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Alteration in contractile G-protein coupled receptor expression by moist snus and nicotine in rat cerebral arteries

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ABSTRACT

The cardiovascular risk for users of use of Swedish snus/American snuff (moist tobacco) has been debated for a long time. The present study was designed to examine the effects of water- or lipid-soluble (DMSO-soluble) snus and nicotine, the most important substance in tobacco, on the expression of vasocontractile G-protein coupled receptors (GPCR), such as endothelin ET_B, serotonin 5-HT_{1B}, and thromboxane A_2 TP receptors, in rat cerebral arteries. Studies show that these vasocontractile GPCR show alterations by lipid-soluble cigarette smoke particles via activation of mitogen-activated protein kinases (MAPK). However, the effects of moist tobacco on the expression of GPCR are less studied.

Rat middle cerebral arteries were isolated and organ cultured in serum-free medium for 24 h in the presence of water-soluble snus (WSS), DMSO-soluble snus (DSS), or nicotine. The dose of snus and nicotine was kept at plasma level of snus users (25 ng nicotine/ml). A high dose (250 ng nicotine/ml) was also included due to the previous results showing alteration in the GPCR expression by nicotine at this concentration. Contractile responses to the ET_B receptor agonist sarafotoxin 6c, 5-HT_{1B} receptor agonist 5-carboxamidotryptamine, and TP receptor agonist U46619 were investigated by a sensitive myograph. The expression of ET_B, 5-HT_{1B}, and TP receptors was studied at mRNA and protein levels using quantitative real-time PCR and immunohistochemistry, respectively.

Organ culture with WSS or DSS (25 ng nicotine/ml) lowered the 5-HT_{1B} receptor-mediated contraction. Furthermore, DSS shifted the TP receptor-mediated contraction curve left-wards with a stronger contraction. High dose of nicotine (250 ng nicotine/ml) increased the ET_B receptor-mediated contraction. The combined 5-HT_{1B} and 5-HT_{2A} receptor-mediated contraction was increased, and both the 5-CT and TxA2 induced contractions were left-ward shifted by WSS, DSS, or nicotine (250 ng nicotine/ml). Only the DSS group showed that the increase of 5-HT_{1B} receptor-mediated contraction occurred at the transcriptional level, demonstrated by an increased mRNA expression for the receptor.

Thus, snus and nicotine alter the GPCR expression in the cerebral arteries, which may be involved in cerebral vascular disease.

Introduction

The use of snus (moist tobacco) is an effective way for Swedes to quit/reduce smoking, and thus reduce health risks of burnt tobacco use. However, this occurs at a cost of local oral exposure and higher nicotine levels in the circulation. The toxic effects of using Swedish moist snus on cerebral and cardiovascular diseases have been debated for a long time. Clinical studies show that snus has (i) mild side-effects on health like few and occasional oral lesions (Andersson et al., 1994), (ii) moderate side-effects such as elevated blood pressure (Bolinder et al., 1992), (iii) rarely, modification of cerebral and cardiovascular diseases, and (iv) seldom larynx/oesophagus cancers (US Department of Health and Human Services, 1986). However, specific and detailed pharmacological studies on snus effects on cerebral arteries are absent.

Nicotine is the addictive substance in snus. In order to reveal possible molecular mechanisms of snus, which could lead to development of cerebrovascular disease, we examined if snus extracts or nicotine could alter GPCR expression and function of contraction in rat cerebral arteries (Hansen-Schwartz et al., 2002; Hoel et al., 2001).

GPCR-mediated vascular smooth muscle contraction, proliferation and apoptosis are important events in the pathogenesis of cerebral and cardiovascular disease (Hansen-Schwartz et al., 2003b). Organ culture of cerebral vessels has been demonstrated as an in vitro method for exploring the molecular mechanisms that lead to changes in GPCR (endothelin type B receptor (ET_B), 5-hydroxytryptamine type 1B receptor (5-HT_{1B}), and thromboxane A_2 prostanoid receptor (TP))

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expression in a way resembling that observed in cerebral ischemia in vivo (Hoel et al., 2001; Rosamond et al., 2008; Hansen-Schwartz et al., 2002; Henriksson et al., 2003). All these three receptors belong to the GPCR family and mediate cerebral vascular contraction and smooth muscle cell proliferation (Coleman et al., 1994; Masaki et al., 1994; Nilsson et al., 1999) and have been observed to be upregulated after a stroke and after organ culture.

The present study has examined the effects of snus and nicotine at plasma level which resemble that seen in snus users (25 ng nicotine/ml) (Benowitz et al., 1994; Foulds et al., 2003). A high dose was also included (250 ng nicotine/ml) due to previous studies showing that higher doses of nicotine may influences GPCR expression (Zhang et al., 2009). "Normal" plasma level of nicotine of WSS, DSS, or nicotine does not affect the receptor expression of endothelin ET_B. Serotonin 5-HT_{1B} and 5-HT_{2A} mediated contraction curve was lowered by WSS and DSS (normal plasma level), while DSS also left-ward shifted the TP mediated contraction curve. The high dose of nicotine of WSS, DSS, or nicotine enhanced the contraction by 5-HT_{1B} and 5-HT_{2A} and TP. High nicotine level increased the endothelin ET_B receptor-mediated contraction curve. The alteration of GPCR expression in the cerebral arteries by snus and nicotine most likely is a molecular mechanism involving cerebral vascular damage. Thus, this suggests that snus and nicotine may have a potential impact on development of cerebrovascular disease.

Materials and methods

Removal of cerebral vessels and organ culture

Studies were approved by the Danish Animal Experiments Committee guidance (no. 2006/561-1139). Male Sprague–Dawley rats (n = number of rats = 78; 300–350 g) (Taconic, Denmark) were sedated with 70% CO_2 in O_2 and decapitated while unconscious. The brains were removed and immediately chilled in ice-cold Na^+–Krebs buffer solution (for composition see below). The right and left middle cerebral arteries were isolated and dissected free of adhering tissue in ice-cold Na^+–Krebs buffer.

Middle cerebral artery segments (1.5–3.0 mm long), rings with intact endothelium, were incubated for 24 h at 37 °C in humidified 5% CO_2 and 95% air in serum-free Dulbecco's modified Eagle's medium (DMEM: 1 mg/ml glucose, 4 mM L-glutamine, 0.11 mg/ml sodium pyruvate) supplemented with an antibiotics mix (10.000 units/ml of penicillin, 10 mg/ml of streptomycin, and 25 ng/ml of amphotericin B). Each middle cerebral artery was bluntly cut into 4–5 segments, each 2.0–3.0 mm long (all in all 8–10 segments per rat) for the myograph bath studies. For real-time PCR and immunohistochemistry studies the middle cerebral artery was dived into 2 segments, each 6.0–7.0 mm long (all in all 4 segments per rat). Middle cerebral artery segments from the rat were used in myograph bath studies for different groups. For real-time PCR and immunohistochemistry experiments middle cerebral artery segments were used for both methods. The anatomical portion of middle cerebral artery segments for myograph bath, real-time PCR and immunohistochemistry studies were interchanged (n values for each group: myograph bath n = 6–10, real-time PCR n = 6–7, and immunohistochemistry n = 4–5). Fresh or organ cultured cerebral artery ring segments were either mounted in myographs for in vitro pharmacology or snap-frozen with dry ice and kept at −80 °C for real-time PCR or immunohistochemistry.

WSS or DSS was added to the DMEM medium at the initiation of the incubation. Vehicle (water or DMSO) was added as control. All organ culture experiments were performed for the duration of 24 h. Water, DMSO, WSS, DSS, or nicotine were added at 0 h, giving a total duration of 24 h. The final volume of DMSO added to the 1 ml DMEM was always kept at 1 μl.

Drugs

Dulbecco's modified Eagle's medium (DMEM: 1 mg/ml glucose, 4 mM L-glutamine, 0.11 mg/ml sodium pyruvate), antibiotics mix (10,000 units/ml penicillin, 10,000 μg/ml streptomycin, and 25 μg/ml amphotericin), and Trizol were from Invitrogen, USA. 1-Bromo-3-chloropropane, dimethyl sulfoxide (DMSO), carbachol, 5-hydroxytryptamine (5-HT), 5-carboxamidotryptamine (5-CT), U46619, absolute ethanol, nicotine and other high grade chemicals were purchased from Sigma-Aldrich, USA. Sarafotoxin 6c was from NeoMPS S.A., France. RNase free water was from Qiagen, USA.

Extraction of water- or DMSO-soluble snus solution (WSS or DSS)

Bags of snus (General brand, Swedish Match # 0200-113114, batch # 52030-462-1) were dissolved for 1 h at 37 °C in either water (WSS) or DMSO (DSS) (tubes were vortexed every 15 min). The Swedish Match company informed that the pH was 8.5 and the nicotine content was 8 mg/g snus. Snus bags were weighted before dissolving in water or DMSO, and the final concentrations were 250 ng nicotine/μl (this was diluted 10 times to give a 25 ng nicotine/μl solution also). One microliter of either of these solutions was added to 1 ml DMEM media for organ culture, giving a final concentration of 25 or 250 ng nicotine pr. ml of DMEM, respectively. The design of the study is then that WSS is the solution containing snus ingredients and nicotine, while DSS contains lipid-soluble snus ingredients. Foulds et al., 2003 showed that one dose of snus results in a plasma nicotine concentration of 15 ng/ml. Our initial studies revealed no effect of this dose; therefore, we increased it to the same level as that seen after cigarette smoking (25 ng/ml) (Benowitz et al., 1994; Foulds et al., 2003).

A previous study has revealed that high doses of nicotine (480 and 960 ng/ml) may affect contractions mediated by endothelin ET_B and 5-HT_{1B} receptor in rat mesenteric arteries (Zhang et al., 2009). The E_{max} of the endothelin ET_B receptor-mediated contraction induced by the specific agonist S6c is increased dramatically by 2-fold when 480 ng/ml nicotine was added to the organ culture, whereas 960 ng/ml nicotine gave a 3-fold increase in E_{max}. The 5-HT curve was right-ward shifted (increase in pEC_{50}) by 60 and 960 ng/ml nicotine. This shows that a higher dose of 5-HT is needed to promote the same 5-HT receptor-mediated contraction, which could be due to fewer 5-HT receptors or lower receptor affinity.

The high doses of nicotine (480 and 960 ng/ml) are about 20–40 times higher than that observed in plasma of snus users and does not have any clinical importance (only a toxic dose); hence, we chose to examine more reasonable doses of 250 ng/ml).

In vitro pharmacology

A sensitive myograph was used to record the isometric tension in isolated cerebral vessel segments (Hogestatt et al., 1983; Mulvany and Halpern, 1977). The vessel segments were threaded on two 40 μm-diameter stainless steel wires and mounted on a Mulvany-Halpern myograph (Danish Myo Technology A/S, Denmark). One of the wires was connected to a force displacement transducer attached to an analog-digital converter unit (PowerLab from ADInstruments, New Zealand), while the other wire was attached to a movable displacement device allowing fine adjustments of vascular tension by varying the distance between the two wires. The measurements were recorded on a computer using the software Chart 5.4.2 (ADInstruments, UK).

The segments were immersed into a temperature-controlled (37 °C) Na^+–Krebs buffer solution (composition in mM/ml; $NaCl$ = 119, $NaHCO_3$ = 15, KCl = 4.6, $MgCl_2$ = 1.2, NaH_2PO_4 = 1.2, $CaCl_2$ = 1.5, and glucose = 5.5). The buffer was continuously gassed with 5% CO_2 in O_2 resulting in a physiological pH at 7.4. The vessels