Investigation of enhancement effects of nicotine on cholinergic neurotransmission in isolated rabbit gastric fundus: role of antioxidants

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Summary

1. Nicotine, which is tobacco alkaloid, still induces interests for researchers because of smokers addiction to nicotine. Nicotine having influence on the neuronal acetylcholine receptors (nAChRs) increases release of most certain neurotransmitters from the nerve endings. Also, nicotine, affecting the mitochondrial respiratory chains, contributes to the formation of reactive oxygen species.

2. In the present study, we investigated the effects of nicotine on smooth muscles of gastric fundus on the electrical field stimulation (EFS) that induces transition contraction via stimulation nAChRs. In addition, we aimed to investigate the interaction between release of acetylcholine, induced by nicotine, and the effects of reactive oxygen species.

3. Therefore, the effects of allopurinol \((10^{-6} - 10^{-5})\) M, deferoxamine \((10^{-4})\) M and mannitol \((10^{-4} - 5 \times 10^{-3})\) M were tested on the transient contraction induced by nicotine.

4. In conclusion, mannitol \((5 \times 10^{-3})\) M significantly reduced contractile response to nicotine on EFS only in high concentration. Whereas in small concentrations mannitol \((10^{-4})\) M statistically did not cause any results. Deferoxamine and allopurinol also did not have any significant response.

Keywords: acetylcholine, electrical field stimulation, neuronal acetylcholine receptors, deferoxamine, allopurinol, mannitol, reactive oxygen species, nicotine

Introduction

Cigarettes are known to contain more than 4700 compounds, and nicotine is the most toxic of these compounds (Barr \textit{et al.}, 2007). Nicotine, which consists of a pyridine and a pyrolidine chain (Guan \textit{et al.}, 2003) still attracts the interest of researchers due to its addictive properties. Nicotine, which poses a threat to human health, has pharmacological, mutagenic, carcinogenic and inflammatory effects on human body cells (Zoghi & Nalbantgil, 2002; Malfertheiner & Schutte, 2006; Rand \textit{et al.}, 2006; Grozio \textit{et al.}, 2007). In contrast to these negative effects, there is insufficient research on the potential positive effects of nicotine in medicine. Recent data indicated that nicotine can have positive effects on the treatment of various diseases, such as Alzheimer’s disease, Parkinson’s disease and ulcerative colitis. Possible mechanisms for the positive effects of nicotine have been defined in the literature, and it is thought that these effects are caused by stimulation of nicotinic receptors and the release of well-known neurotransmitters. Nicotine shows its positive effects by affecting the presynaptic nicotinic receptors and facilitating the release of certain neurotransmitters such as dopamine, serotonin, noradrenaline and other neurotransmitters from nerve endings (Wang \textit{et al.}, 2000; Rao \textit{et al.}, 2003; Gotti & Clementi, 2004). Nicotine was shown to increase acetylcholine release in mouse vas deferens (Cuprian \textit{et al.}, 2005), and stimulated the nicotinic receptors of the myenteric excitatory motor nerve endings and ganglions (Galligan \textit{et al.}, 2000). Nicotine releases acetylcholine by influencing the muscarinic receptors which cause contraction of smooth muscles.
(Schneider & Galligan, 2000). It is known that ganglionic nicotinic receptors play a role in this process, but their mechanisms are poorly understood.

It is also known that nicotine affects reactive oxygen radical oxidation and thus contributes to the formation of superoxide anion (O$_{2}^-$) and hydrogen peroxide (H$_2$O$_2$). Furthermore, nicotine causes tissue damage due to its affect on the antioxidant system (Baskaran et al., 1999). On the other hand, nicotine interacts with complex one of the brain mitochondrial respiratory chain leading to a decrease in reactive oxygen species (ROS) formation (Guan et al., 2003), and can thus have neuroprotective effects in various diseases. Therefore, the interrelation between ROS and the release of acetylcholine caused by nicotine on electrical field stimulation (EFS) has been studied previously. It should be noted that in addition to nicotine, acetylcholine can also induce ROS formation. Oldenburg et al. (2003) demonstrated that atropine inhibited ROS formation caused by acetylcholine.

Nicotine also influences the nicotinic receptors leading to calcium influx into the nerve endings (Brain et al., 2001). An elevation in calcium level triggers the activation of different degradative processes due to ROS formation, and causes impaired energy production and the activation of several hydrolytic enzymes (Liu & Zhao, 2004). Therefore, increased intracellular Ca$^{2+}$ due to stimulation by nicotine can lead to an increase in free radical production. Based on this data, we decided to investigate the effects of antioxidants on the nicotine-induced transient increases in cholinergic neurotransmission.

Materials and methods

Animals

Thirty-five New Zealand albino rabbits weighing 2.5–3.0 kg were used for the experiments. All animals were kept under controlled temperature (23.2 °C) and humidity (55.5%) with a 14 h light and 10 h dark cycle. They were fed standard laboratory chow and given tap water. All experiments were performed in accordance with the ethical regulations of the Helsinki Declaration. This study was approved by Gazi University Ethics Committee for Animals.

Tissues

Animals were sacrificed by exsanguination and their stomachs were rapidly excised, opened lengthwise, and emptied. Adherent fat, gross connective tissues, and gastric mucosa were removed, and uniform longitudinal strips (15 mm × 3 mm) were prepared from the smooth muscle of the gastric fundus.

Organ Chamber Experiments

Each strip was mounted under 1 g isometric resting tension in an organ bath containing 15 ml Krebs-Henseleit solution (composition in mmol l$^{-1}$: NaCl 118.0, KCl 4.7, CaCl$_2$·2H$_2$O 1.3, MgCl$_2$·6H$_2$O 0.5, Na$_2$HPO$_4$·2H$_2$O 0.9, NaHCO$_3$ 24.9, glucose monohydrate 11.0). The pH of the solution was 7.4 after bubbling with a mixture of 95% O$_2$ and 5% CO$_2$, and the solution was maintained at 37 °C. The tissues were allowed to equilibrate for at least 1 h before experimental procedures. Isometric contractions were evoked by EFS through a pair of platinum electrodes with an 8 Hz stimulation frequency by 10 s trains of impulses delivered every 2 min. A stimulator (S48; Grass Instruments, Quincy, MA, USA) delivered 60 V pulses of 1 ms duration. EFS-evoked responses were recorded via Grass isometric force displacement transducers (Grass FT 03) connected to an ink writing oscillograph (Grass 79 E) via a preamplifier. Thirty minutes after the EFS-evoked responses reached a steady state, to test the contribution of the cholinergic component, the tissue was treated with atropine (10$^{-5}$ M), a muscarinic receptor blocker, and neostigmine (10$^{-5}$ M), a reversible anticholinesterase drug. The effects of tetrodotoxin (TTX) (10$^{-6}$ M), deferoxamine (10$^{-4}$ M), allopurinol (10$^{-6}$ M; 10$^{-7}$ M; 10$^{-8}$ M) and mannitol (10$^{-4}$ M; 5 × 10$^{-5}$ M) on the EFS-evoked responses were also tested. To test the effects of nicotine, 10$^{-4}$ M concentration of nicotine were administered to the preparations. To avoid any possible habituation effect or tachyphylaxis, EFS was stopped after seven contractions and the preparations were washed four times every 15 min for 1 h as in our previous study (Ilhan et al., 2007). To investigate the effects of ROS on the nicotine-induced EFS-evoked contractile response alterations, same experimental procedure was repeated in the presence of allopurinol (10$^{-6}$ M; 10$^{-7}$ M), mannitol (10$^{-4}$ M; 5 × 10$^{-3}$ M) and deferoxamine (10$^{-4}$ M).

Drugs

All of the following drugs were obtained from Sigma (St Louis, MO, USA): acetylcholine chloride, nicotine, atropine sulphate, deferoxamine, allopurinol, mannitol, TTX. Stock solutions of drugs were dissolved in distilled water. Solutions were stored at −20 °C. The drugs were diluted in Krebs to the required final concentration on the day of use.

Statistics

Nicotine-induced increases were expressed as % of the control and the average of seven EFS-evoked contractile responses. The value of the last con-
traction before the application of nicotine was taken as the control value.

Experimental values were expressed as the mean ± SEM. Groups were compared statistically using general linear models of analysis of variance (ANOVA) followed by post-hoc analysis with the Bonferroni test.

P values of <0.05 were considered to be statistically significant.

Results

Electrical field stimulation evoked contractile responses in rabbit gastric fundus. Mean amplitude of the EFS-evoked contractile responses was 2.85 ± 0.42 g at 8 Hz of stimulation frequency.

Tetrodotoxin (10^{-6} M), a blocker of Na+ channels, abolished the EFS-evoked contractile responses in rabbit gastric fundus strips (data not shown). However, allopurinol (10^{-6}–10^{-5} M), mannitol (10^{-4}–5 × 10^{-3} M) and deferoxamine (10^{-4} M) did not alter EFS-evoked contractile responses (2.68 ± 0.35 g, 2.52 ± 0.32 g, 2.76 ± 0.45 g, 2.58 ± 0.46 g, 2.65 ± 0.44 g, respectively).

Nicotine (10^{-4} M) increased the EFS-induced contractions transiently (15 166 ± 16.02%) in this study. EFS-evoked contractile responses were abolished by atropine, a muscarinic receptor blocker, at the 10^{-6} M concentration in rabbit gastric fundus strips (data not shown). Neostigmine, a reversible anticholinesterase drug, increases the amplitude of the EFS-evoked contractile responses (data not shown).

Effects of allopurinol, mannitol and deferoxamine on nicotine induced transient neurogenic contractions

Allopurinol at 10^{-6} and 10^{-5} M concentrations did not alter nicotine-induced transient neurogenic contractions (Figs 1 & 2).

Mannitol at 10^{-4} M concentration did not alter nicotine-induced transient neurogenic contractions (Fig. 3) whereas mannitol at 5 × 10^{-3} M concentration significantly inhibited nicotine-induced transient neurogenic contractions (Fig. 4).

Deferoxamine did not alter nicotine-induced transient neurogenic contractions at the concentration of 10^{-4} M (Fig. 5).
cigarette smokers are 60–1200 µg/kg per day, could be achieved in smokers. It is reported that nicotine was found in our laboratory (Vural et al., 2009). In addition to the gastric fundus, nicotine-contracted smooth muscle of isolated rabbit gastric fundus (Ilhan et al., 2007). We now report that allopurinol and deferoxamine have no significant effects, whereas mannitol reduced the nicotine-induced transient increase in EFS contractions only at the highest concentration. In this study, we have used very large doses of nicotine (10^{-4} M). This is a physiologic concentration that could be achieved in smokers. It is reported that nicotine levels in the blood circulation of habitual cigarette smokers are 60–1200 µg/L (Rothem et al., 2009). In addition to the gastric fundus, nicotine-induced transient neurogenic contractions in rabbit myometrium and bladder were previously shown in our laboratory (Vural et al., 2006; Nas et al., 2007). Nicotine produces this effect via presynaptic regulation. Presynaptic regulation is an important mechanism governing neurotransmission, and causes either inhibition or facilitation of transmitter release. Activation of presynaptic neuronal acetylcholine receptors (nAChRs) facilitates or induces the release of neurotransmitters in the central nervous system and autonomic ganglia (Vizi & Lendvayi, 1999; Galligan et al., 2000; Rao et al., 2003). It is known that nAChRs located on the somatodendritic regions of myenteric neurons participate in fast synaptic transmission. Some of these myenteric neurons are motoneurons that project into the smooth muscle layers. According to recent studies, both the nAChR-related mechanism and nicotine caused smooth muscle contraction in non-myenteric plexus preparations. If nAChRs are only found in somatodendritic regions, then isolation of the motor axon and its varicosities derived from myenteric ganglia should abolish nAChR agonist-induced contraction (Schneider & Galligan, 2000). In a recent study, axons projecting into the longitudinal smooth muscle were chemically isolated from myenteric ganglia using the voltage-gated sodium channel blocker TTX. The use of TTX did not abolish nAChRs-mediated contraction of nicotine, but fully abolished only EFS-related contraction. These results indicate that nAChRs are localized on the axons and/or terminals of excitatory motoneurons, and that acetylcholine release was mediated by presynaptic receptors. Our data demonstrated that TTX abolished nicotine-induced acetylcholine release on EFS.

According to the above-mentioned data which was supported by the hypothesis of a correlation between ROS and the release of acetylcholine caused by nicotine on EFS, we tested the effects of antioxidants on the transient contraction induced by nicotine. Recent studies have demonstrated the neuroprotective effects of allopurinol, however, the mechanisms of this neuroprotection are not fully understood. The main effects of allopurinol are based on its inhibition of the xanthine oxidase system. Thus, allopurinol decreases injury in brain cells. However, according to an analysis in the organotypic hippocampal model, allopurinol does not have any neuroprotective effects as endothelial cells would be required (Peeters et al., 2003). For further characterization and more precise elucidation it is necessary to define whether the neuroprotective properties of these agents which are mediated via endothelial cells contribute to this effect. Moreover, it is unclear whether this effect is related to ROS-mediated processes within neuronal cells or if it is dependent on endothelial cells (Rubanyi & Vanhoutte, 1986). Our data shows that allopurinol did not have a significant response on nicotine-induced contraction in EFS. Thus, the effect is probably related to endothelial cells, which we removed from the gastric tissue, to exclude the influence of these cells.

Mannitol is known as a scavenger of OH (Mizoi et al., 1986) and deferoxamine is also known as an iron chelator. In a study of murine cerebral cortical neurons, mannitol increased NO-induced acetylcholine release (Ohkuma et al., 1995). In addition, some authors have hypothesized that acetylcholine-induced vascular relaxation is mediated by OH derived from the interaction between NO and O_2^-. It has been suggested that NO interacts with superoxide anion (O_2^-) to generate peroxynitrite which at physiological pH gives rise to peroxynitrous acid, which rapidly decomposes to hydroxyl radical (OH) and nitrogen dioxide, and OH relaxes isolated aorta. Relaxation produced by the OH generating system was prevented by mannitol (Prasad & Bharadwaj, 1996). Similarly, Asano

**Figure 5** Effects of deferoxamine (10^{-4} M) on nicotine (10^{-4} M)-induced increases in electrical field stimulation (EFS)-evoked contractile responses (n = 7). Each point is expressed as a % of the control and the average of seven EFS evoked contractile responses. All points are given as the mean ± SEM (*P < 0.05).

In all the experiments nicotine’s concentration was 10^{-4} M.

**Discussion**

The present study confirms our previous findings that nicotine transiently increased EFS-induced contractions in a concentration-dependent manner, and suggests that nicotine acts on nicotinic receptors and transiently increases the EFS-induced contractions in the smooth muscle of isolated rabbit gastric fundus (Ilhan et al., 2007). We now report that allopurinol and deferoxamine have no significant effects, whereas mannitol reduced the nicotine-induced transient increase in EFS contractions only at the highest concentration. In this study, we have used very large doses of nicotine (10^{-4} M). This is a physiologic concentration that could be achieved in smokers. It is reported that nicotine levels in the blood circulation of habitual cigarette smokers are 60–1200 µg/L (Rothem et al., 2009). In addition to the gastric fundus, nicotine-induced transient neurogenic contractions in rabbit myometrium and bladder were previously shown in our laboratory (Vural et al., 2006; Nas et al., 2007). Nicotine produces this effect via presynaptic regulation. Presynaptic regulation is an important mechanism governing neurotransmission, and causes either inhibition or facilitation of transmitter release. Activation of presynaptic neuronal acetylcholine receptors (nAChRs) facilitates or induces the release of neurotransmitters in the central nervous system and autonomic ganglia (Vizi & Lendvayi, 1999; Galligan et al., 2000; Rao et al., 2003). It is known that nAChRs located on the somatodendritic regions of myenteric neurons participate in fast synaptic transmission. Some of these myenteric neurons are motoneurons that project into the smooth muscle layers. According to recent studies, both the nAChR-related mechanism and nicotine caused smooth muscle contraction in non-myenteric plexus preparations. If nAChRs are only found in somatodendritic regions, then isolation of the motor axon and its varicosities derived from myenteric ganglia should abolish nAChR agonist-induced contraction (Schneider & Galligan, 2000). In a recent study, axons projecting into the longitudinal smooth muscle were chemically isolated from myenteric ganglia using the voltage-gated sodium channel blocker TTX. The use of TTX did not abolish nAChRs-mediated contraction of nicotine, but fully abolished only EFS related contraction. These results indicate that nAChRs are localized on the axons and/or terminals of excitatory motoneurons, and that acetylcholine release was mediated by presynaptic receptors. Our data demonstrated that TTX abolished nicotine-induced acetylcholine release on EFS.

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et al. (2001) showed that in rabbit intrapulmonary bronchiol, deferoxamine significantly attenuated both the phasic and tonic contractions induced by acetylcholine in the absence of H$_2$O$_2$. However, deferoxamine did not significantly modify the H$_2$O$_2$-inhibited acetylcholine-induced contractions. In addition, Avshalumov et al. (2000) showed that in rat hippocampal preparations, deferoxamine prevented H$_2$O$_2$-induced inhibition of synaptic transmission. However, our data indicated that deferoxamine did not have a significant effect on cholinergic transmission. In contrast, mannitol had a significant effect on cholinergic transmission. The mechanism causing the decrease in acetylcholine release is unknown. The effect on cholinergic transmission by mannitol occurred due to the direct effect of nicotine or acetylcholine which increased NO in smooth muscle. Thus the formed NO interacts with superoxide and may assist in the formation of OH radicals. Thus, OH may reduce contraction induced by nicotine on EFS.

In conclusion, it is suggested that TTX abolished nicotine-induced contraction on EFS, which indicates that acetylcholine release is mediated by presynaptic receptors. Allopurinol and deferoxamine did not have a significant effect on nicotine-induced acetylcholine release or cholinergic transmission. However, mannitol at a high concentration inhibited the transient ability of nicotine to augment EFS-induced neurogenic contractile responses. According to the above-mentioned data, our findings suggest that antioxidants may assist in the nicotine-induced neurotransmitter release mechanism via nAChRs.

Acknowledgments

This work was supported partially by Gazi University Unit of Scientific Research Projects (Project number: 01/2004-77) and by the Scientific and Technological Research Council of Turkey (NATO scholarships programme).

References


(Received 1 October 2009
Accepted 6 October 2009)