

THE ROLE OF NOREPINEPHRINE IN THE REGULATION OF GROWTH, ADHESION AND INVASION OF *PSEUDOMONAS AERUGINOSA* IN HUMAN LUNG CARCINOMA CELL CULTURE

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Abstract: Mammalian hormones are shown to affect not only regulation of mammalian homeostasis but also play roles in cross-talk between microorganisms and their hosts. In our study, the roles of norepinephrine (NE) at two different concentrations on growth alterations, adhesion - invasion of *Pseudomonas aeruginosa* were investigated.

The effects of NE on the growth, adhesion - invasion of *P. aeruginosa* ATTC 27853 were examined in human lung carcinoma (A549) cell culture. We analysed two concentrations (**HNE:** 0.04µg/mL and **LNE:** 0.0017 µg/mL) of NE considering NE's psychological levels in a healthy individual to imitate *in vivo* conditions of the host. Bacterial counts of growth and adhesion-invasion were examined by the colony counting method. Growth of bacterium was significantly reduced in the presence of NE at 90' incubation (p: 0.0004 for high concentration and p: 0.0003 for low concentration); on the other hand, at 180' minutes incubation, only low concentration reduced the growth (p<0.0001). While adhesion was increased in the presence of low NE concentration (p: 0.013), there was no alteration in invasion of *P. aeruginosa* in the presence of NE (p>0.05). Our results suggested that NE has different effects on the growth and adhesion of *P. aeruginosa* and these effects depend on concentration and incubation period.

Keywords: *Pseudomonas aeruginosa*, norepinephrine, growth, adhesion, invasion, A549 cell culture

1. Introduction

It is known that when a microorganism reaches the host tissues, host conditions including hormones have become the microbe's environment. Microbial endocrinology is a research area that aims to determine the roles of mammalian hormones on microbial behaviors during the infection process. Previous studies suggested that different kinds of hormones have effects on the modulation of growth, virulence

properties, antimicrobial susceptibilities and gene expressions (Plotkin and Viselli, 2000; Lyte and Freestone, 2010; Lyte and Cryan, 2014; Fteita et al., 2014; Plotkin and Konakieva, 2017; Lyte et al., 2021).

It seems that microorganisms sense and respond to host hormones. Consisting with this opinion, some authors focused on stress hormones and their metabolic activities. The

ability of hormones to stimulate the growth and the pathogenicity of microorganisms have been shown (Bearson, 2016; Cambronel et al, 2020; Freestone et al., 2007; Gümüş et al., 2019; Lyte et al., 2003; Truccollo et al., 2020). On the other hand, in the intensive care units, it is well known that treatment of patients with catecholamines, especially norepinephrine and dopamine, as inotropic drugs, can be helpful to overcome infectious stages.

Pseudomonas aeruginosa is an opportunistic pathogen and it is responsible for keratitis, cystic fibrosis, ulcers, wound - surgical site infections. Moreover, the bacterium is a common hospital-acquired agent and most strains are resistant to three or more antibiotics including cephalosporins and carbapenems used to treat ordinary *Pseudomonas* infections (Driscoll et al., 2007; Moore and Flaws, 2011).

Therefore, in this study, the role of norepinephrine (NE) in the regulation of growth, adhesion and invasion properties of *Pseudomonas aeruginosa* was examined in human lung cell carcinoma cell (A549) culture as an infection model.

2. Materials and methods

Bacterium, medium and hormone

In the present study, *Pseudomonas aeruginosa* ATCC 27853 strain was used. The bacterial suspension (approximately 10^7 CFU/mL) was prepared in Mueller Hinton broth (MHB) to provide overnight culture.

The effects of norepinephrine (NE) (Sigma) at two concentrations, **low concentration:** 0.0017 and **high concentration:** 0.04 μ g/mL, were chosen to mimic *in vivo* conditions of the host by considering their psychological levels in a healthy individual.

Human lung carcinoma cell line (A549)

The human lung carcinoma cell line (A549) was used for growth, adhesion and invasion assays. Fetal bovine serum (FBS) (10%) (Biowest, S1810-500), 2mM L-glutamine (1%) (Biological Industries, BI03-020-1B) and penicillin/streptomycin (1%) (Biological Industries, 03-031-1B) added into Dulbecco's modified Eagle medium (DMEM) (Sigma, 5546) was used to prepare A549 cell culture.

A549 cells were cultured in 96 and 24-well culture dishes for bacterial growth, adhesion and invasion experiments, respectively. In order to maintain a confluent monolayer cell culture, seeding density was arranged to almost 5×10^4 cells for 24-well and 1×10^4 cells for 96-well culture dishes, then the plates were incubated at 37 °C for 24 hours under 5% CO₂ conditions.

Inoculation of cell culture (A549)

A549 cells were inoculated with overnight cultures of *P. aeruginosa* strain. Prior to inoculation of strain, medium was aspirated and replaced with antimicrobial solution-free DMEM. Then, the two concentrations of NE were added to each well (20 μ L for each well/96-well plate and 50 μ L for each well/24-well plate) for experiments; as controls, cell cultures without hormones were used, for each experiment. The culture dishes were incubated for one hour, at 37°C before experiments, after incubation, A549 cells were inoculated with approximately 10^7 CFU/mL *P. aeruginosa*. Then, plates were incubated at 37°C for different periods according to experiments.

Growth assay

Growth alterations were determined by colony counting method. Cells were inoculated with *P. aeruginosa* strain as mentioned above and incubated for 90 and 180 minutes. After incubation, 20 μ l cell culture suspensions from

each well (with and without hormone at two concentrations) were inoculated on Mueller Hinton agar for comparison of colony numbers. All experiments were independently repeated three times and all conditions were examined thrice.

Adhesion and invasion assays

The effect of NE on adhesion and invasion of bacteria was performed as reported previously (Artini et al., 2011; Castillo et al., 2017). Inoculated A549 cells with bacteria were incubated at 37°C for one hour. After incubation, to remove unbound bacteria, cells in wells were washed three times with phosphate buffer saline (PBS). For lysing cells, 500µl 0.025% Triton X-100 was added to the wells and the plates were incubated for 5 minutes at 37°C under 5% CO₂ conditions. Lysates were homogenized and inoculated on Tryptic Soy agar (TSA) and incubated for 24 hours at 37°C. The real number of adhered bacteria was detected by colony counting (CFU).

For the detection of invasive bacterial numbers, bacteria inoculated-A549 cells were incubated at 37°C, for three hours, then, PBS was used three times for washing inoculated cells and a fresh medium supplemented with gentamycin (200µg/mL) was used for killing extracellular bacteria. After this stage, the plates were incubated for one hour, at 37°C. A549 cells were lysed with Triton X-100 and for quantification of invasive bacteria, homogenized cell lysates were inoculated as mentioned above.

Both determination of adhesive and invasive bacteria (as CFU/mL) obtained from cell lysates of inoculated cell cultures with/without NE (both two concentrations) were compared. All conditions were repeated three times and each experiment was carried out thrice.

Statistical analysis

Differences between results of experimental - and control conditions were statistically analyzed. The analyses were assessed using one-way ANOVA followed by Dunnett's multiple comparisons test for growth alterations. Two-way ANOVA followed by Dunnett's multiple comparisons test was used for the analysis of adhesion and invasion results. All results were presented as mean ± SD. Differences with p values less than 0.05 were considered significant.

3. Results and discussion

Results

The alteration of bacterial growth in the presence of norepinephrine

Both two concentrations of NE were statistically significantly decreased the growth of *P. aeruginosa* strain after 90 minutes incubation (p:0.0004 for HNE and p:0.0003 for LNE) (**Fig. 1**). On the other hand, when incubation was prolonged to 180 minutes, the growth was only statistically significantly reduced in the presence of low NE concentration (p<0.0001).

The alteration of bacterial adhesion and invasion in the presence of norepinephrine

Low NE was shown to be significantly enhanced the adhesion of *P. aeruginosa* strain (p: 0.013). However, the presence of NE has no effect on invasion of *P. aeruginosa* (p>0.05) (**Fig. 2**).

Discussion

Over the years, numerous studies have shown that mammalian hormones have roles not only in the regulation of homeostasis in mammalian hosts but also in the regulation of microbes' behaviors. With the long coexistence of the microbes and their hosts, microbes had to adapt to host conditions.

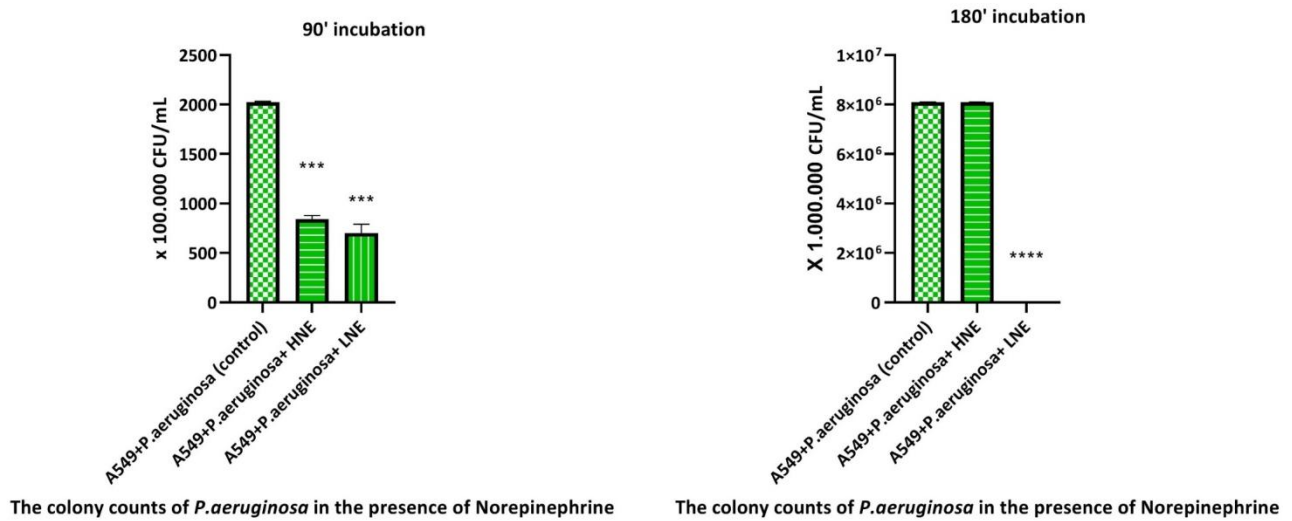


Fig. 1. The alteration of bacterial growth in the presence of NE

The growths of *P. aeruginosa* in A549 cell culture with-without NE were carried out using one-way ANOVA followed by Dunnett's multiple comparisons tests.

HNE: High concentration of norepinephrine (0.04 µg/mL), **LNE:** Low concentration of norepinephrine (0.0017 µg/mL) ***, ****: Significant at p: 0.0004, p: 0.0003 and p<0.0001 levels, respectively

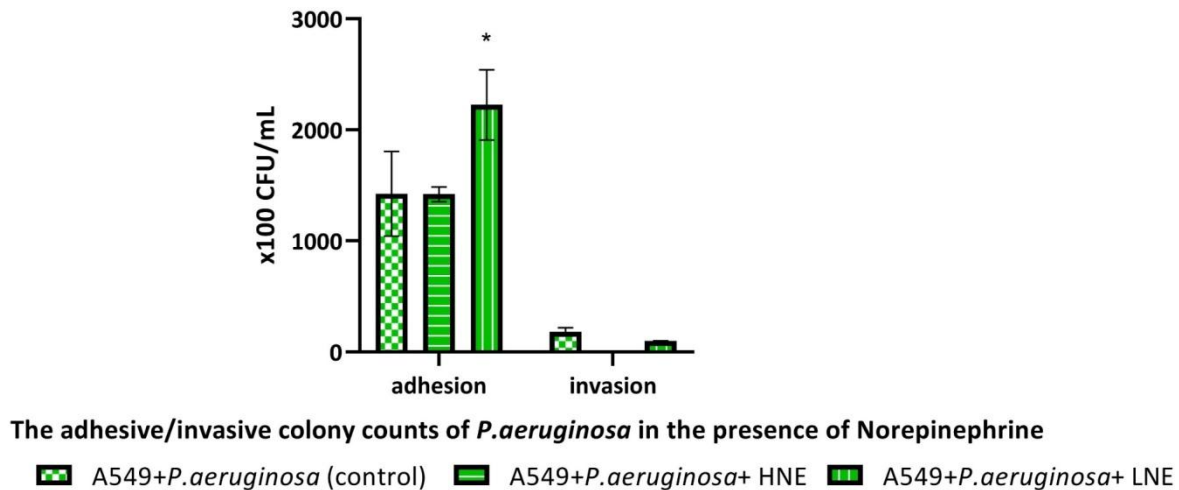


Fig. 2. The alteration of bacterial adhesion and invasion in the presence of NE

The adhesion and invasion of *P. aeruginosa* to A549 cell culture with-without NE were examined using two-way ANOVA followed by Dunnett's multiple comparisons tests.

HNE: High concentration of norepinephrine (0.04 µg/mL), **LNE:** Low concentration of norepinephrine (0.0017 µg/mL) *: Significant at p: 0.013 level

As evolution requires, microorganisms modulate their growth, metabolism, virulence, susceptibilities and gene expressions. Since Lyte and Ernst proposed microbial endocrinology concept as a new approach to understanding the infectious process, many researchers reported that mammalian hormones provide a bi-

directional interaction between host and microorganism via inter-kingdom signaling (Kornman and Loesche, 1982; Lyte and Ernst, 1992; Plotkin and Viselli, 2000; Lyte et al., 2003; Plotkin and Konakieva, 2017; Gonçalves et al., 2020; Truccolloet al., 2020).

Based on this approach, the present study was investigated the role of norepinephrine, a catecholamine, in the regulation of growth, adhesion and invasion of *Pseudomonas aeruginosa* strain in human lung carcinoma cells imitating host conditions as much as possible.

In previous studies, it has been shown that norepinephrine affected the growth of various microorganisms such as *Campylobacter jejuni*, *Escherichia coli*, *Prevotella species*, *Porphyromonas gingivalis*, *Streptococcus mutans*, *Streptococcus pneumoniae*, *Staphylococcus epidermidis*, *Vibrio harveyi*, *Helicobacter pylori*, *Aeromonas hydrophila* and *Pseudomonas aeruginosa* (Lyte et al., 2003; Doherty et al., 2009; Gonzales et al., 2013; Sandrini et al., 2014; Yang et al., 2014; Xu et al., 2015; Boyanova, 2017; Gao et al., 2019; Gümüş et al., 2019). While, most microorganisms' growths were induced (Lyte et al., 2003; Doherty et al., 2009; Gonzales et al., 2013; Sandrini et al., 2014; Yang et al., 2014; Xu et al., 2015; Boyanova, 2017; Gümüş et al., 2019) but some of them were reported to be decreased (Boyanova, 2017; Gümüş et al., 2019) in the presence of NE. In our study, both concentrations of NE decreased the growth of *P. aeruginosa* strain. However, when incubation period was prolonged, the suppressive effect was only detected at low NE concentration. It seems that the inhibitory effect of NE on the growth of *P. aeruginosa* depends on concentration and incubation period.

Host hormones have been reported to interact with several steps of pathogenesis in infectious diseases especially adhesion and invasion. Adhesion which is the first interaction between microorganisms and host tissue is very important and necessary step of the infectious process in the host. Many studies demonstrated the adhesion properties of EHEC O157:H7, adherent-invasive *E. coli*, *C. jejuni*,

S. pneumoniae, *E. faecalis* strains to different host tissues (HeLa S3 cells, HT-29/B6 cells, Caco-2/TC-7 cells, A549 cells, HBMEC cells) were altered in the presence of NE (Bansal et al., 2007; Gonzales et al., 2013; Xu et al., 2015; Cambrone et al., 2020; Xi et al., 2020). It seems that in the presence of NE, whereas adhesion has been affected positively in some microorganisms (Bansal et al., 2007; Xu et al., 2015; Cambrone et al., 2020; Xi et al., 2020; Beata et al., 2021), it could be decreased in some others (Gonzales et al., 2013; Xi et al., 2020). In our study, we found out that, low concentration of NE increased the adhesion of *P. aeruginosa* strain to A549 cells. Considering this result, it is possible to suggest that hormone concentration could determine the virulence properties of microorganisms'.

It seems that, the effects of NE on the invasion to host cells were limitedly investigated. According to previous findings, invasion of *Salmonella Typhimurium*, adherent-invasive *E. coli*, *C. jejuni*, *Salmonella choleraesuis* and EHEC O157:H7, *P. aeruginosa* strains to several cells (Peyer's patch, Caco-2 cells, HT-29/B6 cells, HCT-8 human enterocyte cells) were reported to be modulated in the presence of NE (Green et al., 2003; Brown and Price, 2008; Hegde et al., 2009; Xu et al., 2015a; Xi et al., 2020; Beata et al., 2021). According to many studies, NE acted usually as an inducer for invasion (Green et al., 2003; Brown and Price, 2008; Hegde et al., 2009; Xu et al., 2015; Beata et al., 2021). However, in our study, we did not find any effect on invasion of *P. aeruginosa* in the presence of NE.

The adhesion and invasion are defined as early stages of pathogenesis during infectious processes. In the present study, only low NE concentration was found to increase the adhesion of *P. aeruginosa* and two concentrations of NE did not modulate the invasion of bacterium.

It has been documented that catecholamines can regulate the immune system, norepinephrine, one of the catecholamines is known as a neurotransmitter that can affect the inflammation process directly or indirectly (Oberbeck, 2006; Szelényi and Vizi, 2007). Moreover, as far as is known, under stress and hard conditions, catecholamine secretions can be stimulated (Oberbeck, 2006; Osier and Dixon, 2016; Ma et al., 2020). Considering the roles of NE in both neuro-immune interactions and host-microbe communications, investigations about its influences on the infection process would make sense. According to former studies, it has been observed that the possible action mechanisms of NE are associated with up taking of iron in limited conditions, hormone-mediated induction of auto-inducers, acting as quorum sensing compounds (Lyte and Cryan, 2014; Sandrini et al., 2014; Lyte and Freestone, 2010; Freestone et al., 2007).

Conclusions

In conclusion, the purpose of our study was to investigate the influences of NE on growth, adhesive and invasive properties of a *P. aeruginosa* strain with carrying out to mimic *in vivo* conditions as possible. The results obtained from our study demonstrated that, NE has decreased the growth and increased the adhesion of *P. aeruginosa*. It should be noted that these effects were shown to depend on incubation period for growth and hormone concentration for adhesion.

Conflict of interest

There is no conflict of interest.

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