Comparison of the Effects of YC-1, DEA/NO, Sodium Nitroprusside and Theophylline on the Sheep Sphincter of Oddi

KOYUN ODDI SİFİNTERİ ÜZERİNDE YC-1, DEA/NO, SODYUM NİTROPRUSİYAT VE TEOFİLİLNİN ETKİLERİNİN KARŞILAŞTIRILMASI

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Abstract

Objective: It has been demonstrated that nitric oxide (NO) plays an important physiological role in various parts of the gastrointestinal (GI) tract. This study was designed to show the existence of NO system in the sheep sphincters of Oddi (SO) and to compare the effects of drugs that affect NO system by different mechanisms.

Material and Methods: SO rings were mounted in tissue baths with modified Krebs-Henseleit solution and aerated with 95% oxygen and 5% carbon dioxide. Electrical field stimulation (EFS) responses and concentration-response curves for 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1, 10^-10^-10 M), diethylamine/NO complex (DEA/NO, 10^-6^-10^-4 M), sodium nitroprusside (SNP, 10^-3^-3 x 10^-4 M) and theophylline (10^-5^-10^-4 M) on SO rings precontracted with carbachol (10^-8 M) were recorded isometrically with a Grass polygraph. We also evaluated the effect of YC-1 (10^-8 M) and DEA/NO (10^-7 M and 10^-6 M) on the levels cyclic GMP (cGMP) in isolated SO rings.

Results: EFS-induced contractile responses was decreased by atropine (10^-6 M) and guanethidine (10^-5 M), but potentiated by L-NAMe (3 x 10^-5 M), YC-1 (10^-10^10^-5 M), DEA/NO (10^-7 M), SNP (10^-3^-3 x 10^-4 M) and theophylline (10^-5^-10^-4 M) induced concentration-dependent relaxation of isolated SO rings precontracted with carbachol (10^-6 M). In carbachol precontracted SO rings the order of potency was YC-1>DEA/NO>SNP= theophylline. YC-1 and DEA/NO increased cGMP levels more than control and carbachol groups (p<0.05).

Conclusion: These results show that NO system exists in the sheep SO. YC-1, DEA/NO, SNP and theophylline induce quantitatively different relaxation of SO rings. YC-1 is a more potent relaxant than other drugs and causes more elevation of cGMP levels in isolated SO rings.

Key Words: Sphincter of Oddi, nitric oxide, YC-1 [3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole], nitroprusside, theophylline


Özet

Amaç: Nitrik oksit (NO)‘nin gastrointestinal kanalın değişik böümlerinde önemli bir fizyolojik rol oynadığı gösterilmiştir. Bu çalışma, koyun Oddi sфинkteri (SO)‘nde NO sisteminin varlığını göstermek ve farklı mekanizmalarla NO sisteminin etkileyen ilacların etkilerini karşılaştırarak için planlandık.

Gereç ve Yöntemler: SO halkaları modifeye Krebs-Henseleit sıvıyla ile doldurulmuş ve %95 ıkiksen %5 CO2 ile gazlandırılan doku banyolarına asıldı. Elektriksel alan stimülasyonu (EFS) çevapları ve karbokali (10^-7 M) ile kasıtılmış dokularda 3-(5′-hidroksimetil-2′-furil)-1-benzil indazol (YC-1, 10^-10^-10 M), diethylamine/NO kompleksi (DEA/NO, 10^-6^-10^-4 M), sodium nitroprussiye (SNP, 10^-3^-3 x 10^-4 M) ve teofilin (10^-5^-10^-4 M) konsantrasyon-cevap egrileri izometrik olarak Grass poligrafı kaydedildi. Ayrıca, YC-1 (10^-7 M ve 10^-6 M) ve DEA/NO (10^-7 M ve 10^-6 M)‘nin siklik GMP (sGMP) düzeyleri üzerindeki etkileri ölçüldü.

Bulgular: EFS ile oluşturan kasını çevapları atropine (10^-6 M) ve guanethidine (10^-5 M) ile azaldı, ama L-NAMe (3 x 10^-5 M) ile güçlendi. YC-1(10^-10^-10^-5 M), DEA/NO(10^-7 M), SNP (10^-3^-3 x 10^-4 M) ve teofilin (10^-5^-10^-4 M) karbokali (10^-6 M) ile kasıtılmış SO halkalarında konsantrasyona bağlı gecemeler ortaya çıktı. Karbokali ile kasıtılmış SO halkalarında potansiyel yapıcı YC-1>DEA/NO>SNP= teofilin idi. YC-1 ve DEA/NO sGMP düzeylerini kontrol ve karbokali gruplarından daha fazla arttırdı (p<0.05).

Sonuç: Bu bulgular, koyun SO‘da NO sisteminin bulunduğunü, YC-1, DEA/NO, SNP ve teofilinin SO halkalarında fakir ölçude gecemeleri ortaya çıktığı göstermektedir. YC-1 diğer ilaclarından daha potansiyel bir geceşicidir ve sGMP düzeylerinde daha fazla artışa neden olur.

Anahtar Kelimeler: Oddi sфинkteri, nitrik oksit, YC-1 [3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole], sodium nitroprussiye, teofilin


The sphincter of Oddi (SO), which is a valve that regulates the flow of bile and pancreatic secretions into the duodenal lumen, is innervated by a ganglionated plexus that lies within its wall.1 Anatomical and immunohistochemical
investigations have demonstrated that the SO is richly innervated by cholinergic, adrenergic and nonadrenergic-noncholinergic (NANC) nerves. These nerves in addition to hormones are important in the control of SO motility and function.\textsuperscript{4,5}

Nitric oxide (NO) is synthesized by the activation of neuronal NO synthase (nNOS) in the myenteric plexus.\textsuperscript{6} NO released in response to nerve stimulation of the myenteric plexus regulates the muscle tone of the sphincter in the lower esophagus, pylorus, SO, and anus.\textsuperscript{7} Competitive inhibitors of NOS increase the strength of sphincter contractions, and L-arginine, the substrate for NOS, antagonizes this effect.\textsuperscript{8} Hemoglobin, a potent scavenger of NO, increases the contractile frequency, strength of contractions, and baseline tone of the opossum SO.\textsuperscript{9} Thus, both the inhibition of NO production and prevention of its action alter SO motility.

Topical application of NO donors, such as S-nitroso-N-acetylcyesteine (SNAC), glyceryl trinitrate (GTN) and sodium nitroprusside (SNP), decrease SO motility.\textsuperscript{10-12} Theophylline, a nonselective phosphodiesterase (PDE) inhibitors, inhibits morphine-induced spasm of Oddi’s sphincter in man.\textsuperscript{13} YC-1 (\textsuperscript{3-(5’}-hydroxymethyl-2’-furyl)-1-benzyl indazole), a novel NO-independent soluble guanylyl cyclase (sGC) activator, and diethylamine/nitric oxide (DEA/NO), a recently developed NO donor, are induced concentration-dependent relaxations in various smooth muscles, such as myometrium, corpus cavernosum and carotid artery.\textsuperscript{14-18} There is no available data in the literature related to the compared relaxant effect of YC-1, DEA/NO, SNP and theophylline in isolated sheep SO rings.

The structure of the SO and the effects of drugs that affect NO system varies in different species. Therefore, the aims of this study were to determine the existence of NO system in the sheep SO and, to compare the effects of YC-1, DEA/NO, SNP and theophylline.

**Material and Methods Ethics**

The present experiments conformed to the Guiding Principles for Care and Use of Experimental Animals. In addition, the experimental protocol applied was approved by the local ethical boards of the Cumhuriyet University Medical Faculty.

**Experimental Design and Tissue Preparation**

Adults male Anatolian Akkaraman sheep (n=26), weighing 38-45 kg and aging 12-14 months, were used throughout the study. The sheep were killed by cervical sheers in the slaughterhouse. The abdomen was opened with a midline incision and the SO was rapidly removed and transferred to laboratory in previously aerated (95% O\textsubscript{2} and 5% CO\textsubscript{2}) Krebs’ bicarbonate solution (composition in mmol/L: NaCl, 120; KCl, 4.6; CaCl\textsubscript{2}, 2.5; MgCl\textsubscript{2}, 1.2; NaHCO\textsubscript{3}, 22; Na\textsubscript{2}HPO\textsubscript{4} and glucose 11.5). SO muscle rings were placed in a 10 mL muscle bath, filled with pre-aerated Krebs’ bicarbonate solution at 37°C. The upper end of the preparation was tied to an isometric transducer (Grass FT 03, Quincy, MA, USA) and preloaded with 1.5 g. SO muscle rings were washed in every 15 min and allowed to equilibrate for 60-90 min until a stable baseline was attained.

**Isometric Measurements**

Some of SO rings were placed between stimulating electrodes. Contractions were elicited with electrical field stimulation (EFS). Stimulation was provided by two parallel platinum electrodes and was applied at sequential frequencies of 2, 4, 8, 16 and 32 Hz, as square-wave pulses of 50 V (0.1 ms) delivered at 10-s intervals by a current amplifier and a stimulator (S88, Grass Instruments, Quincy, MA, USA). After control EFS responses, the SO rings were treated with 10\textsuperscript{-6} M atropine (a muscarinic receptor blocker) and 10\textsuperscript{-5} M guanethidine (adrenergic nerve blocker) or 3 x 10\textsuperscript{-5} M L-NAME (an inhibitor of NOS) for 20 min, to eliminate the adrenergic and cholinergic components or nitriergic component, and to determine the relaxation responses to the stimulation of NANC nerves.

Submaximal contraction was obtained with 10\textsuperscript{-6} M carbachol in some of SO rings. Following contraction by carbachol (10\textsuperscript{-6} M), the relaxation responses to YC-1 (10\textsuperscript{-10}-10\textsuperscript{-5} M), DEA/NO (10\textsuperscript{-6}-10\textsuperscript{-4} M), SNP (10\textsuperscript{-8}-3 x 10\textsuperscript{-4} M) and theophylline (10\textsuperscript{-8}-10\textsuperscript{-4} M) were obtained in a cumulative manner. After the addition of each concentration, we waited
until a plateau response was obtained before adding the next one.

**Measurement of cGMP Levels in SO Rings**

SO rings were obtained from male sheeps and equilibrated in Krebs solution (composition as above) continuously gassed with a mixture of 95% oxygen and 5% carbon dioxide at 37°C (pH= 7.40) for 60 min. Five sets of experimental studies were performed except for control group. Tissues were exposed to 10^6 M carbachol for 15 min in the first set. In the 2<sup>nd</sup>, 3<sup>rd</sup>, 4<sup>th</sup> and 5<sup>th</sup> sets tissues were separately exposed to 10^6 M YC-1, 10^5 M YC-1, 10^5 M DEA/NO and 10^4 M DEA/NO after incubation with 10^6 M carbachol for 15 min in organ bath. As a control group, experiments without studied drugs were used. At the end of a total incubation period, the samples were immediately transferred into liquid nitrogen and homogenized with ethanol and incubated for 30 min at 0-4°C. Then, the homogenate was centrifuged at 4°C for 10 min at 12 000 x g. The supernatants poured into a clean test tube and dried in a vacuum at 50°C to remove ethanol. Aliquots of the supernatant were tested for cyclic GMP (cGMP) by radioimmunoassay (RE 110 21 and RE 290 71 respectively; IBL, Hamburg, Germany). The samples were then processed according to the instructions provided with the kits for determination of cGMP levels. Sampling data were divided according to the weight of the SO rings, and the results were expressed in pmol/mg SO.

**Data Analysis**

EFS responses were expressed as a percentage of precontraction by KCl (80 mM). The relaxation responses induced by YC-1, DEA/NO, SNP and theophylline were expressed as a percentage of precontraction by carbachol (10^6 M). Maximum relaxant effect (E<sub>max</sub>) and concentrations of YC-1, DEA/NO, SNP and theophylline producing 50% of the E<sub>max</sub> (EC<sub>50</sub>) were calculated for each concentration-response curve. The pEC<sub>50</sub> was calculated as the negative logarithm to base 10 of the EC<sub>50</sub> for statistical analysis. Experimental values were presented as means ± SEM and analysed by repeated measures of analysis of variance (ANOVA) with the Newman-Keuls test, and Student’s t test when appropriate. A p value of < 0.05 was considered significant. All statistical analyses were performed using Statistica for Windows 6.0. (Statsoft, Inc., Tusla, USA).

**Drugs**

Chemicals used in the current experiments were atropine, guanethidine, L-NAME, carbachol, DEA/NO, SNP and theophylline from Sigma (St. Louis, MO, USA), YC-1 from A.G. Scientific, Inc. (San Diego, CA, USA). All chemicals were dissolved in distilled water except for YC-1, which was dissolved in dimethylsulfoxide (DMSO) and then diluted with distilled water for preparation of decreasing concentrations of this drug. The amount of DMSO used to dissolve the drugs had no effect on sheep SO smooth muscle. Drugs-containing solutions were freshly prepared on the day of the experiments.

**Results**

EFS (2, 4, 8, 16 and 32 Hz, 50 V, 0.1 ms, 10 s) caused contractile responses on basal tension of sheep SO rings. Preincubation (20 min) of SO rings by atropine (10^6 M, n= 10) and guanethidine (10^5 M, n= 10) combination significantly decreased the contractile responses induced by EFS, whereas preincubation (20 min) by L-NAME (3 x 10^5 M, n= 10) significantly increased the contractile responses elicited by EFS (p< 0.05) (Figure 1).

YC-1 (10<sup>-10</sup>-10<sup>-5</sup> M, n= 10), DEA/NO (10<sup>-6</sup>-10<sup>-4</sup> M, n= 10), SNP (10<sup>-9</sup>-10<sup>-4</sup> M, n= 10) and theophylline (10<sup>-8</sup>-10<sup>-4</sup> M, n= 10) induced concentration-dependent relaxations on SO rings precontracted by carbachol (10<sup>-6</sup> M) (Figure 2). In carbachol-precontracted SO rings the order of potency (pEC<sub>50</sub>) was YC-1<sub>-1</sub>&gt;DEA/NO<sub>-1</sub>&gt;SNP<sub>-1</sub>&gt; theophylline (p< 0.05). In carbachol-precontracted SO rings the order of efficacy (E<sub>max</sub>) was YC-1<sub>-1</sub>= DEA/NO<sub>-1</sub>&gt;SNP<sub>-1</sub>&gt; theophylline (p< 0.05) (Table 1).

Carbachol (10<sup>-6</sup> M, n= 6), YC-1 (10<sup>-6</sup> and 10<sup>-5</sup> M, n= 6) and DEA/NO (10<sup>-4</sup> and 10<sup>-4</sup> M, n= 6) significantly increased the cGMP levels compared with the control on SO rings (p< 0.05) (Table 2). In SO rings, the order of increase in cGMP levels was 10<sup>-5</sup>
M YC-1>10^-6 M YC-1>10^-4 M DEA/NO= 10^-5 M DEA/NO>10^-6 M carbachol (p< 0.05) (Table 2).

**Discussion**

In gastrointestinal tract, NANK innervation is important in nerve mediated relaxation and membrane hyperpolarisation and present evidence indicates that NO is a NANK neurotransmitter. It is widely accepted that NO, through cGMP synthesis, induces a sequence of protein phosphorylation that leads to smooth muscle relaxation. It has been shown that nitricergic relaxation of the rabbit SO is accompanied by an increase in both cGMP and cAMP concentration. Theoretically, an increase in tissue cAMP concentration secondary to cGMP-increase might result from an inhibition of cAMP metabolism through inhibition of the enzyme type III (cGMP-inhibited cyclic nucleotide phosphodiesterase: PDE3) phosphodiesterase. Moreover, in addition to its smooth muscle relaxing effect, NO has been proposed to stimulate the release of vasoactive intestinal polypeptide (VIP) or calcitonin gene-related peptide (CGRP) from enteric nerve terminals through presynaptic mechanisms. VIP or CGRP, once released, facilitates further NO

**Table 1.** pEC_{50} and E_{max} values for the relaxant effects of YC-1, DEA/NO, SNP and theophylline in sheep SO rings precontracted by carbachol (10^-6 M). Data are expressed as means ± SEM.

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<tr>
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<th>pEC_{50} (mean ± S.E.)</th>
<th>E_{max} (mean ± S.E.)</th>
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<tr>
<td>YC-1 (n=10)</td>
<td>7.16 ± 0.10* (0.03)</td>
<td>96.4 ± 3.66 (0.02)</td>
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<tr>
<td>DEA/NO (n=10)</td>
<td>6.88 ± 0.08</td>
<td>94.2 ± 3.23 (0.03)</td>
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<tr>
<td>SNP (n=10)</td>
<td>5.80 ± 0.07</td>
<td>80.2 ± 3.6</td>
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<td>Theophylline (n=10)</td>
<td>5.84 ± 0.08</td>
<td>76.4 ± 6.2</td>
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* Statistically different from all other drugs for YC-1 (p< 0.05),  
* Statistically different from SNP and theophylline for YC-1 and DEA/NO (p< 0.05).

**Table 2.** Increase of the cGMP levels by carbachol, YC-1 and DEA/NO compared with the control on sheep sphincter of Oddi rings. Data are expressed as means ± SEM.

<table>
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<th>cGMP levels (fmol/mg)</th>
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<tr>
<td>Control</td>
<td>3.51 ± 0.42</td>
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<tr>
<td>Carbachol (10^-8 M, n=6)</td>
<td>11.25 ± 1.80* (0.03)</td>
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<tr>
<td>Carbachol + YC-1 (10^-8 M, n=6)</td>
<td>25.32 ± 2.43* (0.02)</td>
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<tr>
<td>Carbachol + YC-1 (10^-7 M, n=6)</td>
<td>47.62 ± 5.12 (0.01)</td>
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<tr>
<td>Carbachol + DEA/NO (10^-7 M, n=6)</td>
<td>18.22 ± 2.42 (0.03)</td>
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<tr>
<td>Carbachol + DEA/NO (10^-6 M, n=6)</td>
<td>23.81 ± 3.04 (0.02)</td>
</tr>
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* Statistically different from control (p< 0.05),  
* Statistically different from all other groups (p< 0.05).
synthesis/release through cAMP-dependent pathways, thus, an interplay between NO and these NANC peptides underlie the increase in cGMP and cAMP in the SO.\textsuperscript{25}

Our results have shown that in vitro inhibition of NOS by the L-arginine analogue L-NAME increases the contractile response induced by EFS, whereas in vitro blockage of muscarinic receptors and adrenergic nerves by atropine and guanethidine combination decreases the contractile responses induced by EFS. The augmentation of the contractile response induced by EFS with L-NAME suggests the involvement of the nitrergic system on the regulation of the sheep SO.

YC-1 is a NO-independent sGC activator that provides an about 10-fold increase of enzyme activity. Apart from an increase in the formation of cGMP via the stimulation of sGC, the substance also prevents cGMP degradation.\textsuperscript{14} In the previously study, we shown that YC-1 inhibits spontaneous contractile activity in a concentration-dependent manner in myometrial smooth muscle from pregnant rat.\textsuperscript{10} SNP and DEA/NO are NO donors, but SNP and recently developed DEA/NO do not share the same mechanism for NO release. With regard to SNP, it is thought to produce relaxation, at least in part, by NO-induced guanylyl cyclase activation related to formation of S-nitrosothiol intermediates.\textsuperscript{26} In addition to the thiol requirement, it has been shown that SNP undergoes metabolic activation in smooth muscle by a SNP-directed, membrane-associated, NO-generating activity.\textsuperscript{27} NO/nucleophile adducts DEA/NO complex, differ from SNP in that it spontaneously releases NO in simple aqueous buffer.\textsuperscript{15} Salom et al. demonstrated that SNP and DEA/NO induce relaxation of rabbit carotid artery with different potencies.\textsuperscript{18} Theophylline is a nonsensitive PDE inhibitors. cGMP is catabolized by phosphodiesterases (PDEs), a large family of enzymes that also hydrolyze other cyclic nucleotides (such as cAMP).\textsuperscript{28} At least 50 different PDEs are described in mammalian cells.\textsuperscript{29} Increased intracellular cGMP inhibits smooth muscle contraction. Vedernikov et al. demonstrated in vitro that nonsensitive PDE inhibition was more effective in relaxing rat myometrium at term, whereas zaprinast (a selective cGMP PDE-5 inhibitor) was less effective.\textsuperscript{30} In previous studies, it has been indicated that YC-1, DEA/NO, SNP and theophylline are induced concentration-dependent relaxations in various smooth muscles.\textsuperscript{16,18,30}

In this study, we have compared the relaxant effect of YC-1, DEA/NO, SNP and theophylline in isolated sheep SO rings, precontracted by carbachol. YC-1, DEA/NO, SNP and theophylline induced concentration-dependent relaxation of SO rings, with the following order of potency: YC-1>DEA/NO>SNP= theophylline in carbachol-precontracted rings. The pEC\textsubscript{50} values for YC-1 were significantly greater than those calculated for DEA/NO, SNP and theophylline. This indicates that the actual EC\textsubscript{50} value (i.e., concentration resulting in 50\% of maximal effect) for YC-1 is significantly less than that measured for DEA/NO, SNP and theophylline, and hence, YC-1 is more potent relaxant agents in SO. In carbachol-precontracted rings, the order of efficacy: YC-1=DEA/NO>SNP= theophylline. The E\textsubscript{max} values for YC-1 and DEA/NO were significantly greater than those calculated for SNP and theophylline. This shows that the efficacy of YC-1 and DEA/NO is significantly greater than that of SNP and theophylline, but the efficacy for the YC-1 and DEA/NO or SNP and theophylline was not significantly different in SO rings.

In this study, carbachol, YC-1 and DEA/NO were responsible for a significant increase in cGMP levels of sheep SO rings in a concentration-dependent manner. YC-1 caused high increase in the cGMP levels compared to the other drugs. These increased levels of cGMP by YC-1 and DEA/NO indicate the mediation of cGMP formation in the relaxant effects of these drugs in SO.

In conclusion, results of this study show that NO system exist in the sheep SO. In vitro application of YC-1, DEA/NO, SNP and theophylline induce relaxation of SO rings with different potencies. YC-1 is a more potent relaxant agent and causes more increase in cGMP levels of
SO rings. YC-1 may be suitable choice in the conscious relaxation of the ERCP and help the ERCP procedures. Since our study was performed on sheep SO in vitro, controlled clinical studies are required to determine if the results obtained can be transferred to humans.

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