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Enhancement of salivary secretion and neuropeptide (substance P, α-calcitonin gene-related peptide) levels in saliva by chronic anethole trithione treatment

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Abstract

Anethole trithione, a choleretic, has been reported to be effective in the treatment of dry mouth. We have examined the effects of chronic treatment with anethole trithione on salivary secretion, substance P immunoreactive substance (SP-IS) and α-calcitonin gene-related peptide immunoreactive substance (α-CGRP-IS) concentrations in human saliva. Anethole trithione caused significant increases of saliva SP-IS concentrations from the day 13 (25.3 ± 1.6 pg mL^{-1}) to day 14 (25.8 ± 1.7 pg mL^{-1}) compared with day 1 (19.9 ± 1.9 pg mL^{-1}). Anethole trithione caused significant increase in saliva α-CGRP-IS concentration on day 14 (39.9 ± 4.7 pg mL^{-1}) compared with day 1 (27.7 ± 4.7 pg mL^{-1}). Anethole trithione significantly increased the sialosis volumes from day 11 to day 14 (1.6 ± 0.1–1.7 ± 0.2 mL) compared with the day 1 (1.2 ± 0.2 mL). Simple linear regression of the increase in sialosis volume with saliva SP-IS ($r = 0.94$) and α-CGRP-IS ($r = 0.97$) concentrations was found. These results demonstrated that chronic treatment with anethole trithione affected saliva SP-IS and α-CGRP-IS concentration in human saliva and sialosis volume.

Introduction

Anethole trithione, a choleretic, has been reported to be effective in the treatment of dry mouth. The effectiveness of this medicine in the treatment of patients with Sjögren's syndrome, alleviating the symptoms of dry mouth and increasing the salivary flow rate, has also been reported (Epstein et al 1983). Generally, there are two stimulus-secretion pathways in salivary secretion, one involving cyclic AMP, which primarily regulates enzyme secretion, and the other turnover of phosphatidylinositol, which regulates the fluxes of electrolyte and water excretion (Putney 1986). Previously we reported that treatment with a single dose of anethole trithione raised the concentrations of substance P immunoreactive substance (SP-IS) and α-calcitonin gene-related peptide immunoreactive substance (CGRP-IS) in human saliva (Takeyama et al 1996; Nagano et al 1998a). Ukai et al (1984) demonstrated that chronic treatment with anethole trithione increased the number of muscarinic cholinergic receptors in the submaxillary glands of rats. However, the effects of chronic treatment with anethole trithione on SP-IS and α-CGRP-IS concentration in human saliva are little known. Salivary glands are supplied with nerve fibres that contain neuropeptides, such as substance P and α-calcitonin gene-related peptide (Dawidson et al 1997), and these peptides are known to cause salivation in rat (Ekström 1987). Substance P and α-calcitonin gene-related peptide are known

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also to coexist in a population of sensory neurons in man (Domoto et al 1993), but the difference of the mechanism between substance P and α-calcitonin gene-related peptide is still unclear.

Substance P is a tachykinin that was first detected as a potent sialogogue by von Euler & Gaddum (1931) in extracts from equine intestine and brain. One of the most potent actions of substance P in peripheral tissue is its stimulation of salivation (Ekström et al 1996). In salivary glands numerous substance P-positive fibres can be observed in relation to secretory elements (Hökfelt et al 1977). α-Calcitonin gene-related peptide, which is a 37-amino acid peptide, is formed by modification of the primary RNA transcript of the rat calcitonin gene (Amara et al 1982). It is recognized that CGRP-IS occurs mainly around blood vessels and ducts, and to a minor extent around acini. This peptide is known to coexist with substance P in a population of sensory neurons in man (Domoto et al 1993). However, the action of CGRP-IS on salivary glands is little known. Salivary glands are supplied with nerve fibres that contain neuropeptides, such as substance P and α-calcitonin gene-related peptide (Dawidson et al 1997), and these peptides are known to cause salivation in the rat (Ekström 1987). Substance P and α-calcitonin gene-related peptide significantly increase the blood flow in the salivary glands (Dawidson et al 1997).

In this study, we have examined the effect of chronic treatment with anethole trithione on salivary secretion, and SP-IS and α-CGRP-IS concentration in human saliva. The concentrations of SP-IS and α-CGRP-IS in saliva were measured by a highly-sensitive enzyme immunoassay (EIA) (Takeyama et al 1996; Nagano et al 1998b), along with sialosis volumes before and after chronic treatment with anethole trithione in six healthy male subjects.

Materials and Methods

Materials

Human α-calcitonin gene-related peptide and the fragment (position 8–37) of α-calcitonin gene-related peptide [CGRP(8–37)] were purchased from Peptide Institute Inc. (Osaka, Japan). Synthetic substance P was used. Bovine serum albumin (BSA), polyoxyethylene sorbitan monolaurate (Tween 20), *N*-(ε-maleimidocaproyloxy) succinimide (EMC-succinimide), and 4-methyl-umbelliferyl β-D-galactopyranoside (MUG) were purchased from Sigma Chemical Co. (St Louis, MO). β-D-Galactosidase (β-gal from *Escherichia coli*) and goat affinity purified antibody to rabbit IgG (whole molecule) (55641) were purchased from Boehringer Mannheim Corp. (Mannheim, Germany) and ICN Pharmaceuticals, Inc. (OH), respectively. Antisera to substance P (RA-08-095) and α-calcitonin gene-related peptide (CA1132) were purchased from Genosys Biotechnologies Ltd (London, UK) and Affiniti Research Products Ltd (Nottingham, UK), respectively. Lactose (Merck hoei Co. Ltd, Osaka, Japan) was used as a placebo. Other reagents were extra-pure grade from commercial sources.

Subjects

The Ethics Committee of Oita Medical University approved the study, for which the subjects gave informed consent.

Six healthy male volunteers (23–30 years (mean ± s.d. 27.7 ± 1.4 years), 53–66 kg (median 63 kg)) participated in the study and did not receive any medication for one week before the start of the experiments.

Measurement of sialosis volume

The sialosis volume was measured at 60 min after the second administration of the day by the Saxon test, an oral equivalent of the Schirmer test (Kohler & Winter 1985). This interval was equal to the time with significant increase of neuropeptide concentration in saliva (Takeyama et al 1996; Nagano et al 1998a). Two sterile 5-g absorbent cottons (no. 14, Kawamoto Houtai Zairyou, Co. Ltd, Osaka, Japan) were weighed. After swallowing to remove any existing oral fluid, saliva was collected by setting the two cottons on the vestibule of the mouth for 5 min exactly. The subject then spat out the moist absorbent cottons onto a plastic tray. The sialosis volume produced in 5 min was determined by subtracting the original weight from the weight obtained after setting. Weights were measured on an electron balance (AE240, METTLER Instrumente, Switzerland), which was accurate to 10^{-4} g. The ratio of liquids was most equal to the weight of saliva.

Saliva collection method for enzyme immunoassay (EIA)

Resting whole saliva specimens were used after measurement of sialosis volume by the spitting method according to Navazesh & Christensen (1982). Subjects rinsed their

mouth thoroughly with deionized water and rested for 1 min before saliva collection. After a 30-s practice collection (that was discarded), saliva (2.0 mL) was collected in a test tube (Nunc-Immuno Tube Minisorp 75 × 12, InterMed, Denmark) over 5 min.

Study schedule

Anethole trithione (Felviten tablets, Nippon Shinyaku Co. Ltd, Kyoto, Japan) at a dose of 25 mg or the placebo was orally administered three times daily with water (100 mL) for two weeks. Previously, to examine the effect of a meal, saliva samples had been taken and the substance P and α-calcitonin gene-related peptide levels determined (Takeyama et al 1996; Nagano et al 1998a). The maximum saliva substance P and α-calcitonin gene-related peptide level was reached 10 min after a meal and declined to baseline within 60 min. Thus the study was carried out 120 min after a meal to avoid its effects. The weight of saliva samples, and venous blood samples (10 mL) from a forearm vein, were measured 60 min after the second administration of the day.

Determination of anethole trithione levels in plasma

The concentration of anethole trithione was determined by the modified method of Hayashi et al (1990). Standard anethole trithione was supplied by Nippon Shinyaku Co. Ltd. (Kyoto, Japan). HPLC was carried out using a C18 column (Cosmosil 5C18-AR, Nacalai Tesque, Kyoto, Japan) at room temperature and a UV detector set at 325 nm. Methanol–water (7:3) was used as the mobile phase at a flow rate of 1.0 mL min^{-1}.

Preparation of saliva and plasma extracts

The saliva and blood samples were placed in chilled tubes (4°C) containing aprotinin (500 kallikrein inhibitor U mL^{-1}) and EDTA (1.2 mg mL^{-1}). After centrifugation (1670 *g*, 4°C, 20 min), saliva samples were diluted fivefold with 4% acetic acid, pH 4.0 (adjusted with alkaline solution), and loaded onto Sep-Pak C18 (Millipore Co., MA). After washing with 4% acetic acid, SP-IS or α-CGRP-IS in saliva and plasma were eluted with 60% acetonitrile in 0.5% acetic acid, pH 4.0 (adjusted with alkaline solution). Eluates were concentrated by spin-vacuum evaporation, lyophilized, and stored until use. The recoveries of SP-IS and α-CGRP-IS in saliva with this extracting procedure were more than 91% (data not shown).

EIA for SPIS and α-CGRP-IS

EIA for SP-IS and α-CGRP-IS were performed as described previously (Takeyama et al 1990; Nagano et al 1998b). The EIA were performed by the delayed addition method. Separation of bound and free material was performed using an anti-rabbit IgG coated immunoplate (Nunc-Immuno Module Maxisorp F8, InterMed, Denmark). Substance P and CGRP(8–37) were labelled with β-D-galactosidase by EMC-succinimide according to the method of Kitagawa et al (1981). Test tubes containing antiserum of each peptide and sample (or standard) were incubated, and then enzyme-linked antigen was added. After the test tubes were incubated, the antigen–antibody solution for each sample was added to the secondary antibody-coated immunoplate. The plate was incubated, washed with a buffer (0.01 M phosphate buffer, pH 7.0, containing 0.15 M NaCl and 0.05% Tween 20) and then MUG was added to each well. The plate was incubated again and the fluorescence intensity (λ_{Ex} 360 nm, λ_{Em} 450 nm) of each well was measured with an MTP-100F microplate reader (Corona Electric, Ibaraki, Japan). Precision was estimated at five different concentrations of the two peptides by the above EIA. The coefficients of variation for within- and between-assay were less than 11% (n = 10, data not shown). The detection unit of SP-IS and α-CGRP-IS were 0.40 and 0.08 fmol/well, respectively.

Statistical analysis

Values are expressed as means ± s.d. for n experiments. Statistical analysis was performed using one-way analysis of variance. A value of $P < 0.05$ was regarded as significant. The regressions were calculated by the least-squares method.

Results

The profiles of average saliva SP-IS and α-CGRP-IS concentrations against day are shown in Figure 1. Anethole trithione caused a significant increase of saliva SP-IS concentration from day 13 (25.3 ± 1.6 pg mL^{-1}) to day 14 (25.8 ± 1.7 pg mL^{-1}) as compared with day 1 (19.9 ± 1.9 pg mL^{-1}). Anethole trithione caused a significant increase of saliva α-CGRP-IS concentration on day 14 (39.9 ± 4.7 pg mL^{-1}) compared to day 1 (27.7 ±

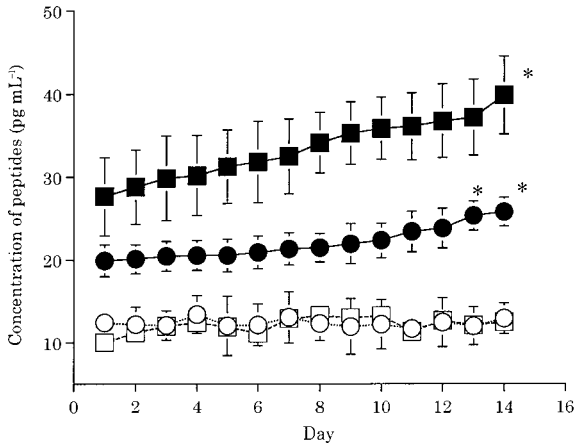

Figure 1 Saliva substance P immunoreactive substance (SP-IS) and α-calcitonin gene-related peptide immunoreactive substance (α-CGRP-IS) concentration after chronic treatment with anethole trithione (SP-IS ●; α-CGRP-IS ■) or placebo (SP-IS ○; α-CGRP-IS □) to healthy volunteers. Mean ± s.d., n = 6. *$P < 0.05$ compared with the result for day 1.

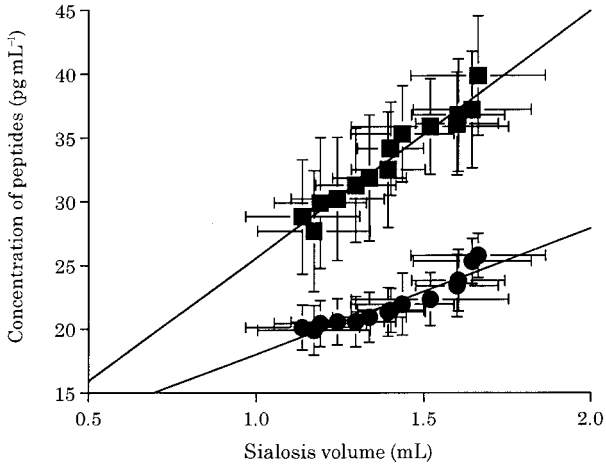

Figure 3 Relationship between the increase in sialosis volume and saliva substance P immunoreactive substance (SP-IS ●) and α-calcitonin gene-related peptide immunoreactive substance (α-CGRP-IS ■) after chronic treatment with anethole trithione to healthy volunteers. Mean ± s.d., n = 6.

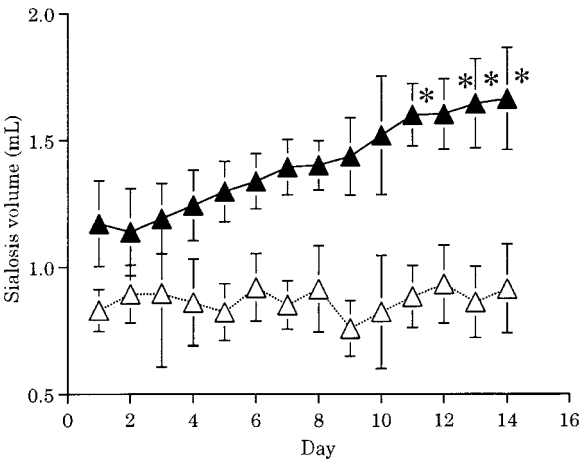

Figure 2 Salivary secretion after chronic treatment with anethole trithione (▲) or placebo (△) to healthy volunteers. Mean ± s.d., n = 6. *$P < 0.05$ compared with the result for day 1.

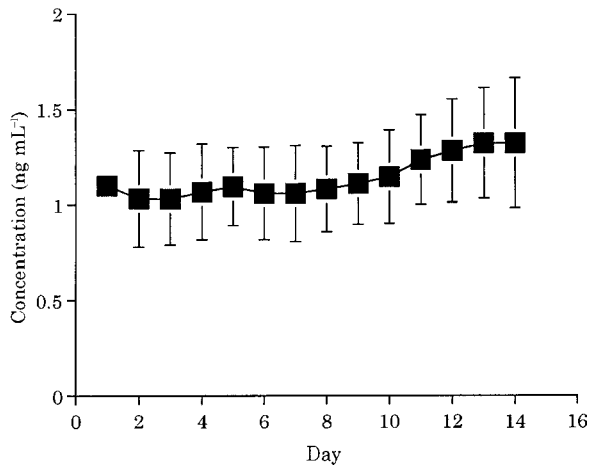

Figure 4 Plasma anethole trithione levels after chronic treatment with anethole trithione to healthy volunteers. Mean ± s.d., n = 6.

4.7 pg mL^{-1}). After a placebo was administered, saliva SP-IS and α-CGRP-IS concentrations appeared constant.

The profiles of average sialosis volumes against day in six subjects after chronic treatment with anethole trithione (25 mg) three times daily for two weeks are shown in Figure 2. Anethole trithione significantly increased the sialosis volumes from day 11 to 14 (1.6 ± 0.1–1.7 ± 0.2 mL) as compared with day 1 (1.2 ± 0.2 mL). After a placebo was administered, sialosis volume appeared constant.

In Figure 3, first degree least-square linear regressions can be found between the increase in sialosis volume and saliva SP-IS or α-CGRP-IS concentration. The regression coefficients of SP-IS and α-CGRP-IS were 0.94 and 0.97, respectively.

The profiles of average plasma anethole trithione concentrations against day are shown in Figure 4. Plasma anethole trithione concentrations increased gradually compared with day 1, but the differences were not significant. Saliva anethole trithione concentrations were less than the detection limit (0.25 ng mL^{-1}) during the study.

Discussion

Ever since anethole trithione was shown to be effective in the treatment of dry mouth caused by antipsychotic drugs with anticholinergic activity, it has been used clinically for patients with this condition and Sjögren's syndrome (Epstein et al 1983). This suggested that there might be other effects of anethole trithione, apart from the stimulation of parasympathetic nerve. Activation of muscarinic receptors has been reported to enhance the turnover of phosphatidylinositol in the salivary glands and to produce changes in cellular calcium transport (Michell 1975). Ukai et al (1989) reported that phosphatidylinositol turnover and cyclic nucleotide accumulation were enhanced by chronic treatment with anethole trithione in rat submaxillary glands. However, the relationship between the increase in sialosis volume and neuropeptide concentrations in saliva, such as substance P or α-calcitonin gene-related peptide, with the chronic treatment with anethole trithione was unclear.

We have studied the effect of chronic treatment with anethole trithione on SP-IS and α-CGRP-IS concentration in human saliva. It became obvious that substance P- and α-calcitonin gene-related peptide-nerves in salivary glands might be influenced by chronic treatment with anethole trithione. As a result, secreted SP-IS and α-CGRP-IS may be involved with sialosis. Sialosis volume increased between day 11 and day 14 (Figure 2). This result coincided with the result of Ukai et al (1988). The ratio of liquids was most equal to the weight of saliva, therefore secreted saliva in this study was almost serous. Figure 3 shows that there was good correlation between the increase of sialosis volume and saliva SP-IS ($r = 0.94$) or α-CGRP-IS ($r = 0.97$) concentrations. Consequently, two neuropeptide concentrations in saliva, SP-IS and α-CGRP-IS, may be closely connected with sialosis volume. With regards to the sex-related difference, no papers have been published dealing with substance P or α-calcitonin gene-related peptide concentrations in saliva. Schifter (1989) reported that the serum level of calcitonin gene-related peptide was unrelated to sex. Due to the fact that male volunteers only were used in this study, the influence of sex on substance P or calcitonin gene-related peptide concentration in saliva could not be investigated. Plasma anethole trithione concentrations were almost the same (Figure 4), and accordingly the possibility of a residual or an accumulative nature of anethole trithione may be low. This report has demonstrated that chronic treatment with anethole trithione affects the saliva SP-IS and α-CGRP-IS concentration in human saliva, and the sialosis volume. Konttinen et al (1992) clarified the survival of the substance P and calcitonin gene-related peptide nerve fibres in patients with Sjögren's syndrome. Therefore, it is suggested that the peptidergic nerve might play important roles in sialosis in chronic treatment with anethole trithione. In addition, the activation of peptidergic nerves (substance P and calcitonin gene-related peptide nerves) may be enhanced by chronic treatment with anethole trithione.

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