Comparison of salivary antimicrobial peptides and upper respiratory tract infections in elite marathon runners and sedentary subjects

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Abstract  The aim of the present study was to examine the relationship between human β-defensin-2 (HBD-2), cathelicidin (LL-37) and upper respiratory tract infections (URTI). In addition, the possible association between salivary cortisol and the salivary antimicrobial peptides was also examined. We hypothesized that the saliva levels of HBD-2 and LL-37 are lower in elite marathon runners; and that saliva cortisol levels might have a negative association with saliva HBD-2 and LL-37. Twenty elite male marathon runners were studied, and twenty additional male subjects were used as sedentary controls. Saliva samples were collected between 12:00 and 14:00 in the afternoon. We selected the cotton swab method of saliva collection. Elite marathon runners tend to have lower concentrations of salivary antimicrobial peptides (HBD-2 and LL-37) than sedentary subjects. Saliva cortisol levels in the elite marathon runners were significantly higher than those in the sedentary subjects. Concentration of saliva cortisol levels showed a negative correlation with saliva HBD-2 and saliva LL-37 concentration levels. Number of URTI in the elite marathon runners was significantly higher than in the sedentary subjects. Number of URTI was negatively correlated with saliva HBD-2 concentration and saliva LL-37 concentration levels. The present findings suggest the relationship between antimicrobial peptides and URTI in elite marathon runners and sedentary subjects. In addition, salivary antimicrobial peptides in the elite marathon runners were significantly lower than sedentary control subjects. It is possible that, while strenuous exercise in elite athletes could partly enhance oral innate immunity, the physical stress could simultaneously restrict the immunological enhancement due to HPA axis activity.

Keywords: Antimicrobial peptides, Human β-defensin-2, LL-37, Cortisol, Upper respiratory infections

Introduction  Strenuous endurance exercise is generally known to decrease resistance to upper respiratory tract infections (URTI). URTI are the most common medical complaint of athletes and can negatively affect training and performance. In addition, elite athletes seem to be more susceptible to URTI than recreationally active or sedentary individuals, with the risk of illness increasing during periods of heavy training and competition. This observation arose from the detection of significant changes in several immunological parameters in various biological samples caused by both a single bout and repeated exercise. Salivary immunoglobulin A (IgA) has been classically recognized as one of the major effectors of host resistance to many pathogens in the oral cavity; and many studies have investigated the impact of transient or repeated exercise on salivary levels. An early finding by Mackinnon and colleagues, of an inverse relationship between salivary IgA and URTI, led to the prevailing belief that salivary IgA is the immune variable most closely associated with URTI. In recent years, a novel class of antimicrobial peptides, such as defensins and cathelicidins, has emerged as potential players in host defense at the oral mucosal surface. These peptides have a broad spectrum of properties for resisting pathogens, including bacteria, fungi, and viruses.

Defensins are cationic peptides which contain six cys-
teines forming three intramolecular disulfide bridges, and can be divided into the following categories: α-defensins (human neutrophil peptides: HNP1-3), β-defensins (HBD-1 to HBD-4) and δ-defensins[17,18]. HBD-2 is expressed in human epithelial cells of the inner and outer surfaces of the human body, including the oral cavity and respiratory tract[19].

LL-37 is the only member of the cathelicidin family in humans which is produced by epithelial cells, macrophages, and neutrophils and secreted into the oral and airway surface fluid[19]. While expression of some antimicrobial peptides is known to be upregulated where active inflammation and infection is present in various mucosal tissues, it has not been fully investigated whether other intrinsic and extrinsic factors, such as exercise associated with physical stress, could also promote or inhibit the production of these peptides. Previous in vitro studies investigated the suppressive effect of glucocorticoid on the expression of HBD-2 in various human cells; although the effect varies among reports[20-25]. In addition, a previous study using a murine skin model demonstrated that an increase in endogenous corticosteroids from psychological stress reduces the levels of antimicrobial peptides including murine β-defensin (mouse β-defensin-3) and murine cathelicidin (cathelin-related antimicrobial peptide) in the epidermis[26]. Previous in vivo studies showed salivary antimicrobial peptide levels to be negatively correlated with increases in salivary cortisol levels from prolonged strenuous exercise[27]. This study raises the intriguing possibility that, although the tissue is different, a rise in endogenous corticosteroids from physical stress, such as prolonged strenuous exercise, as well as psychological stress, might affect the local production of these antimicrobial peptides.

Serum and salivary cortisol levels represent hypothalamic-pituitary-adrenal (HPA) axis adaptation to stress and this hormone is classically used as a biomarker of mental and physical stress including various types of exercise[28,29]. Salivary cortisol levels in elite athletes were shown for participation in the study, which was approved by the Ethics Committee of Osaka City University.

Materials and Methods

Subjects. Subjects included twenty elite male marathon runners (mean ± SD: age 20.2 ± 1.3 yrs; height 170.4 ± 5.9 cm; body mass 62.0 ± 8.8 kg; body fat 9.4±1.7 %; Training volume 200.8 ± 15.5 km/week). All subjects were requested to maintain their normal training and competition programs throughout the 12-month study period. For each training session, type of activity, training distance, duration, and intensity were recorded in a daily training diary. In addition, twenty male subjects were selected as sedentary controls (mean ± SD: age 21.0 ± 1.8 yrs; height 172.4 ± 6.7 cm; body mass 68.5 ± 5.3 kg; body fat 18.3 ± 3.2 %). The sedentary controls also recorded their daily physical activity in a diary. All subjects were reported as 1) being life-long non-smokers[30], 2) not having a history of respiratory and/or allergic diseases, such as asthma, rhinitis and eczema[31], 3) not having experienced any psychological diseases or significant life events, such as death, accident or divorce in the family within the previous 6 months[32]. Subjects who had a respiratory infection, a dental problem or any medication within 4 weeks prior to the study were excluded because of the effect of such conditions on the baseline secretion of the immunological peptides in the oral cavity[33]. All subjects provided written informed consent for participation in the study.

Saliva collection. All saliva samples were collected between 12:00 and 14:00 in the afternoon. Subjects reported at the same time for each collection period after fasting for 2 h (2 hours) and refraining from any strenuous physical activity for 20 h. We selected the cotton swab method of saliva collection[34]. Subjects sat and rinsed out their mouths with sterilized water (30-sec × 3 times). Saliva induction was stimulated by chewing the sterilized cotton swab for one minute at a frequency of 1 chew sec⁻¹. Saliva was separated from the cotton by centrifugation at 3,000 rpm. The saliva volume (ml) was estimated by weighing the total requisite volumes of saliva samples presented as the whole saliva volume. The saliva supernatant was stored at - 80 oC for subsequent assays.

Saliva analysis. An enzyme-linked immunosorbent assay (ELISA) was used for measurements of the saliva concentrations of HBD-2 (Human β-Defensin 2 ELISA Kit, Phoenix Pharmaceuticals Inc., Burlingame, CA), LL-37 (Human LL-37 ELISA Test Kit, Hycult Biotechnology, Uden, The Netherlands), and cortisol (Parameter Cortisol Assay, R&D Systems, Minneapolis, MN), respectively. The present cortisol assay can measure both the free and corticosteroid-binding globulin-bound form of cortisol[35]. Only 14% of cortisol in saliva is bound to carrier protein[36]. The total requisite volumes of saliva samples were 200 µl, 200 µl and 300 µl for the HBD-2, LL-37 and cortisol assays, respectively. The interassay coefficients of variation were less than 15%, 10% and 9.3% for the HBD-2, LL-37 and cortisol assays, respectively. The minimum detection limits for the HBD-2, LL-37 and cortisol
assays were 7.815 pg ml\(^{-1}\), 0.14 ng ml\(^{-1}\) and 0.03 ng ml\(^{-1}\), respectively. All sample measurements were performed in duplicate according to the manufacturers’ instructions.

**Infection and illness reports.** Subjects were required to complete a one-year log in which they recorded any sign or symptom consistent with URTI (including cough, runny nose, and nasal congestion) as well as the number of days that symptoms occurred\(^{17}\). For a URTI episode to be recorded, subjects must have had upper respiratory signs and symptoms for ≥ 48 h.

**Statistical analysis.** All statistical analyses were performed using SPSS for Windows (SPSS Inc., Chicago, IL, USA). All data were normally distributed, and presented as mean ± S.D. The paired Student’s \(t\) test was used to compare each variable within the two groups of subjects - elite marathon runners versus control group. Correlations between variation of HBD-2, LL-37 and cortisol, and number of URTI were examined by determination of Pearson’s correlation coefficients. Differences of \(P < 0.05\) were considered significant for all statistical analyses.

**Results**

**Saliva antimicrobial peptides profile.** Table 1 shows a comparison of the saliva HBD-2, LL-37 and cortisol secretion rate between elite marathon runners and sedentary subjects. Fig. 1 shows a comparison of saliva HBD-2 concentrations between elite marathon runners and sedentary subjects. Elite marathon runners tend to have lower concentrations of saliva HBD-2 than sedentary subjects \((P<0.01)\). Fig. 2 shows a comparison of saliva LL-37 concentrations between elite marathon runners and sedentary subjects. Similarly, concentrations of saliva LL-37 in the elite marathon runners were significantly lower than in sedentary subjects \((P<0.01)\).

**Stress-related hormone assessed by saliva cortisol.** A comparison of saliva cortisol concentrations between elite marathon runners and sedentary subjects is shown in Fig. 3. Saliva cortisol levels in the elite marathon runners were significantly higher than those in sedentary subjects \((P<0.001)\).

**Incidence of URTI.** A comparison of the number of URTI (time/years) between elite marathon runners and sedentary subjects is shown in Fig. 4. The number of URTI in elite marathon runners was significantly higher than in sedentary subjects \((P<0.05)\).

**Correlations of saliva antimicrobial profiles and stress-related hormone (cortisol).** Saliva cortisol concentrations were negatively associated with saliva HBD-2 concentrations.

<table>
<thead>
<tr>
<th></th>
<th>Saliva HBD-2 (pg/min)</th>
<th>LL-37 (ng/min)</th>
<th>cortisol (ng/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marathon runners (n=20)</td>
<td>133.2±23.6</td>
<td>24.6±8.7</td>
<td>6.1±2.9</td>
</tr>
<tr>
<td>Sedentary subjects (n=20)</td>
<td>337.6±60.6</td>
<td>59.9±9.8</td>
<td>2.5±1.7</td>
</tr>
</tbody>
</table>

All values are described as mean ± SD. **\(p<0.01\), ***\(p<0.001\); marathon runners vs. sedentary.

**Fig. 1** Comparison of saliva HBD-2 concentration between marathon runners and sedentary subjects. **\(p<0.01\)**

**Fig. 2** Comparison of saliva LL-37 concentration between marathon runners and sedentary subjects. **\(p<0.01\)**
Fig. 3  Comparison of saliva cortisol concentration between marathon runners and sedentary subjects. ***p<0.001

Fig. 4  Comparison of number of URTI between marathon runners and sedentary subjects. *p<0.05

Fig. 5  Association of saliva cortisol concentrations with those in saliva concentrations of HBD-2 (a), LL-37 (b).

Fig. 6  Association of number of URTI with those in saliva concentrations of HBD-2 (a), LL-37 (b).
Correlations of saliva antimicrobial profiles and number of URTI. The number of URTI were negatively associated with saliva HBD-2 concentrations (URTI vs. HBD-2: $r = -0.682$, $P<0.001$, Fig. 6a) and saliva LL-37 concentrations (URTI vs. LL-37: $r = -0.728$, $P<0.001$, Fig. 6b).

Discussion

The main objectives of the present study were to assess the relationship between antimicrobial peptides and URTI in elite marathon runners and sedentary subjects, and to investigate the association between salivary cortisol and salivary HBD-2 and LL-37. The present study revealed that: 1) Elite marathon runners tend to have lower concentrations of salivary HBD-2 than sedentary control subjects. Similarly, concentrations of saliva LL-37 in the elite marathon runners were significantly lower than in sedentary control subjects. 2) Saliva cortisol levels in the elite marathon runners were significantly higher than those in sedentary control subjects. 3) Numbers of URTI were negatively associated with saliva HBD-2 and saliva LL-37 concentrations. Recently, a variety of humoral immune mediators have been reported to participate in innate defense mechanisms against pathogens targeting the mucosal surface in various tissues including the oral cavity and airways. Particularly, considerable evidence has accumulated that antimicrobial peptides, such as HBD-2 and LL-37, play important roles in oral innate immunity. However few studies have focused on whether saliva levels of these novel peptides are altered during or after exercise. Recent work by Usui T et al. demonstrated that salivary antimicrobial peptide levels were negatively correlated with increases in salivary cortisol levels by strenuous exercise; although previous in vitro studies using a murine skin model demonstrated that an increase in endogenous glucocorticoids by psychological stress reduced mRNA levels of antimicrobial peptides including murine cathelicidin (cathelin-related antimicrobial peptide (CRAMP)) in the epidermis. In human the oral cavity, both of these peptides were detected in salivary glands and gingival epithelial cells. Based on in vitro studies using epithelial culture, the production of HBD-2 is known to be up-regulated by bacterial triggers and pro-inflammatory mediators, such as lipopolysaccharide (LPS), TNF-α and IL-1β in an NF-kB dependent manner. Vitamin D3 and infectious triggers are recognized as major regulators controlling LL-37 expression in humans. Previous studies demonstrated that HBD-2 and LL-37 are both expressed even in normal tissue without infectious and inflammatory conditions, but the precise mechanisms of the exercise-induced transient increases in antimicrobial peptides as observed in the present study are unclear. With regard to LL-37, the previous study by Usui T et al. suggested in the discussion that the exercise-induced rise in the salivary LL-37 levels might be attributed to local and systemic recruitment of neutrophils, which is one of the main sources of LL-37. Of particular interest is negative correlation of increases in the salivary cortisol levels with those in HBD-2 and LL-37 in saliva. Interestingly, previous in vitro or animal studies demonstrated that endogenous or exogenous glucocorticoid can down-regulate the expression of these antimicrobial peptides in various tissue.

Corticosteroids are classically known to have differential effects on lymphocyte subpopulations involved in immunoglobulin biosynthesis by pokeweed mitogen stimulation in vitro where B cell responsiveness is diminished, whereas elevated glucocorticoids are not sufficient to suppress antibody response. Although previous studies investigated the relationship between salivary IgA and URTI leading to the prevailing belief that salivary IgA is the immune variable most closely associated with URTI, it is still unclear what aspects of antimicrobial peptides, such as HBD-2 and LL-37 are responsible for an increased URTI risk in athletes. Previous studies investigated that salivary cortisol levels in elite athletes were higher than in sedentary subjects during resting and after exercise. Prolonged strenuous exercise and a heavy schedule of training have been associated with higher cortisol levels. Endurance athletes experience an increased risk of URTI during intensive training and after competition in prolonged endurance events. Acute and exhaustive exercise also appears to be a risk factor for increased URTI incidence. This relationship between training status and susceptibility to infection has been modeled in the form of a “J”-shaped curve, and is the reason for increased risk of URTI, lower oral immune components and higher salivary cortisol levels. The present findings highlight the variability of stress-induced immunological shifts in response to strenuous exercise among innate immune factors. Furthermore, beyond these immune parameters assessed in the present study, a wide variety of humoral and cellular components in oral mucosa and secretions, including lactoferrin, secretory leukocyte protease (peptidase) inhibitor (SLPI), lysozyme and neutrophils, synergistically act and provide a frontline of defence against pathogens, yielding microbial homeostasis in oral entry. To investigate the impact of stress on innate immunity and subsequent susceptibility or resistance to oral or airway pathogens, it would most likely be informative to elucidate whether and how the stress can or cannot be involved in kinetics of individual local immune components. Furthermore, it is also worthwhile to evaluate the long-term effect of glucocorticoid on the innate immune components as a result of repeated physical stress.

Our work has several limitations. First, we were unable to elucidate how the transient changes observed in the

concentrations (cortisol vs. HBD-2: $r = -0.878$, $P<0.001$, Fig. 5a) and saliva LL-37 concentrations (cortisol vs. LL-37: $r = -0.756$, $P<0.001$, Fig. 5b).
saliva HBD-2 and LL-37 levels could affect the overall antibacterial activity in saliva. In the present study, we only focused on the kinetics of these peptides and its association with changes in saliva cortisol levels as a result of physical stress by prolonged strenuous exercise. Future studies will be needed to examine the antimicrobial effect of exercise-induced changes in these salivary peptides. Secondly, the passive dribbling method might be more appropriate for obtaining physiologically relevant data of saliva contents compared with the present cotton-based method. The amount of IgA (μg) which was pumped to the oral cavity by chewing stimulation (secretion rate) does not necessarily reflect the immune readiness of the oral cavity. Furthermore, the use of cotton-based collection swabs can affect the antimicrobial peptide concentration.

Conclusions

The present findings suggest the relationship between antimicrobial peptides and URTI in elite marathon runners and sedentary subjects. In addition, salivary antimicrobial peptides in the elite marathon runners were significantly lower than in sedentary control subjects. It is possible that, while strenuous exercise in elite athletes could partly enhance oral innate immunity, the physical stress could simultaneously restrict the immunological enhancement due to HPA axis activity.

Acknowledgements and Conflict of interest statement

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References


