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# Bacterial Porins Stimulate Bone Resorption

SAJEDA MEGHJI,[1]* BRIAN HENDERSON,[1] SEAN P. NAIR,[1] AND MARIA A. TUFANO[2]

*Maxillofacial Surgery Research Unit, Division of Surgical Sciences, Eastman Dental Institute, University College London, London WC1X 8LD, United Kingdom,[1] and Instituto di Microbiologia, Facoltá di Medicina e Chirurgia, Seconda Universitá di Napoli, Naples, Italy[2]*

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**Porins are abundant outer membrane proteins of gram-negative bacteria involved in transport of low-molecular-mass molecules. During the past decade, porins from a number of bacteria have also been shown to have proinflammatory activities including inducing the synthesis of proinflammatory mediators (cytokines, platelet-activating factor, and nitric oxide) in cultured cells and inducing inflammation in vivo. With this range of actions, it was possible that porins could also interact with bone cells to cause aberrant bone remodeling and that this could contribute to the bone destruction seen in gram-negative bone infections. By using purified preparations of *Salmonella typhimurium* and *Pseudomonas aeruginosa* porins, in the presence of polymyxin B, it was possible to induce concentration-dependent loss of calcium from cultured murine calvaria at porin concentrations in the range of 1 to 10 nM. The mechanism of action of the porins was determined by the inclusion of inhibitors of cyclooxygenase or inflammatory cytokines in the culture media. The bone-resorbing activity of both porins was not inhibited by the cyclooxygenase inhibitor indomethacin or by neutralizing the activity of tumor necrosis factor. Indeed, relatively high concentrations of these agents produced an unexpected increase in the bone resorption induced by the porins. In contrast, porin-induced bone resorption could be inhibited by relatively high concentrations of the natural inhibitor of interleukin-1 (IL-1 receptor antagonist). It appears that these porins stimulate bone resorption by a mechanism distinct from that of lipopolysaccharide, and the possibility therefore exists that porins play a role in bone destruction in gram-negative bacterial infections of bone.**

Bone pathology is a major cause of morbidity worldwide. Prior to the introduction of antibiotics, bone destruction due to bacterial infection was common in conditions such as osteomyelitis, tuberculosis of bone, and septic arthritis. It is now recognized that one of the major disease groups of bone is the periodontal diseases, i.e., bacterial diseases of alveolar bone, which afflict up to 15% of the world's population (5). It is therefore vital that the mechanisms by which bacteria induce bone destruction are defined. By inhibiting such mechanisms, it may be possible to prevent bone destruction even in circumstances where the bacteria are refractory to other antibacterial therapies.

For many years, the major bacterial constituent believed responsible for tissue destruction in gram-negative bone infections was lipopolysaccharide (LPS). However, during the past decade, it has been established that bacteria produce a number of molecules, mainly proteins, which have the capacity to stimulate the resorption of murine bone in vitro. The available data does not suggest that these various molecules work by a unitary mechanism (reviewed in reference 14). Since the mid-1980s, there has been a general move to study the biological effects of bacterial components other than LPS. This has led to the surprising finding that bacterial porins have a wide range of actions consonant with these molecules being proinflammatory mediators once released from bacteria (reviewed in reference 10). Prominent among these actions is the capacity of porins to stimulate the synthesis of proinflammatory cytokines (7), platelet-activating factor (22, 23), and nitric oxide (25) and to stimulate B- and T-lymphocyte function (18, 24). In vivo studies have supported these culture-based investigations, and porins have been demonstrated to induce paw swelling in the rat (6), to be pyrogenic and induce a Schwartzman reaction in rabbits, and to be lethal to galactosamine-sensitized mice (8).

This range of biological actions ascribed to porins suggested that they may play a role in the pathology of infected bone. In this study, we have addressed the question of whether porins have the capacity to induce the release of calcium from explants of murine calvarial bone.

## MATERIALS AND METHODS

**Preparation of porins.** A strain of *Salmonella typhimurium* SH5014 (provided by M. Nurminen, Central Public Health Laboratory, Helsinki, Finland) and *Pseudomonas aeruginosa* were used as the sources of porins. Porins were extracted by the method described by Nurminen (15). Briefly, 1 g of bacterial envelope was treated with Triton X-100 in 0.01 M Tris HCl (pH 7.5) containing 10 mM EDTA. After addition of trypsin (10 mg/g of envelope), the pellet was dissolved in sodium dodecyl sulfate (SDS) buffer (4%, wt/vol) in 0.1 M sodium phosphate (pH 7.2) and applied to an Ultragel ACA 34 column equilibrated with 0.25% SDS buffer (1). The fraction enriched in protein, identified by absorbance at 280 nm, was extensively dialyzed against distilled water and checked for protein heterogeneity by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (11). Removal of SDS was monitored by the method of Reynolds and Tanford (16). The detectable concentration of SDS in the final porin preparations (0.01 μg/20 μg of porin) did not affect the biological activity of the porins. The porin proteins were lyophilized by standard techniques and reconstituted in the appropriate media for the various tests. Traces of LPS in the porin preparation were identified by means of gel electrophoresis and stained with silver nitrate as described by Tsai and Frash (20) and by means of the *Limulus* amoebocyte lysate assay (19) (BioWhittaker, Walkersville, Md.). SDS-PAGE and subsequent silver nitrate staining of controls showed that the first fraction eluted contained traces of LPS, which were neutralized by polymyxin B (Sigma), as described previously (6, 7, 21–23). A negative *Limulus* test was obtained when porins plus polymyxin B were used as a control. On SDS-PAGE (with a loading concentration of 10 μg of porin), the purified porins from *S. typhimurium* showed the two expected bands with molecular masses of 34 and 36 kDa (8) and the purified porin from *P. aeruginosa* showed one band with a molecular mass of 36 to 38 kDa (Fig. 1).

* Corresponding author. Mailing address: Department of Oral and Maxillofacial Surgery, Eastman Dental Institute, University College London, 256 Gray's Inn Rd., London WC1X 8LD, United Kingdom. Phone: 0171 915 1271. Fax: 0171 915 1259. E-mail: S.Meghji@Eastman.ucl.ac.uk.

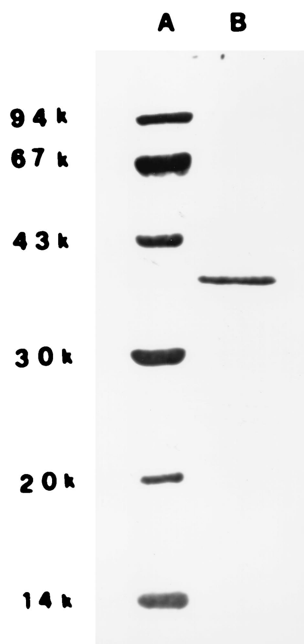


FIG. 1. SDS-PAGE of the purified porin preparation from *P. aeruginosa* stained with Coomassie blue. A single band with a molecular mass of 36 to 38 kDa is present. Lanes: A, molecular mass markers (k, kilodaltons); B, porin preparation.

**Calvarial bone resorption assay.** Bone resorption was assayed by the measurement of calcium release from 5-day-old mouse calvaria in vitro (26). Briefly, after removal of any adherent connective tissue, the calvaria were halved along the sagittal suture and each bone was cultured individually at 37°C on a 1-$cm^2$ stainless-steel grid in 1.5 ml of BGJ modified medium (ICN-Flow) supplemented with penicillin and streptomycin (Gibco; each antibiotic added at 100 U/ml), ascorbate (Sigma; 100 μg/ml), and 5% complement-inactivated rabbit serum (Sigma). After 24 h, the medium was removed from individual cultures and replaced with medium containing various concentrations of the porin preparation (in the log concentration range of 0.01 to 10 μg/ml) made up in medium containing polymyxin B (Sigma; 10 μg/ml). Replicate control cultures had only fresh unsupplemented media (containing polymyxin B) added to determine calcium release from unstimulated bone. After 48 h of culture, the media were removed and the calcium content was measured by automated colorimetric assay (9). In each assay, five replicate calvaria were exposed to 1 μM prostaglandin $E_2$ as a positive control to ensure that the bone was capable of responding to agonists. Porins were tested in three separate assays and gave reproducible dose responses.

**Effects of inhibitors.** To determine the mechanism of porin-induced bone resorption, a concentration of porin defined from the dose-response studies to give approximately 50% of the maximal stimulation (i.e., 0.5 μg/ml) was simultaneously added to bone explants with either indomethacin (Sigma; at log concentrations ranging from 0.001 to 1 μM) to inhibit cyclooxygenase, interleukin-1 receptor antagonist (Synergen; IL-1ra, at 0.01 to 100 μg/ml) to inhibit the action of IL-1, or a neutralizing antibody (TN3-19.12 [Celltech]) to murine tumor necrosis factor alpha (TNF-α, at 0.1 to 100 μg/ml) to block the action of released TNF-α. Inhibitors were examined in three separate experiments. The significance of the results was calculated by use of Student's *t* test.

## RESULTS

**Purity of the porin preparation.** The porin preparation from *S. typhimurium* showed the expected two protein bands at 34 and 36 kDa on both silver-stained and Coomassie blue-stained SDS-PAGE gels. The porin preparation from *P. aeruginosa* demonstrated only one protein band (Fig. 1). The LPS contamination of the *S. typhimurium* porin preparation used in this study has been dealt with in detail in previous publications (6, 7, 22). In the *Limulus* assay, the content of LPS in both porin preparations was of the order of 1 pg/μg of porin.

**Activity of porins in calvarial bone resorption assay.** To determine the potential contribution of LPS to the osteolytic activity of the porin preparations from both bacteria, the preparations were added to calvaria at a concentration of 10 μg/ml in the absence or presence of 10 μg of polymyxin B per ml. Addition of this LPS-binding antibiotic had no inhibitory effect on the osteolytic ability of either porin (Fig. 2). The porin preparations from both bacteria (in the presence of 10 μg of polymyxin B per ml) produced a reproducible stimulation of calvarial bone resorption over the dose range of 100 ng/ml to 10 μg/ml, with a small but significant stimulation of calcium release occurring at 100 ng/ml (Fig. 3).

**Effect of inhibitors.** Indomethacin, at concentrations ranging from 1 nM to 100 nM, had no effect on the bone resorption induced by the porins. However, it was noted in the case of *S. typhimurium* that in two of three assays there was a marked stimulation of calcium release from the calvarial explants at 1 μM indomethacin (Fig. 4). IL-1ra reproducibly inhibited porin-induced bone resorption over the concentration range of 0.1 to 100 μg/ml (Fig. 5). In contrast, neutralization of TNF with antibody TN3-19.12 either failed to inhibit bone resorption or showed some degree of stimulation of calcium release from bone explants (Fig. 6).

## DISCUSSION

Among the outer membrane proteins found in gram-negative bacteria are the abundant porins which form diffusion channels for small molecules such as metabolizable sugars (3). The porins form trimeric structures which are extremely resistant to proteases (3, 15). There is now an extensive literature demonstrating that the porins have proinflammatory properties both with cells in tissue culture and in whole-animal studies (10). Of particular relevance is the finding that preparations of porins which are minimally contaminated with LPS can induce the transcription of the synthesis of cytokines (7), the synthesis of lipid mediators such as platelet-activating factor (22, 23), and the production of the gas nitric oxide (25). These various cell mediators have been implicated in the modulation of bone remodeling (14). It was therefore of interest to determine if porins had any capacity to control the cell biology of bone remodeling. To test this, the murine calvarial bone resorption assay, which is a sensitive and reproducible system for investi-

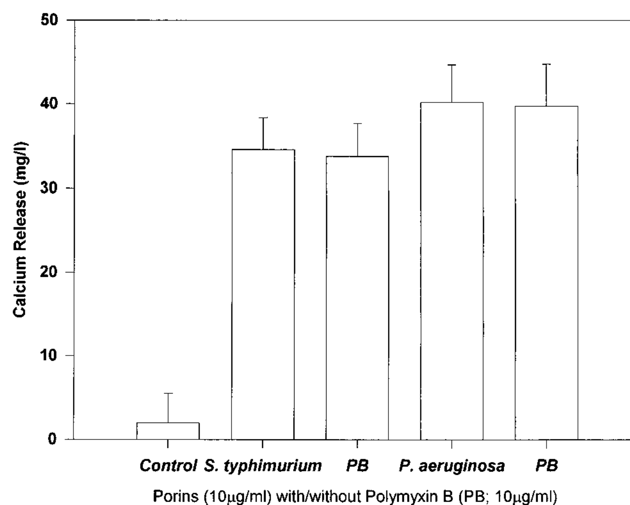


FIG. 2. Effect of adding polymyxin B (PB) at 10 μg/ml on the bone-resorbing activity of purified porins from *S. typhimurium* or *P. aeruginosa* (used at a concentration of 10 μg/ml). Bone resorption is measured by the release of calcium into the medium. The control column is the amount of calcium released from unstimulated bone. Results are expressed as the mean + standard deviation of five replicate cultures.

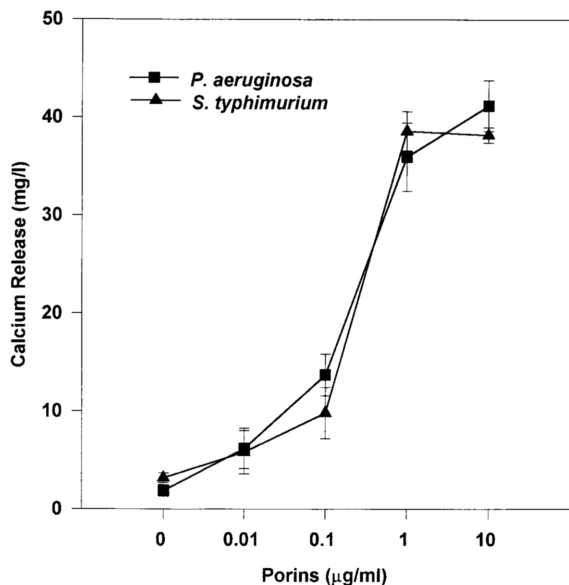


FIG. 3. Typical experiment demonstrating the concentration-dependent stimulation of calcium release from murine calvaria by various logarithmic concentrations of porins from *S. typhimurium* or *P. aeruginosa*. Results are expressed as the mean ± standard deviation of five replicate cultures.

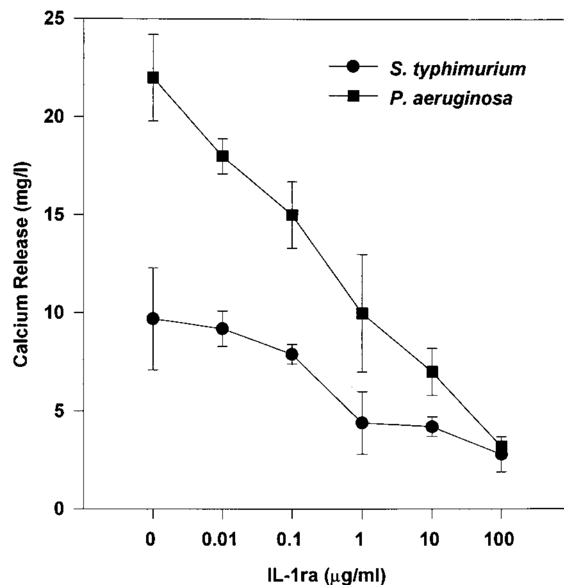


FIG. 5. Effect of increasing concentrations of IL-1ra on the bone resorption induced by the porins from both bacteria which have been added to cultures at a concentration of 0.5 μg/ml. Results are expressed as the mean ± standard deviation of five replicate cultures.

gating putative bone-modulating agents, has been used. Addition of various concentrations of purified porin preparations from *S. typhimurium* or *P. aeruginosa* revealed that these outer membrane proteins had the capacity to induce bone resorption. Such resorption was not due to LPS because (i) the contamination of the porins with LPS was minuscule and (ii) the activity of the porins was not affected by excess polymyxin B. Murine calvarial bone showed reproducible responses to the porins from both bacteria. Porins form trimeric or higher-order structures in the cell membrane. If such structures are the active porin moieties, then the porins are osteolytically active at molar concentrations in the range of 1 to 10 nM, a range as potent as certain proinflammatory cytokines, such as TNF-α, which are believed to be important in various bone pathologies (17).

Bone remodeling is a very complex process involving at least two distinct cell lineages, namely, the mesenchymal osteoblastic lineage and the myeloid osteoclastic lineage, and a range of low- and high-molecular-weight mediators. Many of the bac-

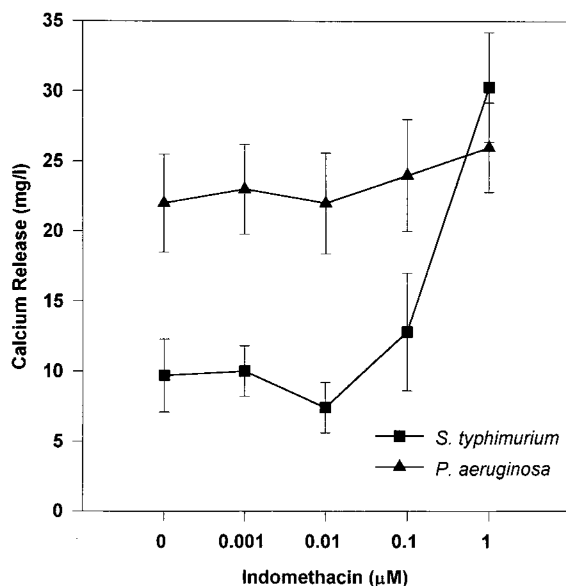


FIG. 4. Effect of indomethacin over the concentration range of 1 to 1,000 nM on the release of calcium from murine calvarial bone explants stimulated by porins from *S. typhimurium* or *P. aeruginosa* added at a concentration of 0.5 μg/ml. Results are expressed as the mean ± standard deviation of five replicate cultures. In two of three experiments, indomethacin stimulated the release of calcium from bone stimulated with *S. typhimurium* porin.

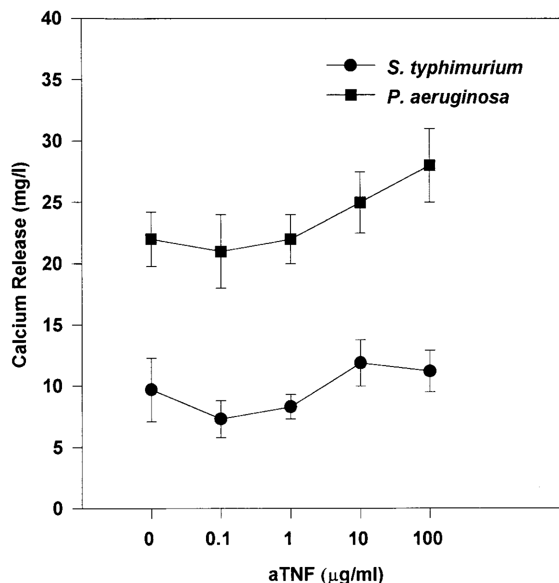


FIG. 6. Effect of increasing concentrations of the hamster anti-murine TNF-α-neutralizing monoclonal antibody TN3-19.12 on the bone resorption induced by porins from *S. typhimurium* or *P. aeruginosa*, which have been added to bone explants at a concentration of 0.5 μg/ml. Results are expressed as the mean ± standard deviation of five replicate cultures.

terial components, such as LPS, which have been shown to stimulate bone resorption can be inhibited by nonsteroidal antiinflammatory drugs which block the activity of the enzyme cyclooxygenase, which produces the prostaglandins (14). We assumed that the osteolytic activity of the porins would be similarly blocked by indomethacin. However, in all experiments, indomethacin failed to inhibit the activity of the porin preparations, and in two of the *S. typhimurium* porin experiments, there was some degree of stimulation of bone resorption at the highest concentration (1 μM) of indomethacin used. This is a concentration at which nonspecific effects are unlikely to be occurring (4). This can only be interpreted as demonstrating that the porin is not acting to induce the synthesis of prostaglandins by, for example, upregulating the transcription of the gene for cyclooxygenase II. However, we have no explanation for the stimulatory effect of the indomethacin in the calvarial explants.

IL-1 and TNF-α are two cytokines which are intimately involved in pathological bone remodeling in infectious and noninfectious bone diseases (14, 17). The activity of IL-1 can be blocked by the natural antagonist IL-1ra, and we have shown that this molecule can be a potent inhibitor of bone resorption induced by certain bacterial constituents (e.g., the surface proteins from *Porphyromonas gingivalis* [12]) but not by others (e.g., the surface proteins from *Staphylococcus aureus* [13]). It proved possible to inhibit the bone resorption induced by both *S. typhimurium* and *P. aeruginosa* porins by IL-1ra. The bone resorption induced by *P. aeruginosa* was inhibited by IL-1ra in a concentration-dependent manner, with significant inhibition at 0.01 to 0.1 μg/ml. *S. typhimurium* porin-induced bone resorption was inhibited at the relatively high concentrations of 1 μg/ml and above. In contrast, inhibition of TNF-α bioactivity by the hamster anti-mouse TNF-α monoclonal antibody TN3-19.12 failed to inhibit bone resorption and in some experiments actually caused a slight increase in calcium release, a phenomenon not seen in the dozens of previous experiments we have conducted with this antibody both in vitro (14) or with anti-TNF antibodies generally (2). In conclusion, the purified preparations of porins from *S. typhimurium* and *P. aeruginosa* have been shown to be able to induce calcium release from murine calvarial explants at nanomolar concentrations. The mechanism of action of these porins appears to be rather different from that of other bacterial mediators of bone resorption, in particular, LPS, whose activity on bone can be blocked by indomethacin, IL-1ra, and TN3-19.12 (12, 14). Thus, given the large amount of porins present in gram-negative bacteria, their resistance to proteases, and their actions on bone, it is conceivable that in gram-negative bone infections (including the osteomyelitis which can accompany *S. typhimurium* infections), the porins play a chronic role in causing bone destruction.

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