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Inhibitory effects of alendronate on cholinergic responses in rat lower esophageal sphincter

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Abstract

Alendronate is a potent inhibitor of osteoclast-mediated bone resorption, but its use results in serious esophageal damage. In order to clarify the latter, we examined the effects of alendronate on electrical field stimulation-induced responses in the rat lower esophageal sphincter. Electrical field stimulation induced atropine-sensitive contraction. Alendronate inhibited electrical field stimulation-induced contraction in a concentration-dependent manner. In the presence of N^G-nitro-L-arginine (L-nitroarginine), electrical field stimulation elicited a strong cholinergic contraction. This contraction was also inhibited by alendronate, to a similar extent as that seen in the absence of L-nitroarginine. In lower esophageal sphincter contracted by prostaglandin $F_{2\alpha}$ and treated with atropine, electrical field stimulation induced L-nitroarginine-sensitive relaxation. Alendronate did not affect relaxation. These results suggest that alendronate decreases the tone of lower esophageal sphincter by inhibiting cholinergic nervous activity.

Keywords: Alendronate; Electrical field stimulation-induced contraction; Lower esophageal sphincter; NO-mediated relaxation

1. Introduction

Alendronate is a nitrogen-containing bisphosphonate that is effective in preventing osteoporotic fractures (Liberman et al., 1995; Watts et al., 1999). Since alendronate was found to be an effective agent for drug-induced osteoporosis in addition to both senile and postmenopausal osteoporosis (Adachi et al., 2001; Liberman et al., 1995), it has been approved in over 90 countries and used by more than 4.5 million patients (Fleisch, 2000; Stevenson et al., 2005). Though it was reported that the benefit/risk ratio of alendronate remains highly favorable, one of the major side effects of oral alendronate therapy was gastrointestinal intolerability, particularly esophageal irritation and ulceration (Lowe et al., 2000). The effects of alendronate observed in the gastrointestinal tract have been reported to be due to inhibition of proliferation or apoptosis of the epithelial cells or a local irritation that cause inflammation through neutrophil infiltration and oxidative damage in the tissues (Sener et al., 2005; Suri et al., 2001). However, exact details of the mechanism remained to be clarified.

The lower esophageal sphincter relaxes briefly in order to allow the passage of food during swallowing, and also functions as an antireflux barrier protecting the esophagus from the caustic gastric content (Castell et al., 2004). The two typical examples of dysfunction of the lower esophageal sphincter are achalasia and gastro-esophageal reflux disease (Boeckxstaens, 2005). Of these diseases, gastro-esophageal reflux disease results from failure of the antireflux barrier, with increased exposure of the esophagus to gastric acid, leading to esophagitis (Nguyen and Holloway, 2005). Thus, agents that inhibit the proper functioning of the lower esophageal sphincter have the potential of causing esophageal irritation.

It was reported that both the inhibitory and the excitatory neuronal pathways exert tonic effects in the lower esophageal sphincter. Electrical field stimulation of lower esophageal sphincter isolated from the human and the guinea pig induced both the excitatory response which was abolished by atropine, and the relaxation which was inhibited by nitric oxide

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synthase (NOS) inhibitors (Gonzalez et al., 2004; Yuan et al., 1998). In the present study, we examined the effects of alendronate on electrical field stimulation-induced excitatory and inhibitory responses in the rat lower esophageal sphincter and report that alendronate can decrease the tone of lower esophageal sphincter by the inhibition of cholinergic neural activity.

2. Materials and methods

Male Wistar Hannover GALAS rats (200–300 g) obtained from Clea Japan, Inc. (Osaka, Japan) were lightly anesthetized with ether and then stunned by a blow to the head and bled via the carotid artery. Animal maintenance and experimental procedures were performed in accordance with the guidelines of the ethics committees of Osaka Prefecture University. The stomach together with the esophagus and ileum were removed and placed in Tyrode solution consisting of (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.1, NaH_2PO_4 0.42, $NaHCO_3$ 11.9 and glucose 5.6 (pH 7.4). The preparations were opened following the lesser curvature of the stomach. The contents in the excised segments were discarded and the mucosal layer removed with a scissors using an inverted microscope. Muscle strips (2×10 mm) of lower esophageal sphincter were prepared by cutting the wall of the gastroesophageal junction between the gastric fundus and the esophagus in the direction of the circular muscle layer. The circular and longitudinal muscle strips of fundus, and the ileum were prepared as described previously (Toyoshima et al., 2006; Waseda et al., 2005).

2.1. Recording of responses of lower esophageal sphincter to electrical field stimulation

Lower esophageal sphincter was suspended in an organ bath containing 2 ml of Tyrode solution maintained at 37 °C and bubbled with a mixture of 95% O_2: 5% CO_2. One end of each strip was attached to an isometric transducer (UL-10GR, Minebea, Tokyo, Japan) and the other end mounted on an anodal electrode placed at the bottom of the bath. Changes in tension of the strips were recorded on a pen recorder (U-228, Nippon Denshi Kagaku Co., Tokyo, Japan) via a preamplifier (DC Strain amplifier 6M92, San-ei, Tokyo, Japan). The strip was subjected to a resting load of 1.0 g. After an equilibration period of 30 min, responses of lower esophageal sphincter to electrical field stimulation with trains of 30 and 100 pulses of 0.5-ms width (3–10 Hz frequencies) and 20 V intensity for 10 s were recorded isometrically with a 10-min interval between tests. Electrical field stimulation was applied by means of an electrical stimulator (SEN-3301, NIHON KOHDEN, Tokyo, Japan). Drugs were added to the bathing fluid, after the responses to electrical field stimulation became reproducible. In another series of experiments, responses to exogenously added acetylcholine were recorded. The responses of circular and longitudinal muscle strip of fundus and ileum to electrical field stimulation were also recorded using the same method.

2.2. Statistical analysis

The effect of alendronate on the electrical field stimulation-induced contraction was expressed as the percentage of that immediately before the treatment with alendronate. The numbers of experiments were represented as number of animals. Data are expressed as means±S.E.M. Results were analyzed by Student's *t*-test for paired values and a P value of <0.05 was regarded as significant.

2.3. Drugs

Acetylcholine, alendronate, atropine and tetrodotoxin were purchased from Wako Pure Chemicals, Osaka, Japan. N^G-nitro-L-arginine (L-nitroarginine) was from Sigma Chemical Co., St. Louis, U.S.A. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was from Funakoshi Co., Osaka, Japan. All other chemicals were of analytical grade. $PGF_{2\alpha}$ was dissolved in ethanol at a final ethanol concentration of 0.1%, this ethanol concentration did not have any effect on tissue preparations. $PGF_{2\alpha}$ was prepared as a stock solution that was diluted to the final concentration used in individual experiments. Other drugs were added as redistilled-water solutions in volumes of less than 1.0% of the bathing solution. A similar volume of redistilled water alone had no effect on the muscle activity.

3. Results

3.1. Effects of alendronate on responses of lower esophageal sphincter to electrical field stimulation

Electrical field stimulation at 10 Hz induced a contraction consisting of two components in the rat lower esophageal sphincter (Fig. 1). A slow phase of contraction immediately after the start of electrical field stimulation was followed by a rapid contraction. Electrical field stimulation at 3 Hz produced a very small contraction in lower esophageal sphincter. Thus, the

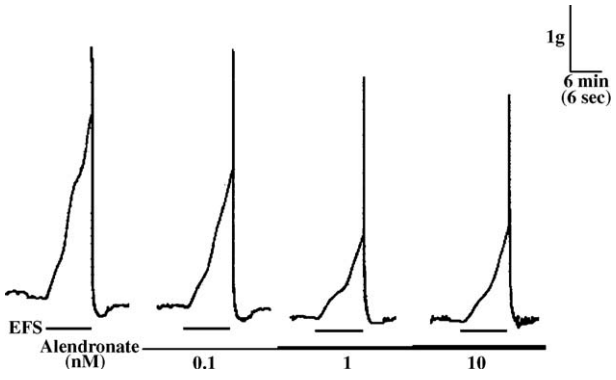

Fig. 1. Effects of alendronate on electrical field stimulation-induced contraction in rat lower esophageal sphincters. When electrical field stimulation-induced contractions became constant, alendronate was added cumulatively. Alendronate was applied into organ bath 10 min before electrical field stimulation. After recording normal spontaneous movements, the chart was run at a fast speed immediately before the stimulation to make the contractile response clear. Electrical field stimulation; 0.5 ms, 20 V, 10 Hz for 10 s.

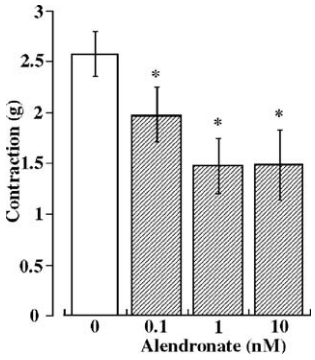

Fig. 2. Summarized results of effects of alendronate on electrical field stimulation-induced contraction. Columns and bars are the means and S.E.M. for 4–9 experiments. *Significantly from the value of contraction in the absence of alendronate, $P<0.05$.

effects of alendronate on contraction induced by electrical field stimulation at 10 Hz were studied. When the electrical field stimulation-induced contraction became constant, alendronate was added cumulatively to the bath solution (Fig. 1). Alendronate inhibited the contraction in a concentration-dependent manner (Fig. 2). At 0.1 nM, electrical field stimulation-induced contraction was inhibited to 68.5±5.1% of control (in the absence of alendronate). The maximal inhibition was obtained at 10 nM (44.8±7.8% inhibition). Electrical field stimulation-induced contraction was restored by a washout of alendronate (104.5±17.3% of control, $n=5$), suggesting that the effect of alendronate was reversible. Alendronate did not affect resting tone. Atropine and tetrodotoxin abolished electrical field stimulation-induced contraction (data not shown).

It was reported that electrical field stimulation stimulated the inhibitory neuron mediated by nitric oxide (NO) in addition to the activation of the excitatory cholinergic neurons. Therefore, the effects of alendronate were examined in the presence of a NOS inhibitor. In the presence of L-nitroarginine, 30 μM,

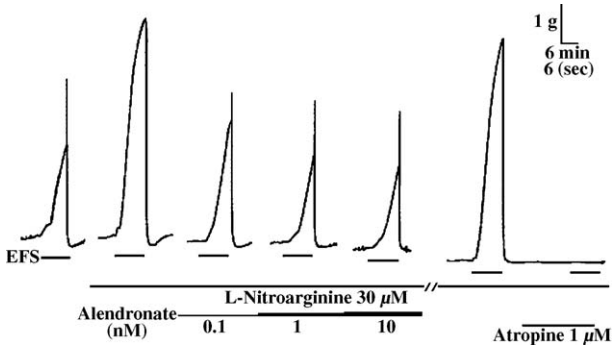

Fig. 3. Effects of alendronate on electrical field stimulation-induced contraction of rat lower esophageal sphincter in the presence of 30 μM L-nitroarginine. When electrical field stimulation-induced contractions became constant, alendronate was added cumulatively. Alendronate was applied into organ bath 10 min before electrical field stimulation. Atropine (1 μM) inhibited completely electrical field stimulation-induced contraction in the presence of L-nitroarginine. After recording normal spontaneous movements, the chart was run at a fast speed immediately before the stimulation to make the contractile response clear. Electrical field stimulation; 0.5 ms, 20 V, 10 Hz for 10 s.

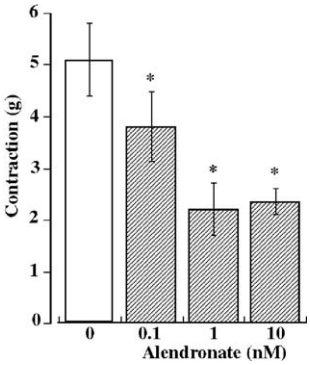

Fig. 4. Summarized results of effects of alendronate on electrical field stimulation-induced contraction in the presence of 30 μM L-nitroarginine. Columns and bars are the means and S.E.M. for 4–5 experiments. *Significantly different from the value of contraction in the absence of alendronate, $P<0.05$.

electrical field stimulation-induced contraction increased to 206.2±18.0% of that in the absence of L-nitroarginine (Fig. 3). Atropine abolished electrical field stimulation-induced contraction in the presence of L-nitroarginine. Alendronate at concentrations between 0.1–10 nM inhibited electrical field stimulation-induced contractions in the presence of L-nitroarginine in a concentration-dependent manner (Figs. 3 and 4). At 10 nM, electrical field stimulation-induced contraction was inhibited to 43.0±2.3% of control.

Acetylcholine, added exogenously, induced a contraction of the rat lower esophageal sphincter in a concentration-dependent manner. Alendronate at 10 μM did not affect the contraction induced by acetylcholine (Fig. 5).

In order to study the effects of alendronate on the relaxant responses induced by electrical field stimulation, the preparations were treated with atropine. In the presence of atropine, electrical field stimulation did not induce a change of tension in lower esophageal sphincter (results not shown). Thus, the preparation was precontracted by the use of $PGF_{2\alpha}$ at a concentration of 1 μM. In the precontracted condition, electrical field stimulation induced the relaxation which was inhibited by

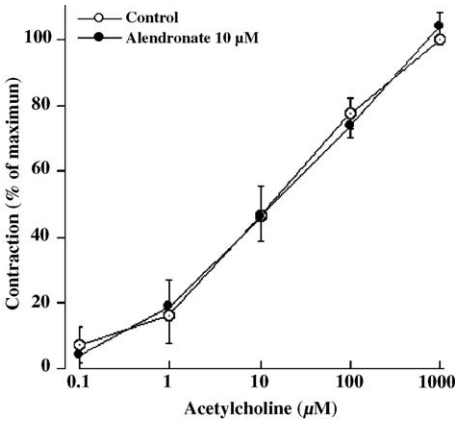

Fig. 5. Effects of alendronate on acetylcholine-induced contraction in rat lower esophageal sphincter. Contractions are expressed as a percentage of the contraction induced by 1 mM acetylcholine in the absence of alendronate. Points and bars are the mean and S.E.M. for 4 experiments.

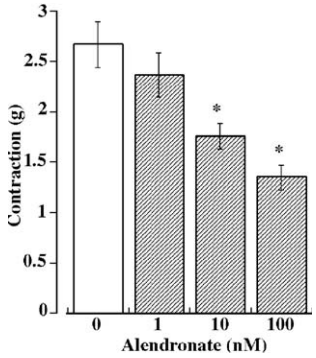

Fig. 6. Effects of alendronate on electrical field stimulation-induced contraction of the rat ileum in the presence of 30 μM L-nitroarginine. Columns and bars are the means and S.E.M. for 3–5 experiments. *Significantly different from the value of contraction in the absence of alendronate, $P<0.05$.

L-nitroarginine at 30 μM. Alendronate at 10 nM did not affect electrical field stimulation-induced relaxation ($n=2$).

3.2. Effects of alendronate on electrical field stimulation-induced contraction in ileum and circular and longitudinal muscle strip of fundus

Since alendronate inhibited the contraction mediated by acetylcholine in the lower esophageal sphincter, we studied whether this effect of alendronate on cholinergic neurotransmission was also present in other sites of the gastrointestinal tract than the lower esophageal sphincter. Atropine completely inhibited the contraction induced by electrical field stimulation in the rat fundus and ileum (data not shown, $n=3$–4). Alendronate inhibited the electrical field stimulation-induced contraction of the ileum in the presence of L-nitroarginine (30 μM) in a concentration-dependent manner (54.2±3.0% inhibition at 100 nM) (Fig. 6). Electrical field stimulation-induced contraction was restored by a washout of alendronate (74.3±11.3% of control, $n=4$). In contrast, the contractile responses of circular and longitudinal muscle of the rat fundus in the presence of L-nitroarginine were not affected by alendronate at 100 nM (circular muscle, 105.0% of control ($n=2$); longitudinal muscle, 104.8% of control ($n=2$)).

4. Discussion

Although alendronate which inhibits osteoclast-mediated bone resorption is an effective agent in the prevention and the treatment of osteoporosis, an important side effect is the development of erosive esophagitis (Watts et al., 1999). In the present study, we found, for the first time, that alendronate inhibited the electrical field stimulation-induced contraction in the rat lower esophageal sphincter. Since the contraction induced by electrical field stimulation was abolished by tetrodotoxin and atropine, it may be mediated by the activation of cholinergic neurons. These results suggest that alendronate has an inhibitory effect on acetylcholine-mediated contraction. The inhibitory effect of alendronate was observed at a low concentration of 0.1 nM. It was reported that oral bioavailability of alendronate was low (0.76%) (Gertz et al., 1995). Therefore, the effect of alendronate on the lower esophageal sphincter described in the present study may be of clinical significance.

It has been reported that in the lower esophageal sphincter inhibitory nitrergic neurons are activated by electrical field stimulation at the same time as the cholinergic neurons, thus counteracting both responses (Gertz et al., 1995; Gonzalez et al., 2004; Richards and Sugarbaker, 1995). Thus, the possibility exists that alendronate exerts an increasing effect on a NO-mediated response. In the present study, electrical field stimulation-induced contraction was augmented in the presence of L-nitroarginine. Alendronate, however, inhibited the contraction to a similar extent in both the absence and the presence of L-nitroarginine. In addition, NO-mediated relaxation was not affected by alendronate. We conclude from these results that alendronate may directly inhibit acetylcholine-mediated contraction without influencing NO-mediated responses.

To study the site of action of alendronate on the cholinergic responses, we examined the effect of alendronate on acetylcholine-induced contraction. Alendronate did not affect on the contraction of smooth muscle stimulated by acetylcholine. The results in the present study suggest that alendronate inhibits the release of acetylcholine from the cholinergic neurons in the rat lower esophageal sphincter. In recent, alendronate was reported to have an inhibitory effect on the isoprenoid biosynthetic enzyme, resulting in a disruption of isoprenylation that is involved in the modulation of regulatory proteins including the small GTPases (Reszka and Rodan, 2004), and to regulate activity of a nonselective cation channel (Shao et al., 2005). It seems likely that these actions of alendronate in addition of the effect on calcium homeostasis are involved in the modulation of acetylcholine release (Bonabello et al., 2001). Since, however, there are few reports concerning an effect of alendronate on the neuronal activity, further investigation will be necessary.

The lower esophageal sphincter has intrinsic myogenic properties that keep the sphincter closed during basal conditions (Chang et al., 2003). Furthermore, both the inhibitory and the excitatory neurons regulate the basal tone (Chang et al., 2003). Suppression of cholinergic neurons decreases lower esophageal sphincter pressure due to the unopposed action of nitrergic inhibitory neurons. Thus, the inhibition of cholinergic nervous activity of lower esophageal sphincter with alendronate may result from the opening of the gastroesophageal junction between the esophagus and the stomach leading, in turn, to a reflux of gastric acid to the esophagus. In a recent report, it was speculated that since alendronate at acidic pH was more damaging to the esophageal epithelium than either alendronate at neutral pH or acidic pH alone, its association with esophagitis requires gastric acid for expression (Dobrucali et al., 2002). Therefore, alendronate may produce severe injury to esophageal epithelium through a combination of the direct action on the epithelium and the inhibitory effect on the lower esophageal sphincter.

The inhibitory effect of alendronate on cholinergic neurotransmission was also observed in the rat ileal preparation. It was shown that some of the adverse events of alendronate in clinical trials were constipation and diarrhea (Jeal et al., 1997).

Thus, a part of side effect of alendronate may be explained from the action on the cholinergic nervous activity. In contrast, alendronate did not have any effects on electrical field stimulation-induced contraction in the rat fundus preparations. These results suggest that the effect of alendronate on cholinergic neurotransmission differs in the region from the region of the gastrointestinal tract. The cause of regional difference needs further investigation.

In conclusion, we described for the first time the underlying mechanisms involved in the esophageal damage caused by alendronate administration. It was reported that the maximum plasma concentration was 40.94±19.60 ng/ml (about 66–186 nM) following the oral administration of 70 mg of alendronate sodium to human (Yun and Kwon, 2006). Since alendronate is used at a clinical dose of 5–10 mg (Watts et al., 1999), it is expected that the plasma concentration of alendronate in patients is a range of concentration from 5 to 27 nM that is an effective concentration indicated in the present study. This is of clinical significance as it may lead to ways of preventing the most common adverse event associated with alendronate use.

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