Peptidoglycan derived from *Staphylococcus epidermidis* induces Connexin43 hemichannel activity with consequences on the innate immune response in endothelial cells

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Gram-positive bacterial cell wall components including PGN (peptidoglycan) elicit a potent pro-inflammatory response in diverse cell types, including endothelial cells, by activating TLR2 (Toll-like receptor 2) signalling. The functional integrity of the endothelium is under the influence of a network of gap junction intercellular communication channels composed of Cxs (connexins) that also form hemichannels, signalling conduits that are implicated in ATP release and purinergic signalling. PGN modulates Cx expression in a variety of cell types, yet effects in endothelial cells remain unresolved. Using the endothelial cell line h.End5, a 6 h challenge with PGN induced IL-6 (interleukin 6), TLR2 and Cx43 mRNA expression that was associated with enhanced Cx43 protein expression and gap junction coupling. Cx43 hemichannel activity, measured by ATP release from the cells, was induced following 15 min of exposure to PGN. Inhibition of hemichannel activity with carbenoxolone or apyrase prevented induction of IL-6 and TLR2 mRNA expression by PGN, but had no effect on Cx43 mRNA expression levels. In contrast, knockdown of TLR2 expression had no effect on PGN-induced hemichannel activity, but reduced the level of TLR2 and Cx43 mRNA expression following 6 h of PGN challenge. PGN also acutely induced hemichannel activity in HeLa cells transfected to express Cx43, but had no effect in Cx43-deficient HeLa OHIO cells. All ATP responses were blocked with Cx-specific channel blockers. We conclude that acute Cx43 hemichannel signalling plays a role in the initiation of early innate immune responses in the endothelium.

Key words: Connexin43, hemichannel, innate immunity, peptidoglycan, *Staphylococcus epidermidis*.

INTRODUCTION

The highly responsive endothelial lining of the vasculature system plays a central role in regulating vascular behaviour, including vascular tone and responses to inflammatory agents [1,2]. Central to endothelial function is the ability of the cells of the endothelial layer to co-ordinate responses and exchange regulatory information between themselves (homocellular communication) or between endothelial cells and their neighbours such as smooth muscle cells (heterocellular communication) [3], or transiently between endothelial cells and cells of the immune system [4]. Such intimate and direct cell–cell communication is provided by a network of gap junction intercellular communication channels that permit the exchange of small regulatory molecules and metabolites between neighbouring cells [5]. The constituent proteins of these channels are a highly conserved family of proteins, the Cxs (connexins), of which 21 subtypes have been identified in humans, with Cx43 being the dominant Cx expressed in the vascular system. Cxs share a common topology spanning the membrane four times with two highly conserved extracellular loops and cytoplasmically located N- and C-termini and one intracellular loop. Six Cxs oligomerize to form a connexon, or hemichannel, that is trafficked in a closed state to the plasma membrane where they laterally accrete to align and dock with a hemichannel from a neighbouring cell [5]. Hemichannels not only are inert precursors of the gap junction unit, but also can be induced to open by a diverse range of ‘environmental’ stimuli resulting in the release of paracrine signalling molecules such as ATP, NAD, glutamate and prostaglandins [6]. ATP is important in purinergic signalling especially in endothelial cells where it has a vital role in vascular tone, synaptic transmission and cell death [7]. ATP also increases leucocyte locomotion and adhesion to endothelial cells via purinergic receptors [7] and augments the immune response by inducing cytokines such as IL-6 (interleukin 6) [8].

LPS (lipopolysaccharide), a major cell wall component of Gram-negative bacteria, induces pro-inflammatory cytokines and the expression of molecules on endothelial cells [9]. This inflammatory mediator modulates Cx expression and gap junction communication in aortic endothelium [10], astrocytes and microglia [11]. Furthermore, stimulation of cells with LPS induces a transient release of ATP, associated with hemichannel activity and downstream purinergic signalling events [7,12]. Among the Gram-positive bacteria, the staphylococci are prevalent pathogens and are responsible for a number of infections. A major aetiological agent of deep-seated abscesses, e.g. in brain, is *Staphylococcus aureus*. *S. aureus* has been reported to differentially influence Cx-mediated signalling events in astrocytes and microglia [13,14]. *Staphylococcus epidermidis* is particularly noted for its association with medical-device-related infections through its ability to form biofilms on the surface of devices, and is the causative agent of infective endocarditis associated with artificial heart valves, characterized by growth of a cardiac vegetation and localized endothelial inflammation.
Two of the Gram-positive bacterial cell wall components, namely PGN (peptidoglycan) and LTA (lipoteichoic acid), are potent pro-inflammatory mediators in many cell types, including endothelial cells, and PGN extracted from *S. aureus* influences Cx expression in astrocytes and microglia [13,14]. However, the consequences of these pro-inflammatory mediators on the endothelial gap junction network and Cx-mediated signalling events remain unresolved.

In the present study, we have explored the impact of PGN, extracted from *S. epidermidis* NCIMB 40896 that was previously isolated from a patient with infective endocarditis [17], on Cx expression and hemichannel signalling events in the b.End5 model endothelial cell line. Our data show that within 15 min of challenge, PGN induced Cx43 hemichannel opening and a rapid post-translational modification of Cx43 protein. Further exposure (6–12 h) resulted in a transient increase in *IL-6*, *TLR2* (Toll-like receptor 2) and Cx43 mRNA expression. Attenuation of the acute hemichannel activity with a panel of Cx-specific hemichannel blockers prevented PGN-induced *IL-6* and *TLR2* mRNA expression, but had no effect on the induction of Cx43 mRNA expression. In contrast, inhibition of TLR2 expression affected Cx43 mRNA expression, but had no effect on acute hemichannel opening. Taken together, the results suggest an acute role for hemichannel signalling events in the initiation of early innate immune responses.

**MATERIALS AND METHODS**

**Cell culture**

A murine endothelial cell line (b.End5) derived from murine brain endothelial cells (ECACC 96091930) [18], HeLa OHIO (wild-type) cells and HeLa cells transfected to express Cx43 (HeLa43) were used in these studies. All cells were grown in DMEM (Dulbecco’s modified Eagle’s medium), with 4.5 g/l glucose (Cambrex), supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine and 50 units/ml penicillin/streptomycin, with HeLa43 cells supplemented further with 0.5 μg/ml puromycin [19,20]. Cells were incubated at 37°C and 5% CO₂ and maintained and used at ~80% confluence.

**Transfection studies**

HeLa OHIO cells were transfected with GFP (green fluorescent protein)-tagged Cx43 in the expression vector backbone pEGFP-N1 (Clontech), prepared as described previously [21]. Subsequently, 12-well plates of HeLa OHIO cells (2 × 10⁵ cells) were transfected with 0.5 μg of pEGFP-N1 or Cx43–GFP cDNA using Lipofectamine according to the manufacturer’s instructions (Invitrogen) according to the manufacturer’s instructions [21], and functional assays were performed 48 h after transfection as required.

**TLR2 siRNA (short interfering RNA) transfection**

Pre-designed siRNA duplexes for TLR2 were purchased from IDT DNA Technologies and transfection conditions were optimized using a TYE 563™-labelled transfection control RNA duplex. A scrambled universal negative control duplex was used in all experiments. Cells were transfected with 0.1 nM siRNA using TrifECTin according to the manufacturer’s instructions (IDT DNA Technologies). At 24 h after transfection, cells were challenged and functional assays were performed as required.

**Extraction of bacterial cell wall components**

PGN was isolated from *S. epidermidis* NCIMB 40896, as described previously [17]. Briefly, bacterial cell wall sacculi comprising PGN and covalently linked WTA (wall teichoic acid) were prepared by boiling a whole-cell suspension for 30 min in a solution of SDS followed by thorough washing [22]. WTA was released from the protein- and lipid-free cell wall sacculi with 0.1 M NaOH, and the PGN was solubilized with lysostaphin (Sigma) [17]. Purified PGN was freeze-dried and dissolved in pyrogen-free water to 10 mg/ml (Versol® water for irrigation; Laboratoire Aguettant). Levels of contaminating LPS in the PGN preparation was measured by the quantitative chromogenic LAL (*Limulus* amoebocyte lysate) assay (QCL-1000; BioWhittaker) according to the manufacturer’s instructions. Responses to PGN were compared with that of LPS isolated from *Escherichia coli* O111:B4 by phenol extraction (Sigma).

**Challenge of cells**

Cells were challenged with PGN at concentrations from 0.1 to 10 μg/ml for times ranging from 5 min to 24 h in the presence or absence of a variety of known hemichannel blockers, including CBX (carbenoxolone) (100 μM) [23], LnCl₃ salt (200 μM) [23], 18α-GA (18α-glycyrrhetinic acid) (50 μM) [24] and the CX mimetic peptide Gap26 (100 μM) [24,25]. In some experiments, cells were exposed to the PKC (protein kinase C) inhibitor chelerythrine (50 mM) for 15 min before PGN challenge. In other experiments, cells were pre-exposed to apryrase (40 units/ml) for 30 min before experiments as described previously [21]. At set time points following challenge, supernatants and cell extracts were harvested for further analysis as required. Cells were assayed for viability using Trypan Blue exclusion and LDH (lactate dehydrogenase) levels in the medium were measured using a colorimetric assay according to the manufacturer’s instructions (Sigma).

**ELISA**

b.End5 cells (2 × 10⁵) were plated on to 12-well plates and challenged with PGN (0.1–10 μg/ml) for up to 24 h. Culture supernatants were harvested and assayed for IL-6 levels by ELISA (Peprotech) according to the manufacturer’s instructions.

**RT (reverse transcription)–PCR**

Total RNA was isolated using a Mini RNA Isolation Kit (Zymo Research; R1031) as per the manufacturer’s instructions. Contamination with DNA was avoided by treating total RNA with DNase I (Promega), and cDNA was synthesized using an M-MLV kit (Promega). Standard PCRs were subsequently performed to determine gene expression of Cx45, Cx43, Cx40 and Cx37 using specific primer sequences (Table 1) in an Applied Biosystems 2720 Thermo cycle. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control. The target sequence was amplified following an initial denaturation step at 95°C for 10 min, followed by 30 cycles of 95°C for 30 s, 30 s of annealing at a temperature dependent on the primer set (Table 1), an extension step of 72°C for 30 s, and a final extension time of 72°C for 10 min. All samples were analysed by electrophoresis on 1% agarose gels. Mouse genomic DNA was used as a positive control and DNase/RNase-free water was used as a negative control.
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Table 1 Primer and probe sequences and annealing temperatures of Cxs, IL-6 and GAPDH sequences

The real-time probes all had 5′-FAM (6-carboxyfluorescein) and 3′-TAMRA (6-carboxytetramethylrhodamine) ends.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type</th>
<th>Primer (5′ → 3′)</th>
<th>Tm (°C)</th>
<th>Band size (bp)</th>
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</thead>
<tbody>
<tr>
<td>Cx37</td>
<td>Sense</td>
<td>GGCTGGGACCATCGAGGCCTGT</td>
<td>56</td>
<td>421</td>
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<tr>
<td>Cx40</td>
<td>Sense</td>
<td>TTTGCCAGCTCAGGCGAGGG</td>
<td>56</td>
<td>311</td>
</tr>
<tr>
<td>Cx43</td>
<td>Sense</td>
<td>TACAGGCAACCCCGCC</td>
<td>56</td>
<td>407</td>
</tr>
<tr>
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<td>Sense</td>
<td>AAAGAGCAGAGGACCAACA</td>
<td>56</td>
<td>313</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense</td>
<td>TGTTCTACCCCCAATGTGT</td>
<td>72</td>
<td>350</td>
</tr>
<tr>
<td>Real-time primers</td>
<td></td>
<td></td>
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<tr>
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<td>Sense</td>
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<td>60</td>
<td>156</td>
</tr>
<tr>
<td>IL-6</td>
<td>Probe</td>
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<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense</td>
<td>CACATGGCCTCCAAGGAGTAA</td>
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<td>GAPDH</td>
<td>Antisense</td>
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Real-time PCR

To quantify changes in Cx43 and IL-6 expression, real-time PCR analysis was performed. Primers and probes were designed with sequence data from GenBank® and the real-time RT–PCR probe/primer design software Primer Express (version 1.0, PerkinElmer), which optimized the sequences for use in RT–PCR (Table 1). PCR conditions were optimized with respect to concentrations of MgCl₂, probe and both primers to maximize PCR (Table 1). PCR conditions were optimized with respect to MgCl₂, probe and both primers to maximize PCR (Table 1). PCR was performed in an Opticon 2 unit (Peqlab), primers (100 nM) and probe (100 nM) and cDNA (400 ng). Real-time PCR was performed in an Opticon 2 unit under the following cycling conditions: 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The mRNA levels were obtained from the value of threshold cycle (Cₜ) for each specific gene and normalized against the Cₜ of GAPDH (ΔΔCₜ method).

Western blot analysis

Cells, grown to confluence on six-well plates before challenge with PGN, were harvested in 100 μl of ice-cold lysis buffer [1% (v/v) SDS, 1 mM Na₂VO₄ (Sigma), 1 mM DTT (dithiothreitol) (Sigma), 100 μM PMSF (Sigma) and 0.01% protease inhibitor cocktail (Sigma)]. Total protein (25 μg) was run on a SDS/10% polyacrylamide gel and transferred on to a nitrocellulose membrane (GE Healthcare) in transfer buffer (25 mM Tris/HCl, 200 mM glycine and 20% methanol) as described previously [26]. Membranes were blocked with 5% (w/v) dried non-fat skimmed milk powder in TBS-T (Tris-buffered saline with Tween 20: 200 mM Tris/HCl, 1.5 mM NaCl and 0.1% Tween 20), and probed with primary antibodies targeted against Cx43 (r-Cx43, kindly provided by Dr E. Rivedal [27]), Cx43 Ser368 (New England Biolab; 35115), TLR2 (Abcam) or β-tubulin (Sigma) at dilutions of 1:10000, 1:500, 1:200 and 1:2000 respectively at 4°C overnight. Primary antibodies were visualized using HRP (horseradish peroxidase)-conjugated antirabbit or anti-mouse secondary antibodies (1:2000 dilution) followed by chemiluminescent detection (ECL, Plus, GE Healthcare) and exposure to Hyperfilm followed by quantification using a densitometer with the Quantity One program as described previously [26].

Microinjections

Cells were grown to confluence in 60-mm-diameter Petri dishes and challenged with PGN for 6 or 24 h. Individual cells were microinjected with Alexa Fluor® 488 (570 Da) (Invitrogen) using an Eppendorf 5120 femtotransject injection system linked to a Cairns monochromator with ~50 cells injected per experimental group [26]. Following injection, cells were incubated at room temperature (20°C) for 5 min and fixed in 3.7% (v/v) formaldehyde and the number of cells the dye had transferred to were counted. Results are presented as the mean ± S.E.M. extent of dye spread from individually injected cells. Experiments were repeated in triplicate.

Hemichannel functionality

Cells (2 × 10⁵) were plated on 12-well plates and were challenged the following day in 1 ml of medium containing 0.1 μg/ml PGN in the presence or absence of the hemichannel blockers outlined above. As a positive control, cells were also challenged with DTT (10 mM) [23]. Medium was harvested (500 μl from 1000 μl) and snap-frozen on solid CO₂. Cells were washed in ice-cold PBS and any excess liquid was removed before harvesting in 500 μl of 5% (v/v) TCA (trichloroacetic acid). The total amount of protein in each sample was subsequently measured using an Bio-Rad Laboratories DC protein assay kit following the manufacturer’s instructions. To measure extracellular ATP, the samples were thawed on ice, and levels of ATP in the medium were determined using the Sigma luciferin–luciferase assay as described previously [24]. For standardization, results were calculated as the concentration of ATP (nM) per μg of protein and are presented as the percentage of ATP release relative to controls.

To measure levels of NAD present in the medium, samples were collected following treatments and analysed using an NAD/NADH assay kit from Abcam following the manufacturer’s...
and analysed for IL-6 expression by ELISA (n = 3). (B) mRNA was extracted 0, 3, 6, 12 and 24 h after treatment with 0.1 μg/ml PGN. Real-time PCR analysis determined that IL-6 mRNA expression was increased following 6 and 12 h exposure to PGN (n = 4). *P < 0.05; **P < 0.01; ***P < 0.001.

Statistical analysis
Experiments were performed in triplicate per setting and repeated three times. Statistical analysis was performed using GraphPad Prism. Results are expressed as means ± S.E.M. and Student’s t tests were used to determine statistical significance at P < 0.05.

RESULTS
PGN induces a pro-inflammatory response in b.End5 cells
LPS contamination of PGN extracts is often associated with commercially sourced PGN [28]. LAL assays determined that our purified PGN extracts from S. epidermidis NCIMB 40896 had less than 0.4 unit/ml LPS, significantly lower than the commercial supply, thereby confirming its purity. To confirm that PGN triggered a pro-inflammatory response, b.End5 cells were initially challenged with 0.1, 1 or 10 μg/ml PGN and the level of IL-6 produced was determined by ELISA. A sequential increase in IL-6 production occurred with 0.1 μg/ml inducing a significant protein response following 24 h of exposure to PGN (Figure 1A). Taking 0.1 μg/ml as the lowest concentration to induce a response, a real-time PCR time course showed that IL-6 mRNA expression was induced 6–12 h following challenge (Figure 1B) and a 6 h time point was used in subsequent experiments to monitor IL-6 mRNA expression levels. Similar effects were seen for TNFα (tumour necrosis factor α) (results not shown).

Impact of PGN on Cx expression and gap junction functionality
RT–PCR detected Cx43 and Cx45 transcripts, but not Cx40 or Cx37 in the b.End5 cells (Figure 2A). Following exposure of b.End5 cells to 0.1 μg/ml PGN, real-time PCR analysis determined that PGN induced a transient increase in Cx43 mRNA expression, peaking at 6 h (Figure 2B). This increase in mRNA expression correlated with an increase in Cx43 protein expression and a change in phosphorylation levels following 6 h of exposure to PGN as determined by Western blot analysis (Figure 2C). Upon further exposure to PGN, Cx43 expression levels returned to basal levels. In subsequent experiments, a 6 h time point was used to monitor Cx43 mRNA expression levels. Gap junctional functionality was also transiently increased. Under control conditions the ability of cells to transfer Alexa Fluor® 488 following microinjection of the dye was restricted to three or four neighbouring cells; in contrast, following challenge with PGN for 6 h, a significant increase in the number of cells transferring the small fluorescent dye Alexa Fluor® 488 was observed (Figure 2D). Following 24 h of challenge with PGN, dye transfer was still significantly higher than for controls, although lower than at the 6 h time point (Figure 2D). Trypan Blue exclusion assays confirmed that cells were still viable.

Effect of PGN on hemichannel activity in b.End5 cells
To determine whether PGN affected hemichannel activity, we performed a range of ATP and NAD release assays in the presence and absence of a variety of established hemichannel blockers. To validate that hemichannel activity could be readily assayed, b.End5 cells were challenged for 15 min with the reducing agent DTT in the presence or absence of the generic Cx blocker 18α-GA. Under control conditions, a limited amount of ATP was detected in the medium; however, following exposure to 10 mM DTT, an increase (4-fold) in ATP was found, a stimulation that was reduced by co-treatment with 18α-GA (Figure 3A), in agreement with similar studies by Retamal et al. [23]. A time course of PGN exposure revealed a transient increase in ATP release (2-fold), peaking at 15 min, with little significant differences to mock challenged cells at time points thereafter (up to 4 h) (Figure 3B). In all subsequent hemichannel assays, cells were challenged with PGN for 15 min. To confirm that this acute release of ATP was associated with hemichannel opening, cells were challenged with PGN following pre-exposure to the hemichannel blockers LnCl2, CBX or the Cx mimic peptide Gap26 for 1 h. Under control conditions, i.e. in the absence of PGN, none of the blockers had any significant effect on the level of ATP in the medium compared with mock-challenged cells (Figure 3C). In contrast, challenge with PGN resulted in at least a 2-fold increase in ATP levels that was significantly attenuated with each of the blockers (Figure 3C). Following PGN challenge, an increase in NAD levels in cell supernatants was recorded that was attenuated by treatment with both Gap26 and CBX (Figure 3D), agreeing with reports that hemichannel opening can also trigger release of NAD from cells [23]. Furthermore, a dose-response to CBX in b.End5 cells illustrated that concentrations lower than 50 μM failed to significantly inhibit ATP release (Figure 3E). LDH levels were maintained at basal levels under the different treatments, thereby confirming cell viability (Figure 3F).

The acute hemichannel response was not associated with any rapid changes in Cx43 mRNA expression (Figure 2B). To investigate whether post-translational modification of Cx43 occurred, we performed Western blots on b.End5 cells exposed to PGN for 15 min. This correlated with an acute increase in Cx43 expression and phosphorylation (Figure 4A). Interaction
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Figure 2 Impact of PGN on Cx43 expression and gap junction functionality

(A) Cx mRNA expression profile in b.End5 (b), HeLa OHIO (O) and HeLa43 (43) cells. In each case, mouse genomic DNA was used as a positive control. (B) Real-time PCR analysis determined that Cx43 mRNA expression was induced following 6 h of exposure to PGN (n = 4). (C) An increase in the phosphorylation status of Cx43 occurred following 6 h of challenge with PGN returning to control levels by 18 h (P, PGN; C, Control). β-Tubulin was used as an internal control. Representative blot (n = 3). (D) Effect of PGN on dye coupling in b.End5 cells. Monolayers of cells were exposed to control conditions or PGN for 6 and 24 h after which individual cells were microinjected with the fluorescent tracer Alexa Fluor® 488. Dye transfer was recorded following visualization by fluorescence microscopy. Results are mean ± S.E.M. numbers of cells to which the dye transferred (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001.

Effect of PGN on hemichannel activity in HeLa cells

To clarify a role for Cx signalling in these events, we performed further experiments in HeLa OHIO cells (which do not express Cx43 and are gap junction communication-deficient) and in HeLa cells stably transfected to express Cx43 (HeLa43). RT–PCR analysis confirmed that the HeLa43 cells had the same Cx expression profile as the b.End5 cells; in contrast, only Cx45 was detected in HeLa OHIO cells (Figure 2A). When HeLa43 cells were challenged with PGN, a 3-fold increase in levels of extracellular ATP was detected that was blocked with 50 μM CBX (Figure 5A). In contrast, challenge of the Cx43-deficient HeLa OHIO cells with PGN did not induce an ATP response (Figure 5A). Furthermore, transient transfection of HeLa OHIO cells with a plasmid expressing Cx43–GFP, hence overexpressing Cx43, induced a 6.5-fold increase in extracellular ATP levels that was blocked by 50 μM CBX. In contrast, cells transfected with pEGFP-N1 did not respond to PGN stimulation (Figure 5B). Transfection efficiencies for the two constructs were comparable, as determined by GFP fluorescence. Taken together, these findings suggest that the observed ATP release caused by PGN is due to acute activation of Cx43 hemichannels.

Attenuation of Cx43 hemichannel activity inhibits induction of IL-6 and TLR2 expression

Following challenge of the b.End5 cells with PGN for 6 h, TLR2 and IL-6 expression were both induced at the mRNA level (Figures 1B and 6A respectively), with enhanced protein expression readily detected by ELISA (IL-6, measured 24 h after challenge) (Figure 1A) and Western blot analysis (TLR2) where a band of 84 kDa corresponding to TLR2 was detected following 12 h of exposure to PGN (Figure 6B); however, this antibody had to be used at low titres and no protein was detected at earlier time points. Cx43 mRNA and protein expression were also induced following 6 h exposure to PGN (Figure 2B). To determine whether the acute hemichannel signalling observed following PGN challenge had any effect on the expression profile of these genes, hemichannel activity was blocked by pre-exposure to CBX. The blocker was removed and cells were challenged for 15 min with PGN before collection of medium for ATP analysis or further exposure to PGN for 6 h before RNA was extracted. Exposure to PGN for 15 min induced an ATP response that was negated by pre-treatment with CBX, as seen in all other experiments (Figure 7A). Following pre-exposure to CBX, i.e. absence of ATP release via Cx43 hemichannels, Cx43 mRNA expression was still induced following 6 h exposure to PGN (Figure 7B). In contrast, TLR2 and IL-6 expression were not induced when the initial hemichannel activity was blocked (Figures 7C and 7D). Addition of apyrase...
Figure 3  PGN induces hemichannel activity in b.End5 cells

(A) b.End5 cells were exposed to DTT in the presence or absence of 18α-GA for 1 h and extracellular ATP levels were monitored. (B) b.End5 cells were exposed to PGN for up to 4 h. Maximal ATP release occurred 15 min after challenge that was inhibited by the hemichannel blocker CBX. This time point was used in all future ATP release assays. (C) A range of hemichannel blockers, including Gap26, LnCl3 (LN) and CBX inhibited PGN-induced ATP release in b.End5 cells. (D) Challenge of b.End5 cells with PGN also induced NAD release that was attenuated by co-treatment with Gap26 and CBX. (E) Dose response of b.End5 cells to CBX, concentrations of CBX lower than 50 μM failed to inhibit the PGN-stimulated ATP release. (F) LDH levels were unaltered in b.End5 cells following PGN challenge. For all panels, n = 3. *P < 0.05; **P < 0.01; ***P < 0.001.

to the medium, thereby degrading extracellular ATP levels, also attenuated the acute PGN-induced ATP release (Figure 7E) and the later PGN-induced expression of IL-6 and TLR2, but had no effect on Cx43 mRNA expression levels (Figures 7F–7H). Similar effects to IL-6 were seen for TNFα expression (results not shown).

Inhibition of TLR2 expression has no effect on PGN-induced hemichannel activity

Finally, we sought to explore the role of TLR2 in the observed responses. Transfection of b.End5 cells with siRNA targeted to TLR2 efficiently reduced the induction of TLR2 expression by 6 h of exposure to PGN (Figure 8A). The ability of 6 h of exposure to PGN to induce Cx43 mRNA expression was also inhibited (Figure 8B). However, decreased TLR2 expression had no effect on the ability of PGN to induce hemichannel activity following 15 min of challenge (Figure 8C).

DISCUSSION

The present study illustrates that challenge of endothelial cells with PGN isolated from S. epidermidis elicits an acute Cx43 hemichannel response that is required for the induction of TLR2 and IL-6 expression. These findings add to the range of bacterial cell wall components and pro-inflammatory agents that illicit Cx-mediated signalling events and establishes a central role for acute hemichannel signalling in the induction of the innate immune response in endothelial cells.

The impact of pro-inflammatory mediators including PGN and Gram-negative-derived LPS on cellular events is often studied at 6–24 h time points following challenge (e.g. [14,17]). Using a mouse endothelial model cell line (b.End5), we determined that PGN induced IL-6 and TLR2 mRNA expression within 6 h of exposure, demonstrating the potent pro-inflammatory effect of PGN that is observed in diverse cell types including microglia, astrocytes and synovial fibroblasts where staphylococci

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Figure 4 PGN acutely induces Cx43 protein expression

(A) b.End5 cells were treated with PGN for 15 min and total Cx43 protein expression was determined by Western blot analysis. (B) Pre-treatment of cells with chelerythrine (Chl) before PGN challenge for 15 min had no effect on total Cx43 protein expression (r-Cx43). Cx43 phosphorylation of Ser368 was not affected by PGN challenge and was efficiently removed following chelerythrine treatment (Cx43-ser368). β-Tubulin served as an internal loading control (representative blots n = 3). (C) Exposure of b.End5 cells to chelerythrine before PGN challenge reduced the level of ATP released (n = 6). **P < 0.01.

are prevalent pathogens. The impact of such pro-inflammatory mediators on Cx expression and function has highly cell-specific effects. In astrocytes, PGN inhibits gap junction communication and Cx43 expression following 6 h of challenge; in contrast, in microglial cells, S. aureus-derived PGN induced Cx43 expression and gap junction communication [13,14]. In the present study, we have demonstrated that PGN isolated from S. epidermidis transiently enhanced Cx43 mRNA and protein expression, peaking at 6 h, which was associated with increased gap junctional intercellular communication in our endothelial cell model. Thus our results clearly show that PGN influences Cx43 expression and function in endothelial cells at 6 h. Increased Cx43 protein expression was associated with an increase in the overall phosphorylation of the protein. The phosphorylation status of the C-terminal tail of Cx43 is influenced by subtle localized cellular events and is under the influence of PKC, c-Src and MAPK (mitogen-activated protein kinase) signalling pathways [31]. Changes in the phosphorylation status of Cx43 have profound and cell-type-specific effects on both gap junctional intercellular communication and hemichannel signalling events. For example, rapid Ser368 phosphorylation of Cx43 during hypoxia is associated with inhibition of Cx43 hemichannel activity in

Figure 5 PGN induces hemichannel activity in HeLa cells expressing Cx43

(A) HeLa OHIO cells which do not express Cx43 and are communication-incompetent showed no ATP release following exposure to PGN for 15 min. In HeLa43 cells, PGN induced a significant release of ATP compared with non-treated cells that was attenuated with CBX (n = 3). (B) HeLa OHIO cells were transfected with Cx43-GFP or EGFP (enhanced GFP) cDNA for 30 h before exposure to PGN (n = 6). **P < 0.01; ***P < 0.001.

Figure 6 PGN induces TLR2 expression in b.End5 cells

(A) Real-time PCR showed that TLR2 mRNA expression was induced following 6 h of exposure to PGN (n = 3). (B) Western blot analysis detected TLR2 protein expression 12 h after challenge with PGN. P, PGN; C, control. Representative blot (n = 3). *P < 0.05; ***P < 0.001.
endothelial cells [32]. The extracellularly located PGN initiates its pro-inflammatory response by interaction with TLR2 receptors on the cell surface that are located in caveolin-A-associated lipid raft domains where Cx43 also resides [33,34]. An early event in TLR2 signalling is activation of c-Src and PKC that could explain the rapid phosphorylation of Cx43 observed following 15 min of PGN challenge and may influence Cx43 signalling within the localized vicinity. Our data indicate that this was not linked with changes in the level of Ser368 phosphorylation. However, other phosphorylation changes cannot be ruled out, particularly as TLR2 activation has been closely linked with induction of Cx43 expression and phosphorylation in airway epithelial cells [35].

Under normal physiological extracellular calcium concentrations, hemichannels are closed [6]. Increasing evidence suggests that pathophysiological conditions can alter hemichannel activity, releasing ATP, NAD and other extracellular signalling molecules that will affect purinergic and downstream signalling pathways. Following exposure to PGN for periods of 15 min, we determined that b.End5 cells acutely released ATP and NAD into the medium, which is suggestive of hemichannel opening. ATP release via hemichannels is an area of increasing interest due to the downstream pathological events this can lead to. ‘Leaky hemichannels’ arising in Cx26 and Cx30 mutations are associated with profound skin disorders often leading to apoptosis [36–38]. Ischaemia and hypoxic insult are reported to open Cx43 hemichannels in cardiac myocytes [24] and microglial cells [39], whereas hypoxic insult inhibits hemichannel activity in endothelial cells [32]. LPS and pro-inflammatory cytokines have also been shown to elicit a hemichannel response in astrocytes [12].

Until recently, Cxs were considered to be the only family of proteins involved in gap junction intercellular communication in vertebrates. However, a second protein family, the pannexins (Panx1, Panx2 and Panx3) has been identified [40]. These glycosylated mechanosensitive channels also result in the release of ATP, with associated purinergic signalling events in diverse cell types and considerable controversy over the precise role of Cxs compared with Panxs in hemichannel signalling has emerged [40–43]. The b.End5 cells expressed Panx1 mRNA, and limited Panx1 protein was detected by Western blot with limited diffuse intracellular staining detected by immunofluorescence (results not shown). A range of highly selective inhibitors are now available to distinguish between these two channels [40,44]. LDH assays determined that PGN challenge did not damage cellular integrity, and the ATP and NAD release was attenuated by the Cx mimetic peptide Gap26. Gap26 has been widely used as a highly specific inhibitor of Cx function in mammalian cells both in vitro and in vivo [12,45–48]. The tervalent cation La3+ also attenuated ATP release that has been robustly shown by others to attenuate Cx, but not Panx, hemichannels in a variety of cell types and assays (reviewed by [44]). Panx1 hemichannel activity is also more sensitive to the blocker CBX than Cx hemichannels [49]. At doses of 50 μM, CBX attenuated ATP release in b.End5 cells, i.e. at levels reported to attenuate Cx and Panx channels. However, doses below 25 μM CBX had no effect on ATP release, confirming further the Cx-mediated response that PGN was delivering. We also performed experiments using Cx43-deficient HeLa OHIO cells [19,20], where limited endogenous Panx protein expression has been reported previously [50]. PGN challenge elicited an ATP response in HeLa cells stably expressing Cx43, but not in non-transfected HeLa OHIO cells. In HeLa OHIO cells transiently transfected to overexpress Cx43–GFP, a large ATP response was induced, all of which were attenuated by 50 μM CBX. The fact that, in vertebrates, Cxs and Panxs are co-expressed in many cell types and are evolutionarily distinct implies that both protein families have their own specific function. Our data...
Peptidoglycan enhances Connexin43 expression and hemichannel activity

Transfection of b.End5 cells with siRNA targeted to TLR2 attenuated (A) PGN-induced TLR2 mRNA expression and (B) PGN-induced Cx43 mRNA expression. (C) Transfection of b.End5 cells with siRNA targeted to TLR2 had no effect on PGN-induced hemichannel activity \((n = 3)\). ***\(P < 0.001\).

Figure 8 Inhibition of TLR2 expression has no effect on PGN-induced hemichannel activity

Transfection of b.End5 cells with siRNA targeted to TLR2 attenuated (A) PGN-induced TLR2 mRNA expression and (B) PGN-induced Cx43 mRNA expression. (C) Transfection of b.End5 cells with siRNA targeted to TLR2 had no effect on PGN-induced hemichannel activity \((n = 3)\). ***\(P < 0.001\).

point towards Cx hemichannel activity being the initial trigger following challenge of endothelial and HeLa cells with PGN.

Extracellular ATP can initiate downstream purinergic signalling responses leading to pro-inflammatory cytokine release. Recent evidence indicates that ATP released from LPS-activated microglia signals through P2X receptors thereby augmenting pro-inflammatory cytokine production [51]. A further study suggested that Shigella infection of epithelial cells triggers ATP release through Cx26 hemichannels, resulting in the activation of purinergic receptors on neighbouring cells and bacterial dissemination [52]. Thus we hypothesized that the acute PGN-induced ATP release would trigger purinergic signalling events ultimately leading to the enhanced gene expression observed. Blockage of the acute ATP release by pre-treating the endothelial cells with CBX or removing extracellular ATP by apyrase, followed by continued exposure to PGN for 6 h, prevented the PGN-induced TLR2 and IL-6 expression. However, inhibition of hemichannel activity had no impact on the PGN-induced expression of Cx43 mRNA. These results suggest that localized membrane responses to PGN acutely activate Cx43 hemichannels causing a transient ATP release that alerts cells of bacterial challenge, thus providing a local way of priming cells of infection, possibly via purinergic signalling events. Inhibition of PKC with chelerythrine caused a significant reduction in PGN-induced ATP release, suggesting that PKC pathways play a role in triggering the response, although, as discussed, this was not due to Ser168 phosphorylation. PGN interacts directly with TLR2, leading to a sequence of events that ultimately activates signal transduction pathways, including those involved in NF-κB (nuclear factor κB) and MAPK [53]. This, in turn, would induce changes in Cx expression and gap junction coupling. TLR2 regulation of Cx expression and gap junctional intercellular communication has recently been reported in airway epithelial cells [35] and in the intestinal epithelial barrier [54], supporting our observations. To clarify this link in our studies, we transfected b.End5 cells with siRNA targeted to TLR2 and performed PGN challenges 24 h after transfection. This prevented PGN-induced TLR2 and Cx43 mRNA expression, reinforcing a role for TLR2 signalling in the changes in Cx43 expression and gap junction function observed following 6 h of exposure to PGN. However, following 15 min of challenge with PGN, cells transfected with TLR2 siRNA still elicited an acute ATP response. This may be due to residual endogenous TLR2 protein being present in the membrane and sufficient to trigger localized membrane responses resulting in hemichannel signalling.

In conclusion, our findings clearly illustrate, for the first time, that Gram-positive bacterial cell wall components elicit an innate immune response that is associated with acute activation of Cx43 hemichannels. These events are likely to be driven by localized early responses at the plasma membrane and occur before an increase in Cx43 expression and gap junction coupling that are associated with TLR2 activity. The findings support further the emerging concept that Cx hemichannel activity is intricately linked to infection and inflammatory conditions in diverse tissues including the endothelium (the present study), lung epithelia [47] and the gut [52,55]. As Cxs emerge as proteins amenable to direct therapeutic targeting by siRNA strategies [56] and highly specific peptides that can inhibit [47,48,55] or enhance [26] coupling, further work defining the signalling cascades involved is an area of important future research.

AUTHOR CONTRIBUTION

Jennifer Robertson performed the experimental work and data analysis supervised by Patricia Martin and Sue Lang. Sue Lang and Peter Lambert were involved in PGN preparations and experimental design. The paper was written by Jennifer Robertson and Patricia Martin with contributions from Sue Lang and Peter Lambert.

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