Published in final edited form as:
Cell Microbiol. 2008 April ; 10(4): 863–875. doi:10.1111/j.1462-5822.2007.01089.x.

ATP scavenging by the intracellular pathogen *Porphyromonas gingivalis* inhibits $P2X_7$-mediated host-cell apoptosis

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Summary

The purinergic receptor $P2X_7$ is involved in cell death, inhibition of intracellular infection and secretion of inflammatory cytokines. The role of the $P2X_7$ receptor in bacterial infection has been primarily established in macrophages. Here we show that primary gingival epithelial cells, an important component of the oral innate immune response, also express functional $P2X_7$ and are sensitive to ATP-induced apoptosis. *Porphyromonas gingivalis*, an intracellular bacterium and successful colonizer of oral tissues, can inhibit gingival epithelial cell apoptosis induced by ATP ligation of $P2X_7$ receptors. A *P. gingivalis* homologue of nucleoside diphosphate kinase (NDK), an ATP-consuming enzyme, is secreted extracellularly and is required for maximal suppression of apoptosis. An *ndk*-deficient mutant was unable to prevent ATP-induced host-cell death nor plasma membrane permeabilization in the epithelial cells. Treatment with purified recombinant NDK inhibited ATP-mediated host-cell plasma membrane permeabilization in a dose-dependent manner. Therefore, NDK promotes survival of host cells by hydrolysing extracellular ATP and preventing apoptosis-mediated through $P2X_7$.

Introduction

Bacteria that initiate infections at host epithelial surfaces are, in many instances, capable of entry into and survival within in host cells. Intracellular bacterial pathogens have evolved sophisticated mechanisms that enable them to prevail over host defences, replicate and successfully colonize host tissues (Rhoades and Ullrich, 2000; Amieva *et al*., 2002; Phalipon and Sansonetti, 2007). Many of these pathogens, including *Helicobacter pylori*, *Salmonella enterica* serovar Typhimurium and *Mycobacterium* and *Chlamydia* species are also known to prolong the integrity of their host-cell environment by inhibiting host-cell death. The relationship between bacterial microorganisms and apoptotic processes is a regulated and complex phenomenon, many aspects of which are yet to be explored. Epithelial cells, which are often the first line of defence and target sites of microbial infections, can show an elevated or repressed apoptotic response depending on the nature of the microbial challenge (Monack and Falkow, 2000; Byrne and Ojcius, 2004; Knodler *et al*., 2005).

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Porphyromonas gingivalis, a Gram-negative anaerobe, is a major pathogen in severe and chronic forms of periodontal disease and has recently been identified as a risk factor in severe systemic conditions such as coronary heart disease (Socransky and Haffajee, 1992; Herzberg and Weyer, 1998; Kuramitsu *et al*., 2001). *P. gingivalis* can replicate, survive and later disseminate intercellularly within and through the epithelial cells that line host gingival tissues (Lamont *et al*., 1995; Yilmaz *et al*., 2004; 2006). Gingival epithelial cells (GECs), an important arm of the innate immune response of oral tissues, are among the first host cells colonized by *P. gingivalis* (Schroeder and Listgarten, 1997; Weinberg *et al*., 1998; Nisapakul-torn *et al*., 2001; Lamont and Yilmaz, 2002). The organism modulates a variety of signalling pathways and phenotypic properties of the epithelial cells, including inhibition of interleukin-8 secretion, modulation of intracellular calcium concentrations, and activation of integrin receptor-associated signalling pathways (Darveau *et al*., 1998; Yilmaz *et al*., 2002; Zhang *et al*., 2005). More importantly, *P. gingivalis*, which replicates to high levels intracellularly, does not induce host-cell death; instead, the infected GECs are resistant to apoptosis induced by potent pro-apoptotic agents (Yilmaz *et al*., 2004; Mao *et al*., 2007). Previously, it has been shown that *P. gingivalis* promotes host-cell survival by inhibiting mitochondrion-dependent apoptosis, activating the phosphatidylinositol 3-kinase (PI3K)/AKT (protein kinase B) pathway, inhibiting caspase-3 activation through upregulation of JAK/Stat signalling, and balancing the expression of pro-apoptotic Bax and anti-apoptotic Bcl-2 (Nakhjiri *et al*., 2001; Yilmaz *et al*., 2004; Mao *et al*., 2007). *P. gingivalis* thus uses diverse mechanisms to modulate cell death pathways to ensure its intracellular survival. Nevertheless, the physiological upstream modulators of inhibition of apoptosis in the infected cells remain to be characterized.

The purinergic receptor, $P2X_7$, has emerged recently as an important mediator of apoptosis, in addition to its role in initiation of inflammatory responses, control of infection by intracellular bacteria and stimulation of cell proliferation (Chen and Brosnan, 2006; Lister *et al*., 2007). Thus, extracellular ATP (ATP_e) ligation of $P2X_7$ receptors on macrophages produces a variety of cellular effects, including activation of an inflammasome, maturation and release of interleukin-1β, and induction of macrophage death by apoptosis and/or necrosis. ATP released from infected cells or stressed cells at sites of inflammation is thus viewed as a generic ‘danger signal’ that can alert the innate immune system to the presence of infection (Surprenant *et al*., 1996; Schwiebert and Zsembery, 2003; Khakh and North, 2006; Mariathasan and Monack, 2007).

A number of studies have shown that some intracellular bacterial pathogens such as *Mycobacterium tuberculosis* and *Chlamydia psittaci* that can cause persistent infections have the ability to inhibit $P2X_7$-mediated apoptosis of their host cells (Lammas *et al*., 1997; Zaborina *et al*., 1999; Coutinho-Silva *et al*., 2001; Fairbairn *et al*., 2001; Franchi *et al*., 2007). In most cases, the bacterial factors responsible for inhibition of host-cell death have not been identified. In addition, the studies were conducted with infected macrophages, which are not the preferred host cells for many pathogens. The ability of infection to modulate $P2X_7$ activity in epithelial cells remains poorly characterized, partly due to a functional $P2X_7$ deficiency in the favourite epithelial cell line used to study many infections, the cervical carcinoma HeLa (Feng *et al*., 2006).

The present report shows for the first time that primary GECs express purinergic P2X receptors, including a functional $P2X_7$, and are sensitive to ATP_e-induced apoptosis. Consistent with the need of *P. gingivalis* to proliferate within the same cells, ATP_e-mediated GEC apoptosis is inhibited by infection with *P. gingivalis*. No *P. gingivalis* factors that can inhibit apoptosis had been identified until now. This study demonstrates that a putative *P. gingivalis* ecto-nucleoside diphosphate kinase (NDK), which can scavenge ATP_e, is involved in inhibition of $P2X_7$-mediated apoptosis. An *ndk*-deficient mutant was no longer able to inhibit ATP-induced

apoptosis of infected GECs and a recombinant *P. gingivalis*-NDK significantly inhibits one of the early events of ATP_e-mediated $P2X_7$ activation, plasma membrane permeabilization, in uninfected GECs and GECs infected with *ndk*-deficient mutant.

Results

Expression of the $P2X_7$ receptor in primary GECs

To determine whether GECs express the ionotropic purinoreceptor family of receptors ($P2X_{1–7}$), particularly the $P2X_7$ receptor, the cells were analysed by reverse transcription polymerase chain reaction (RT-PCR) and immunofluorescence microscopy. The RT-PCR analysis showed that amplicons of the expected sizes were present for at least five P2X receptors: $P2X_2$, $P2X_4$, $P2X_5$, $P2X_6$ and $P2X_7$ (Fig. 1A). Amplification of $P2X_6$ gave an additional smaller band, previously shown to be the result of alternative splicing (Coutinho-Silva *et al.*, 2005a). $P2X_1$ and $P2X_3$ were not expressed in GECs, indicating tissue specificity for these receptors. Immunofluorescence microscopy confirmed the uniform surface expression of the $P2X_7$ receptor throughout the GEC plasma membrane (Fig. 1B), although a large proportion of the receptors appeared to be distributed in granular compartments, reminiscent of endocytic or secretory vesicles. Thus, the GECs, which comprise the initial lining of the gingival mucosa, express most P2X receptor family members, and particularly $P2X_7$.

Apoptosis of GECs Due to treatment with extracellular nucleotides

Ionotropic P2X receptors, such as $P2X_7$, are linked to pores that switch their conformation from closed to open on binding of ATP, allowing ions such Ca^{++} and K^+ to flow into cells. In macrophages, prolonged ATP_e treatment leads to $P2X_7$-dependent necrosis or apoptosis (Lemaire *et al.*, 2006; Lister *et al.*, 2007). In order to evaluate whether the $P2X_7$ receptor may be functional, we examined the sensitivity of GECs to ATP_e-mediated apoptosis. GECs were incubated with control buffer or 5 mM of the indicated nucleotides for 1 h in Mg-free buffer phosphate-buffered saline (PBS). The buffer was then removed and cells incubated in GEC culture-medium for an additional 24 or 48 h. Apoptosis was quantified by Annexin-V/propidium iodide (PI) staining and analysed by cytofluorimetry (Fig. 2A). Treatment with the $P2X_7$ agonist ATP caused a significant increase in the level of apoptosis compared with control samples, whereas nucleotides such as ADP and UTP had no effect on the level of apoptosis (Fig. 2A). These initial results indicate that GECs are sensitive to ATP_e-induced apoptosis and suggest a role for the $P2X_7$ receptor. However, in the light of a recent report (Solini *et al.*, 2007) demonstrating that the $P2X_4$ receptor could also mediate ATP-induced apoptosis, we examined $P2X_4$ protein expression in GECs by immunofluorescence microscopy and detected very low levels of $P2X_4$ protein (not shown). Thus, we can not exclude the possibility that $P2X_4$, although expressed at much lower levels than $P2X_7$ protein, could contribute to some degree to ATP-induced apoptosis of GECs.

Effect of $P2X_7$ receptor agonists and antagonists on GEC apoptosis

The specificity of the receptor for ATP was further evaluated by measuring the effect of various $P2X_7$ agonists and antagonists on apoptosis. ATP-induced apoptosis of GECs could be significantly blocked by pretreatment with the irreversible $P2X_7$ antagonist oxidized (ox) ATP (0.3 mM); and incubation with the selective $P2X_7$ agonist, benzoylbenzoyl (BzATP, 2 mM), stimulated a large level of apoptosis in GECs (Fig. 2B). Thus, the results suggest that $P2X_7$ receptors in GECs are functional and are likely to be responsible for most of the apoptosis mediated by ATP_e.

Inhibition of ATP-induced apoptosis by P. gingivalis infection

In macrophages, infection with mycobacteria or chlamydiae inhibits partially $P2X_7$-mediated host-cell death (Lammas *et al*., 1997; Coutinho-Silva *et al*., 2001). In order to determine whether infection of epithelial cells with an intracellular pathogen may have a similar effect, we examined ATP_e-induced apoptosis in GECs infected with *P. gingivalis*. Hence, the epithelial cells were pre-infected with *P. gingivalis* for 12 h before 1 h treatment with 5 mM ATP_e followed by a 24 or 48 h infection. The percentage of apoptotic cells was measured by Annexin-V/PI staining. Consistent with previous findings (Yilmaz *et al*., 2004), infection by itself for 24 or 48 h induced only a low level of apoptosis (Fig. 3), but *P. gingivalis* infection suppressed considerably the pro-apoptotic effect of ATP (Fig. 3).

Identification of a P. gingivalis protein with homology to NDK

A BLAST similarity search of the *P. gingivalis* W83 proteome (Nelson *et al*., 2003), using the *Bacillus anthracis* NDK protein NP843987 as query, revealed a weak similarity (e = 0.002) to an unannotated hypothetical protein PG1018. A subsequent BLAST search of the non-redundant protein database using PG1018 as query revealed additional weak similarities to NDK homologues from a number of other bacillus strains and a putative protein from *Giardia lamblia* that has strong similarities to NDK proteins. The 102 amino acid sequence of PG1018 was aligned with a number of NDK proteins from organisms ranging from bacteria to humans (Fig. 4). The known NDK proteins, each containing ~150 amino acids, showed strong sequence similarity to each other, with only a small amount of heterogeneity at the N- and C-termini. Optimal alignment of the PG1018 sequence required several gaps to be inserted into the alignment. However, significant amino acid conservation between the PG1018 and NDK proteins was observed. Importantly, Tyr34, Asn96 and His101 were conserved with residues in the NDK family of proteins that have been shown to be critical for kinase function (Schneider *et al*., 2001) (Fig. 4). This analysis suggests that PG1018 could be classified as a distantly related kinase belonging to the NDK family of kinases. In addition, recombinant PG1018 protein derived from *P. gingivalis* was able to hydrolyse ATP with a K_m of 8.9 mM and a K_{cat}/K_m equal to 1.62 min^{-1} μM^{-1} (not shown), similar to the values reported for *M. tuberculosis* (Kumar *et al*., 2005) and reinforcing its classification as an NDK enzyme.

Inhibition of ATP-mediated cell death by the P. gingivalis homologue of NDK

Supernatants from *M. tuberculosis*, another successful persistent intracellular bacterium, have been shown to contain the ATP-scavenging enzyme, NDK, and to inhibit ATP-induced death of macrophages (Zaborina *et al*., 1999). To establish a role for the *P. gingivalis* NDK homologue more directly, we therefore constructed an isogenic *ndk* mutant strain of *P. gingivalis* 33277 by allelic exchange, naming the *ndk*-deficient strain ΔPG1018. To ensure that any difference in the apoptotic properties of the mutant are not the result of changes in invasion efficiency, intracellular levels of ΔPG1018 were quantified by fluorescence microscopy, as we previously described (Yilmaz *et al*., 2006). Infection by both the wild-type and mutant strains showed similar numbers of intracellular bacteria at both early time points (1 h, 6 h, 12 h and 24 h) post infection (data not shown) and later time points (48 h) (Fig. 5A), confirming that invasion and infection efficiency was similar for both strains. Subsequently, the culture supernatants and pellets from the wild-type and ΔPG1018 strains were assayed for ATPase activities of the secreted and intracellular forms of the *P. gingivalis* NDK protein, using ATP as a substrate. The rate of ATP hydrolysis was found to be significantly lower in the mutant strain, compared with the wild-type strain (Fig. 5B), demonstrating that the *P. gingivalis* NDK homologue is secreted and consumes ATP.

Next, in order to assess the relevance of *P. gingivalis*-NDK for protection against apoptosis induced by ATP_e, GECs were infected with the wild-type or ΔPG1018 strain in the presence of ATP or control buffer (no ATP) for 24 or 48 h. Uninfected cells were also treated with ATP,

UTP or ADP for 24 or 48 h (not shown). Induction of apoptosis by ATP was confirmed using the TUNEL technique, which measures single-strand breaks in DNA. In agreement with the level of apoptosis measured by Annexin-V/PI staining, we found that incubation with ATP, but not UTP or ADP, leads to apoptosis in > 45% of the uninfected cells (Fig. 6A), and 12 h preinfection with the wild-type strain protects cells against apoptosis induced by subsequent incubation with ATP for 24 or 48 h. In contrast, ΔPG1018 infection by itself induced a low level of apoptosis after 24 or 48 h of infection, and 12 h preinfection with the mutant strain did not protect GECs against apoptosis following subsequent incubation with ATP (Fig. 6B). In fact, GECs preinfected with ΔPG1018 were more sensitive to ATP-induced apoptosis than uninfected cells as observed by the larger amount of cells dying after infection with the mutant in the presence of ATP (Fig. 6B). In order to verify the specific effect of the mutagenesis, the mutant strain was complemented by the wild-type *ndk* allele in *trans*. GECs that were infected with the complemented strain and incubated with ATP displayed similar levels of apoptosis induced by the wild strain (Fig. 6B). Thus, the results suggest a major role for the NDK homologue in the survival of ATP-treated host cells.

Effect of ndk mutation on ATP-induced membrane permeabilization

The *P. gingivalis*-NDK homologue could either inhibit a $P2X_7$-dependent apoptotic pathway in the cell or deplete extracellular ATP before ligation of $P2X_7$. To discriminate between these possibilities, we therefore measured the effects of infection by wild-type and *ndk*-deficient strains on an early event following $P2X_7$ ligation, namely plasma membrane permeabilization. In this regard, $P2X_7$ ligation leads to opening of ion channels that are selective for small ions, followed by the opening of larger non-specific pores that are permeable to molecules smaller than 900 Da (Surprenant *et al*., 1996; Khakh and North, 2006). We investigated whether *P. gingivalis* infection could block $P2X_7$-mediated permeabilization by using a 457 Da membrane-impermeant fluorescent dye, Lucifer Yellow (LY). As shown in Fig. 7A, cells treated with ATP showed a significant time dependent increase in LY uptake, whereas cells infected with the wild-type strain showed no significant increase in LY uptake and also demonstrated a dramatic ability to decrease LY uptake in response to ATP treatment. Approximately 83% of cells were stained with LY upon 1 h ATP_e treatment followed by 48 h incubation, whereas the wild type-infected cells were only 20% positive for dye uptake after treatment with ATP_e (similar to basal levels). Conversely, cells infected with the ΔPG1018 strain and incubated with ATP under the same conditions exhibited a significantly reduced level of inhibition of permeabilization (approximately 59% of the cells were positive for LY uptake after treatment with ATP). Accordingly, the results indicate that the NDK homologue of *P. gingivalis* interferes at an early step of $P2X_7$-dependent signalling, suggesting that it acts before downstream apoptotic signalling events.

The ability of NDK to interfere with $P2X_7$ ligation was confirmed by examining the effect of recombinant NDK (rNDK) on the $P2X_7$-mediated dye uptake in uninfected cells. ATP-evoked LY uptake was significantly inhibited in a concentration dependent manner within 15 min and 1 h exposure to rNDK in uninfected GECs (Fig. 7B). Moreover, incubation of rNDK with the *ndk*-deficient strain (ΔPG1018) infected cells during the ATP_e treatment greatly inhibited the ATP-induced dye uptake in the infected cells (Fig. 7C). The results decisively support the concept that the NDK homologue of *P. gingivalis* serves as a critical molecule for the inhibition of ATP-induced cell death in GECs.

Discussion

Epithelial cells represent a major line of initial defence against pathogenic bacteria that colonize mucosal tissues, but at the same time represent an initial site for host invasion (Ganz, 2002). An increasing number of intracellular bacterial pathogens are now known to modulate

apoptotic pathways in epithelial cells to establish persistent infection (Monack and Falkow, 2000). *P. gingivalis*, a successful oral colonizer, is capable of intracellular replication and remains viable for extended periods in primary GECs (Rudney *et al*., 2001; Yilmaz *et al*., 2006). Interestingly, despite the burden of large numbers of intra-cellular bacteria, infected GECs do not undergo cell death and are instead resistant to chemically induced apoptotic cell death. *P. gingivalis* infection modulates several anti-apoptotic pathways in addition to interfering with other cell-signalling pathways and disseminating intercellularly through actin-based membrane protrusions (Yilmaz *et al*., 2004; 2006; Mao *et al*., 2007).

The $P2X_7$ receptor has been studied primarily in cells of haemopoietic origin (Lister *et al*., 2007), but recent studies indicate that it is also expressed in some epithelial cells (Schwiebert and Zsembery, 2003; Pastore *et al*., 2006), although its function in epithelial cells remains largely uncharacterized. In macrophages, the receptor is involved in a number of essential cellular events including regulation of apoptosis, control of intracellular bacterial infection, and modulation of inflammatory immune responses (Coutinho-Silva *et al*., 2001; Fairbairn *et al*., 2001; Chen and Brosnan, 2006; Mariathasan and Monack, 2007). $P2X_7$ receptor activation requires high concentrations of extracellular ATP (e.g. 100 μM-10 mM), but these concentrations can be attained at sites of bacterial infection and inflammation (Khakh and North, 2006; Idzko *et al*., 2007). $P2X_7$-deficient mice are resistant to development of anticollagen-induced arthritis (Labasi *et al*., 2002) and accumulate lower levels of IL-1β in inflamed foot pads induced by injection of Freund's complete adjuvant (Chessell *et al*., 2005), suggesting that ATP concentrations sufficiently high to stimulate $P2X_7$ can be produced *in vivo* under physiological conditions. ATP can also be released extracellularly in a regulated manner, and its release into the extracellular space from necrotic cells is expected to result in the activation of $P2X_7$ receptors (Khakh and North, 2006). ATP released into the external milieu can thus serve as a 'danger signal' for other components of immune system and initiate a prompt innate immune response by triggering secretion of IL-1β, generating reactive oxygen species, inducing apoptosis and killing of intracellular pathogens following ligation of the danger signal receptor, $P2X_7$. Conversely, these special signalling events can be modulated by intracellular bacteria to persist in the host cells (Coutinho-Silva *et al*., 2001; Fairbairn *et al*., 2001; Franchi *et al*., 2007). Several intracellular pathogens including *Chlamydia* and *Mycobacterium* species can enhance their survival by partially inhibiting host-cell apoptosis. In the case of *M. tuberculosis*, supernatant from the mycobacteria containing the NDK protein decreases the activity of the $P2X_7$ receptor (Zaborina *et al*., 1999).

In the present study, we demonstrate for the first time that an intracellular pathogen can interfere with $P2X_7$-dependent apoptosis of epithelial cells, and identify a likely mechanism. Primary GECs, which form an initial interactive interface with colonizing oral bacteria, express functional $P2X_7$ purinergic receptors and are sensitive to apoptosis induced by the $P2X_7$ agonists ATP and BzATP. Moreover, treatment of GECs with control nucleotides such as ADP and UTP did not induce apoptosis, while the specific $P2X_7$ antagonist, oxATP, blocked ATP-induced death. Collectively these results further corroborate the role of $P2X_7$ in ATP-induced apoptosis and establish the functionality of this mechanism in epithelial cells.

The host-adapted pathogen *P. gingivalis* suppresses $P2X_7$-ATP-mediated apoptosis. This effect is mediated by a putative NDK homologue of *P. gingivalis*, as evidenced by a significant reversal of apoptosis suppression in cells infected with an *ndk*-deficient strain and the ability of rNDK to inhibit significantly ATP-induced plasma membrane permeabilization in both uninfected cells and cells infected with the *ndk*-deficient strain. In addition, analysis of the levels of intracellular bacteria during the 48 h infection period of GECs in the absence of ATP_e treatment for wild-type versus *ndk*-deficient strain demonstrates comparable values, suggesting that the main role of the. NDK homologue of *P. gingivalis* is to interfere with host-cell apoptosis due to stimulation with extracellular ATP. In a similar manner, previous work

on macrophages infected with the *Mycobacterium bovis* Bacille Calmette-Guérin has shown that induction of apoptosis with a Fas ligand (H_2O_2) did not kill the intracellular bacterium even though it caused the death of the host cells. However, promoting of apoptosis with ATP through $P2X_7$-signalling stimulated the death of both the bacteria and the host cells (Molloy *et al*., 1994; Fairbairn *et al*., 2001). These results highlight the need of intracellular bacteria such as mycobacteria and *P. gingivalis* to evolve specialized mechanisms for interfering with $P2X_7$ ligation.

In conclusion, our results suggest that NDK is secreted by *P. gingivalis* and consumes ATPe, which is released at sites of infection, thus preventing activation of $P2X_7$ receptors by ligation with ATP. The consequent inhibition of $P2X_7$-mediated apoptosis and extension of GEC viability would allow *P. gingivalis* to survive for prolonged periods in the gingival epithelium and contribute to disease when other host and bacterial factors are conducive to the processes of tissue destruction. Moreover, a study (Xia *et al*., 2007) examining the quantitative proteomics of intracellular *P. gingivalis* demonstrates the secretion of the NDK homologue during *P. gingivalis* infection in GECs. Interestingly, in the absence of exogenous ATP_e, infection with the mutant lacking NDK results in a higher level of GEC apoptosis than infection with the wild-type strain, suggesting that some ATP may be released from the infected cell. It had been previously reported that ATP is released from macrophages infected with mycobacteria (Sikora *et al*., 1999), implying that the presence of ATP_e may be a general phenomenon associated with many infections with intracellular pathogens. It is thus tempting to speculate that other intracellular bacteria may also produce secreted ATP-scavenging enzymes as a strategy for protecting the host cell. Although future investigations remain to elucidate the exact mechanisms and outcomes of the interaction of NDK with host cells, the enzyme may represent a novel virulence factor of *P. gingivalis*, and a potential target for therapeutic intervention.

Experimental procedures

Culture of primary GECs

Primary cultures of GECs were generated as described previously (Lamont *et al*., 1995). Briefly, healthy gingival tissue was obtained after oral surgery, and surface epithelium was separated by overnight incubation with 0.4% dispase. Cells were cultured as monolayers in serum-free keratinocyte growth medium (KGM) (Lonza) at 37°C in 5% CO_2. GECs were used for experimentation at 75−80% confluence and cultured for 48 h before infection with bacterial cells or exposure to other test reagents in KGM.

Bacteria, growth conditions, mutant and complementation strain construction

Porphyromonas gingivalis ATCC 33277, the nucleoside diphosphate kinase (*ndk*)-deficient mutant and the complementation strain were cultured anaerobically for 24 h at 37°C in trypticase soy broth supplemented with yeast extract (1 mg ml^{-1}), haemin (5 μg ml^{-1}) and menadione (1 μg ml^{-1}). Erythromycin (10 μg ml^{-1}) was added to the media for culture of the mutant strain. The media of the complemented strain was supplemented with erythromycin (10 μg ml^{-1}) and tetracycline (3 μg ml^{-1}). All bacteria were grown for 24 h, harvested by centrifugation at 6000 *g* and 4°C for 10 min, washed twice, and resuspended in Dulbecco's Phosphate-buffered saline (Sigma) pH 7.3 before they were incubated with host cells. The number of bacteria was determined using a Klett-Summerson photometer.

Construction of mutant strain: Mutation in the PG1018 gene was obtained by allelic replacement, using a PCR fusion technique. A DNA sequence containing 1000 bp upstream of the PG1018 initiation codon was amplified from *P. gingivalis* ATCC 33277 chromosomal DNA using primers PG1018A (5′ACG GCTCTTCGGTTCAATTTGATCTTCTGT3′) and

PG1018B (5′AT TTGCGGATAATCATATAGAACCATGCTA3′). A 1000 bp region downstream of the PG1018 stop codon was amplified using primers 1018C (5′ TGTATATCAGGCCAAGCAAAGCCACCA CAT3′) and 1018D (5′ TCAGAGAAAAAAGTATACTTAGGAAAG AAG3′). To replace the PG1018 gene, an ermF cassette was constructed from pVA3000 using primers ermF-f (5′CATGGTTC TATATGATTATCCGCAAATATGACAAAAAAGAAATTGCCCGTT CG3′) and ermF-r (5′ATGTGGTGGCTTTG CTTGGCCTGATATA CACTACGAAGGATGAAATTTTTCAGGGAC3′). A fusion PCR amplicon was produced using the technique previously described (Kuwayama *et al*., 2002). The final fusion product was cloned into the pCR2.1-TOPO vector (Invitrogen) and sequenced through the fusion region using the ermF start (5′ATGAACAGTAA GAAACCCCT3′) and ermF stop (5′ CTGTCAAATCAGCCCT GTTA3′) sequencing primers. Once the construct was confirmed, the plasmid was linearized with BamHI and introduced into *P. gingivalis* by electroporation (Simionato *et al*., 2006). A double crossover recombination event was selected with erythromycin (10 μg ml^{-1}). Insertion of the replacement allele was confirmed by PCR and Southern hybridization.

Construction of complementation strain: A DNA sequence containing 1500 bp upstream of the PG1018 initiation codon and PG1018 gene was amplified from P. gingivalis ATCC 33277 chromosomal DNA using primers, NdkUp (5′-GCGCGGATCCGA TGGACTGCGAGAGCATCTG-3′) and Nd kDaown 3′-CGCGTC GACTCAGAGAGGCAGAGGGCGGTCAATC-5′) which introduced SalI and BamHI sites. The PCR product was digested with SalI and BamHI and ligated into the corresponding sites of shuttle vector plasmid pT-COW (kindly provided by N. Shoemaker, University of Illinois), to create pT-1018, which was introduced into Ndk mutant by conjugation. Erythromycin (10 μg ml^{-1}) and tetracycline (3 μg ml^{-1})-resistant transconjugants were selected, and the presence of plasmid was confirmed by PCR.

Reverse transcription PCR analysis for P2X receptors in primary GECs

Total RNA was isolated using RNeasy kit (Qiagen) following the manufacturer's instructions. Total RNA was converted into cDNA by standard reverse transcription with M-MLV-Reverse Transcriptase (Promega). cDNAs were amplified using the MJ, Mini (BIO-RAD laboratories) in a 50 ml reaction mixture containing one-fifteenth of the cDNA generated from reverse transcription reaction, 1 × PCR buffer, 2.5 mM $MgCl_2$, 0.25 mM (each) dNTPs, 0.5 μM forward and reverse primers, and 1 U GoTaq DNA polymerase (Promega). The sequences of the primers used for GAPDH, $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$ and $P2X_7$ are listed in Table 1 (Coutinho-Silva *et al*., 2005a). The PCR cycling protocol for all primers was 94°C at 45 s, 60° C at 45 s, 72°C at 45 s. The protocol was conducted for 40 cycles and included an initial 10 min enzyme activation step at 95°C and a final 10 min extension step at 72°C. PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining. The intestinal epithelial cells were used as positive controls for the primers for $P2X_1$ and $P2X_3$, which we previously detected in the intestinal cells (Coutinho-Silva *et al*., 2005b). The PCR products were sequenced to confirm authenticity.

Surface expression analysis of the $p2x_7$ and $p2x_4$ receptor by immunofluorescence microscopy

Gingival epithelial cells were grown on 4-well chambered glass slides (Nalge-Nunc International), washed with ice-cold PBS, and fixed with 10% neutral buffered formalin for 1 h at room temperature. After washing twice with PBS, the cells were treated with permeabilization solution (0.1% Triton X-100) for 15 min. Samples were then washed twice with PBS and incubated with antibody against an extracellular domain of the human $P2X_7$ receptor (Chemicon) or antibody against human $P2X_4$ (Santa Cruz Biotechnology) and

detected with Alexa-Fluor 594 secondary antibody (Invitrogen). Samples with no primary antibody incubation were included as control. The samples were treated with 4,6-diamidino-2-phenylindole (DAPI) 1 μg ml^{-1} (Sigma) to visualize the nuclei. Finally, the samples were washed twice with PBS and analysed using a Zeiss Axio imager A1 fluorescence microscope equipped with band pass optical filter sets appropriate for imaging of the dyes and a cooled CCD camera (Qimaging). Single exposure images were captured sequentially and saved by Qcapture software v.1394.

Infection of primary GECs with P. gingivalis and treatment with extracellular nucleotides

Gingival epithelial cells were infected at a multiplicity of infection (moi) of 100 with *P. gingivalis* for 24 or 48 h at 37°C in a 5% CO_2 incubator. All time points for the infections were carried backwards, so that all incubations could be stopped and assayed at the same time at the end of 48 h. For measurement of nucleotide-induced apoptosis and inhibition, GECs were incubated with 5 mM concentrations of ATP, ADP, UTP, 2 mM benzoylbenzoyl ATP (BzATP) (Sigma) for 1 h or 0.3 mM oxidized ATP (oxATP) (Sigma) in Mg^{++} free PBS for 2 h at 37°C in a 5% CO_2 incubator. The medium was then removed and replaced with cell culture medium. The cells were incubated for the indicated time points (24 and 48 h). For some assays, cells were infected 12 h with P. gingivalis before ATP treatment in cell culture medium for the additional 24 or 48 h incubations.

Analysis of apoptosis by annexin-v and PI staining by cytofluorimetry

Early apoptotic changes were identified using fluorescein isothiocyanate (FITC)-conjugated Annexin-V-fluos (Roche), which binds to PS exposed on the outer leaflet of apoptotic cell membranes. PI (Sigma) was used for discrimination of necrotic cells from the annexin-V-positively stained cell cluster (Yilmaz *et al*., 2004). Briefly, GECs were grown on six-well plates (Falcon) at a density of 2×10^5 per well, incubated with the indicated treatments as described above, and dissociated using 0.05% trypsin/0.53 mM EDTA (Gibco BRL). Since apoptotic and necrotic cells could be present in the supernatant, both adherent GECs and cells in suspension were collected. The samples were then washed in cold phosphate-buffered saline (PBS), and resuspended in 100 μl Annexin-V-Fluos binding solution containing 20 μl Annexin-V-Fluos labelling reagent per 1000 ml Hepes buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$) and 1 μg ml^{-1} PI. After 15 min incubation in the dark at room temperature, 400 μl incubation buffer was added to each sample and the cells were analysed by flow cytometry using 488 nm excitation, a 515 nm band pass filter for fluorescein detection, and a 585 nm filter for PI detection. Cells incubated in the binding buffer with only Annexin-V or PI separately served as controls. For each dye, appropriate electronic compensation of the instrument was performed to avoid overlapping of the two emission spectra.

DNA fragmentation assay by TUNEL

DNA strand breaks induced during apoptosis were identified using a terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay kit following the manufacturer's protocol (Roche Applied Science). Briefly, GECs were grown on 4-well chambered slides, washed with ice-cold PBS, and fixed with 10% neutral buffered formalin for 1 h at room temperature. After washing twice with PBS, the cells were treated with permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. Samples were then washed twice with PBS and incubated with the TUNEL reaction mixture, containing FITC-labelled dUTP and terminal deoxynucleotidyl transferase, for 1 h at 37°C. Control cells were incubated in the absence (negative control) or presence (positive control) of DNAse (Roche Applied Science), in addition to control samples incubated without deoxynucleotidyl transferase. After incubation, the cells were washed twice with PBS and analysed using the

fluorescence microscope system described above. An average of 150 cells per sample was studied from at least 2 separate experiments per condition.

Measurement of Lucifer Yellow (LY) uptake by fluorescence microscopy

Cell membrane permeabilization was visualized by the differential uptake of LY (457 Da) in uninfected GECs or cells infected with the wild-type *P. gingivalis* or the mutant strain (ΔPG1018) grown on 4-well chambered slides that were treated with 0.25 mg ml^{-1} LY with and without 5 mM ATP at 37°C for 10 min. The samples were then washed 3 times with PBS and examined under the Zeiss Axio imager A1 microscope described above. LY uptake in the acquired images was then quantified with NIH ImageJ analysis software. Samples treated with 5 mM ATP for 10 min and 2 h served as positive controls. The threshold of fluorescence intensity was determined with sample that was uninfected and untreated. At least 300 cells per sample from 2 separate experiments were analysed to determine the percentage of cells positively stained for LY.

Analysis of P. gingivalis and ndk mutant (ΔPG1018) invasion efficiency by immunofluorescence microscopy

Immunofluorescence labelling and microscopy for determining the level of infection were performed as previously described (Yilmaz *et al*., 2003; 2006). Briefly, GECs cultivated on the 4-well chambered cover-glass slides were infected with *P. gingivalis*, ΔPG1018, and the complemented strain at an moi of 100 at 37°C for 1, 6, 12, 24 or 48 h. The samples were incubated with anti-*P. gingivalis* 33277 antibody and reacted simultaneously with Oregon Green 488 secondary antibody (Invitrogen), and DAPI 1 μg ml^{-1} (Sigma). The samples were visualized using the fluorescence microscope system described above. Acquired images were analysed for the intensity of fluorescence emitted from the infected samples with NIH ImageJ analysis software. An average of 150 cells per sample was studied from at least 2 separate experiments.

Purification of recombinant NDK

Recombinant *P. gingivalis*-NDK (rNDK) was produced by cloning the PCR-amplified putative *ndk* coding sequences from *P. gingivalis* into the pET30 expression system (Novagen). After induction in *E. coli*, rNDK was purified by chromatography over a Ni^{+2} metal chelating resin and eluted with imidazole. The eluted samples were subjected to SDS-PAGE, and staining with Coomassie blue (not shown) indicated greater than 95% purity.

ATPase activity assay of P. gingivalis strains and rNDK

ATPase activity was measured using an ATPase colorimetric assay kit (Innova Biosciences). Samples were prepared from log phase *P. gingivalis* cells in addition to purified rNDK. Briefly, bacteria were centrifuged at 6000 *g* for 10 min. Both the supernatant and bacterial pellet were collected. The unused bacterial growth media used as control. Supernatants were filtered (0.22 μm pore size) whereas the pellets were lysed in 50 mM Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl, 0.1% SDS and 1% Triton X-100. Protein levels were determined (BIO-RAD) before the ATPase activity assay. The assay contained purified P_i –free ATP to ensure lowest possible background signals. Inorganic phosphate (P_i) standards provided by the company were used to calculate a standard curve of the enzymatic activity. The results were determined by calculating the amount of enzyme that catalyses the reaction of 1 μmol ATP substrate per minute.

Acknowledgements

This study supported by NIDCR R01DE16593 and R01DE11111 grants.

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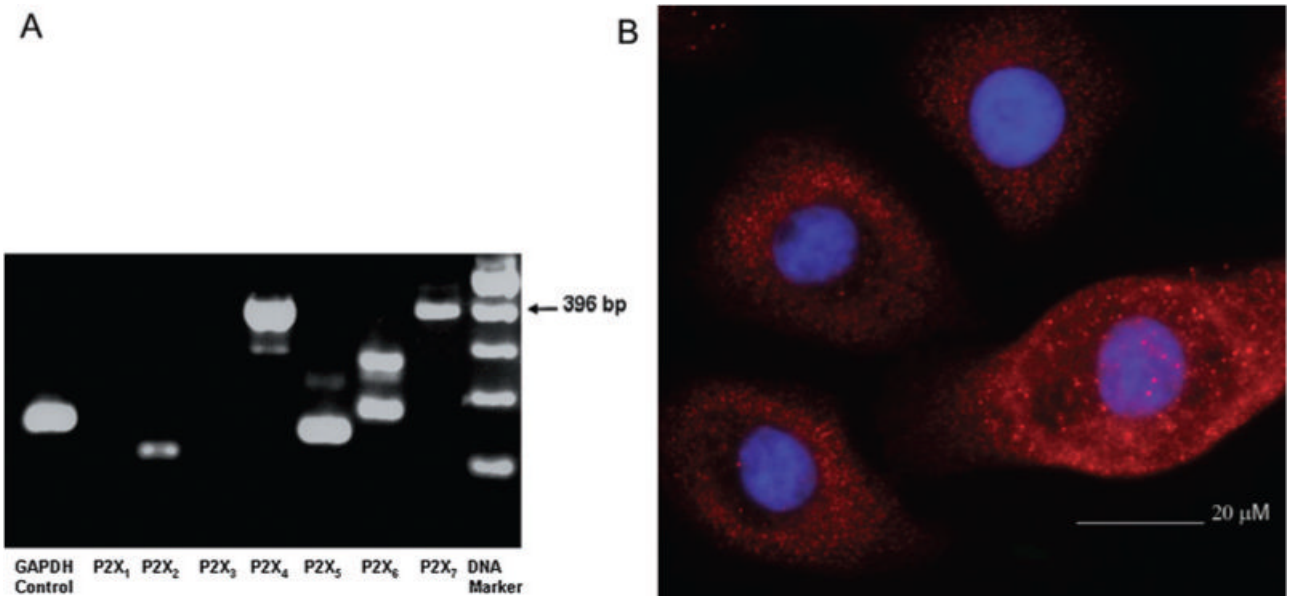

Fig. 1. Detection of P2X receptor(s) in GECs

A. RT-PCR measurement of receptor mRNA. mRNA expression of $P2X_7$ and other P2X receptors was verified by RT-PCR. Amplicons with predicted sizes were present for $P2X_2$, $P2X_4$, $P2X_5$, $P2X_6$ and $P2X_7$. The arrow indicates the predicted 396 bp transcript for $P2X_7$ receptor. B. Localization of $P2X_7$ by immunofluorescence microscopy. GECs were fixed and incubated with anti-$P2X_7$ antibody followed by Alexa-Fluor 594 conjugated secondary antibody (red). DAPI (blue) was used for staining the nucleus. The staining displayed strong and uniform expression of $P2X_7$ receptors in the GECs, along with some punctuate staining consistent with lysosomes or endosomes.

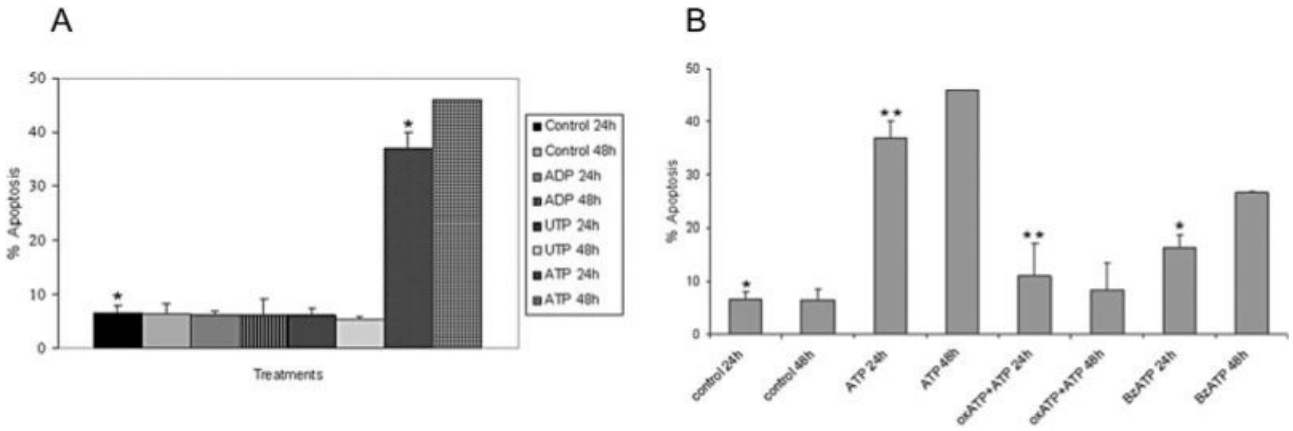

Fig. 2. Effect of $P2X_7$ agonists and antagonists on GEC apoptosis

A. Effect of ATP and other nucleotides on apoptosis. GECs were incubated with control buffer or 5 mM of the indicated nucleotides for 1 h in Mg-free buffer (PBS). The buffer was then removed and cells were incubated in GEC culture medium for an additional 24 or 48 h. Apoptosis was quantified by cytofluorimetry using AnnexinV/PI staining. Error bars represent the standard deviations of at least two independent measurements (**P* = 0.02 Student *t*-test). B. Effect of oxATP and BzATP on apoptosis. GECs were incubated with the $P2X_7$ agonist, BzATP (2 mM), for 1 h or alternatively with the antagonist oxidized ATP, oxATP (0.3 mM), for 2 h. Then, the antagonist was removed and ATP (5 mM) in Mg-free buffer (PBS) added for 1 h, followed by 24 or 48 h incubation with GEC culture medium. Apoptosis was quantified by cytofluorimetry using AnnexinV/PI staining. Error bars represent the standard deviations (± SD) of at least two independent measurements (***P* = 0.01 and **P* < 0.1 Student *t*-test).

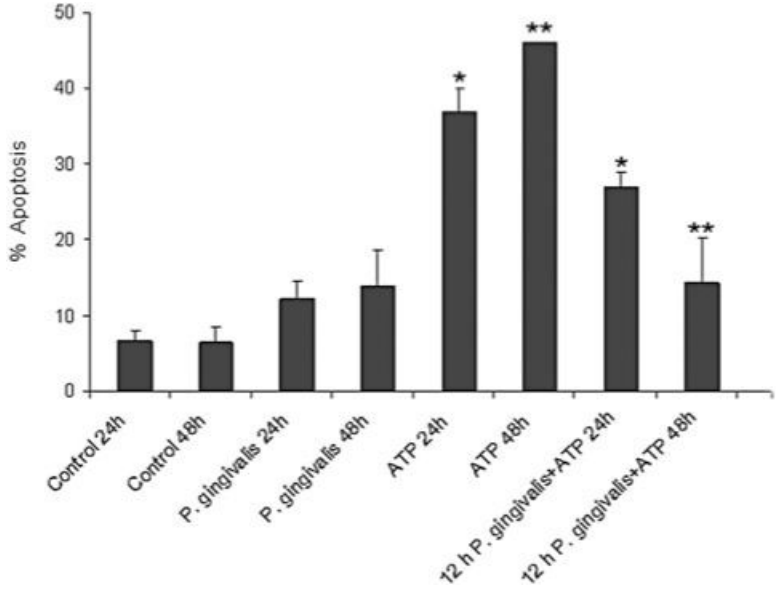

Fig. 3.

Porphyromonas gingivalis infection inhibits ATP-induced apoptosis in GECs. GECs were preinfected with *P. gingivalis* for 12 h before they were treated with 5 mM ATP in Mg-free buffer (PBS) for 1 h, followed by a 24 or 48 h infection period in GEC culture medium. Apoptosis was quantified by cytofluorimetry using AnnexinV/PI staining. Error bars represent the ± SD of at least two independent measurements. Asterisks (* and **) denote statistical significance ($P < 0.05$ Student *t*-test) between 12 h *P. gingivalis* + ATP treated and samples treated with ATP alone, respectively, for 24 and 48 h.

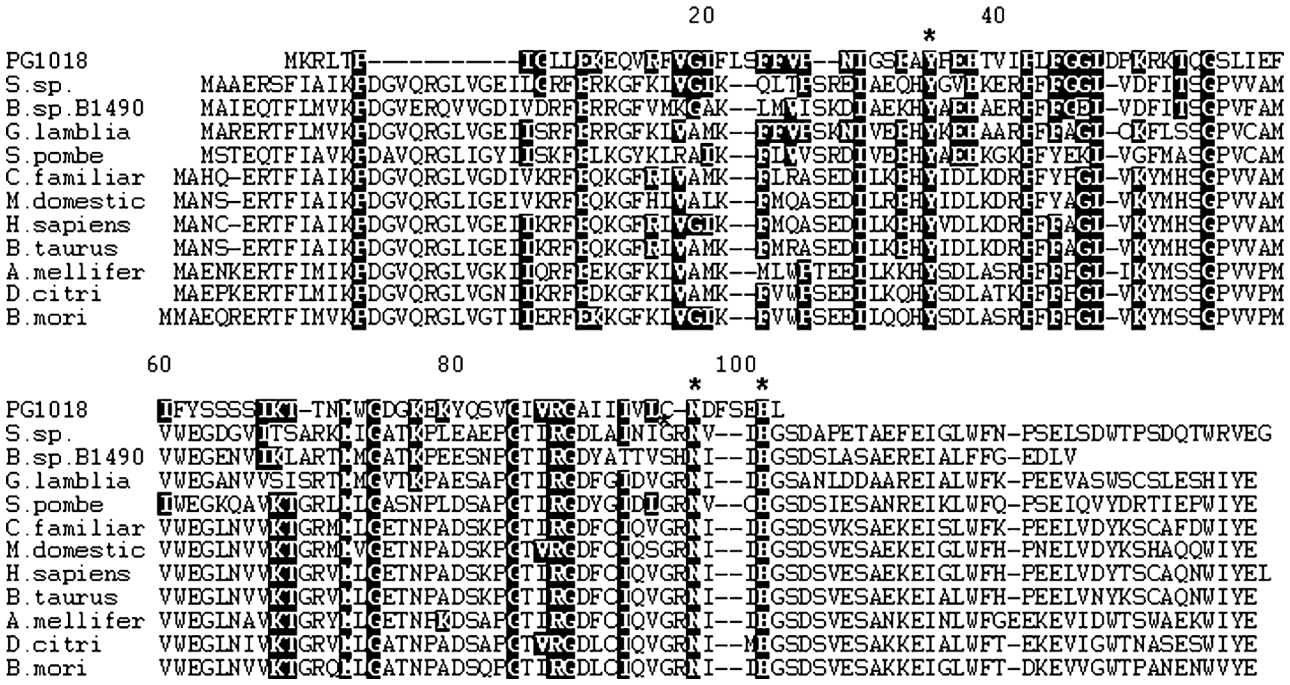

Fig. 4.

Sequence comparison of the putative *P. gingivalis*-NDK with NDK from different organisms. In the sequences an asterisk indicates residues implicated in NDK kinase activity: Y35, N96 and H101. Highly conserved residues are indicated by dark background and gaps are indicated by dashes. Accession numbers for the sequences are *P. gingivalis* PG1018 NP905239.1; *G. lamblia* XP771399.1; *Bacillus* sp. B14905ZP01725465.1; *B. mori* ABF51506.1; *B. taurus* NP991387.1; *Schizosaccharomyces pombe* NP592857.1; *D. citri* ABG81980.1; *P. pinaster* CAC84493.1; *A. mellifera* XP393351.2; *Synechococcus* sp. CC9311 YP731929.1; *Heliobacillus mobilis* AAC84038.1; *C. familiaris* NP001019809.1; *Mus domestica* XP001363771.1; *Homo sapiens* NP000260.1. Sequence alignment of the PG1018 protein and representative NDK proteins from organisms ranging from bacteria to humans was performed using ClustalW.

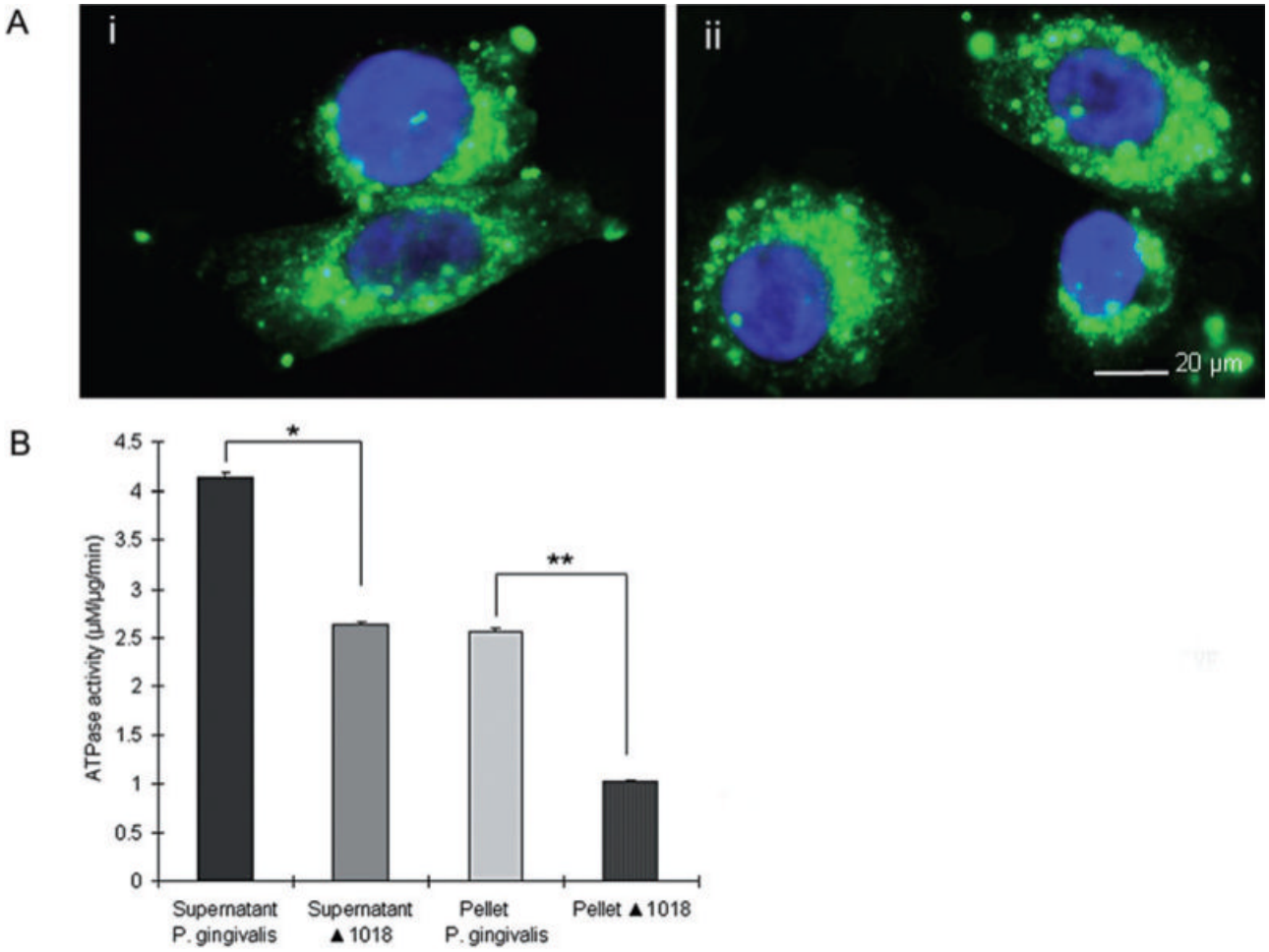

Fig. 5. Infection and ATPase Activity of wild-type and *ndk*-deficient *P. gingivalis* strains

A. Immunofluorescence microscopy of invasion (48 h) by the wild type (i) and the mutant ΔPG1018 (ii) strains. GECs were infected with wild type or ΔPG1018 (*ndk*-deficient mutant) at an moi of 100. The samples were fixed and stained with *P. gingivalis* antibody and then incubated simultaneously with Oregon Green 488 secondary antibody (green) and DAPI (blue) to visualize the bacterial infection. Results are representative images of 150 cells studied per sample from at least two individual experiments performed in duplicate. B. ATPase activity of the wild-type and mutant strains. The enzymatic activities were measured in the samples containing 0.01 mg ml^{-1} protein from the supernatants and 1 mg ml^{-1} protein from the pellets of the wild-type and mutant strains by calculating the μM P_i generated per minute. (*$P < 0.01$; and **$P < 0.001$ Student *t*-test, $n = 4$).

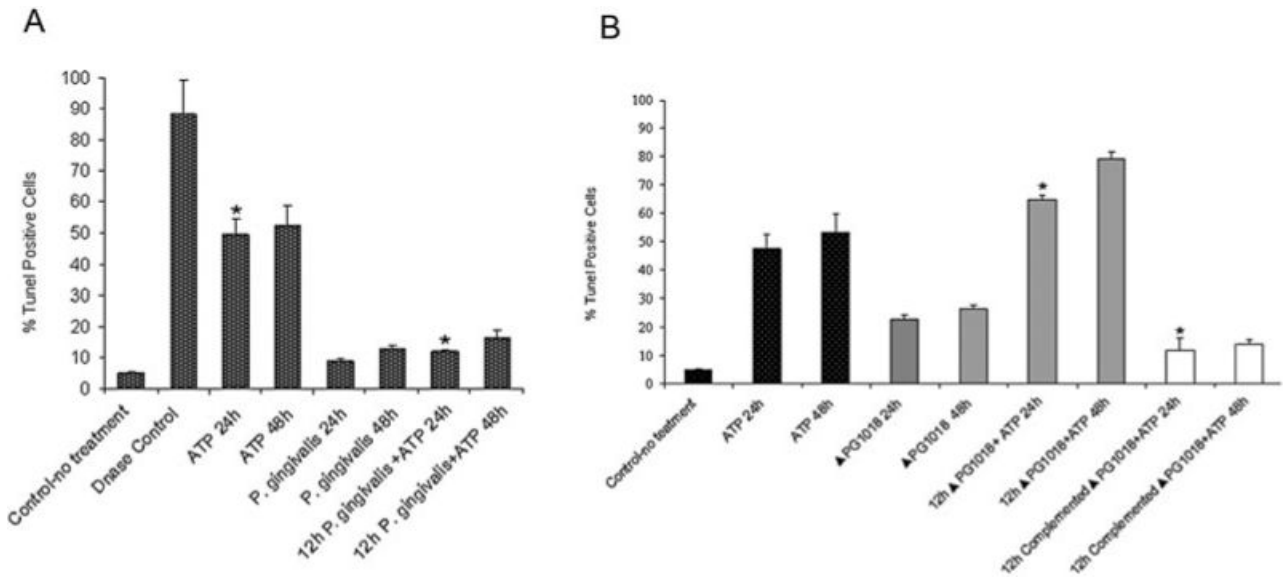

Fig. 6. DNA fragmentation of cells infected with the wild type, *ndk*-deficient and complemented strains

A. TUNEL assay determining the level of DNA fragmentation following treatment of wild-type *P. gingivalis*-infected cells with and without ATP. GECs were fixed and stained with TUNEL reagents and visualized by fluorescent microscopy to determine the level of DNA fragmentation. Samples incubated with DNAse and without any treatment, and without TUNEL enzyme (not shown) served as controls (**P* < 0.05, Student *t*-test, *n* = 2).

B. TUNEL assay determining the level of DNA fragmentation following treatment of *ndk*-deficient mutant (ΔPG1018) and the complemented strain-infected cells with ATP. DNA fragmentation in GECs was measured as described in Fig. 6A. For both A and B, results represent means ± SD of approximately 150 cells studied per sample from at least two individual experiments performed in duplicate (**P* < 0.005, Student *t*-test, *n* = 2).

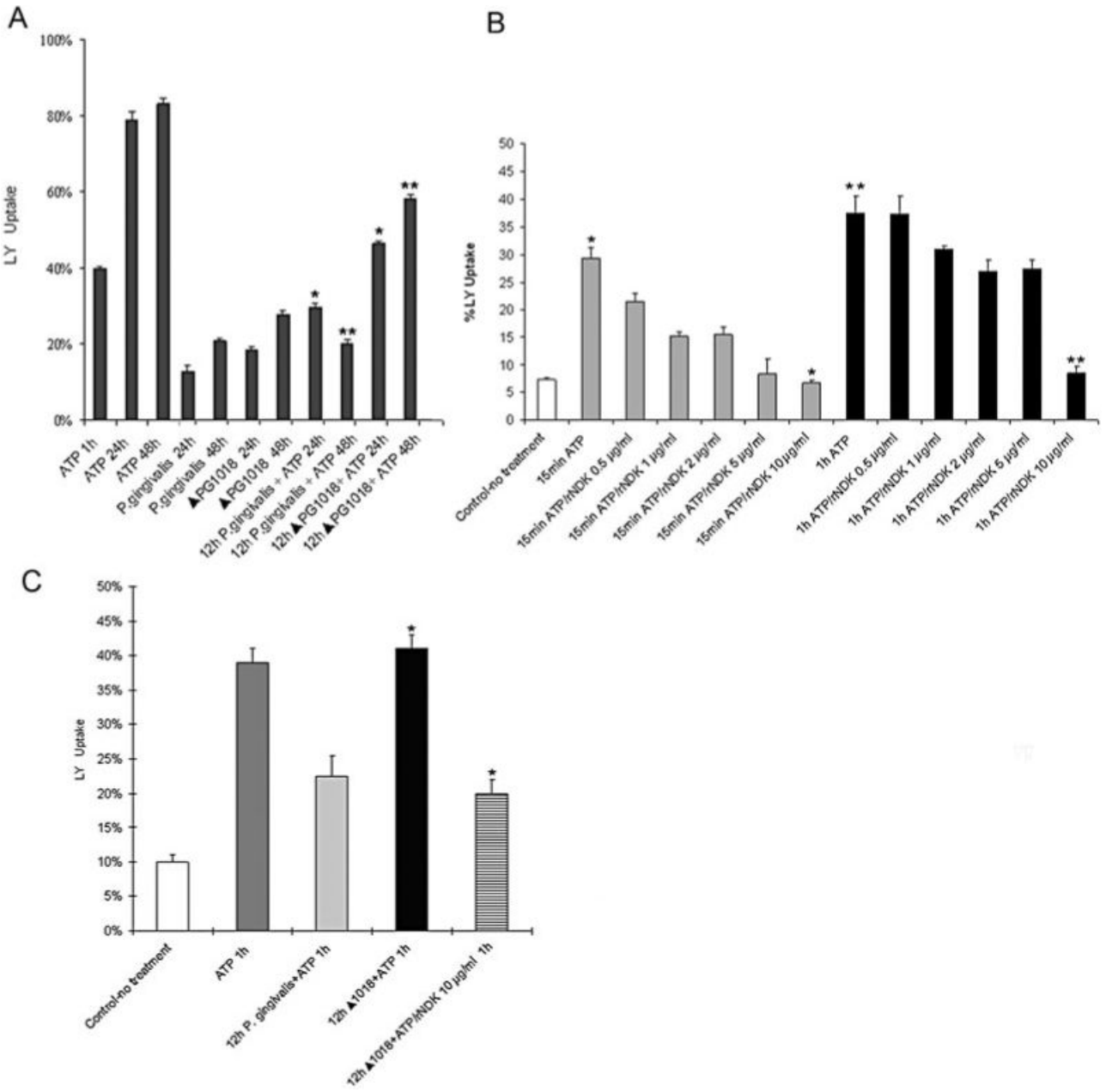

Fig. 7. Plasma membrane permeabilization of GECs infected with wild-type *P. gingivalis* and the *ndk* mutant strain following treatment with rNDK

A. Differential internalization of LY (457 Da) by GECs during infection by *P. gingivalis* and *ndk*-deficient mutant (ΔPG1018) was visualized by fluorescence microscopy. The threshold of fluorescence intensity was determined with use of a sample that was uninfected and untreated. Values represent means ± SD of approximately 300 cells studied per sample from at least two individual experiments performed in duplicate (**P* < 0.005; and ***P* < 0.01, Student *t*-test).

B. Differential internalization of LY by GECs following treatment with ATP and rNDK. LY uptake in GECs was measured as described in Fig. 7A. GECs were treated with 5 mM ATP and the varying concentrations of rNDK for 15 min or 1 h in Mg-free buffer (PBS). The starting

concentration of rNDK was determined from the ATPase activity assay of the enzyme (**P* = 0.01; and ***P* = 0.001, Student *t*-test).

C. Differential internalization of LY by *ndk*-deficient strain (ΔPG1018) infected GECs following treatment with ATP and rNDK. The results were obtained as described in Fig. 7A. GECs were infected with *ndk*-deficient strain for 12 h and then incubated with rNDK in the presence of 5 mM ATP for 1 h in Mg-free buffer (PBS) (**P* = 0.03, Student *t*-test).

Table 1

Primers used P2X receptor amplification.

Gene	Primer sequence, forward (5′-3′)	Primer sequence, reverse (3′-5′)
$P2X_1$	GCTGGTGCGTAATAAGAAGGTG	ATGAGGCCGCTCGAGGTCTG
$P2X_2$	AGGTTTGCCAAATACTACAAGATC	GCTGAACTTCCCGGCCTGTC
$P2X_3$	CTTCACCTATGAGACCACCAAG	CGGTATTTCTCCTCACTCTCTG
$P2X_4$	GATACCAGCTCAGGAGGAAAAC	GCATCATAAATGCACGACTTGAG
$P2X_5$	GGCATTCCTGATGGCGCGTG	GGCACCAGGCAAAGATCTCAC
$P2X_6$	AGCACTGCCGCTATGAACCAC	AGTGAGGCCAGCAGCCAGAG
$P2X_7$	TGATAAAAGTCTTCGGGATCCGT	TGGACAAATCTGTGAAGTCCATC
GAPDH	AACGGATTTGGTCGTATTGGGC	CTTGACGGTGCCATGGAATTTG