Induction of Nitric Oxide Production by Polysides from the Cell Walls of *Streptococcus mutans* OMZ 175, a Gram-Positive Bacterium, in the Rat Aorta

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The cardiovascular dysfunctions associated with septic shock induced by gram-negative or gram-positive bacteria (gram-positive or gram-negative septic shock) are comparable. In gram-negative septic shock, lipopolysaccharide (LPS) induces nitric oxide (NO) synthase, which contributes to the vascular hypotension and hyporeactivity to vasoconstrictors. The role of NO in gram-positive septic shock and the nature of the bacterial wall components responsible for the vascular effects of gram-positive bacteria are not well known. This study investigated the vascular effects of cell wall serotype polysides, rhamnose glucose polymers (RGPs), from *Streptococcus mutans*, in comparison with lipoteichoic acid (LTA) from *Staphylococcus aureus*, on the induction of NO synthase activity in the rat aorta. We show that 10 μg of both RGPs and LTA per ml induced hyporeactivity to noradrenaline, l-arginine-induced relaxation, increases of 2.2- and 7.8-fold, respectively, of cyclic GMP production, and increases of 7- and 12-fold in nitrite release. All of these effects appeared after several hours of incubation and were inhibited by N^\text{\textcircled{2}}\text{-nitro-L-arginine methyl ester (L-NAME)}, an inhibitor of NO synthase. Electron paramagnetic resonance spin trapping experiments demonstrated directly that RGPs and LTA induced NO overproduction (four- to eightfold, respectively) in rat aortic rings; this production was inhibited by L-NAME and prevented by dexamethasone. These results demonstrate directly the induction of NO production in vascular tissue by LTA and show that another, chemically different component of gram-positive bacteria can also have these properties. This result suggests that different components of the gram-positive bacterial wall could be implicated in the genesis of cardiovascular dysfunctions observed in gram-positive septic shock.

Septic shock is an urgent medical problem, with more than 200,000 cases occurring each year with an associated mortality rate of 20 to 80% in the United States (39). Septic shock can be induced by gram-negative as well as by gram-positive bacteria. Up to now, the importance of gram-negative organisms in the genesis of sepsis has been emphasized. However, recent studies show an increasing incidence of gram-positive sources of sepsis (6).

Lipopolysaccharide (LPS; the glycolipid part of endotoxin), a component of the bacterial cell wall (41), is considered the single contributor involved in eliciting gram-negative sepsis. Administration of endotoxin in animals (37) or humans (51) reproduces the signs and symptoms of sepsis. LPS by itself and/or through releasing a number of cytokines causes expression of inducible nitric oxide synthase (i-NOS) in a wide variety of cells and tissues. Overproduction of nitric oxide (NO) as a result of i-NOS activity in vascular tissue contributes greatly to hypotension and hyporeactivity associated with endotoxin shock (49, 52).

The cardiovascular dysfunctions, the inflammatory responses, and the level of mortality associated with gram-positive- or gram-negative-bacterium-induced sepsis (gram-positive or gram-negative sepsis) seem to be comparable in spite of the absence of LPS in the gram-positive bacterial cell wall (1, 7, 24). Enhanced formation of NO may also contribute to the circulatory failure in gram-positive sepsis. This is supported by findings demonstrating that (i) killed *Staphylococcus aureus* (10) can induce the expression of i-NOS in macrophages and (ii) lipoteichoic acid (LTA), a component associated with the membrane of gram-positive bacteria (60), can induce the expression of i-NOS in cultured vascular smooth muscle cells (31) or in macrophages (30) in vitro. In addition, the delayed circulatory failure caused by LTA in the rat was prevented by continuous infusion of N^\text{\textcircled{2}}\text{-nitro-L-arginine methyl ester (L-NAME); an inhibitor of NO synthase} (12). LTA has therefore been implicated as the major component of the gram-positive bacterial cell wall which is itself and/or via cytokine release responsible for the induction of i-NOS.

Some data suggest that in addition to LTA, some other component(s) of the gram-positive bacterial wall may contribute directly or indirectly to the induction of i-NOS. Another bacterial wall component, peptidoglycan G, does not induce i-NOS but enhances greatly the effect of LTA (13). Bacterial serogroup polysides of *Streptococcus mutans* OMZ 175 (rhamnose glucose polymers [RGPs]) induce the release of several cytokines, including tumor necrosis factor alpha (TNF-α) (3), which is known to be a powerful inducer of i-NOS activity (18, 36, 37, 45).

While indirect data suggest that i-NOS activity can be induced by LTA in vascular tissue, actual elevated NO levels in this situation have not been measured directly. In this study, we applied the electron paramagnetic resonance (EPR) spin trapping approach to characterize semiquantitatively NO production (34, 56) in order to investigate NO level in LTA-treated vascular tissue. The long-term influence of RGPs on the contractile property of vessels is not known. Using both indirect
Materials. Drugs were obtained from Sigma, St. Louis, Mo. All cell culture media used were obtained from Gibco BRL Life Technology, Cergy Pontoise, France, except for LPS (Escherichia coli O55:B5) used was from Difco, Detroit, Mich. LTA from S. aureus was obtained from Sigma.

RGPs were prepared by lyophilization Streptococcus mutans OMZ 175 (serotype I) by the method of Hamada et al. (20), modified by Benabdelmoumen et al. (3). Briefly, polysaccharides were obtained from supernatant of autoclaved bacterial cells, and charged components were eliminated by chromatography on a DEAE-Trisacryl M column equilibrated with a 0.01 M Tris-HCl buffer (pH 8.2). Unabsorbed fractions were assayed for proteins, carbohydrate, and phosphorus, and samples containing only carbohydrates were incubated with CNBr-pronase (24 h at 25°C) and further chromatographed on a DEAETrisacryl column as described above (3). The serotype polysaccharides used in this study had a molecular weight of 6 × 10^3, contained >98% neutral sugars by weight, were free of endotoxin (< 0.00003%) by EPR spectroscopy, and had only trace amounts of protein (<0.05%) and phosphorus (<0.01%).

Functional studies. (i) Effects of RGPs on the contractile response to NA of rat aortic rings. Male Wistar rats (11 weeks old) were killed by cervical dislocation. The thoracic aorta was removed and cleaned of adherent tissues, and 2-mm-long rings were prepared. Rings with endothelium were incubated for 4 h with RGPs (10 μg/ml) in Dulbecco's modified Eagle's medium at 37°C in a humidified incubator gassed with 5% CO2 in air. The endothelium was removed just before the rings were mounted in organ bath chambers as previously described (26). The absence of functional endothelium is checked by the failure of acetylcholine (1 μmol/liter) to cause a 60% relaxation on LTA-incubated rings.

(ii) Effect of RGPs on the vasomotor response of precontracted aortic rings to l-arginine. When the maximal tension was stable (stable tension after the addition of 3 μmol of NA per liter), the effect of 1 mmol of l-arginine per liter was tested. The relaxation induced by l-arginine on precontracted rings is an index of the induction of i-NOS (49). When the tension after l-arginine addition was stable, 1 mmol of l-NAME per liter was added. Therewith, when this latter effect of l-NAME was stabilized, 5 μmol of methylene blue (MBr, an inhibitor of the activation of the soluble guanylyl cyclase) per liter was added. Results are expressed as tension developed (grams) per milligram of dried tissue, and values shown are the means ± standard errors of the means of n experiments.

(iii) Influence of the endothelium on the effects of RGPs on the vasomotor response of NA of rat aortic rings. Rat aortic rings with or without endothelium were incubated for 4 h with RGPs (10 μg/ml) as described above. Rings with endothelium were gently rubbed with forceps in order to remove the endothelium just before they were mounted in organ bath chambers. The contractile response to NA and the effect of l-arginine on precontracted rings were tested at t1, t2, t3, t4.

Biochemical effects of RGPs and LTA on rat aortic rings. (i) Determination of tissue cyclic GMP content. Rat aortic rings with endothelium were incubated for 4 h in MH10 (minimal essential medium-Ham F10 [vol/vol]) with saline, RGPs (10 μg/ml), LTA (10 μg/ml), or LPS (10 μg/ml). The endothelium was then removed, and rings were placed in baths with bubbled Krebs Henseleit (in millimoles per liter: NaCl 118.4; KCl 3.7; MgSO4.7H2O, 1.2; CaCl2, 2.5; KH2PO4, 2.2; NaHCO3, 24.9; and glucose, 10) with or without l-NAME (1 mmol/liter) for 4 h. Isobutylmethylxanthine (a nonspecific inhibitor of phosphodiesterases; 100 μmol/liter) was added to rings 30 min before t4. At t4, rings were frozen in liquid nitrogen and then placed in 1 ml of 0.1 N HCl. Following homogenization with a glass-glass potter and sonication, the samples were centrifuged. Pellets and supernatants were frozen and kept at −20°C. The cyclic GMP content was determined in the supernatant by using a radioimmunoassay previously described (9), modified by separation of free cyclic GMP with activated charcoal. Protein content was determined in the pellet according to the Bradford method (8), with bovine serum albumin as the standard.

(ii) Assay of NO production. Rat aortic rings with endothelium were incubated for 16 h in Dulbecco's modified Eagle's medium at 37°C in a gassed (95% air–5% CO2) humidified incubator with saline, RGPs (10 μg/ml), LTA (10 μg/ml), or LPS (10 μg/ml). The incubation medium was used for nitrite determination, which was established to measure NO production by spin trapping followed by EPR spectroscopy.

(iii) Nitrilo assay. The accumulation of nitrite was measured in the incubation medium by the Griess reaction. One hundred microliters of Griess reagent [1% sulfanilamide (0.1 M in 5% phosphoric acid) and 0.5% n-ethyl-n-hydroxylamine hydrochloride in 2.5% phosphoric acid] was added to 100 μl of medium. After 10 min of incubation at room temperature, the absorbance of the mixture at 545 nm was measured. Concentrations were determined relative to a standard curve, using sodium nitrite. The amount of nitrite produced was expressed as nanomoles/milligram of dried tissue per 16 h.

(ii) Spin trapping of NO and EPR spectroscopy. For direct detection of NO in rat aortic rings, we used a previously described NO spin trapping approach based on the ability of Fe(DETC)3 to react with NO to form NO-Fe(DETC)2 adducts, which are characterized by an axial EPR signal with g-values = 2.055 and g-values = 2.056 of 1.3 mT at g = 2.055 (35, 56). Briefly, after 16 h of incubation, rings were placed in 1 ml of fresh medium and incubated with sodium diethyldithiocarbamate (DETC; 5 mmol/liter), FeCl3, 7H2O (50 μmol/liter), and sodium citrate (1 mmol/liter) at 37°C for 1 h. Rings were then frozen and lyophilized in liquid nitrogen. EPR spectra were recorded on a Varian E-100E spectrometer at 77 K, using a Dewar flask (Wilmad). EPR settings were 10 mW of microwave power, 0.61 mT of amplitude modulation, a 9.45-GHz microwave frequency, and a 100-kHz modulation frequency. For quantification of NO-Fe(DETC)2 adducts, spectra were analyzed by comparison with standard spectra of paramagnetic (NO)2Fe(S2O3)2+. Concentrations were determined by linear regression, with the standard curve expressed as a percentage of Fe(DETC)2 formed in tissue, a solution of paramagnetic (NO)2Fe(S2O3)2+.

Results. Effects of RGPs and LTA on the contractile response to NA and L-arginine. In vitro, both LPS (26) and LTA (31) are able to decrease the contractile response of the rat aorta to NA. We have investigated the effects of RGPs in a similar experimental setup. In preliminary experiments, RGPs induced a reproducible vascular hyporeactivity at a concentration of 10 μg/ml. This concentration was previously shown to induce the secretion of cytokines from monocytes (48) and was therefore used throughout this study. After incubation of rat aortic rings for 4 h in vitro with 10 μg of RGPs per ml, the contractile response on control was shifted to the right (Fig. 1a). The pD2 for NA after RGP treatment was significantly decreased (8.6 ± 0.1 and 7.9 ± 0.1 for control and treated rings, respectively; P < 0.01; n = 7 for each group). However, the Emax obtained with 3 μmol of NA per liter was not modified by RGPs (3.6 ± 1.2 and 3.5 ± 0.1 g·mg of dried tissue−1 for control and treated rings, respectively).

L-Arginine (1 mmol/liter) induced a significant relaxation on rings treated by RGPs (6.3% ± 2.3% versus 24.9% ± 3.4% for control and treated rings, respectively; P < 0.01 [Fig. 1b]). This l-arginine-induced relaxation was completely reversed by lNAME (1 mmol/liter), an inhibitor of NO synthase, or by McB (3 μmol/liter), an inhibitor of the activation of the soluble guanylyl cyclase by NO.

LTA induced a significant decrease of the pD2 for NA (8.6 ± 0.1 and 7.1 ± 0.2 for control and LTA-incubated rings, respectively; P < 0.01; n = 5 to 7 [data not shown]) and of Emax (3.6 ± 0.2 versus 2.9 ± 0.2 g·mg of dried tissue−1 for control and LTA-incubated rings, respectively; P < 0.05 [data not shown]). The addition of l-arginine induced approximately 56% relaxation on LTA-treated rings.

Influence of the endothelium on the effects of RGPs. The presence of the endothelium during the incubation time increased significantly the effects of RGPs on the contractile response to NA. The pD2 for NA of RGP-treated rings with endothelium was significantly lowered (8.5 ± 0.1 and 7.8 ± 0.1 for control and RGP-treated rings, respectively; P < 0.01 [results not shown]). When rings were incubated without endothelium, RGPs caused only a small, nonsignificant decrease of the pD2 and NA (8.5 ± 0.1 versus 8.2 ± 0.1 g·mg of dried tissue−1 for control and RGP-treated rings, respectively). There was no effect of RGPs on the Emax for NA, whether the endothelium was present or not.

L-Arginine (1 mmol/liter) induced a significant relaxation of RGP-treated rings with endothelium (2.8% ± 0.8% and 26.3%
tion (respectively 7.8- and 7.5-fold increases of cyclic GMP production). The contractile response to cumulative addition of NA (1 nmol/liter) was totally inhibited by an inhibitor of NO synthase, L-NAME (Fig. 2).

Effect of RGPs and LTA on NO generation in aortic rings. (i) Effect on cyclic GMP tissue content. Rat aortic rings with endothelium were incubated with RGPs (10 μg/ml) for 4 h. Intracellular content of cyclic GMP was assayed 8 h after the first contact with the bacterial product. RGPs induced a significant 2.2-fold increase of cyclic GMP level (12.6 ± 3.6 and 28.3 ± 4.7 pmol/mg tissue for control and treated rings, respectively; n = 6 to 8).

In parallel, LTA (10 μg/ml) and LPS (10 μg/ml) induced, respectively 7.8- and 7.5-fold increases of cyclic GMP production (P < 0.01 compared to the control for each treatment) which were totally inhibited by l-NAME (Fig. 2).

(ii) Effect on nitrite production. Incubation of rat aortic rings with endothelium for 16 h with RGPs (10 μg/ml) induced a sevenfold increase in nitrite production (P < 0.01) which was prevented by dexamethasone (10 μmol/liter) (Fig. 3).

LTA or LPS also caused an increase in nitrite formation (12- and 14-fold, respectively; P < 0.01) which was also prevented by dexamethasone (Fig. 3).

(iii) EPR spin trapping experiments: measurement of NO production. Aortic rings with endothelium incubated for 16 h with RGPs (10 μg/ml) and then exposed for 1 h to DETC (5 mmol/liter) and FeSO₄ (50 μmol/liter) exhibited an axial EPR signal with g⊥ = 2.035 and g∥ = 2.02 and triplet structure at g⊥ (Fig. 4). This signal is characteristic of NO-Fe(DETC)₂ complexes, and its amplitude reflects the amount of NO formed (25, 34, 35, 56). Quantitative analysis showed that RGPs induced approximately a fourfold increase in NO-Fe(DETC)₂ complex formation (Fig. 5; P < 0.01) which was prevented by dexamethasone (Fig. 5a; P < 0.01) and l-NAME (Fig. 5b; P < 0.001). LTA (10 μg/ml)- and LPS (10 μg/ml)-treated rings exhibited the EPR signal of the NO-Fe(DETC)₂ complex (Fig. 4). They induced a four- to eightfold increase of NO-Fe(DETC)₂ content, which was prevented by dexamethasone and l-NAME (Fig. 5).

DISCUSSION

NO overproduction has been implicated in the vascular hypotension and hyporeactivity to vasoconstrictors observed in septic shock induced by gram-negative bacteria (49). In an in vivo model, this induction of NO production in the vascular wall causes a decrease both of the mean arterial blood pressure and of the pressor response to vasoconstrictors such as NA (40, 52, 53); in vitro, NO synthase induction causes a decrease of
the contractile effect of NA (17, 26). All of these effects are inhibited by an inhibitor of NO synthase or by dexamethasone, which prevents the induction of i-NOS in the vascular wall (42).

In gram-positive septic shock, the type of bacterial wall components responsible for the vascular disorders and their cellular effects are not well established. It has been shown that different types of killed gram-positive bacteria or LTAs are able to induce NO synthase activity in vascular smooth muscle cells (11), in macrophages (10, 11), or in an in vivo situation (29, 43, 57, 59). Some capsular polysides from the bacterial wall of Streptococcus mutans OMZ 175, RGPs, activate human monocytes to release cytokines (48). Their vascular effects were not known. We therefore decided to explore the effects of RGPs on the NO synthase activity in the vascular wall. First, indirect approaches were used: functional studies, measurement of a stable end product (nitrite), and measurement of mediators generated by NO (cyclic GMP). We have shown that both RGPs and LTA induced in blood vessels hyporeactivity to NA, L-arginine-induced relaxation, increased cyclic GMP production, and nitrite release. All of these effects were inhibited by L-NAME, an inhibitor of NO synthase; the production of nitrite was also prevented by dexamethasone, an inhibitor of the induction of i-NOS (42). These data suggest strongly that RGPs induce NO synthase activity in the vessel wall as well as LTA. Some of the effects of LTA described here have been observed in different experimental systems. Lonchampt et al. have shown an increase in cyclic GMP production by smooth muscle cells and a decrease of the contractile response to NA of rat aortic rings (2, 31); LTA induces nitrite production in vascular smooth muscle cells in culture (11), and in vivo, LTA induces a hypotension and a hyporesponsiveness to NA that seem to be due to the induction of NO synthase activity in the vascular wall (12). For the first time, however, our results provide all of these indirect pieces of evidence in one single homogeneous blood vessel model.

However, these results lacked direct evidence of NO production. Therefore, we used the EPR spin trapping technique to directly detect NO. We show that RGPs, LTA, and LPS induce the delayed increase in NO formation in the vascular wall, thus confirming the indirect evidence described before. The amount of NO produced after contact with RGPs or LTA and the inhibitory effect of L-NAME or dexamethasone were comparable with the effects of LPS observed in the same system.

Up to now, the only component of the gram-positive bacterial cell wall whose action on the induction of NO synthase was well characterized was LTA. We show that a second component of gram-positive bacteria, RGPs, is able to induce NO synthase activity. In contrast, peptidoglycan G, another cell wall component, is not effective as a single component but only increases the effects of LTA (13).

A putative contamination by LPS could explain the present results. However several lines of evidence suggest that it is not the case: (i) the cell wall of Streptococcus mutans does not contain endotoxin in its structure; (ii) sterile conditions and apyrogenic reagents were used throughout the purification procedures, so that LPS contamination during this purification process seems unlikely; and (iii) a Limulus assay performed on the final RGP preparation was negative, indicating that the
endotoxin level is less than 0.125 endotoxin unit/ml (≤0.01 ng/ml). LTA contamination is also ruled out by the low phosphorus content of the RGP s (<0.01%).

The effect of RGPs can be observed only after 8 h, indicating a delayed cellular process and suggesting the induction of protein synthesis. Indeed, the effects of RGPs on nitrite and NO production were abolished by incubation with dexamethasone, which is known to prevent the induction of i-NOS by endotoxin (42) and by LTA (12). The delayed action of RGPs seems to be dependent, at least in part, on the presence of the endothelium. After 8 h, the hyporeactivity to NA and the relaxant effect of l-arginine were significant only when rings were incubated with their endothelium. This suggests that, if not essential for the induction of vascular hyporeactivity, the endothelium was able to sensitize the rat aortic rings to RGPs. Fleming et al. have shown that the presence of endothelium accelerated the induction of i-NOS by LPS and also increased the sensitivity of the preparation to LPS (17). It would be interesting to study the mechanism(s) by which endothelium increases the sensitivity of blood vessels to RGPs. It has recently been shown that capsular polysaccharides of S. aureus bind specifically to human monocytes and induce the release of TNF-α, interleukin-1β (IL-1β), IL-6, and IL-8 (47). RGPs could also induce the release of cytokines by the endothelial cells; those cytokines would accelerate NO synthase induction in the vascular wall, as already shown for TNF-α (18, 36, 45) and IL-1 (27, 28).

What is the importance of this study in understanding the mechanisms of altered vascular reactivity observed in septic shock? LTA has been implicated as the major mediator of gram-positive shock (12). In our study, we observed that the effects of RGPs on NO synthase activity were comparable to those of LTA. It thus seems conceivable that RGPs could be another component of gram-positive bacteria playing an important role in the genesis of the vascular disorders in gram-positive shock. The underlying mechanisms of the vascular effects of RGPs in septic shock might be similar with those induced by LTA and/or LPS; both have been shown to cause directly, but also through cytokine release, the induction of i-NOS in the vessel wall (16, 23, 29, 38, 54, 58). RGPs also cause the release of various cytokines from monocytes (48), thus supporting this hypothesis. In gram-positive septic shock, the cytokines released seem to be comparable to those released during gram-negative septic shock (14, 19, 23, 29, 33, 44, 46, 55). An antibody to TNF and an antagonist of the platelet-activating factor (PAF) receptor have similar protective effects in gram-positive (14, 23) and gram-negative (16, 21, 32, 54) septic shock. However, the mechanisms of action of cytokines might be different in the two types of shock. For example, in gram-positive shock, it seems that intracellular PAF is implicated in vascular disorders, while in gram-negative shock, it is the PAF released from cells (14). We observed that the kinetics of induction of NO synthase by RGPs was slower than that of induction by LTA: the contractile response to NA was significantly decreased 6 h after the first contact with LTA but not with RGPs (data not shown). However, this difference disappeared with longer times of incubation. Also, RGPs induced a lower increase in cyclic GMP accumulation than LTA (Fig. 2). This result suggests that the mechanisms activated by RGPs could differ from those of LTA at one or several stages. It has been reported that the induction of NO synthase by some gram-positive bacteria can be long lasting (4, 5, 50), whereas it can be a short-lasting process with some others (10, 61), suggesting that various mechanisms are implicated.

The identification of the compounds implicated in the pathological activity of the gram-positive bacteria will allow us to explore more precisely the cellular membrane receptors and the intracellular mechanisms activated. The cellular effects of gram-positive bacterial products may (15) or may not (47) be dependent on the presence of serum. Heumann et al. observed that the stimulating activity of the peptidoglycan and the glycan-teichoic acid complex on monocytes required a serum- and/or plasma-derived factor(s) (22). Results described here were obtained in the absence of serum. It has already been determined that on monocytes, the CD14, the C3, and the C1q receptors bind RGPs: the CD14 receptor mediates cytokine release; the C3 receptor may mediate the activation of the complement system as well as the clearance of the polysaccharides as does the C1q receptor (48). The membrane receptors involved in the vascular effects of RGPs remain to be investigated.

Our data provide indirect and direct evidence, through functional studies and the EPR spin trapping technique, that both RGPs, polysaccharides from the bacterial wall of Staphylococcus mutans, and LTA induce NO synthase activity in the vascular wall. This work supports the hypothesis that different wall components of gram-positive bacteria are involved in the pathogenesis of the vascular disorders observed in septic shock. In this respect, it would be interesting to investigate the complementary vascular effects of various gram-positive bacterial cell wall components.

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