RELAXATIONS INDUCED BY NITRERGIC NERVE STIMULATION AND NITROXYL IN MOUSE CORPUS CAVERNOSUM

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SUMMARY

Effect of nitroxyl anion donor, Angeli's salt has been investigated, and compared with that of electrical field stimulation (EFS) in the mouse corpus cavernosum. Electrical field stimulation (40V, 0.5 msec, 1, 2, 4, 8, 16 Hz for 15 sec) and Angeli's salt (10-8 M-10-4 M) produced frequency and concentration dependent relaxation. EFS-induced relaxations were 16.42.8 %, 31.33.9 %, 49.23.7 %, 68.75.2 % and 84.76.1 % at 1, 2, 4, 8 and 16 Hz respectively. 1H-(1,2,4) oxadiazolo (4,3-a) quinoxalin-1-one (ODQ, 10-5 M), a soluble guanylyl cyclase inhibitor completely inhibited both of the relaxations. Nitric oxide radical oxidizing agent, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (carboxy-PTIO, 3x10-4 M) did not inhibit EFS-elicited relaxation, but reduced Angeli's salt-induced relaxation. Nitric oxide scavenger, hydroxocobolamine (3x10-4 M) diminished the relaxations by nitroxyl and EFS. However, 10-4 M hydroxocobolamine failed to reduce both of them. L-cysteine (3x10-3 M) substantially reduced Angeli's salt-induced relaxation but had an inhibitory effect on EFS-induced relaxation at only 16 Hz. L-Ascorbic acid (10-3 M) conspicuously inhibited nitroxyl but not EFS-induced relaxations. These results could suggest that Angeli's salt can be considered as a cavernosal smooth muscle relaxant. However, based on the findings the nature of the nitrergic neurotransmitter still remains elusive since it is resistant to the inhibitors that easily inhibited the effects of exogenously applied nitroxyl anion, suggesting that the nitrergic neurotransmitter may, at least in part, have different characteristics from those of nitroxyl anion.

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2003;3:213-220

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2003;2:213-220

Keywords: *Angeli's salt, corpus cavernosum, electrical field stimulation, nitrergic neurotransmission, nitric oxide, nitroxyl, ODQ*

ÖZET

FARE KORPUS KAVERNOZUMUNDA NİTRERJİK SİNİR STİMÜLASYONU VE NİTROKSİL ANYONU İLE OLUŞTURULAN GEVŞEMELER

Nöronal nitrik oksid sentaz (nNOS) enziminden üretilen son ürünün NO radikali yerine nitroksil anyonu (NO-) olabileceği bildirilmektedir. Dolayısıyla bu çalışmada fare korpus kavernozumunda elektriksel saha stimülasyonu (ESS) ile oluşturulan gevşemelerle NO- donörü Angeli tuzunun indüklediği gevşeme yanıtları karşılaştırıldı. ESS (40V, 0.5 ms, 1, 2, 4, 8, 16 Hz, 15 sn süreyle) ve Angeli tuzu (10-8 M-10-4 M) frekansa ve konsantrasyona bağımlı olarak gevşemeler oluşturdu. ESS ile indüklenen gevşemeler 1, 2, 4, 8 ve16 Hz frekanslarda sırasıyla % 16.42.8, % 31.33.9, % 49.23.7, % 68.75.2 ve % 84.76.1 idi. Çözünür guanilil siklaz inhibitörü 1H-(1,2,4) oxadiazolo (4,3-a) quinoxalin-1-on (ODQ, 10-5 M), hem ESS hem de NO- ile oluşturulan gevşemeleri ortadan kaldırdı. Nitrik oksid radikalini oksidleyen bir ajan olan 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (carboxy-PTIO, 3x10-4 M) ESS ile elde edilen gevşemeleri değil fakat Angeli tuzu ile indüklenen gevşemeleri azalttı. Nitrik oksid yakalayıcısı olan hidroksokobalamin (3x10-4 M) nitroksil ve ESS gevşemelerini inhibe etti. Bununla birlikte 10-4 M hidroksokobalamin her iki gevşemeyi de etkilemedi. L-cysteine (3x10-3 M) Angeli tuzu ile indüklenen gevşemeleri esaslı olarak azalttı fakat yalnızca 16 Hz frekansta ESS ile indüklenen gevşemeleri inhibe edebildi. L-Askorbik asid (10-3 M) ESS ile indüklenen gevşemeleri etkilemezken, nitroksil ile indüklenen gevşemeleri belirgin olarak inhibe etti. Bu sonuçlara göre Angeli tuzunun kavernozal düz kas gevşeticisi olduğu önerilebilir. Diğer taraftan nitrerjik nörotransmitterin doğası hala belirsiz kalmıştır, çünkü nitrerjik cevaplar eksojen olarak uygulanan nitroksil anyonunun etkilerini kolaylıkla inhibe edebilen maddelere dirençlidir. Bu da nitrerjik nörotransmitterin nitroksil anyonundan en azından kısmen farklı karekteristiklerinin olduğunu önerebilir.

Anahtar kelimeler: *Angeli tuzu, korpus kavernozum, elektriksel saha stimülasyonu, nitrerjik nörotransmisyon, nitrik oksid, nitroksil, ODQ*

INTRODUCTION

The end product of the neuronal nitric oxide synthase (nNOS) has been proposed to be nitroxyl anion rather than nitric oxide radical itself (1). This has been put forward by the finding that the stochiometry of the NADPH used in the L-arginine: NO pathway was miscalculated as 1.5 mol. Instead, it has been proposed to be 1 mol, leading the suggestion that the ultimate product from NOS may be one-electron reduced species of nitric oxide radical (NO.), i.e. nitroxyl anion (NO-). Although

NO- is not capable of activating soluble guanylyl cyclase (2), nitroxyl anion donor such as Piloty's acid and Angeli's salt are potent vasorelaxants (3-6). Consequently, nitroxyl must be anyhow oxidized by the tissue to active form, nitric oxide radical. Although the major pathway of the conversion of nitroxyl to nitric oxide has yet to be identified, the proposed bioconversion mechanisms involve SOD (7), Cu2+ (6), benzoquinone and, co-enzyme Q0 and ferricytochrome c (8).

Li and Rand (9) suggest that the nitrergic neurotransmitter may not be free radical NO, although some others propose that it may be free NO in the canine ileocolonic junction (10). Therefore, the precise nature of the nitrergic neurotransmitter is still debatable. Since the first challenging proposal with regard to final metabolite of L-arginine:NO pathway has been made in the experiments where neuronal NOS was examined (1), we compared neuronal relaxant responses with nitroxyl anion-induced relaxations in the isolated mouse corpus cavernosum. For this comparison, we employed particular NO scavengers, such as hydroxocobolamine and carboxy-PTIO, and soluble guanylyl cyclase inhibitor, ODQ as well as L-ascorbic acid and L-cysteine which were earlier used to probe nitrergic neurotransmitter, EDRF and nitroxyl anion-induced relaxations (11, 12), and thus we goaled to gain an insight into the nature of the nitrergic neurotransmission.

MATERİALS AND METHODS

Tissue Preparation:

This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of Mersin University Centre for Experimental Medicine.Male Balb c mice weighing 30-35 g were sacrificed by servical dislocation. Cavernosal tissue was obtained as described elsewhere (13). Briefly, penises were removed and placed in a Petri dish containing Krebs solution (composition mM: NaCl 118, KCl 4.8, CaCl2 2.5, MgSO4 1.2, NaHCO3 25, NaHPO4 1.2, glucose 11). The glans penis and urethra were excised and adherent tissues were carefully removed keeping the tunica albuginea intact. Cavernosal strips were suspended through two platinum ring electrodes in organ baths maintained at 37o C, filled with Krebs solution, gassed with 95 % O2 and 5 % CO2 under 0.5 g initial tension. Tension was recorded isometrically with a force transducer (COMMAT, Ankara, Turkey) and displayed on a Biopac acquisition system (Biopac Systems Inc., CA, USA). Tissues were allowed to equilibrate for 45 min before experiments were carried out, during which the resting tension was re-adjusted to 0.5 g as required, and every 15 min the bath was replaced with fresh Krebs solution.

Experimental Procedure:

Following equilibration, cavernosal strips were submaximally precontracted with 5x10-5 M phenylephrine. After a steady state of contraction, electrical field stimulation (EFS, 40 V, 0.5 msec) was delivered for 15 sec at the frequencies of 1, 2, 4, 8, 16 Hz at 2 min interval via 2 platinum ring electrodes connected to the Biopac stimulator (CA, USA). Consequently, first series of responses was yielded. Thereafter, the tissue was rinsed with fresh Krebs solution and allowed to incubate for 45 min and then second series was obtained in the same manner. Chemicals were applied for 15 min at the incubation period of 45 min between the first and the second series, and the second series of responses was obtained in the presence of the scavengers or inhibitors.

In Angeli's salt experiments, it was applied cumulatively from 10-8 M to 3x10-5 or 10-4 M on phenylephrine-induced tone. In some

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experiments, chemicals were applied between the first and the second series for 15 min.

Drugs:

L-Phenylephrine hydrochloride, L-ascorbic acid and hydroxocobolamine hydrochloride were obtained from Sigma (St Lois, MO, USA), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (carboxy-PTIO) and 1H-(1,2,4) oxadiazolo (4,3-a) quinoxalin-1-one (ODQ) from Tocris Cookson Ltd (Bristol, UK). Angeli's Salt was purchased from Cayman Chemical (MI, USA). All chemicals except for Angeli's Salt and ODQ were dissolved in distilled water. ODQ was dissolved in DMSO, and Angeli's Salt in 0.01 M NaOH. Angeli's salt stock solution (10-2 M) was stored at -20o C. Further dilution of Angeli' salt was also made with 0.01 M NaOH. The dilution was prepared on the everyday of experimentation.

Statistical Analysis:

Relaxation to EFS and Angeli's Salt was expressed as percentage of phenylephrine-induced contraction and shown as means ±S.E.M. Comparisons were made by one way of ANOVA, followed by Bonferroni post hoc test with the help of a computer-based program (Graph-Pad, Prism, CA, USA). A P value of less than 0.05 was considered as significant.

RESULTS

Effects of electrical field stimulation and Angeli's salt on the cavenosal reactivity:

Electrical field stimulation (EFS, 40 V, 0.5 msec, 1, 2, 4, 8, 16 Hz, 15 sec) and Angeli's salt (10-8 -10-4 M) produced frequency and concentration dependent relaxations in the mouse corpus cavernosum. EFS-induced relaxations were 16.42.8 %, 31.33.9 %, 49.23.7 %, 68.75.2 % and 84.76.1 % at 1, 2, 4, 8 and 16 Hz respectively. Angeli's salt-induced relaxation was nearly 100 % of phenylephrine-evoked tone at 3x10-5 M in control series.

*Fig. 1 Effects of 1H-(1,2,4) oxadiazolo (4,3-a) quinoxalin-1-one (ODQ, 10-5 M, n=4, upper panel) and hydroxycobolamine (10-4 M, central panel; 3x10-4 M, lower panel, n=5-12) on Angeli's salt (10-8 M-10-4 M, left column) and electrical field stimulation (EFS, 40 V, 0.5 msec, 15 sec, 1, 2, 4, 8, 16 Hz, right column)-induced relaxations. Inhibitor compounds were incubated for 15 min, thereafter the second series was obtained. Each point is the mean s.e.mean. *: P<0.05; **: P<0.01; ***: P<0.001, different from control values.*

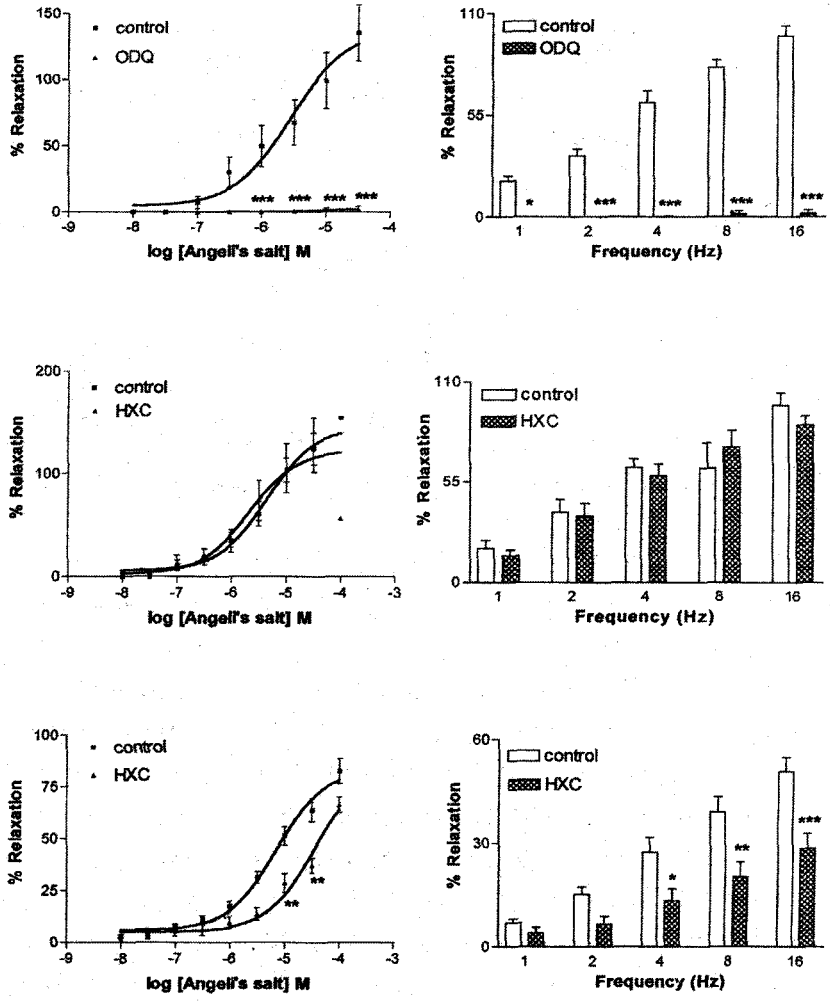
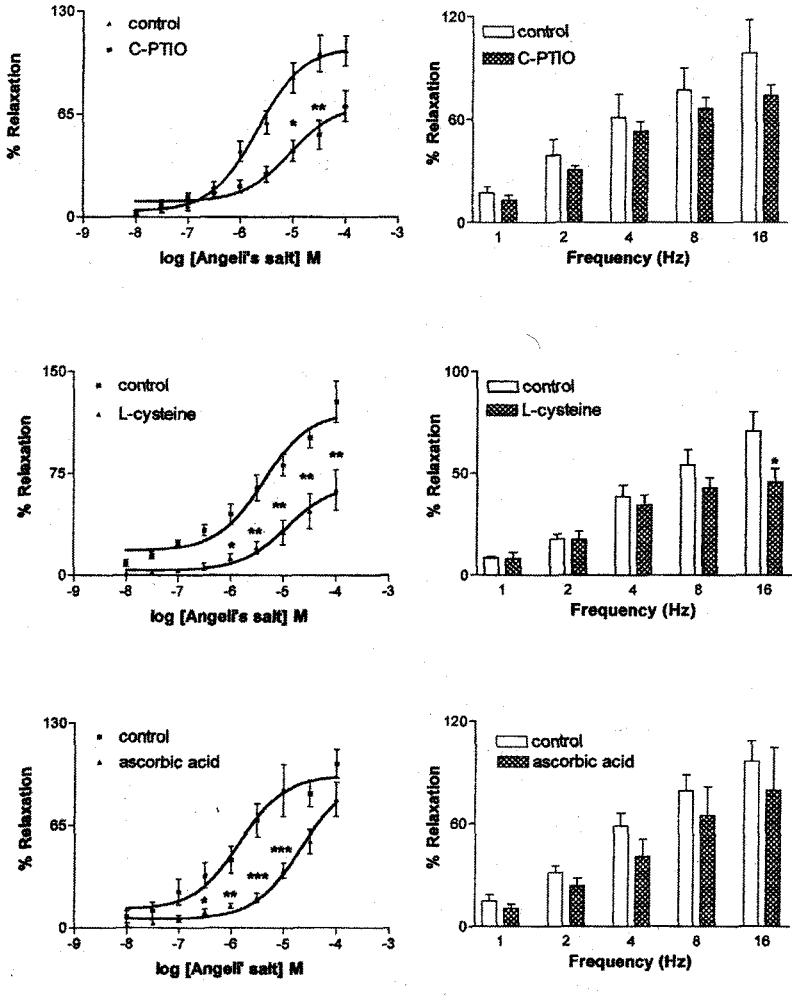


*Fig. 2: Effects of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (carboxy-PTIO, 3x10-4 M, n=8-10, upper panel), L-cysteine (3x10-3 M, central panel, n=7) and ascorbic acid (10-3 M, lower panel, n=8-9) on Angeli's salt (10-8 M-10-4 M, left column) and electrical field stimulation (EFS, 40 V, 0.5 msec, 15 sec, 1, 2, 4, 8, 16 Hz, right column)-induced relaxations. Inhibitor compounds were incubated for 15 min, thereafter the second series was obtained. Each point is the mean s.e.mean. *: P<0.05; **: P<0.01; ***: P<0.001, different from control values.*



Effects of ODQ, hydroxocobolamine, C-PTIO, L-cysteine and L-ascorbic acid on the EFS-induced relaxations:

10-5 M ODQ completely blocked both EFS and Angeli's salt-mediated relaxations (Fig. 1). 10-4 M hydroxocobolamine (HXC) did not affect both of the relaxation (Fig. 1). However, 3x10-4 M HXC significantly reduced the relaxations (Fig. 1). C-PTIO at 3x10-4 M failed to decrease EFS-induced relaxation but inhibited Angeli's salt-elicited relaxation (Fig. 2). 3x10-3 M L-cysteine significantly diminished the relaxation by nitroxyl but reduced EFS-induced relaxation only at 16 Hz (Fig. 2). L-ascorbic acid at 1 mM concentration has no effects on EFS-induced relaxation; however, it shifted the concentration-response curve to the right (Fig. 2).

DISCUSSION

In the present study, we investigated characteristics of Angeli's salt induced relaxations, and compared them with the electrical field stimulation-induced relaxant responses to gain an insight into the hypothesis that instead of nitric oxide radical, nitroxyl could be the final product of neuronal NOS (nNOS) in the isolated mouse corpus cavernosum. It has been well established that electrical field stimulation-induced relaxation is nitrergic in origin, i.e., mediated by nitric oxide or related species in this tissue (13, 14).

Nitroxyl is one-electron reduced species of nitric oxide, which can be produced from the intermediate compound, NG-hydroxyl-L-arginine in the pathway from L-arginine to nitrogen monoxide, or directly from the amino acid under the influence of NOS (1, 3). Since the nitroxyl anion in the form of HNO is quite unstable, its donors namely Piloty's acid and Angeli's salt (disodium trioxodinitrate) are used in experimental studies because those compounds can decompose to nitroxyl (4).

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Because Angeli's salt more readily donates nitroxyl anion than Piloty acid does, we chose Angeli's salt as nitroxyl donor in this study. EFS and nitroxyl both induced reproducible relaxation, and these relaxations were abolished by a selective soluble guanylyl cyclase inhibitor, ODQ (15), pointing out the same site of action of the nitrergic neurotransmitter and nitroxyl anion. Although C-PTIO is known to selectively scavenge only the free radical of nitric oxide (NO.), it also inhibited nitroxyl-induced relaxation. This has been explained by Ellis et al (11) that nitroxyl is oxidized to NO. by C-PTIO, and formed NO. radical is scavenged by the compound, or nitroxyl is oxidized to NO. by the cell, which is then inactivated by C-PTIO. Inability of C-PTIO to inhibit neurogenic responses may be due to the insufficient penetration of the compound to the neuroeffector junction, or too short pathway traversed by the nitrergic neurotransmitter to be inactivated by C-PTIO. In addition, oxidative capacity of C-PTIO may be diminished by a mechanism, which is conducted by the nitrergic nerve stimulation (i.e. release of a reductive compound to the biophase such as ascorbic acid) (16). Likewise, it has been reported that the relaxation to the nitrergic neurotransmitter but not NO. radical, is resistant to C-PTIO in mouse anococcygeus muscle, and porcine anococcygeus and retractor penis muscle (9, 16). Another NO. scavenger, hydroxocobolamine reduced both nitrergic and nitroxyl-induced relaxation similarly, consistent with the notion that nitroxyl and the nitrergic neurotransmitter might have similar nature. On the other hand L-cysteine has been reported to inhibit NO. and EDRF responses at low concentration (5 M) (17, 18). However at milimolar concentrations, it enhances or prolongs NO. responses (17, 19, 20) by acting as a NO-carrier upon S-nitrosothiols produced by NO. and L-cysteine. In the present study, however, at 3x10-3 M, L-cysteine substantially inhibited nitroxyl-induced relaxation but reduced partly nitrergic relaxation, particularly at 16 Hz. Inhibition of nitroxyl-induced relaxation by high concentration of L-cysteine may be due to the fact that nitroxyl anion may be reduced to N2O or some other species which has little or no relaxant activity. This consistent with previous study in rat aorta where L-cysteine inhibited the responses to acetylcholine, ATP or A-23187 suggesting that a component of the response to EDRF may be mediated by nitroxyl anion (11). Using NO-sensitive electrode, we have obtained that Angeli's salt-generated NO signal was abolished in the presence of 3x10-3 M L-cysteine in tris-buffered Krebs (data not shown), supporting that finding. However, the fact that EFS-induced relaxation was significantly inhibited by L-cysteine only at 16 Hz but not at other frequencies may rise the possibility that the nitrergic neurotransmitter(s) might not be nitroxyl. However this does not necessary mean that the first species produced from L-arginine by NOS is not nitroxyl as it can readily oxidized to nitric oxide radical by the generator and/or target cells. Likewise, L-ascorbic acid greatly inhibited exogenous nitroxyl responses but little or no effects on EFS-mediated relaxation. Similar to L-cysteine, ascorbic acid may reduce nitroxyl to ineffective species. However, inability of ascorbic acid to inhibit endogenous nitrergic relaxation may indicate either that nitrergic neurotransmitter might be different from nitroxyl or that it may be indeed nitroxyl but tightly escorted with some compounds from the prejunctional site (generator cell) to postjunctional site (target cell) against both reductive and oxidative agents. The fact that an oxidative compound, C-PTIO and the reductive substances, L-ascorbic acid and L-cysteine have

no effects on EFS-mediated responses in this study might strengthen this interpretation.

In conclusion, if Schmidt's hypothesis (1) is true, the nitrergic neurotransmitter must be nitroxyl anion, which is readily oxidized to NO. by the generator, and possibly by the target cell, at least in part. On the other hand, whatever the primer molecule produced by NOS from L-arginine is, the effector molecule, responsible for tissue relaxation must be NO. which diffuses out of the cell by itself or carried as packaged (e.g. as S-nitrosothiols) (14, 21). Or some proportion of the neurotransmitter might be in the form of nitroxyl, which escapes out of the generator cell from the saturated intracellular oxidative mechanisms. Perhaps, the controversy over the nature of nitrergic neurotransmitter in the literature may be resulted from this difference in oxidative capacity among tissues. In order to clearly discriminate NO-, NO. and the nitrergic neurotransmitter, more direct evidence should be needed.

Another conclusion of the study is that Angeli's salt can be considered as a cavernosal smooth muscle relaxant in vitro.

Acknowledgements

This study has been supported by the Turkish Academy of Sciences, in the framework of the Young Scientist Award Program (K.B-TÜBA-GEBİP/2002-1-5).

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