Shiraz University

IJVR

ISSN: 1728-1997 (Print)
ISSN: 2252-0589 (Online)

Vol. 19 No. 3 Ser. No. 64 2018

IRANIAN JOURNAL OF VETERINARY RESEARCH

The roles of potassium channels in contractile response to urotensin-II in mercury chloride induced endothelial dysfunction in rat aorta

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(Received 25 Nov 2017; revised version 17 Feb 2018; accepted 3 Mar 2018)

Summary

Urotensin-II (U-II), the most potent vasoconstrictor that has recently been recognized as a new candidate in cardiovascular dysfunction, might exert vasoconstriction through, at least partially, potassium channels that are predominant in both endothelial and vascular smooth muscle cells (VSMCs). The present study was designed to evaluate the roles of potassium channels in vascular responses to U-II in intact and mercury induced endothelial dysfunction in rat aorta. The study involved pre-incubation of rat aortic rings with potassium channels blockers: charybdotoxin (chtx), tetraethylammonium (TEA), barium chloride ($BaCl_2$), glibenclamide, 4-aminopyridine (4-AP) and clotrimazole. Then vascular responses to increased concentrations of human U-II (hU-II) were applied to each group in the presence and absence of mercury chloride ($HgCl_2$). Urotensin-II efficacy was significantly increased in chtx, TEA and $BaCl_2$ treated groups, while significantly decreased in glibenclamide and clotrimazole treated groups as compared with the control group. In the presence of mercury, hU-II efficacy was significantly changed in all groups except clotrimazole treated group. The novel findings were that potassium channels modulated the vascular contractile responses to hU-II in isolated rat aorta and mercury treatment increased hU-II efficacy and deteriorated potassium signaling.

Key words: Endothelial dysfunction, Mercury, Potassium channels, Urotensin-II

Introduction

Under pathological conditions, U-II has been considered as a new candidate for cardiovascular dysfunction including atherosclerosis, hypertension and renal failure (Russell, 2008; Bianca *et al*., 2012). Urotensin-II, a cyclic peptide which was firstly isolated from goby fish as an osmoregulator in the neurosecretory system (Loirand *et al*., 2008) is known to play a crucial role in vascular tone synergistically with the rest of endothelial-derived vasoactive factors including vasorelaxants and vasoconstrictants (Pantan *et al*., 2014; Huo *et al*., 2015). Urotensin-II receptors have been cloned in different vascular beds and tissues including vascular endothelial and smooth muscle cells, brain, liver, kidneys, spinal cord, and visceral tissues (Rego *et al*., 2008; Maguire *et al*., 2008). Urotensin-II exerts vasoconstrictive effects on rat thoracic aorta through endothelial-independent pathways through activation of Gαq/11 UT-2 receptors and subsequently activation of phospholipase C (PLC), inositol triphosphates (IP3), RhoA signaling pathways (Proulx *et al*., 2008). Urotensin-II also exhibits vascular relaxation effects in coronary and renal arteries through calcium-independent enhancement of endothelial nitric oxide synthase (eNOS) activity (di Villa Bianca *et al*., 2012) and increased nitric oxide (NO) availability. Endothelial cells integrity is the vital defense line that enhances blood flow, vascular tone and remodeling (Bernatova, 2014) through secreting variety of vasoactive factors that keep the vascular tone within physiological boundaries. Potassium channels are the most common proteins existing in both endothelial and vascular smooth muscle cells (VSMCs) that play important roles in maintaining vascular tone (Wrzosek, 2009), regulating membrane potential and calcium signaling (Su *et al*., 2013). Some potassium channels, including voltage-sensitive potassium channels (K_v) are involved in the vasoconstriction effects of endothelin-1 and angiotensin-II in VSMCs via calcium-independent protein kinase C (PKC) activation (Su *et al*., 2013). These channels are exclusively sensitive against reactive-oxygen species (ROS) that mediated impairment of endothelial and VSMCs membrane permeability for potassium ions (Wrzosek, 2009) that are necessary for subcellular signaling involved in regulating vascular tone. On the other hand, endothelial dysfunction has been addressed in most cardiovascular diseases that causes imbalance between the vasoactive agents (Ellinsworth, 2015) and are predominantly alters vascular responses toward vasoconstriction. One of the potent causes of endothelial dysfunction known to date is free radicals (Almenara *et al*., 2013; Angeli *et al*., 2013; Nagaraj *et al*., 2013) that typically exist in physiological levels in health and within catastrophic levels in certain pathological circumstances including diabetes, hypertension, renal diseases and vascular injury (Behm *et al*., 2008). It has been published that exposure of endothelial cells to mercury in micro molar shifts the

vascular reactivity toward constriction state and impairs vascular response against vasorelaxant agents accompanied by a remarkable increased in free radicals (Almenara *et al*., 2013). As described in certain studies, inorganic mercury, $HgCl_2$, has biphasic effects; an endothelial-dependent vasorelaxation effects in nano molar concentrations (Omanwar *et al*., 2014) and endothelial-independent vasoconstriction effects in micro molar concentrations (Houston, 2011). According to our knowledge, no published evidence is available yet to explain roles of potassium channels in vascular responses to U-II. This study is an attempt to find out roles of potassium channels in vascular response to U-II in both intact and mercury induced endothelial dysfunction in VSMCs which will provide a helpful guide to further understand underlining mechanisms of U-II vascular actions.

Materials and Methods

Chemicals

Human urotensin-II and chtx were purchased from Bachem (Bubendorf, Switzerland). N^G-nitro-L-arginine methyl ester (L-NAME), TEA, $BaCl_2$, nifedipine, $HgCl_2$, clotrimazole, methylene blue (MB), 4-AP, and glibenclamide were provided by Scharlab S.L/Sentmenat Spain.

Animals

Male albino rats weighing (200-250 g) were purchased from Directorate of Duhok Veterinary-Duhok, Iraq. Animals were housed in animal house and were kept in a standard conditions according to the laboratory animal care guide prepared by the scientific committee in College of Science, Salahaddin University. The study was carried out in Biology department, College of Science from November 2016 to July 2017, and the Animal Care Committee approved the study.

Aortic rings preparation

152 aortic rings from 38 male albino rats were used in this study. Animals were anaesthetized by ketamine: xylazine mixture (90 mg/kg, i.p. and 10 mg/kg, i.p., respectively) (Rameshrad *et al*., 2016). From the proximal descending thoracic aorta (Chatenet *et al*., 2013), immediately next to the left subclavian branch, 12 mm length from thoracic aorta was isolated in cold Krebs bicarbonate solution, then excess surrounding tissues were removed and 4 aortic rings each about 3 mm in length were prepared.

Vascular reactivity assay

The prepared aortic segments were held by stainless steel hooks into 10 ml organ bath (Automatic Organ Bath-Panlab Harvard Apparatus-USA, AD instrument PowerLab 8/35-Australia) filled with Krebs bicarbonate solution (in mM/L:119 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 1.5 $CaCl_2$, 25 $NaHCO_3$, 11 glucose, pH = 7.4). The bath solution was maintained at 37°C and bubbled with a mixture of about 95% O_2 and 5% CO_2. The aortic rings were loaded by 2 g tension force and allowed to equilibrate for at least 1 h during which the Krebs solution was replaced every 15 min, and the tension was continuously readjusted to the optimum force. For the functional integrity of the prepared aortic segments, KCl (60 mM) (Qu *et al*., 2014) was used and the maximum contraction developed was considered as standard percentage contractile response. After the maximum contraction by KCl was reached to plateau, the aortic rings were washed and re-stabilized at the optimum tension for at least 30 min before applying any vasoactive substances. In one group, the endothelium was removed by gently rubbing the lumen of the aortic rings with a narrow forceps tip, and the denuded rings were assessed by adding acetylcholine (10 µM) to pre-contracted aortic segments with phenylephrine (1 µM).

Experimental procedures

To evaluate effects of $HgCl_2$ on vascular response to hU-II, cumulative doses of hU-II (10^{-11}-10^{-8} M) were used in the presence and absence of $HgCl_2$ and results were considered as control groups. To find out roles of endothelial cells in aortic ring responses to hU-II, increased concentration doses of hU-II were applied in endothelial intact and denuded aortic rings.

To investigate roles of different potassium channels in hU-II elicited VSMCs contraction, aortic segments were incubated for 20 min with the big conductance calcium-activated potassium channels (BK_{ca}) blocker; chtx (1 µM) and intermediate conductance calcium-activated potassium channels (IK_{ca}) blocker; clotrimazole (30 µM), a non-selective calcium-activated potassium channel blocker; TEA (1 mM), delayed inward rectifier potassium channels (K_{ir}) blocker; $BaCl_2$ (0.2 mM), adenosine triphosphates-sensitive potassium channels (K_{ATP}) blocker; glibenclamide (10 µM), voltage sensitive potassium channels (K_v) blocker; 4-AP (0.5 mM), in endothelial intact groups. Then, increased concentrations of hU-II were applied in all groups. In another set of the experiment, to study effects of $HgCl_2$ on potassium channels involvement in the vascular responses to hU-II, aortic rings were incubated with $HgCl_2$ (1 µM) for 45 min before cumulative concentration doses of hU-II were applied in the presence of each of the above blockers, separately. To investigate the role of endothelial nitric oxide (eNO) on hU-II elicited contraction, intact aortic rings were incubated for 20 min with irreversible eNOS inhibitor; L-NAME (200 µM) in both $HgCl_2$ treated and untreated groups. To find out possible roles of guanylate cyclase (GC) system and L-type-calcium channels in vascular responses to hU-II in presence and absence of mercury, some aortic rings were incubated with MB (0.8 mM); soluble GC inhibitor or nifedipine (20 µM). At the end of each mercury treated group, acetylcholine (10 µM) was applied to aortic ring to check the endothelial impairment. Loss of vascular response to acetylcholine is considered as an indication of successful endothelial impairment induced by mercury.

Statistical analysis

The vascular contraction induced by hU-II was expressed as percentage of tension generated by KCl (60 mM). To differentiate the effect of blockers or inhibitors on vascular responses to hU-II in aortic segments from both control and $HgCl_2$ treated groups, some results were expressed as the differences in area under curves (dAUC). AUCs were calculated from individual concentration-response curve plots; differences were expressed as the percentage of AUC of the control group. Data are expressed as mean±SE of means (SEM) of the number of aortic rings used in all groups. Results were analyzed using independent Student's t-test for comparing dAUC and potency difference (pD_2) between the studied groups. Two-way analysis of variance (ANOVA) for comparison between control and studied drugs was applied and Sidak post hoc test was used to compare individual means. Dunnett-test was also applied to compare pD_2 between all groups with the control. Differences were considered statistically significant at $P<0.05$.

Results

The prepared aortic rings were contracted to cumulative concentration doses of hU-II (10^{-11}-10^{-8} M) (Fig. 1A). Aortic segments toward abdominal part exhibited no or feeble responses to hU-II. All mercury treated aortic rings lost response to acetylcholine at the end of each experiment indicating that endothelial layer was totally impaired in mercury treated groups.

To assess the endothelial involvement in contractile response of aortic rings to hU-II, hU-II was applied to both intact and denuded aortic segments. The results, as shown in (Fig. 1B), revealed that the maximum response of denuded rings was significantly increased (Emax: 115 ± 3.887) as compared with the intact segments (Emax: 74.83 ± 2.46), but the potency of hU-II showed no significant differences (pD_2: -8.782 ± 0.077, control *vs* -8.654 ± 0.072, denuded). The results showed that incubation of aortic rings with $HgCl_2$ for 45 min remarkably increased the efficacy of hU-II (Table 1), while the pD_2 value significantly remained unchanged.

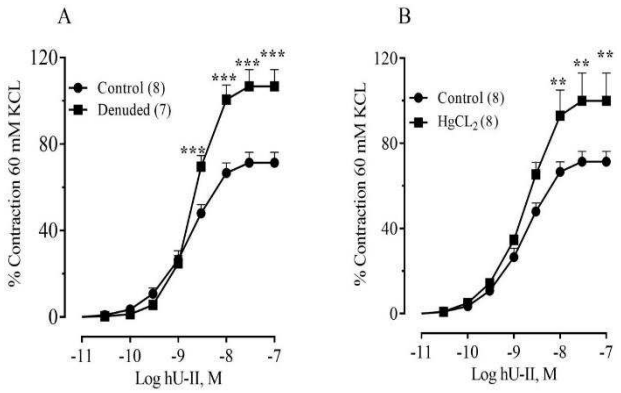

Fig. 1: The vascular responsiveness to hU-II from rat thoracic aorta. (**A**) The effects of endothelial denudation and (**B**) the effects of $HgCl_2$ 1 µM on the cumulative doses of hU-II. ** Represents statistical differences at $P<0.01$, and *** represents statistical differences at $P<0.001$. Number of animals used is indicated in parentheses

The effects of calcium-activated potassium channels on vascular response to hU-II

One of the novel findings of the present study was that non-selectively blocking of the calcium-activated potassium channels by TEA, accentuated the vasoconstriction response to hU-II in both mercury-treated and untreated groups as shown in (Fig. 2A), and increased the contraction altitudes (Emax: 111.8 ± 6.45 in mercury-untreated and 143 ± 6.68 in mercury-treated vs 74.83 ± 2.46 in control). This result was confirmed by dAUC illustrated in (Fig. 2A), the similar statistical differences reflect the vital roles of these channels in the vascular responses to hU-II in both groups, intact and endothelial dysfunction induced group. The present data indicated that chtx (1 µM) increased the maximum response to hU-II (Emax: 99.02 ± 5.18) in comparison with the control group (Emax: 74.83 ± 2.46) with no significant increase in potency (pD_2: -8.533 ± 0.118), as shown in (Table 1). In contrast, mercury chloride shifted hU-II concentration-response curve to the right, altering

Table 1: The potency (pD_2) and the maximum response (Emax) from the thoracic aortic rings responses to hU-II in the presence and absence of $HgCl_2$

Groups	Un-treated			$HgCl_2$ treated		
	n	Emax (% KCl)	pD_2	n	Emax (% KCl)	pD_2
control	8	74.83 ± 2.46	-8.782 ± 0.077	8	105.2 ± 5.392	-8.744 ± 0.119
TEA	7	111.72 ± 1.69***	-8.572 ± 0.12	7	153.3 ± 6.64.57***	-8.533 ± 0.11
chtx	6	99.02 ± 5.18***	-8.533 ± 0.118	8	70.11 ± 5.045**	-8.514 ± 0.157
clotrimazole	8	53.70 ± 1.98**	-8.953 ± 0.097	8	89.06 ± 5.7	-8.497 ± 0.138
$BaCl_2$	7	178 ± 7.117***	-8.447 ± 0.081	8	54.07 ± 6.6***	-9.194 ± 0.38
glibenclamide	7	31.68 ± 2.33***	-7.969 ± 0.114***	8	40.65 ± 2.833***	-8.271 ± 0.132
4-AP	7	76.58 ± 3.09	-8.614 ± 0.09	8	205.2 ± 11.4***	-8.707 ± 0.12
MB	8	97.41 ± 3.39***	-8.245 ± 0.064**	8	38.06 ± 4.678***	-8.861 ± 0.30
L-NAME	7	108.6 ± 5.73***	-8.355 ± 0.105*	8	152.1 ± 8.230***	-8.581 ± 0.115
nifedipine	8	53.40 ± 2.018**	-8.725 ± 0.087	8	75.15 ± 2.693**	-8.448 ± 0.073

The studied groups were compared with the control group (ANOVA was applied with Dunnett-test). * Significant differences between the studied groups vs control group at $P<0.05$, ** Significant differences between the studied groups vs control group at $P<0.01$, and *** Significant differences between the studied groups vs control group at $P<0.001$

the maximum response (Emax: 70.11 ± 5.04) but hU-II potency (pD_2: -8.514 ± 0.157) remained statistically without change. The dAUC shown in the (Fig. 2B) referred to highly significant alterations in vascular responses to hU-II in mercury-treated group in the presence of chtx. To evaluate whether vascular exposure to mercury alters the involvement of IK_{ca} channels in the vascular responses to hU-II, a set of prepared aortic segments was incubated with clotrimazole (30 µM). As illustrated in (Fig. 3A), clotrimazole significantly reduced the maximum response to hU-II, while no statistical changes were observed in hU-II potency (Table 1) in both mercury-untreated and treated groups. In the dAUC graph (Fig. 3A), similar statistical differences of hU-II potency between control and mercury-treated groups were observed.

The effects of K_{ir} channel on vascular response to hU-II in mercury-treated aortic rings

To find out the role of the K_{ir} channels on vascular response to hU-II, $BaCl_2$, a selective K_{ir} channel blocker was used. Figure (3B) showed that $BaCl_2$ noticeably magnified the vasoconstriction response to hU-II with no significant changes in hU-II potency (Table 1). While in the presence of $HgCl_2$, $BaCl_2$ exhibited highly significant reducing effect on hU-II efficacy, however, hU-II potency remained significantly unchanged. The vascular response to hU-II exhibited, according to the dAUC graph shown in (Fig. 3B), high significant change in the presence of mercury indicating that these channels were, in particular, sensitive to mercury and can alter the vascular behavior.

The effects of K_{ATP} and K_v channels on vascular response to hU-II in the presence and absence of mercury chloride

The data analysis of the present study showed a putative role of K_{ATP} channels in altering the vascular reactivity of hU-II in presence and absence of mercury. As it can be seen in (Fig. 4A), blocking of K_{ATP} channels by glibenclamide (30 µM) abolished maximum responses in $HgCl_2$-treated (Emax: 40.65 ± 2.833) and untreated groups (31.68 ± 2.33). The dAUC showed similar attenuating effects of glibenclamide on the vascular responses to hU-II and reducing the potency of hU-II (pD_2: -7969 ± 0.114, mercury-untreated), but with no significant change in the potency (pD_2: -8.271 ±

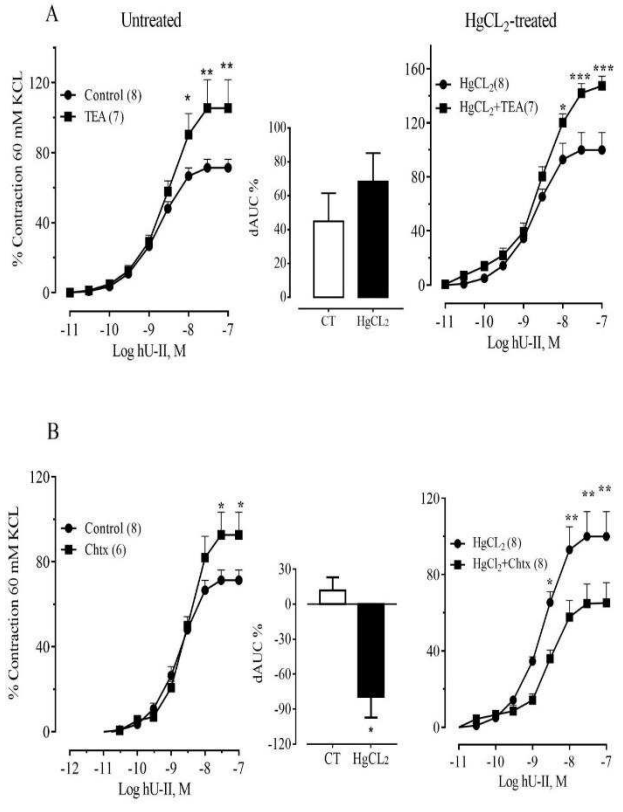

Fig. 2: The effects of $HgCl_2$ on the vasoconstrictor responses to hU-II in proximal thoracic aorta. (**A**) Effect of TEA 1 mM, and (**B**) Effect of chtx 1 µM on the vasoconstrictor responses to hU-II in $HgCl_2$-treated and untreated aortic rings. The inset graphs show differences in area under the concentration-response curve (dAUC). * Represents statistical differences at P<0.05, ** represents statistical differences at P<0.01, and *** represents statistical differences at P<0.001 versus the corresponding control group. Number of animals used is indicated in parentheses

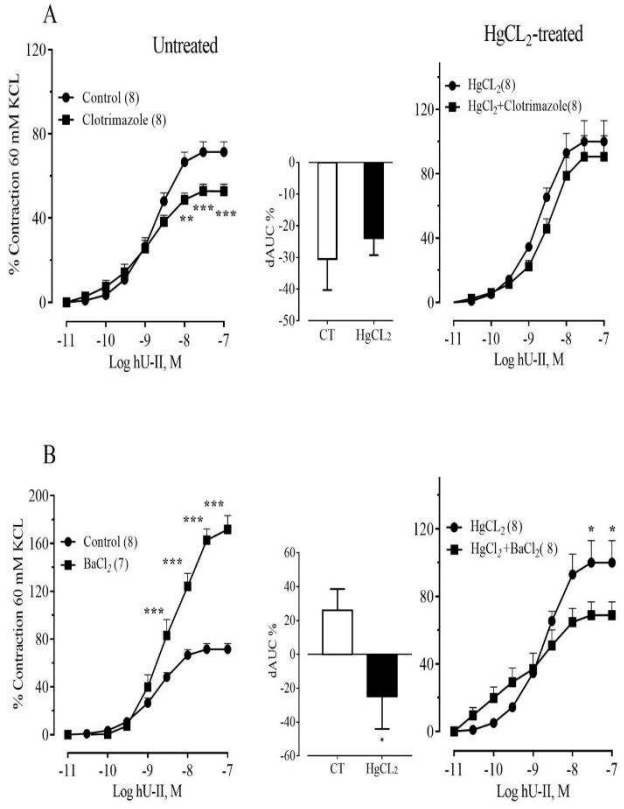

Fig. 3: The effects of $HgCl_2$ on the vasoconstrictor responses to hU-II in proximal thoracic aorta. (**A**) Effect of clotrimazole 10 µM, and (**B**) Effect of $BaCL_2$ 0.2 mM on the vasoconstrictor responses to hU-II in $HgCl_2$-treated and untreated aortic rings. The inset graph shows differences in area under the concentration-response curve (dAUC). * Represents statistical differences at P<0.05, and *** represents statistical differences at P<0.001 versus the control group. Number of animals used is indicated in parentheses

0.132) in mercury-treated group (Table 1). The present study showed that blocking of K_v channel by 4-AP showed no significant differences in both efficacy and potency of hU-II in the absence of mercury, while mercury treatment, as shown in (Fig. 4B), greatly increased hU-II efficacy (Table 1) but the pD_2 value statistically remained without change. The dAUC shown in (Fig. 4B) illustrated highly significant effects of 4-AP in the vascular responses to hU-II in the presence of mercury.

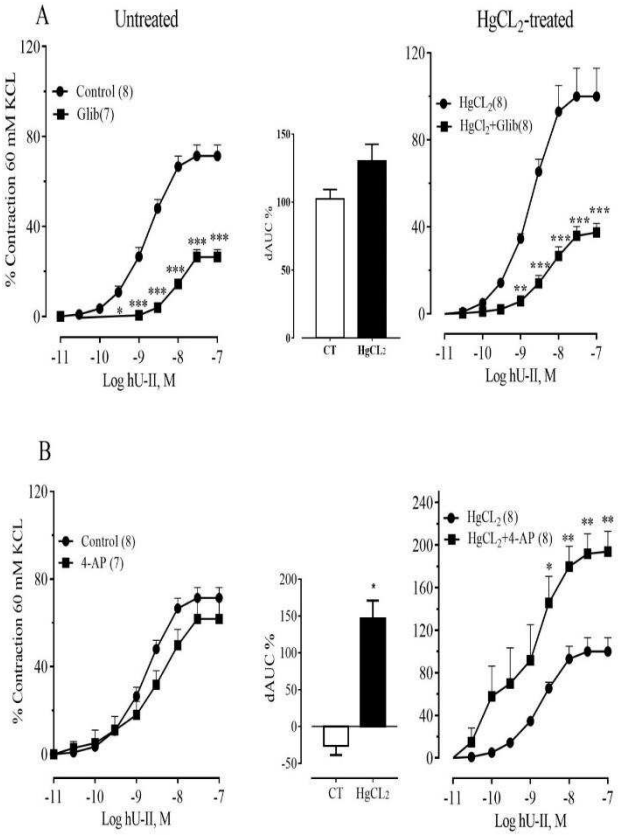

Fig. 4: The effects of $HgCl_2$ on the vasoconstrictor responses to hU-II in proximal thoracic aorta. (**A**) Effect of Glib 30 µM, and (**B**) Effects of 4-AP 0.5 mM on the vasoconstrictor responses to hU-II in aortic rings from control and $HgCl_2$-treated. The inset graph shows differences in area under the concentration-response curve (dAUC). * Represents statistical differences at P<0.05, ** represents statistical differences at P<0.01, and *** represents statistical differences at P<0.001 versus control group. Number of animals used is indicated in parentheses

The roles of L-type calcium channels in vascular responses to hU-II

To investigate the effects of mercury on hU-II vascular actions and the possible role of L-type calcium channel, nifedipine 20 µM was used. The results showed that altitude of the vascular response to hU-II was significantly declined in the presence of nifedipine in both mercury-treated and untreated groups (Fig. 5A) and shifted the concentration-response curve to the right with no significant changes in hU-II potency in both groups. The dAUC graph in (Fig. 5A) showed that nifedipine in the presence of mercury exerted more significant impact on the vascular response compared with the control group.

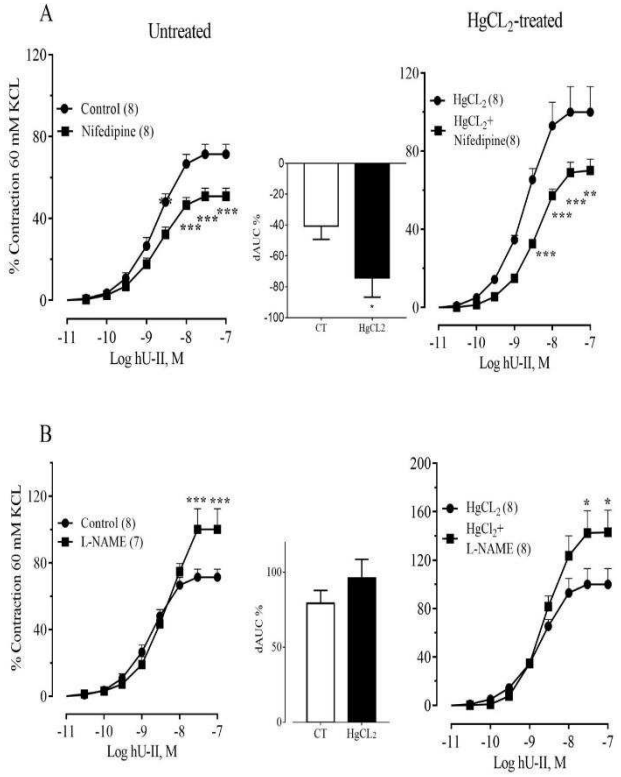

Fig. 5: The effects of $HgCl_2$ on the vasoconstrictor responses to hU-II in proximal thoracic aorta. (**A**) Effect of nifedipine 20 µM, and (**B**) Effect of L-NAME 200 µM on the vasoconstrictor responses to hU-II in aortic rings from control and $HgCl_2$-treated. The inset graph shows differences in area under the concentration-response curve (dAUC). * Represents statistical differences at P<0.05, ** represents statistical differences at P<0.01, and *** represents statistical differences at P<0.001 versus the control group. Number of animals used is indicated in parentheses

The effects of eNO on vascular responsiveness to hU-II

To investigate the role of eNO in vascular responses to hU-II, and whether mercury could modulate hU-II vasoreactivity in the presence or absence of NO, the aortic rings were incubated with L-NAME (200 µM) for 20 min before applying cumulative doses of hU-II in both $HgCl_2$-treated and untreated groups. Our results showed that inhibiting eNOS significantly potentiated hU-II potency in mercury-untreated group and significantly increased the vascular maximum responses to hU-II (Table 1) in both mercury treated and untreated groups. The results of dAUC in (Fig. 5B) showed a similar vascular effect of eNOS inhibitor in the presence and absence of mercury.

The effects of soluble GC on vascular responsiveness to hU-II

To investigate if the soluble GC, as a subcellular signaling system, might share the vasoconstriction exerted by hU-II, incubation of aortic segments with MB

showed a significant increase in the maximum response (Table 1) in mercury untreated group and the potency remarkably increased with noticeable significant decline in maximum response in the group treated with mercury. The dAUC graph in (Fig. 6) showed the great impact of mercury on the vascular response to hU-II as compared with control group.

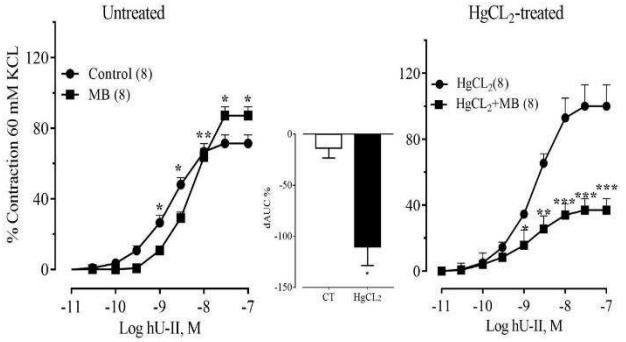

Fig. 6: The effects of $HgCl_2$ on the vasoconstrictor responses to hU-II in proximal thoracic aorta. Effect of MB 0.8 mM on the vasoconstrictor responses to hU-II in aortic rings from un-treated and $HgCl_2$-treated groups. The inset graph shows differences in area under the concentration-response curve (dAUC). * Represents statistical differences at P<0.05, ** represents statistical differences at P<0.01, and *** represents statistical differences at P<0.001 versus the corresponding control group. Number of animals used is indicated in parentheses

Discussion

The present study, for the first time, addressed the effects of potassium channels on contractile responses to hU-II in VSMCs and the endothelial impairment altered both the vascular reactivity to hU-II and potassium channels function. In the present study, removal of endothelial layer potentiated the vasoreactivity of hU-II indicating the endothelial-independent vasoconsctriction of the peptide and was inconsistent with Peng *et al*. (2013) and Al Kindi *et al*. (2014) who demonstrated that hU-II exerts endothelial-independent vasoconstriction and also reflecting that endothelial vasorelaxant factors, to some extent, modulate the efficacy of hU-II and play a significant role in modulating the peptide actions. In the impaired endothelial layer induced by mercury, the efficiency of hU-II was increased possibly through inducing oxidative stress (Furieri *et al*., 2011) that further potentiated hU-II maximum response by abolishing NO availability and eNOS activity and also deteriorated potassium channels actions (Ko *et al*., 2010), accordingly, loss of vascular relaxative capacity and shifting vascular responsiveness toward vasoconstriction.

The roles of calcium-activated potassium channels in vascular response to hU-II in the presence and absence of mercury

The new finding of the present study was blocking of calcium-activated potassium channels potentiated the altitude of the vascular contraction exerted by hU-II in both mercury untreated and treated groups. Seemingly, TEA through blocking most of the calcium-activated potassium channels obstructed an effective relaxative route in endothelial and VSMCs. It is well documented that blocking of these potassium channels augments the vascular basal tone and contractile responses to phenylephrine in rat aorta (Grgic *et al*., 2009). However, the dAUC in (Fig. 2A) uncovered the impaired endothelial impact on magnifying the peptide effects when most calcium-activated potassium channels were blocked by TEA, the condition that clarified the positive relationship between the endothelial dysfunction and hU-II vasoconstriction capacity. Furthermore, these findings indicated that under normal conditions, calcium-activated potassium channels play a fundamental role in regulating vascular tone and modulating effects of vasoactive agents released by endothelial and VSMCs.

According to the dAUC in (Fig. 2B), chtx elicited different vascular response to hU-II in the presence of mercury, possibly due to the direct effects of mercury on the selected potassium channels. These effects might reflect the existence of some underlining mechanisms that modulate the toxin vascular action resulted from the involvement of free radical-induced by mercury treatment as previously described (Furieri *et al*., 2011). In other words, free radicals produced by mercury may target the subcellular enzymes and modify their actions and decline the vascular responses to hU-II. However, it was documented that chtx can also block IK_{ca} in cultured endothelial cells and has inhibitory effects on endothelial-driven hyperpolarizing factors (EDHF) signaling (Wulff and Castle, 2010). Moreover, an early voltage-clamp study by (Ko *et al*., 2010) revealed that chtx blocked K_v channels in the same manner of blocking the calcium-activated potassium channels. Accordingly, these types of potassium channels seemed to be involved in vascular actions of hU-II and endothelial impairment deteriorated these potassium channels behavior.

Blocking of IK_{ca} channels by clotrimazole, interestingly, decreased the vascular response to the peptide uncovering some underlying mechanisms that interferred with its blocking properties. However, because clotrimazole selectively inhibits cytochrome-P_{450} (Crowley and Gallagher, 2014) it is reasonable to interfere with the biosynthesis of the vasoactive agents produced by the endothelial or VSMCs. Also, clotrimazole inhibits COX pathways and TXA_2 synthesis in various cellular systems through suppressing the cytochrome-p450-dependent pathways of arachidonic acid metabolism (Tep-Areenan and Sawasdee, 2010). Moreover, clotrimazole inhibits calcium transport in isolated tissues through inhibiting of SERCA-ATPase and reducing the contractile capacity through decreasing affinity of SERCA to calcium ions (Elam *et al*., 2011). Accordingly, our finding that clotrimazole attenuated the vascular responses to hU-II was probably via decreasing prostanoids biosynthesis, a mechanism which provided evidence of whether hU-II might exert some of its effects

through enhancing production and release of vasoactive prostanoids. However, in the presence of mercury, the endothelial cells were the primary targets for the mercury toxicity and thereby selectively impaired NO pathways and increased ROS production as reported by (Fernandes Azevedo *et al*., 2012). Based on the available evidence, mercury abolished clotrimazole effects by deteriorating the NO signaling pathways and retained hU-II efficacy.

The vascular response to hU-II was augmented by blocking of K_{ir} channels by barium chloride referring to the vital roles of these channels in the vascular tone regulation and the significant roles of these channels in modulating the vascular actions of hU-II. On the other hand, endothelial impairment induced by mercury also interfered with these channels responses. These channels are predominant in endothelial cells and play crucial roles in endothelial-dependent vasodilatation (Qu *et al*., 2014). Accordingly, these channels have been used as pharmacological targets to study vasorelaxant agents signaling pathways. However, different cellular actions of barium ions also have been reported in both *in vivo* and *in vitro* studies (Konduru *et al*., 2014). Barium has a direct intracellular interaction with contractile and regulatory proteins and enters rat aortic smooth muscles through calcium channels and mobilizes intracellular calcium pool and may even cause desensitization of some intracellular factors (Konduru *et al*., 2014). Additionally, oxidative stress also reported for barium in rat exposed to the metal (Chukwunonso Obi *et al*., 2016).

Another strange finding of the present study was the vascular response to hU-II in the presence of the K_{ATP} blocker, glibenclamide, was shifted to the right, that is opposite to the expected role of these channels and the blocking capacity of this drug. Anyway, vascular response to this drug has not necessarily been limited to blocking of these potassium channels and a sort of subcellular mechanisms might participate and alter hU-II vascular actions. A recent study reported that glibenclamide exhibited a significant reduction in malondialdehydes concentration and improved the activities of antioxidant enzymes (Chukwunonso Obi *et al*., 2016). Furthermore, a study in rat aorta suggested that glibenclamide had noticeable inhibitory effects on VSMCs besides its pharmacological property as K_{ATP} channels blocker (Dixon *et al*., 2011).

Even though blocking of K_v channels by 4-AP did not alter the vascular response to hU-II, but mercury treatment decreased the vascular sensitivity to the peptide which indicated that endothelial dysfunction changed the vascular behavior and, in turn, reflected to what extent endothelial dysfunction could deteriorate the vascular response to the vasoactive agents. It has experimentally proven that 4-AP enhances the vascular reactivity and stand against vasorelaxant agents through blocking of K_V channels. A study in patch clamp experiments on isolated cells revealed that the vascular effects of 4-AP were limited by its ability to change intracellular pH that indirectly altered ion channels activities (Tep-Areenan and Sawasdee, 2010). However, providing some possible scenarios to explain the vascular behavior against some selected channel blockers may probably hide the real effect of the studied channels in vascular reactivity.

The roles of L-Type calcium channels, NO signalling and GC system on vascular response to hU-II in the presence and absence of mercury

Our study demonstrated that L-Type calcium channels were involved in the contraction actions of hU-II and indicated that calcium influx, in rat aorta, could be one of the main vasoconstriction strategies of hU-II. L-type calcium channels have limited roles in calcium mobilizing processes and antagonists of these channels have been demonstrated as a pharmacological route in VSMCs relaxation (Onsa-ard *et al*., 2013). Interestingly, the antioxidant properties of these channels blocker, nifedipine, were also reported. A study by (Ersoy *et al*., 2008) found that nifedipine enhanced NO availability in isolated tissues by increase in superoxide dismutase enzyme activities. Taking these findings into consideration, we suggested that contraction lowering effects of nifedipine in the present study were due to its antioxidant effects besides its blocking of calcium entry that highlighted the roles of calcium channels in the VSMCs response to h-UII. On the other hand, in the presence of mercury, blocking of calcium channels also exhibited more significant effects illustrated in dAUC (Fig. 5A).

Under normal physiological conditions NO signalling pathways attempt to attenuate or modulate the vascular actions of hU-II, but in contrast, the vascular responses to hU-II will be amplifed due to lack of NO signalling and most often under pathological circumstances. Mercury treatment also showed an increase in hU-II contraction power in the presence of L-NAME, possibly because of at least two logical reasons:

Firstly, both L-NAME and mercury had augmented the vasoconstriction capacity of each other seemingly throughout their direct abolishing effects on NO availability.

Secondly, because mercury *per se* induced the release of vasoactive prostanoids (Furieri *et al*., 2011) that further altere vascular responses toward constriction. In the present study, the applied dose of $HgCl_2$ (1 µM) exerted the vasoconstriction effects probably through overproduction of ROS (Fernandes Azevedo *et al*., 2012; Macirella *et al*., 2016), and also via decreasing NO availability (Furieri *et al*., 2011).

It is not surprising that blocking of GC pathway by MB further augmented hU-II vasoconstriction. However, in some studies, the cellular actions of MB might be shifted to its antioxidant behavior (Schirmer *et al*., 2011; Xiong *et al*., 2017) and its potent ability to reduce ROS (Jena and Chainy, 2008). Furthermore, the antioxidant activity of MB in the present study might have augmented its GC inhibitory action in the presence of mercury.

In the present study, for the first time the roles of potassium channels in the vascular responses to hU-II from rat thoracic aorta have been highlighted. The

current study also addressed that endothelial impairment potentiated the vascular responses to hU-II with its concomitant impairment of potassium signalling. Despite its exploratory nature, this study offers some pharmacological tools to modulate the peptide behavior under pathological cases in particular.

Acknowledgements

Great thanks to Prof. Dr. A. M. Rasheed for Lab support, Dr. B. J. Abdullah and Mr. H. D. Najeeb for their technical assistance, Ch. M. Jawdat and A. Z. Umer for organ bath training and Sh. Z. Abdulqadir for technical support.

Conflict of interest

I do not have any conflict of interest or any other relevant connection or shared interest.

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