

Chronic ingestion of *Porphyromonas gingivalis* induces systemic nitric oxide response in mice

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Introduction: *Porphyromonas gingivalis* induces nitric oxide (NO) production in various cells, systemic NO elevation being expected in chronic oral challenge.

Methods: Groups of BALB/c mice were inoculated orally with either live *P. gingivalis* ATCC 33277 or sterile broth on days 0, 2 and 4, with or without later administration of the inducible nitric oxide synthase (iNOS) inhibitor 1400W. Plasma and tissues were harvested on day 42 for assays of tumor necrosis factor- α (TNF- α), nitrite and nitrate (NOx) and tissue NO, or histology and iNOS immunohistochemistry.

Results: No signs of gingivitis were observed, but plasma NOx was significantly elevated ($P = 0.028$) as was TNF- α ($P = 0.079$) in *P. gingivalis*-inoculated animals compared with controls, NOx being reduced when 1400W was used. NO production in organs showed a similar trend, with significant elevation in liver ($P = 0.017$) and kidneys ($P = 0.027$), whereas concomitant treatment of inoculated animals with 1400W caused significant reductions in NO in aorta ($P = 0.008$) and kidneys ($P = 0.046$). Sham-inoculated 1400W-treated animals had significantly increased plasma NOx ($P = 0.004$) and liver NO ($P = 0.04$). NOx in plasma correlated significantly with NO production in lungs (0.35, $P = 0.032$) and kidneys (0.47, $P = 0.003$). Immunohistochemistry demonstrated iNOS activity in many tissues in all groups.

Conclusion: Repeated oral administration of *P. gingivalis* induced systemic NO and NOx production in mice, probably by activating iNOS as suggested by the response to 1400W.

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Key words: *Porphyromonas gingivalis*; systemic nitric oxide; 1400W; chronic oral challenge; mice

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Swallowing and aspiration of bacteria from the oral cavity are common sequelae of periodontal disease (36, 37). *Porphyromonas gingivalis*, or its lipopolysaccharide (LPS) alone, can interfere with the cytokine network modulating a host response in distant tissues (26). *P. gingivalis* LPS is reported to induce nitric oxide (NO) formation in both immune and non-immune cells by inducing the expres-

sion of inducible nitric oxide synthase (iNOS) (6, 9, 21, 38); however, during acute oral *P. gingivalis* ATCC 33277 challenge, systemic NO seems to be reduced (33). The aim of this study was to determine whether the systemic NO response was the same in mice exposed to a more chronic oral challenge with the recognized periodontal pathogen *P. gingivalis* ATCC 33277.

Materials and methods

Animals

Thirty-five specific pathogen-free female BALB/c mice between 2 and 3 months of age were included in the study. All animals received standard laboratory chow (Teklad Global 16% Protein Rodent Diet, Harlan Italy, San Pietro al Natisone, Italy) before and during the experiment and boiled

water (containing 2% glucose to enhance dental plaque accumulation) *ad libitum* following Veterinary Administration of the Republic of Slovenia approved animal protocol No. 323-02-187/2004/2.

P. gingivalis culture

The *P. gingivalis* ATCC 33277 was maintained frozen in a sterile vial containing porous beads, which serve as carriers to support microorganisms (Microbank; Pro-lab Diagnostics, Richmond Hill, ON, Canada), and was kept at -70°C . Bacteria were cultured as described previously (12, 14) by inoculation on brain–heart infusion agar (Oxoid Ltd, Basingstoke, UK) plates, supplemented with 10 mg/ml [weight/volume (W/V)] yeast extract, 0.15 mg/ml (W/V) haemin (Sigma-Aldrich Inc., St Louis, MO), 0.075 ml/ml (V/V) menadione (Sigma-Aldrich), and sheep blood 5% (V/V). Cultures were incubated for at least 72 h in an anaerobic environment (GENbox anaer; bioMerieux, Marcy l’Etoile, France). Challenge material was prepared on brain–heart infusion (Oxoid Ltd) liquid medium supplemented by 10 mg/ml (W/V) yeast extract, 0.15 mg/ml (W/V) haemin, 0.075 ml/ml (V/V) menadione. The cell concentration was adjusted to approximately 10^9 bacterial cells per millilitre (10^9 colony-forming units/ml).

Experimental design

The mice were randomly divided into six groups housed separately as detailed in Table 1 and were given a week for accli-

matization before being orally inoculated with 0.3 ml sterile broth [brain–heart infusion liquid medium supplemented with 10 mg/ml (W/V) yeast extract, 0.15 mg/ml (W/V) haemin, 0.075 ml/ml (V/V) menadione] or the same type of broth containing *P. gingivalis*. For this purpose the broth was mixed with carboxymethylcellulose to enable retention of the slurry on oral surfaces (14). Inoculation was performed on days 0, 2 and 4 (i.e. at 48-h intervals) using a 20G blunt cannula and 1-ml plastic syringe. The slurry was slowly delivered to the buccal region, avoiding aspiration.

Groups 4 and 6 (Table 1) were given the selective iNOS inhibitor 1400W (((N-(3-aminomethyl)benzyl)acetamide), dihydrochloride; Sigma-Aldrich), 10 mg/kg intraperitoneally, in 0.1 ml water for injection (11, 28) every 6 h for the last 5 days of the experiment.

When used, the spin trap required for electron paramagnetic resonance (EPR) measurements was administered in two parts (5). Diethyldithiocarbamate (DETC; diethyldithiocarbamic acid.sodium salt.trihydrate; Alexis Biochemicals, Alexis Corporation, Lausen, Switzerland) was given intraperitoneally at a dose rate of 500 mg/kg body weight in 0.1 ml water for injection (Aqua ad iniectionabilia; B. Braun Melsungen AG, Melsungen, Germany) immediately followed by subcutaneous injection of a mixture of ferrous sulphate (Johnson, Matthey & Co. Limited, London, UK) 100 mg/kg and sodium-citrate (Alkaloid, Skopje, Macedonia) 500 mg/kg, again in 0.1 ml water for injection. The

components are distributed throughout the body entering the tissues where $\text{Fe}(\text{DETC})_2$ is created. This reacts with any NO produced in the tissues, especially in cell membranes, and results in the formation of a stable $\text{FeNO}(\text{DETC})_2$ adduct which gives a measurable EPR signal with a typical g-factor ($g = 2.04$) (5, 20, 47, 48). Spin trap was given 60 min before sacrifice (33).

EPR measurements

Mice were sacrificed 42 days after the first inoculation and the organs (lungs, thoracic aorta, heart, liver, spleen, kidneys and brain) were harvested immediately thereafter as previously described (33) and frozen in liquid nitrogen until the EPR measurements were performed. The EPR spectra (5) were recorded on an X-band EPR spectrometer Bruker ESP 300 (Bruker Instruments, Inc., Billerica, MA), at 130°K . The EPR spectra intensity, suggestive of the amount of NO in tissues, was measured as the height of the triplet $\text{FeNO}(\text{DETC})_2$ signal (40, 43). Signal heights were normalized with respect to the mass of each sample and the relative intensities were recorded as adjusted units (AU).

Plasma tumor necrosis factor- α and nitrite/nitrate assay

Blood was taken immediately post mortem by cardiac puncture and plasma preparation was performed as previously described (33). The measurements of tumor necrosis factor- α (TNF- α) concentration and combined nitrite and nitrate (NOx) concentration in plasma were performed by a blinded observer. Plasma TNF- α concentration was determined on samples from sham-inoculated and *P. gingivalis*-inoculated mice using an enzyme-linked immunosorbent assay test (Assay Designs Inc., Ann Arbor, MI).

NOx concentration was determined in samples from both control and experimental mice, from groups that had or had not received iNOS inhibitor, using a colorimetric non-enzymatic assay (Neogen Corporation, Lexington, KY) following deproteinization of samples with zinc sulphate and conversion of nitrate to nitrite using metallic cadmium, nitrite then being measured using Griess reagent (8, 45).

Oral bacteria sampling

Oral swabs were obtained from the mice immediately post mortem for inoculation directly on to brain–heart infusion agar

Table 1. Experimental groups and the number of animals included in each experimental group

Group	Procedure	Number of mice
1	Native mice. Immediate sacrifice.	3
2	Spin trap-treated ¹ control group. Sacrificed 60 min after spin trap injection.	8
3	Sterile broth inoculation (0.3 ml) + 0.2 ml 2% CMC ² per os on days 0, 2 and 4, spin trap administered 60 min before sacrifice on day 42.	5 (Originally 6 but 1 died during experiment)
4	Sterile broth inoculation (0.3 ml) + 0.2 ml 2% CMC per os on days 0, 2 and 4, 1400W ³ on days 37 to 42 every 6 h, spin trap administered 60 min before sacrifice on day 42.	6
5	<i>Porphyromonas gingivalis</i> inoculation (0.3 ml 10^9 colony-forming units/ml) + 0.2 ml 2% CMC per os on days 0, 2 and 4, spin trap administered 60 min before sacrifice on day 42.	6
6	<i>P. gingivalis</i> inoculation (0.3 ml 10^9 colony-forming units/ml) + 0.2 ml 2% CMC per os on days 0, 2 and 4, 1400W on days 37 to 42 every 6 h, spin trap administered 60 min before sacrifice on day 42.	6

¹The spin trap consisted of diethyldithiocarbamate (DETC) 500 mg/kg in saline intraperitoneally and a mixture of iron sulfate 100 mg/kg plus sodium citrate 500 mg/kg in saline subcutaneously.

²CMC, carboxymethylcellulose (a thickening agent).

³1400W (((N-(3-aminomethyl)benzyl)acetamide), dihydrochloride) – highly selective iNOS inhibitor; 10 mg/kg in 0.1 ml water for intraperitoneal injection.

(Oxoid Ltd) plates, supplemented with 10 mg/ml (W/V) yeast extract, 0.15 mg/ml (W/V) haemin, 0.075 ml/ml (V/V) menadione and sheep blood 5% (V/V). Plates were incubated for at least 72 h in an anaerobic environment and evaluated for the presence of black-pigmented anaerobic bacteria.

Histology of the jaws

The jaws were fixed in 10% neutral buffered formalin immediately following organ harvest, and then demineralized for 3–4 days in Osteosoft solution (Osteosoft, Merck KGaA, Darmstadt, Germany). Mandibles and maxillae were separated following demineralization. Jaws were then embedded in paraffin, oriented in the buccolingual plane, sectioned at 5 μ m, adhered to glass slides and stained with haematoxylin & eosin (H&E; right maxilla and mandible) or toluidine blue (TB, pH 2; left maxilla and mandible). Histology of gingival tissues was evaluated microscopically on H&E-stained sections and mast cells were counted in the gingiva of TB-stained sections.

Immunohistochemistry of lungs, livers and kidneys

Left lung lobes, left liver lobes and right kidney were fixed immediately after harvesting using Bouin's solution, changing to 70% ethanol after 24 h. Organs were then routinely embedded in paraffin and 5- μ m sections were prepared. Organ slides were dewaxed, rehydrated through graded ethanol, washed in Tris-buffered saline (TBS), treated with 3% H₂O₂ in TBS for 20 min to quench endogenous peroxidase activity and washed once in TBS. Antigen retrieval was performed by incubating slides in boiling 0.01 M sodium citrate (pH 6) for 20 min in a microwave oven operating at full power (750 W) and allowed to cool for 20 min before being washed once in TBS. Blocking of non-specific sites was performed with normal goat serum (Millipore, Billerica, MA) in TBS (1 : 5 dilution) for 60 min at room temperature and tissue sections were thereafter incubated with specific primary antibody (anti-iNOS rabbit polyclonal antibodies, anti-iNOS/NOSII, NT, Millipore, 1 : 100 dilution) or normal goat serum (control slides) at 4°C overnight. After thorough rinsing with TBS, the sections were incubated with a secondary antibody (goat antirabbit antibodies; Jackson Immunoresearch, West Grove, PA, 1 : 100 dilution in TBS) at room temperature for 30 min, washed twice in TBS and then incubated with peroxidase-antiperox-

idase complex (PAP, Jackson Immunoresearch) at room temperature for 30 min. Slides were washed twice in TBS before specific binding with diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich) in the presence of 0.01% H₂O₂ for up to 20 min (depending on the organ) was detected. The slides were counterstained with haematoxylin, dehydrated through a graded ethanol series, cleared in xylene and mounted with Pertex mounting medium (Medite, Burgdorf, Germany) before being examined microscopically.

Statistical analysis

A statistical software package (SAS 9.00, SAS Institute Inc., Cary, NC) was used for analysis of the results, values of $P < 0.05$ being regarded as significant. Normality of distribution was tested by univariate analysis of NOx, NO, TNF- α and mast cell number 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. Additionally, Fisher's exact test was used to detect any differences in iNOS immunoreactivity (described as positive or negative) in different regions of kidneys of mice from different groups.

Results

Culture and histology of the gingival tissues

No black-pigmented anaerobic bacteria were cultured from any of the oral cavity swabs.

A few polymorphonuclear cells were present in the subepithelial vascular plexus but no subepithelial round-cell infiltrates were observed in the gingival tissues of mice in any of the groups. The junctional epithelium was normal and there was no detectable epithelial attachment loss, therefore all the specimens were described as having clinically healthy gingiva (51). No differences were observed between mice in different groups regarding the occurrence of mast cells in gingival submucosa (data not shown).

TNF- α and NOx in plasma

TNF- α was elevated in *P. gingivalis*-inoculated animals compared with sham-inoc-

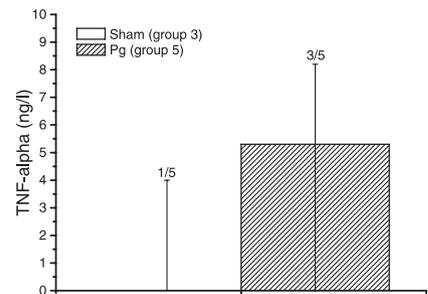


Fig. 1. Tumor necrosis factor- α (TNF- α) plasma levels of experimental mice. Columns indicate medians, bars indicate ranges. Numbers above bars indicate positive and number of actually tested plasma samples per group.

ulated mice, though the difference did not reach statistical significance ($P = 0.079$) (Fig. 1).

There was a detectable level of NOx in all plasma samples of native and spin-trap-treated native mice with no statistically significant difference between the two groups. When NOx levels in plasma samples from sham-inoculated mice from group 3 (Table 1) were compared with NOx plasma levels from *P. gingivalis*-inoculated mice from group 5, there was significant ($P = 0.028$) elevation of NOx plasma concentration in *P. gingivalis*-inoculated animals. When NOx plasma levels were measured in 1400W-treated *P. gingivalis*-inoculated mice (group 6), a reduction of NOx levels was observed, although the drop was not statistically significant in comparison with *P. gingivalis*-inoculated mice and did not reach the basal (group 3) NOx levels. When sham-inoculated mice were treated with the iNOS inhibitor only (group 4), a statistically significant ($P = 0.004$) increase in NOx plasma levels was observed compared with the sham-inoculated mice (group 3) (Fig. 2).

EPR spectra of organs

There was an EPR-detectable NO level in all the organs of native and sham-inoculated spin-trap-treated mice, although the signal was not present in all the mice. However, the signal typically appeared in most animals and was always higher in the organs of *P. gingivalis*-inoculated mice, reaching statistical significance in livers ($P = 0.017$) and kidneys ($P = 0.027$) when comparing these with the sham-inoculated mice from group 3. In *P. gingivalis*-inoculated animals the signal was reduced in all organs except the spleen when the iNOS-selective inhibitor 1400W was used; when 1400W was used in sham-inoculated animals, the NO signal typically increased, reaching statistical significance in livers

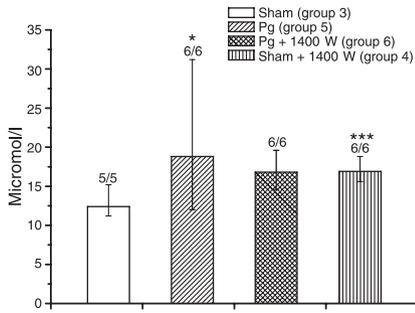


Fig. 2. Nitrite and nitrate (NOx) plasma levels of experimental mice. *Statistically significant difference between sham-inoculated and *Porphyromonas gingivalis*-inoculated mice. ***Statistically significant difference between sham-inoculated and sham-inoculated/1400W-treated mice. Columns indicate medians, bars indicate ranges. Numbers above bars indicate positive and number of actually tested plasma samples per group.

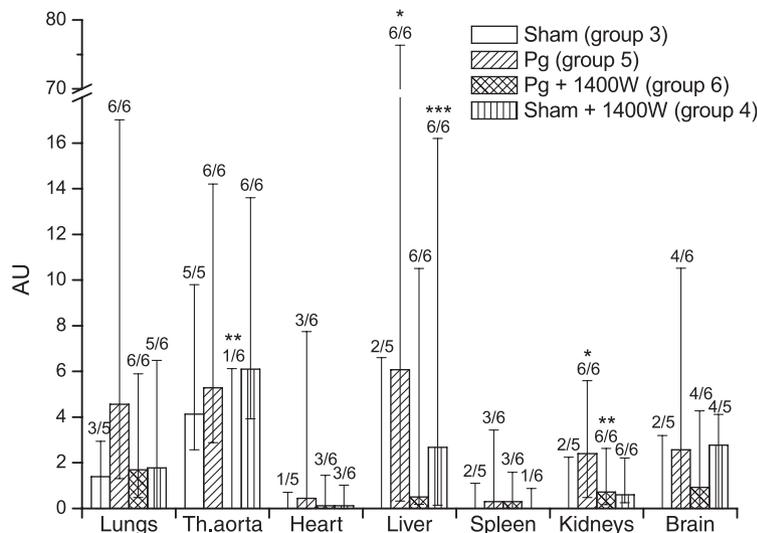


Fig. 3. Production of nitric oxide (NO) in organs from experimental mice. *Statistically significant difference between sham-inoculated and *Porphyromonas gingivalis*-inoculated mice (i.e. between basal and stimulated NO levels). **Statistically significant difference between *P. gingivalis*-inoculated and *P. gingivalis*-inoculated/1400W-treated mice. ***Statistically significant difference between sham-inoculated and sham-inoculated/1400W-treated mice. Columns indicate medians, bars indicate ranges. Numbers above bars indicate positive and number of actually tested organs per group (damaged samples were excluded).

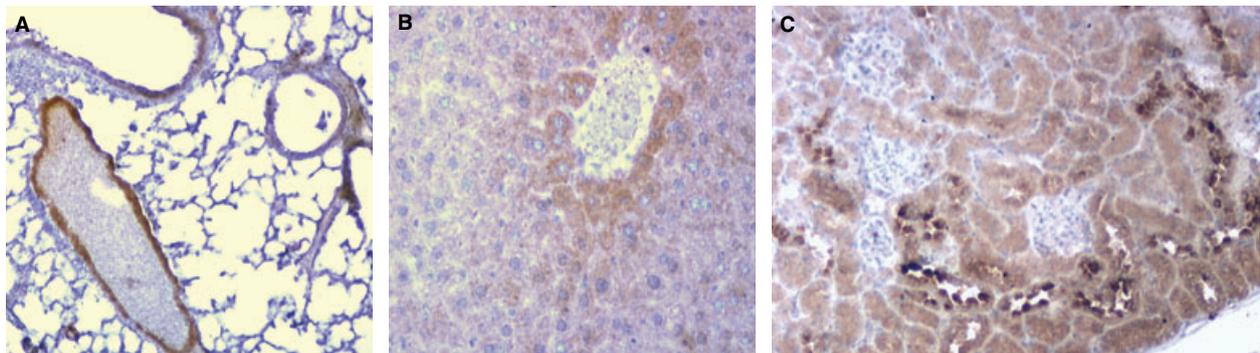


Fig. 4. Inducible nitric oxide synthase (iNOS) immunoreactivity in (A) lungs, magnification 40 ×; (B) livers, magnification 200 ×; and (C) kidney-cortex, magnification 100 ×. Nuclei were counterstained with haematoxylin.

($P = 0.04$) (Fig. 3). The NO level reduction in *P. gingivalis*-inoculated animals was statistically significant in thoracic aorta ($P = 0.008$) and kidneys ($P = 0.046$), but it only reached basal (group 3) level in the aorta.

The correlation between NOx in plasma and NO in organs was statistically significantly positive for lungs (Spearman correlation coefficient = 0.35, $P = 0.032$) and kidneys (0.47, $P = 0.003$) and marginal for heart (0.30, $P = 0.069$) and liver (0.30, $P = 0.069$).

Immunohistochemistry of lungs, livers and kidneys

No differences in immunostaining were observed between the lungs, livers and

kidneys of mice from different experimental groups.

There were scattered round-cell infiltrates throughout the lung parenchyma in mice from both control and *P. gingivalis*-inoculated groups. These did not express any iNOS immunoreactivity, but distinct iNOS immunoreactivity was observed in the muscular layer of the large vessels in all lung samples (Fig. 4A).

Weak iNOS immunoreactivity was observed in hepatocytes from all animals in the study, the staining sometimes being more prominent around the central vein (Fig. 4B) and on the periphery of lobes.

The majority of kidneys from mice in all groups were immunopositive for iNOS and there was no difference between the groups regarding the proportion of iNOS-positive samples. Cells exhibiting iNOS immunoreactivity were present in the outer cortex (Fig. 4C), outer medulla and, in up to 50% of cases, also in the papilla.

Discussion

Triple peroral *P. gingivalis* exposure after a course of antibiotic therapy is described to induce periodontal disease at 42 days in mice (3, 14), but in our study, where we wanted to avoid any potential interference with NO formation by the use of any drugs before or during the experiment, we failed to induce any histologically evident periodontal disease or even gingivitis. This might be a result of the absence of any live black-pigmented anaerobic bacteria in the mouth at 42 days, although the sampling method may not have detected them if only low numbers of organisms were present (14). It is, however, important to know, that *P. gingivalis* strain ATCC 33277 can survive within epithelial cells in a non-cultivable stage but capable of inducing an inflammatory response (7).

There was a significant increase in NO and NO_x production in *P. gingivalis*-inoculated mice, with the NO at least partially deriving from iNOS, as determined by the partial reduction in NO and consequently NO_x formation with the use of the iNOS selective inhibitor 1400W. There was a mild pneumonia observed in both control and *P. gingivalis*-inoculated mice. This was most likely a sequel of stress from moving the animals from specific pathogen-free housing to the new environment of the experimental animal house where they would be exposed to airborne environmental organisms. The body's response to this exposure is probably the main cause of the 'basal' NO production detected in control animals, although no iNOS immunoreactivity was observed in round-cell lung infiltrates.

As mice were carefully inoculated to avoid aspiration and there was no detectable periodontal response, the observed increase in systemic NO and NO_x production seen following oral *P. gingivalis* exposure was most likely the result of swallowing the bacterium and/or its products/components. Several sites of bacterial/antigen exposure are possible. Besides the inflammatory reaction of cells in which *P. gingivalis* is living intracellularly (7), initial reactions to plaque involve stimulation of the junctional epithelium by bacterial products and components resulting in cellular production of inflammatory mediators (51). Both viable and sacrificed *P. gingivalis* can induce gingival fibroblasts to produce various proinflammatory cytokines (39). Additionally, the LPS of gram-negative oral anaerobes is reported to stimulate a mast cell response (1, 27, 50). However, gingival mast cell involvement in our study is unlikely because there were no differences observed in occurrence, location or degranulation of mast cells in the gingival tissues of animals from different groups as determined by toluidine blue staining (50).

Oropharyngeal lymphoid tissues play an important role in initiating immune responses against antigens entering the body through the mouth (4, 25). The gastrointestinal tract is replete with mast cells (27) and unstimulated intestinal epithelial cells also express the Toll-like receptors TLR2 and TLR4, which are important for sensing pathogens, with these receptors being upregulated after LPS challenge (41). Although gastrointestinal tract infection is unlikely as *P. gingivalis* ATCC 33277 is acid-sensitive (42), LPS might reach the intestines, thereafter being delivered to the blood-

stream with the potential to induce systemic effects as described for the LPS of enteric bacteria (53). It is therefore possible that intestine with its extensive mucosal immune system plays an important role in the induction of innate immunity in *P. gingivalis* infections as has been suggested for peroral infection of mice with *Toxoplasma gondii* (10) or as with commensal bacteria and/or their products (19).

Regardless of the bacterial/antigen exposure site, it is important to realize that at the time of sampling there was still an active infection stimulating the systemic host response. This was suggested also by the elevated plasma TNF- α , which very rapidly disappears from the circulation (46) and is also an important activator of iNOS (17).

Body excretory systems (mainly kidneys and liver) are responsible for the elimination of LPS and TNF- α from the circulation (46, 52). NO upregulation in kidneys and liver after oral *P. gingivalis* challenge may therefore play a role in this mechanism, although many other functions of NO are possible (30, 35). Although NO most likely derives from iNOS activity in the kidneys, as suggested by the use of the iNOS selective inhibitor, this source does not seem to be the sole or most important source (24) in most organs because NO did not fall to basal levels after inhibiting iNOS in *P. gingivalis*-inoculated animals. It is, however, possible that the inhibitor had an incomplete effect, but at the same time, no changes in immunopositive signal for iNOS were observed between *P. gingivalis* challenged and non-challenged mice. Although iNOS is reported to be expressed in normal human liver, distributed mainly periportal (29), it is not reported as a normal finding in the liver of mice (13). As immunohistochemistry is not a quantitative method, only detecting the presence of the protein, it does not give any information about the changes of enzyme activity and also provides a relatively poor detection signal of iNOS protein in kidneys (16), other methods, especially enzymatic activity measurements, would be necessary to detect any differences in iNOS activity between experimental groups. However, it is also possible, that iNOS is activated elsewhere, NO produced being stabilized, stored, transported to kidneys and liver with subsequent release to have remote effects as suggested previously (22, 32). Further studies are needed to determine the site of NO production and its effects in kidneys and liver after chronic oral *P. gingivalis* challenge and potentially in periodontal disease.

Our data also suggest that the iNOS inhibitor 1400W acts differently in inoculated and healthy animals/tissues, promoting NO and NO_x formation in healthy animals/tissues. This may be a sequel of NOS cross-talk disturbance (34), iNOS being constitutively expressed in certain cells (24, 35), as was also suggested by the immunohistochemistry of control tissue samples in our study. However, it could also be a sequel of non-enzymatic NO production as described associated with the use of some other NOS inhibitors (31) or even result from repeated intraperitoneal injection of 1400W itself, which requires further evaluation.

Nitrite and nitrate are stable end products of NO metabolism that are present in blood and urine (45). Plasma/serum nitrate is reported to reflect the level of NO produced in tissues, so it has been proposed as an index of immune system activation after LPS inoculation (8, 15, 18, 44, 49) although this is not universally accepted (23, 45) and increased nitrate production may only be associated with certain pathogens or sites of infection (2). As we have demonstrated that plasma NO_x concentration correlated well with NO produced in the organs, NO_x would appear to be a useful biomarker of NO production in experimental *P. gingivalis* infections and potentially even for naturally occurring periodontal disease.

It is difficult to compare the results of the present study with those of our previously described study on acute NO response to *P. gingivalis* inoculation (33) mainly because of differences in the inoculation method. However, looking at the systemic NO production it seems likely that host response to *P. gingivalis* occurs with chronic stimulation only, while in the acute phase reduced NO production could indicate tolerance of the body to the presence of *P. gingivalis* (26).

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