required. Organs were harvested into quartz tubes and frozen in liquid nitrogen within 10 min of death. Sampling order (lungs, thoracic aorta, heart, liver, spleen, kidney, and brain) and the segments of the larger organs used were kept consistent to minimize variables.

EPR measurements

EPR spectra were taken at 130°K on an X-band EPR spectrometer Bruker ESP 300 (Bruker Instruments, Inc., Billerica, MA, USA) with spectrometer settings of: center field 330 mT, microwave frequency 9.3 GHz, modulation amplitude 0.3 mT, modulation frequency 100 kHz, microwave power 13 dB, and scan range 20 mT (2).

The EPR spectra intensities were measured as the height of the triplet signals (Fig. 1). Any detected $Cu(DETC)_2$ signal was subtracted from the $FeNO(DETC)_2$ signal as recommended (27). The signal height was normalized with respect to the mass of the sample and recorded as adjusted units (AU) to permit valid statistical comparisons between samples.

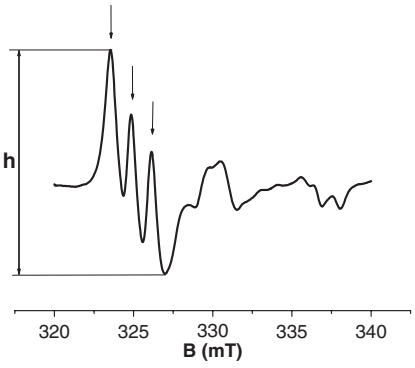

Fig. 1. Typical $FeNO(DETC)_2$ electron paramagnetic resonance spectrum (arrows) superimposed on the basal spectrum of the liver from mouse (28) following peroral inoculation with 10^8 colony-forming units (CFU) *Porphyromonas gingivalis.* (Measurement at 130°K, $g = 2.04$, h – signal height, B – magnetic field).

Three tissue sample tubes that broke during freezing were excluded from study.

Nitrite/nitrate assay

The measurement of plasma concentration of combined nitrite and nitrate (NOx) was performed by a blinded observer using a colorimetric non-enzymatic assay (Neogen Corporation, Lexington, KY, USA).

Statistical analysis

A statistical software package (SAS 9.00, SAS Institute Inc., Cary, NC, USA) was used for analysis of the results; values of $P < 0.05$ were regarded as significant. Normality of distribution was tested by univariate analysis of NOx and NO results. Since neither the distribution nor its log transformation was normal, the results are presented as medians plus minimum and maximum values. Non-parametric Wilcoxon's rank-sum one-sided two-sample and Spearman rank-order correlation tests were used to check for differences between groups (samples were independent) and to confirm correlations between NOx in plasma and NO in organs.

Results

Nitrite/nitrate in plasma

NOx was detected in plasma samples from all the control mice (groups 1 and 2), with no statistically significant difference between these two groups. Reduced NOx levels were found at all time intervals in the *P. gingivalis*-inoculated animals (group 3), the drop being statistically significant ($P = 0.017$) when compared with mice from group 2 at 7, 13, and 25 h after inoculation (Fig. 2A), with a minimum 7 h postinoculation.

EPR spectra of organs

The basic spectra from group 1 organs varied as previously described (28). After spin trap treatment (group 2) a $FeNO(DETC)_2$ signal was superimposed on the basic organ spectra (e.g. liver; Fig. 1) in most mice. A $FeNO(DETC)_2$ signal was detected consistently in the lungs and livers of *P. gingivalis*-inoculated animals (group 3). In other organs the proportion of animals with positive signals was typically greater as time progressed. The median signal was generally lower following *P. gingivalis* inoculation compared with non-inoculated animals (Fig. 2B–H) but the differences were not statistically significant.

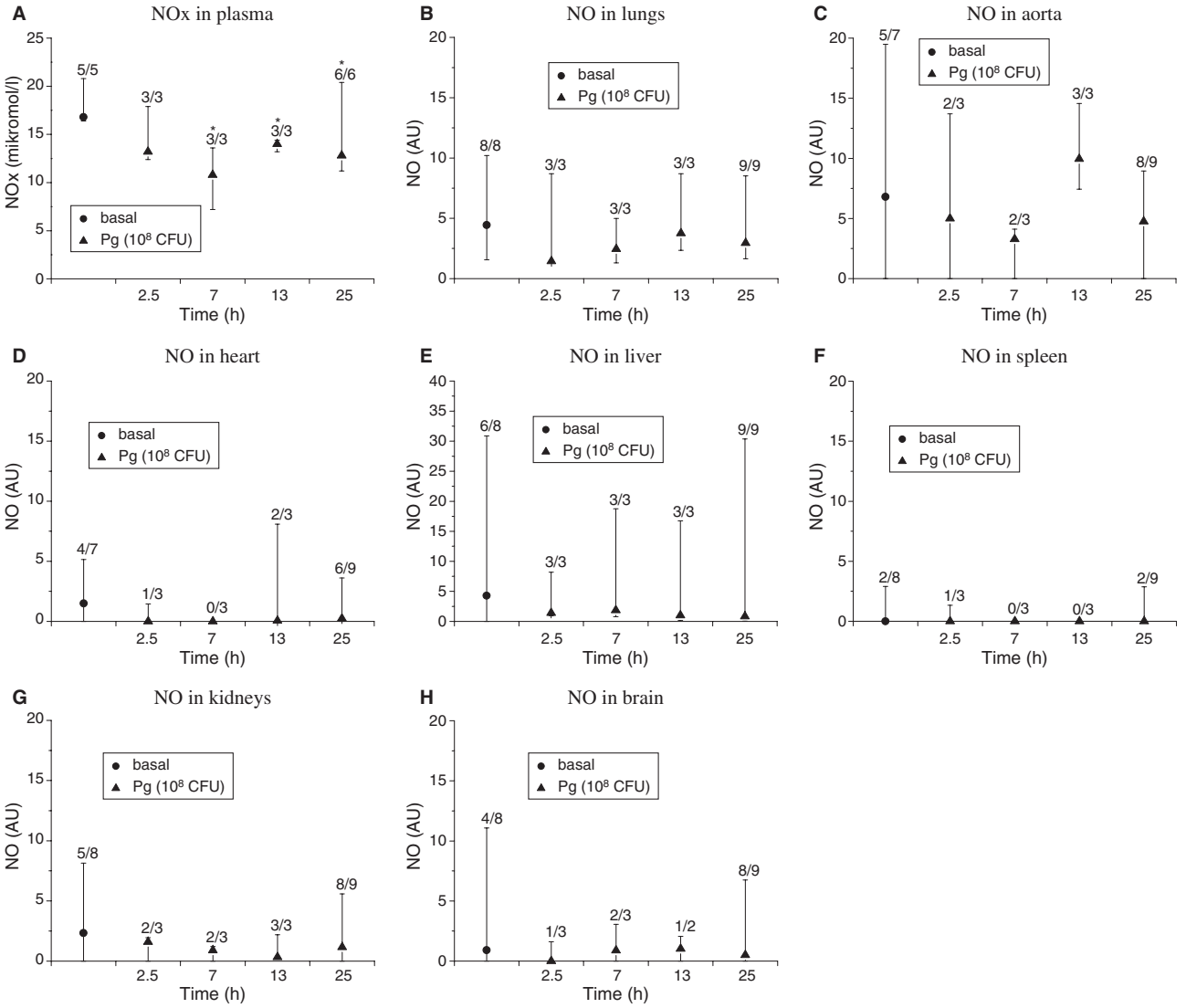

Fig. 2. The time-dependent production of nitrite and nitrate (NOx) in plasma (A) and of nitric oxide (NO) in organs (B–H) from inoculated mice. (B) NO in lungs; (C) NO in aorta; (D) NO in heart; (E) NO in liver; (F) NO in spleen; (G) NO in kidneys; (H) NO in brain. Asterisks indicate statistically significant difference between non-inoculated and inoculated mice (i.e. between basal and stimulated NO levels). Circles and triangles indicate medians, bars indicate ranges. Numbers above bars indicate positive and number of actually tested organs per group (damaged samples were excluded).

The correlation between NOx in plasma and NO in organs was positive for liver (Spearman correlation coefficient = 0.81, $P = 0.0025$) and marginal for kidneys (0.58, $P = 0.059$).

Discussion

During periodontal disease bacteria are swallowed and sometimes aspirated (22), so oropharyngeal lymphoid tissues, gastrointestinal tract, and lungs are possible sites of infection or antigen exposure in addition to the oral tissues. Oral gavage of conscious mice was used in this study to simulate natural exposure while avoiding any interference with cytokine responses from anesthetic agents, that would be required for intratracheal/intranasal inoculation of mice (18, 20, 30). A preliminary radiographic study showed that oral gavage of 0.1 ml of radiographic contrast medium (Omnipaque 300; Amersham Health, Carrigtohill, Cork, Ireland) resulted in swallowing of a significant part of the dose in all animals with less consistent aspiration. Gastrointestinal tract infection is unlikely, *P. gingivalis* ATCC 33277 being acid-sensitive (29), but LPS reaches the intestines, potentially inducing systemic effects as described for enteric bacterial LPS (33).

The end-products of NO metabolism present in blood are primarily nitrates with small amounts of nitrites (NOx) (6, 32), so systemic NO levels were evaluated by measuring NOx in plasma. All mice were fed the same diet to avoid any effects of food on plasma nitrate (6, 8) and the similar levels of NOx recorded in groups 1 and 2 show that NO trapping did not influence plasma NOx. A downward trend of NOx levels was observed after *P. gingivalis* inoculation, reaching the lowest value and statistical significance 7 h after inoculation. Earlier changes were probably not detected because of the relatively long half-life of nitrate in plasma (1.54 h) (32).

Nitrate in plasma/serum has been proposed as an index of immune system activation after LPS inoculation reflecting NO production in tissues (6, 31), though not everyone agrees (14). Plasma NOx levels are considered to be indicative of the amounts of nitric oxide synthase (NOS)

induced *in vivo* (32), although NO can also be formed non-enzymatically (2). To determine organ NO production and its contribution to plasma NOx, we measured $FeNO(DETC)_2$ triplet EPR signal intensities (Fig. 1), which are proportional to the amount of tissue-derived NO (2, 3, 27). As previously found, NO was detected in organs of spin-trap-treated control mice (5). This may derive from constitutive NOS isoforms, inducible NOS in unrecognized inflammation, or non-enzymatic NO production (2). In inoculated animals the trend for organ NO formation was a decrease, consistent with that seen affecting plasma NOx levels, although the correlation was only statistically significant for liver. It therefore seems that single peroral inoculation of mice with *P. gingivalis* reduces the systemic NO response in the acute phase. This may be a result of the enhanced NO consumption/breakdown or of down-regulation of NO production (17).

Endotoxin-induced S-nitrosylation of target cell proteins (17) could lead to a decrease in EPR-detectable NO, but other metabolic pathways may be involved (6, 16).

Lipoprotein–LPS complexes attenuate hepatocellular responses to proinflammatory cytokines, reducing NO production in liver; this effect was detected 2 h after exposure, peaked after 6 h and was indistinguishable from the controls after 40 h (11), similar to the NO trends observed in our study.

A single intratracheal challenge of mice with sonicated *P. gingivalis* ATCC 33277 enhanced the soluble tumor necrosis factor-α receptor ratio (sTNFR2 : sTNFR1) in serum 2 h after inoculation, which may suppress TNF-α effects (20) and it is suggested that sTNFR can diminish NO production (9). *P. gingivalis* LPS is also a poor activator of interleukin-1β and TNF-α production (15, 21), which are major activators of iNOS (12).

As NO from iNOS is an important element of the host defense against *P. gingivalis* (1), reduced levels probably have debilitating effects on the acute host response to infection with *P. gingivalis*, facilitating bacterial colonization and resulting in tolerance of the body to the presence of bacteria (15).

At later time points a greater proportion of animals showed NO production in different organs, which might indicate a delayed, but still ineffective, host response, explaining why *P. gingivalis* remains recalcitrant to elimination by the immune system (15). It will therefore be interesting to see if there is a systemic NO response at later time points or with chronic stimulation as the local pathogenesis of periodontal disease involves production of large amounts of NO (25).

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