THE ROLE OF ENDOTHELIUM-DERIVED RELAXING FACTOR IN THE REGULATION OF SNP INDUCED VASORELAXATION: SODIUM-POTASSIUM-CHLORIDE COTRANSPORTER

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Abstract:

Vascular endothelium plays a key role in the local regulation of vascular tone and vascular architecture. However, it is unclear whether the combination of NKCC cotransporter with endothelial mediated factor and potassium channel in rat aortic ring contributes in regulation of the vascular activity. In the current study, the potential role of endothelium-mediated relaxing factors and potassium channel in SNP induced vasorelaxation in precontracted isolated rat aortic rings in the presence of Bumetanide as NKCC blocker were investigated. The maximum vasorelaxation induced by SNP significantly blocked by the presence BUM. SNP induced relaxation was affected by combination of BUM with Indomethacin, Clotrimazole. TEA and BaCl2 significantly blocked of SNP induced relaxation in aorta preincubated with all blockers used, but BaCl2 showed a more potent effect as compared with others. These results indicate that SNP – induced vasorelaxation was mediated via EETs, Prostaglandin, BKCa and KIR pathways which ultimately enhanced the contribution of chloride transporter in aortic smooth muscle cells hyperpolarization and subsequent relaxation.

Key words: NKCC, Bumetanide, SNP, Rat Aorta, EDHF1

INTRODUCTION

Diverse ion channels, exchangers and transporters are able to modulate the membrane potential and the tension of vascular smooth muscle cells (Jayakumar et al., 2008). Kreye et al. (1981) were the first who showed the relevance of sodium potassium chloride (NKCC1) cotransporter with vascular smooth muscle contractility. Since then several studies have shown the possibility that the NKCC1 co-transporter intervenes in blood pressure regula-tion and normal vascular tone (Palacios et al., 2006). Previous review summarizes the data on the functional significance of ubiquitous (NKCC1) isoforms of electroneutral sodium, potassium and chloride cotransporters. These carriers contribute to the pathogenesis of hyper-tension via regulation of intracellular chloride concentration in vascular smooth muscle. NKCC1 is inhibited by Bumetanide. However, the chronic use of this compound for the treat-ment of hypertension may has diverse side-effects due to suppression of myogenic response in microcirculatory beds (Orlov et al., 2015).

The vascular tone is regulated by several mechanisms that implicate the participation of hormonal, neuronal and endothelial factors (Orshal and Khalil, 2004). Among them, NO, prostanoids and reactive oxygen species play pivotal roles in regulating the vascular tone through their vasoactive properties as well as regulating cell proliferation (Wang et al., 2014, Atochin and Huang, 2010). Nitric oxide plays an important role in many physiological processes such as the regulation of vascular system, neuro-transmission and various homeostatic events (Stankevicius et al., 2003). Prostaglandin I_2 which is a powerful vasodilator and inhibitor of platelet aggregation is a major product of arachi-donate metabolism by vascular tissue (Giles et al., 2012). In addition, potassium channels also play a fundamental role in regulation of mem-brane potential and cell excitability (Shieh et al., 2000)

Thus, the current study was an attempt to explore the role of EDHF on the responses of NE precontracted rat aortic muscle and to evalu-ate the involvement of the most important endo-thelial mediator relaxating factors and potassium channels in SNP induced aortic relaxation. Fur-thermore, special emphasis was paid to the effect of Bumetanide in combination with other NKCC blockers on aortic relaxation.

MATERIALS AND METHODS

The present investigation was performed ac-cording to the Biology Community guidelines for animal ethical care Guide.

Animals

Wister Albino rats of either sex, weighing 250–350 g used during the present study were reared in Animal House of the Department of Biology, College of Science, University of Salahaddin. Animals were kept in a well-ventilated environement, received standard animal chow and water *ad libitum.* Prior to experiments, they were fasted overnight with access to water.

Chemicals and Drugs

Norepinephrine (NE), Sodium nitroproside (SNP), Bumetanide, Clotrimazole, L-NAME, Indomethacin, BaCl2, TEA, Glibenclamide and Fresh physiological (Krebs) solution used in the experiments were prepared daily.

Isolated Aorta Preparation and Experimental Protocol

Prior to the isolation of aorta, the animals were injected intraperitoneally with heparin (2000 units/ 200 gm) and left for 30 min, to avoid blood clotting and possible damage of endothelium of the aorta. Then the animals were anaesthetized with Ketamine (40 mg/kg) and zyalxine (10 mg/Kg) intraperitoneally. The chest cavity was opened, the heart and lung were removed and th aorta, free from surrounding tissue and adventitia was isolated according to (Shekha and Al-Habib, 2012). The aorta was transferred to a beaker containing Krebs solution well aerated with carbogen (95% O2 and 5% CO2) and kept at 37 ^{o}C. The aorta was cut into rings approximately 3.5 mm wide, and only the first four segments distal to the aortic arch were used.

The aorta was mounted with two stainless steel wires, one of them was anchored to the base of 25 ml glass tissue chamber containing 10 ml of oxygenated kreb's solution (PanLab, Model SP3922) and set at 37 ^{o}C. Other wire was connected to a force transducer (Model MLT0420 Force Transducer 20 g) coupled to the transbridge amplifier (Model FE224, Quad Bridge Amp) and PowerLab Data Acquisition System (PL3508B5/C-V Panlab 4 Chamber Organ Bath System, AD instrument , Sydney, Australia) and computer running chart software. Labchart Pro (Version 7) was used for measurement of isometric tension.The Physiological salt solution (PSS) used contained: NaCl, 118.0 mM; KCl, 25 mM; CaCl2, 25 mM; MgSO4 , 1.2 mM; and glucose, 11.0 mM.

After preincubation of aortic rings with noradrenaline (10^{-6} M) in normal Krebs' solution for 20 min, a controlled contractile response was obtained and expressed as a percentage of of contraction.

Statistical Analysis and Potency Measurement

All dose-response curves were fitted with a Hill equation, from which the half maximal inhibitory concentration (IC_{50}) values were given as geometric mean with 95% confidence intervals (95% CI). The potency of a nitric oxide donor was commonly quantified as the PIC_{50}, which is used as standard to compare chloride channel blocker potencies. A computer statistics program, GraphPad Prism 6.07, was used to measure PEC_{50} and PEC_{50} of 95% CI values (Motulsky and Christopoulos, 2003). PEC_{50} and PEC_{50} of 95% CI values were measured after establishing a dose - response curve for each blockers and the SEM was calculated for each group of aortic smooth muscles.

The statistical analysis of the data was performed using two-way analysis of variance (ANOVA) supported by Bonferroni test when carrying out pair wise comparison between the same doses of different groups using Graphpad Prism program. ANOVA for repeated measurements was applied for data consisting of repeated observations at successive time points. P-values less than 0.05 (P<0.05) were considered as statistically significant. In all Figures the symbols (*, ** and ***) representing mean differences are significant at the 0.05, 0.01 and 0.001 levels, respectively.

Results
a. Role of Sodium Potassium Chloride Cotransporter

To identify the types of NKCC involved in SNP-induced relaxation, aortic rings were preincubated for 20 minutes with BUM (10-5), DIDS. The maximum vasorelaxation induced by SNP significantly blocked by the presence BUM (P<0.01) (Figure 1A). As shown in (Figure 2B), preincubation of aortic rings with BUM alne significantly reduced (P<0.01) PIC_{50} caused by SNP.

Role of Endothelium-derived hyperpolarizing factor in rat aortic ring preincubated with Bumetanide:
Role of Endothelium-derived relaxing factor in SNP induced vasorelaxation preincubated with Bumetanide

Dose response curves for the effect preincubation of the combination of BUM with L-NAME, Indomethacin and Clotrimazole, on SNP induced relaxation against NE induced contraction are shown in figures (2A, 3A, 4A).

SNP induced relaxation of aorta preincubated with combinations of BUM with either Indomethacin or Clotrimazole was significantly blocked. On the other hand, PIC50 of aortic ring preincubated with the combination of BUM and L-NAME significantly enhanced the relaxation as compared to control (Figure 1B). In addition, a significant inhibition in PIC50 was identified during preincubation with a combination of BUM+ Indomethacin
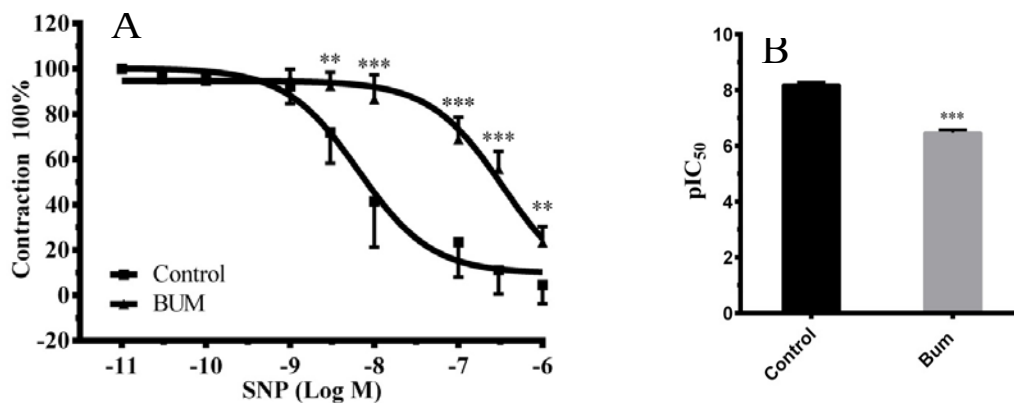

Figure 1: Cumulative dose-response curves for the vasorelaxant effects of SNP on control and aortic rings preincubated with BUM (3×10^{-5}M), precontracted with NE (10^{-6} M) (A) and a comparison between PIC50 of both groups (B).
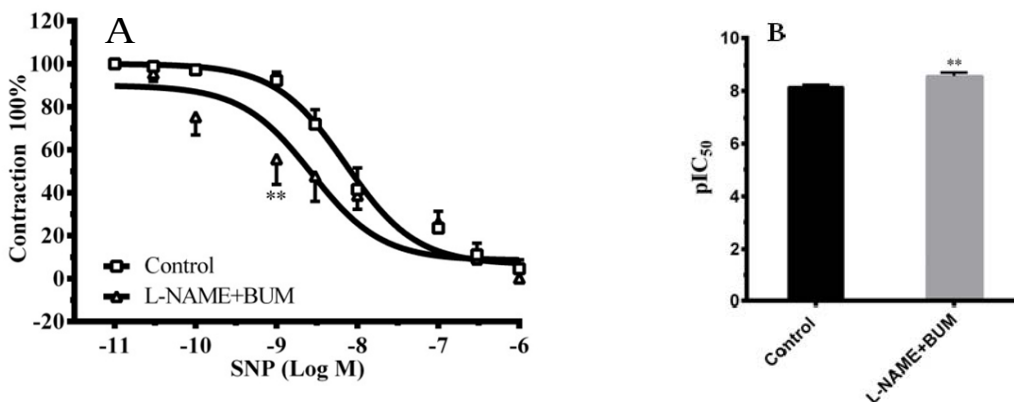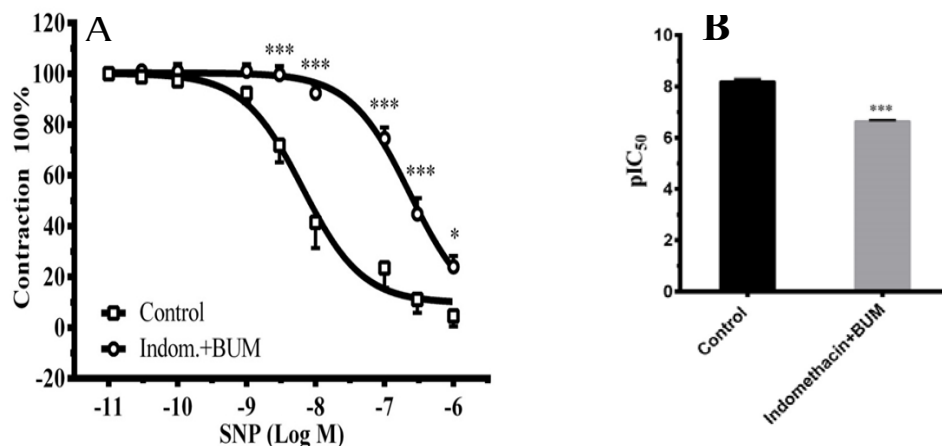

Figure 2: Cumulative dose-response curves for the vasorelaxant effects of SNP on control and aortic rings preincubated with a combination of L-NAME and BUM, precontracted with NE (10^{-6} M) (A) and a comparison between PIC50 of both groups (B).

Figure 3: Cumulative dose-response curves for the vasorelaxant effects of SNP on control and aortic rings preincubated with a combination of Indomethacin and BUM, precontracted with NE (10^{-6} M) (A) and comparison between PIC_{50} of both groups (B).
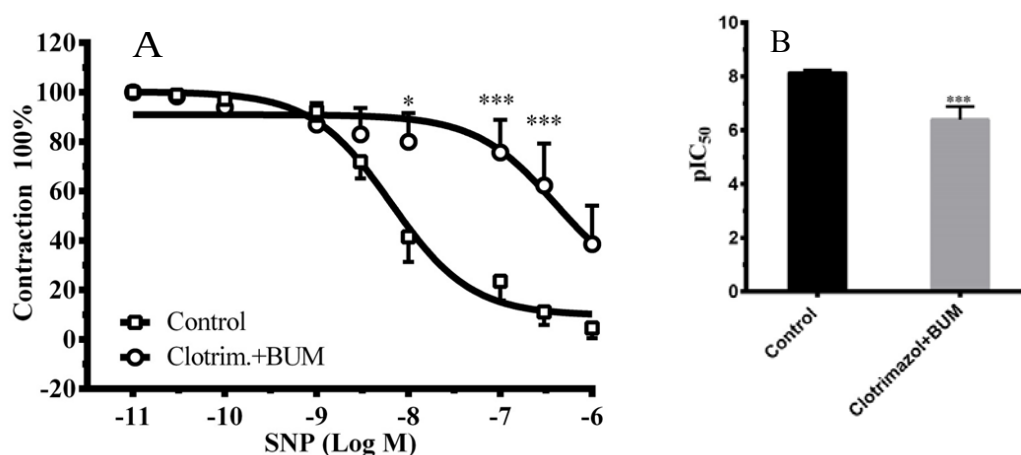

Figure 4: Cumulative dose-response curves for the vasorelaxant effects of SNP on control and aortic rings preincubated with a combination of Clotrimazole and BUM, precontracted with NE (10^{-6} M) (A) and a comparison between PIC_{50} of both groups (B).

Role of Potassium channel in SNP induced vasorelaxation preincubated with Bumetanide

Dose response curves for the effect preincubation of the combination of TEA and $BaCl_2$ on SNP against NE induced contraction are shown in figures (5& 6).

SNP induced relaxation was significantly blocked by combinations of BUM+TEA and BUM+ $BaCl_2$,but the the presence of $BaCl_2$ in the combination showed a more potent inhibitory effect than TEA.

On the other hand PIC_{50} of aortic ring preincubated with combinations of BUM+ TEA or BUM $+BaCl_2$ significantly increased as compared to control (figures (5B and 6B).
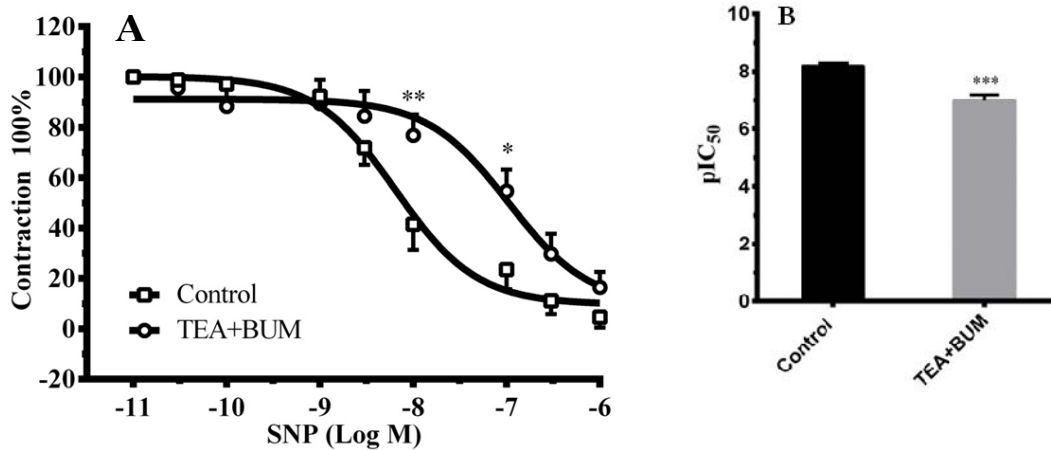
Figure 5: Cumulative dose-response curves for the vasorelaxant effects of SNP on control and aortic rings preincubated with a combination of TEA and BUM, precontracted with NE (10^{-6} M) (A) and a comparison between PIC$_{50}$ of both groups (B).

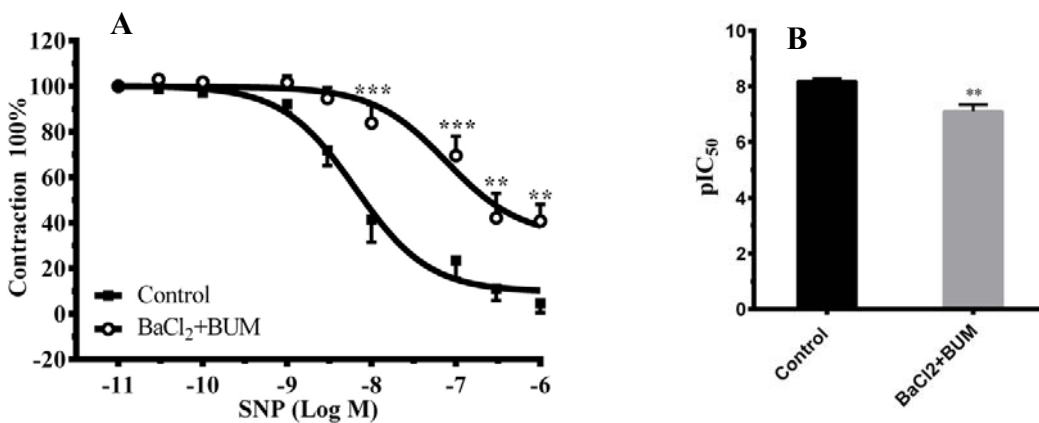**Figure 6:** Cumulative dose-response curves for the vasorelaxant effects of SNP on control and aortic rings preincubated with a combination of BaCl$_2$ and BUM, precontracted with NE (10^{-6} M)(A) and a comparison between PIC$_{50}$ of both groups (B).

Discussion

The results of the present study showed that vasodilation induced by NO donor is partially depended on the activation Cl channel and co-transporter. Despite the available evidence for the presence of Cl⁻ current in isolated cells, there is a dearth of informations about the role of Cl⁻ channel in contractile mechanisms in SMCs (Bulley and Jaggar, 2014). However, recent evidences revealed the accumulation of intracellular Cl⁻ in SMCs via NKCC proteins, and there are several contractile agonists induced Cl⁻ exit with a subsequent depolarization (Loewen and Forsyth, 2005).

The tested chloride transport inhibitors, bumetanide strongly inhibited the vasorelaxant responses to nitroprusside in isolated rat aorta; and bumetanide antagonizes the vasorelaxant responses to genistein in isolated rat aorta, suggesting that intracellular chloride plays an important role in vasorelaxation (Valero et al., 2006).

Chloride accumulation in vascular smooth muscle cells creates a electrochemical gradient (Chipperfield and Harper, 2000), which is dissipated by several vasoconstrictors, via the opening of calcium-dependent chloride channels (Loewen and Forsyth, 2005) which, permitting an influx of extracellular Ca2+ that causes muscle contraction (Hughes, 1995).

The data obtained by (Koltsova et al., 2009) showed that in smooth muscles, inhibition of Na+,K+,2Cl cotransport (NKCC) by bumetanide decreased intracellular Cl⁻ content ([Cl⁻]i) and suppressed the contractions triggered by diverse stimuli. In cultured vascular smooth muscle cells, NaHCO₃ almost completely abolished inhibitory actions of bumetanide on transient de-

polarization and $[Ca^{2+}]i$ elevation triggered by PE.

SNP induced relaxation was not affected by combination of BUM with either (L-NAME and Clotrimazole), which reflects the antagonized impact for the mentioned combinations, while significant blocking of SNP recorded in preincubation with BUM and indomethacin. These results are also in agreement with previous studies showing that endothelial prostanoids could be involved in the improvement of NKCC1 function in PE induced contraction in rat aortic rings (Palacios et al., 2006). Mtabaji et al., (1976) demonstrated that bumetanide decreased the response of the rat mesenteric vascular bed to norepinephrine by inhibiting prostaglandin synthesis. In addition, it has been proposed that all vasodilation is caused by decreased activity of NKCC1 (Greenberg et al., 1994) and/or prostaglandin release (Pickkers et al., 1997).

The novel results of the current work showed that aortic rings pre-incubated with clotrimazole (an Epoxyeicosatrienoic acids inhinbitor) with Bumetanide significantly reduced SNP-induced vasorelaxation which may be due metabolism of Bumetanide by cytochrome P450 pathways (Brater, 1991), because EETs are important regulators of vascular tone and homeostasis (Pfister et al., 2010).

Furthermore, the results of the current study also indicate that both TEA and $BaCl_2$ when combined with bumetanide significantly reduced SNP-induced relaxant effect in aortic smooth muscle cells. This suggests that SNP-mediated relaxation is dependent on activation of BK_{Ca} and KIR channels, respectively.

Conclusion

It was concluded that EETs, Prostaglandin, BK_{Ca} and KIR pathways are involved in SNP-induced vasodilation and that the contribution of chloride contransporet rat aortic rings was enhanced.

Acknowledgment

References

BRATER, D. C. 1991. Clinical pharmacology of loop diuretics. *Drugs,* 41 Suppl 3**,** 14-22.

BULLEY, S. & JAGGAR, J. H. 2014. Cl(−) channels in smooth muscle cells. *Pflugers Arch,* 466**,** 861-72.

CHIPPERFIELD, A. R. & HARPER, A. A. 2000. Chloride in smooth muscle. *Prog Biophys Mol Biol,* 74**,** 175-221.

GREENBERG, S., MCGOWAN, C., XIE, J. & SUMMER, W. R. 1994. Selective pulmonary and venous smooth muscle relaxation by furosemide: a comparison with morphine. *J Pharmacol Exp Ther,* 270**,** 1077-85.

HUGHES, A. D. 1995. Calcium channels in vascular smooth muscle cells. *J Vasc Res,* 32**,** 353-70.

KOLTSOVA, S. V., LUNEVA, O. G., LAVOIE, J. L., TREMBLAY, J., MAKSIMOV, G. V., HAMET, P. & ORLOV, S. N. 2009. HCO3-dependent impact of Na+,K+,2Cl-cotransport in vascular smooth muscle excitation-contraction coupling. *Cell Physiol Biochem,* 23**,** 407-14.

LOEWEN, M. E. & FORSYTH, G. W. 2005. Structure and function of CLCA proteins. *Physiol Rev,* 85**,** 1061-92.

MOTULSKY, H. J. & CHRISTOPOULOS, A. 2003. *Fitting Models to Biological Data Using Linear and Nonlinear Regression. A Practical Guide to Curve Fitting. GraphPad Software Inc.* , San Diego, CA.

MTABAJI, J. P., MANKU, M. S. & HORROBIN, D. F. 1976. Vascular actions of furosemide and bumetanide on the rat superior mesenteric vascular bed: interactions with prolactin and prostaglandins. *Canadian Journal of Physiology and Pharmacology,* 54**,** 357-366.

PALACIOS, J., ESPINOZA, F., MUNITA, C., CIFUENTES, F. & MICHEA, L. 2006. Na+ -K+ -2Cl- cotransporter is implicated in gender differences in the response of the rat aorta to phenylephrine. *Br J Pharmacol,* 148**,** 964-72.

PFISTER, S. L., GAUTHIER, K. M. & CAMPBELL, W. B. 2010. Vascular pharmacology of epoxyeicosatrienoic acids. *Adv Pharmacol,* 60**,** 27-59.

PICKKERS, P., DORMANS, T. P., RUSSEL, F. G., HUGHES, A. D., THIEN, T., SCHAPER, N. & SMITS, P. 1997. Direct vascular effects of furosemide in humans. *Circulation,* 96**,** 1847-52.

SHEKHA, M. S. & AL-HABIB, O. A. M. 2012. Vasorelaxant, antispasmodic and Cardiotonic effect of the Chloroform Fraction of Crataegus aronia on isolated rat muscles.

International Journal of Biological and Biomedical Sciences, 1**,** 6-11.

VALERO, M. S., GARAY, R. P., GROS, P. & ALDA, J. O. 2006. Cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel and Na-K-Cl cotransporter NKCC1 isoform mediate the vasorelaxant action of genistein in isolated rat aorta. *European journal of pharmacology,* 544**,** 126-131.

پوخته:

ناوپۆشی بۆریەکانی خوێن رۆڵێکی گرنگیان هەیە لە رێکخستنی چاڵاکی و شێوازی خانه، بەڵام کاریگەریەکانی کۆترازسپۆرتەرکان NKCC و ئەو پێکهاتانەی کە لە ناوپۆشه خانه دەردەچن و کەناڵەکانی پۆتاسیۆم بە یەکەوە زۆر رۆن نین لە سەر رێکخستنی چاڵاکیەکانی شاه خوێنبەری جورج دەکەن. بۆیه هەڵساین بە ئەنجامدانی ئەم توێژینەوەیه بۆ دەرخستنی رۆڵی شاراوەی ئەو پێکهاتانەی کە لە ناپۆشەخانه دەردەچن لەگەڵ کەناڵەکانی پۆتاسیۆم لە سەر SNP هاندانی خاوبونەوەی لولەکان لە بازنەی شاه خوێنبەری دابراو لە جورج لە حاڵەتی هەبوونی BUM کە بلۆکەری NKCC یه. بەرزترین بلۆک کردنی خاوبونه بە SNP رووێدا لە حاڵەتی کرکەوتنی بە BUM. هەروەها ئەو SNP کە دەبێته هاندانی خاوبونەوه کاریگەری تێکرا بە تێکەڵەی BUM و ئیدومیثازین و کلۆتریمازۆل.

هەروەها TEA و BaCl2 رێگری بەرچاویان کرد لەو SNP یەی که دەبێته هۆی خاوبونەوەی شاه خوێنبەری که کرکراو بەم بلۆکەرانه. بەڵام BaCl2 کاریگەریەکی زۆرتری هەبوو به بەراوردکردن به ئەوانی تر.

ئەنجامەکانمان ئەوەیان بە دیارخست ئەو خاوبونەوەی لولەکان که بە SNP روودەدات لە ژێر کاریگەری EETs و پرۆستاگلاندین و کەناڵەکانی BKCa و Kir که لە کۆتاییدا هەرهەمووویان بەشدارن له هایپەرپۆلەرایزێشنی ماسولکه لوسەکانی شاه خوێنبەری دابراو و خاوبونەوەکانی تر.

الخلاصة:

البطانة الغشائية للأوعية الدموية تلعب دورا رئيسيا في هيكلية الاوعية الدموية و تنظيم نشاطها. مع ذلك فإنه من غير الواضح تأثيرات الجمع بين ناقلات المشتركة NKCC مع المواد المفرزة من قبل البطانة الغشائية و قنوات البوتاسيوم في حلقة الشريان الأبهر على تنظيم فعالية الشريان الابهر.

في الدراسة الحالية، وقد تم التحقيق في دور المحتمل لعوامل الاسترخاء المفرزة من قبل البطانة الغشائية وقناة البوتاسيوم على SNP المسببة للارتخاء الوعائي مسبقا لتحفيز تقلص حلقات الأبهر المعزولة من الجرذان في تواجد بوميتانيد (المثبطة NKCC). أظهرت تواجد البيوتاميد تأثيرا تثبيطيا معنويا عاليا على ارتخاء الوعائي الناجمة عن SNP . تأثر SNP الناجم للاسترخاء بمزيج من BUM مع الإندوميثاسين و كلوتريمازول.

أظهر TEA و BaCl2 تأثيرا تثبيطيا على SNP الناجم للاسترخاء الشريان الابهر الحاضن من قبل مع المثبطات، ولكن أظهرت تأثير BaCl2 أكثر فعالية مقارنة مع الآخرين. وتشير هذه النتائج إلى أن SNP المحفزة للارتخاء الوعائي تتم من خلال EETs، البروستاگلاندين، BKCa ومسارات Kir مما عزز في النهاية مساهمة فرط نقل الكلوريد في خلايا عضلات الملساء للشريان الابهر والاسترخاء المتعاقب