RESEARCH PAPER

L-arginine and tetrahydrobiopterin modulate endothelin-1A receptor activity in isolated rat aorta

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A B S T R A C T:

Endothelin-1 (ET-1), is a potent endogenous vasoconstrictor secreted by endothelial cells. It acts as the natural counterpart of the vasodilator effect of nitric oxide (NO). ET-1 exert it is the vasoconstrictor activity through two types of receptors, ET-1A and ET-1B receptors that are located on vascular smooth muscle cells (VSMCs). The present study was designed to evaluate the effect of L-arginine (LA), tetrahydrobiopterin (BH4), and their combination on ET-1A receptor activity in rat aortic rings. The study involved pre-incubation of rat aortic rings with LA, BH4, and their combination. Then, the vascular response to a cumulative dose of the ET-1A receptor antagonist (BQ-123) and in the second set of experiment cumulative doses of Acetylcholine (ACh) were applied to each group. BQ-123 potency increased after LA pre-incubation, but LA and BH4 in combination significantly potentiated BQ-123 potency and maximum response. In the second set of experiment, ACh potency does not change, while ACh efficacy markedly increased during pre-incubation of LA, BH4, and their combination. In conclusion, LA and BH4 may offer some pharmacological tools to modulate the ET-1A receptor activity and treat cardiovascular disease beyond pulmonary arterial hypertension.

KEY WORDS: Acetylcholine; BQ-123; Endothelin-1; L-arginine; Rat aorta; Tetrahydrobiopterin.

1. INTRODUCTION:

Endothelial cell and other cells in the body produce ET-1, a 21-amino acid peptide which is acts as one of the most powerful vasoconstrictors identified to play a vital role in the generation of hypertension (Hamad et al., 2016), that was recognized by Yanagisawa et al. (1988). ET-1A and ET-1B are the two pharmacological receptors for ET-1. VSMCs have ET-1A and ET-1B receptors while endothelial cells have only ET-1B receptors (Vignon-Zellweger et al., 2012). ET-1 regulates its activity by binding to either ET-1A or ET-1B receptors, which are receptors with G protein-coupled to Gαq/11. After binding ligand to its receptor, there are secondary messenger system activated within VSMCs involves Gq, Gs and Gi small G proteins (In addition to the heterotrimeric G proteins, other forms of G proteins play important roles in cell function. These proteins belong to a large superfamily often referred to as “small G proteins” based on their low molecular weight (20,000 to 35,000) prompting provoke activation of phospholipase C. The back to back creation of inositol triphosphate (IP3) and diacylglycerol (DAG) expands the convergence of

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calcium ion (Ca2+) within the cell which is released from the sarcoplasmic reticulum by stimulation of the IP3 receptor, and outer Ca2+ rushes into the sarcoplasm through the opening of the Ca2+ channels on the sarcolemma (Barrett et al., 2010). The enzymes NADPH oxidase and/or uncoupled nitric-oxide synthase (NOS) which are located on blood vessels is activated by increased levels of ET-1, then the production of superoxide and constriction of blood vessels occur while the vasodilation might be regulated by endothelial ET-1B receptors through releasing NO (Kowalczyk et al., 2015).

BQ-123 is a powerful and specific blocker for ET-1A receptors in various distinctive tissues and cells. The capacity of BQ-123 to stop the impacts of ET-1 in rabbit aorta, Schneider proposes that the contractile impacts of ET-1 are incitement because of ET-1A receptors (Schneider et al., 2007). Preliminary studies on BQ-123 showed that BQ-123 inhibited angiotensin II-induced contractions in isolated rabbit aorta (C Kowala et al., 2004). The effects of ET-1 that are mediated via ET-1A receptors are sensitive to blockade by BQ-123, whereas those that are mediated via ET-1B receptors are BQ-123-insensitive (Martínez-Revelles et al., 2012).

The chemical precursor of NO is a semi-essential amino acid which is known as LA, causes vasodilation of blood vessels so NO termed vasodilator (Soderman, 2013). Endothelial NOS (eNOS) is the enzyme that mediates the conversion of LA to L-citrulline and NO (Endemann and Schiffrin, 2004). NO activates the enzyme guanylyl cyclase which is regarded as a soluble receptor for NO (Friebe and Koelsing, 2003). ACh can mobilize a stored NO pool, which, synergistically with prostacyclin, can relax precontracted aortic rings Patients with hypercholesterolemia and atherosclerosis get benefit from supplementation of LA amino acid which enhances NO production and reestablish endothelial function (Tsuboi et al., 2018) but other studies recorded that LA does not improve endothelial function in rabbit aortic rings (Sagach et al., 2006).

Tetrahydrobiopterin (BH4) act as eNOS cofactor, the coupling and stability of NOS homodimer maintains by binding two BH4 to the two NOS homodimer because it provides stability of the homodimer through numerous hydrogen bonds and it makes intimate coupling between LA substrate and the heme site of the eNOS oxygenase domain that binds to the oxygenase domain adjacent to the heme active site (Chen et al., 2010). The relaxation of blood vessels which is stimulated by ACh, augmented by BH4 (100 mM) (Jiang et al., 2000). The using LA with BH4 together shows a synergistic effect and elevates the production of NO and restore endothelial function (Tratsiakovich et al., 2013b). To the best of our knowledge, combined administration of LA and BH4 with the purpose to decrease the ET-A receptor has never been tested before. Therefore, the present study aimed to investigate the impact of NO precursor, BH4 and their combination of ET-1A receptor activity.

2. MATERIALS AND METHODS

2.1: Chemicals

ET-1 was purchased from Bachem (Bubendorf, Switzerland); BH4 from Nootropic (Arizona, USA); BQ-123 selective ET-1A Receptor antagonist from Selleckchem (Houston, Texas, USA); ACh from Sigma-Aldrich, LA was provided by Scharlab S.L/Sentmenat Spain.

2.2: Animals

Twenty male albino rats weighing 200-300 gm were housed and kept in standard laboratory conditions of light and temperature about 12 hours light: 12 hours dark photoperiod and 22 ± 4 °C. The experimental protocol was approved by the ethics committee and animal care committee in the College of Science; Salahaddin University – Erbil. The study was carried out in the Biology department, College of Science from March/ 2018 to October/ 2018.

2.3: Aortic rings preparation

Rats were anaesthetized by intraperitoneal injection of a mixture of ketamine: xylasine 90 mg/kg: 10 mg/kg, respectively (Rameshrad et al., 2016). Thoracic aorta was immediately ectomized, set in a cold Krebs solution and cleaned from surrounding adipose and connective tissues, then cut into 4-segments, each about 2-2.5mm in length.
2.4: Vascular reactivity measurement

Two metal rods were passed through the lumen of the rings, one of the hooks was fixed to the bottom of organ bath chamber, and the other was connected to the force transducer through a thread to record vasoreactivity in organ bath (Automatic organ bath-Pan lab, Harvard apparatus USA, AD Instrument Power lab 8/35-Australia) that is filled with 10-ml of Krebs bicarbonate solution in (mM/L NaCl,119; MgSO₄, 1.2; CaCl₂, 1.5; NaHCO₃; KCl, 4.7; KH₂PO₄ 1.2; glucose 11.1. The solution in the organ bath was kept up at 37 °C and aerated with a mixture of about 95% O₂ and 5% CO₂. After 60 to 90 minutes of equilibration, each aortic rings was exposed to KCl (60mM) as a viability test. Each ring was washed with a fresh medium and re-equilibrate for at least 30 minutes before adding any substances.

2.5: Experimental protocol

Prepared aortic rings have been used to evaluate the possible contribution of some substances to vascular action of BQ-123 and ACh. Two sets of experiments were carried out as follows:

**Experiment 1:** The prepared aortic rings were incubated for 45 minutes with LA (1µM), BH₄ (100Mm), and their combination, then pre-contracted by ET₁ (0.01µM), and after stabilization of contractile response. The BQ-123 relaxation -response curves were generated (from 10⁻¹¹ to 10⁻⁶ M). The relaxations are expressed in percent of pre-contractile tone

**Experiment 2:** After pre-incubation of aortic with LA (1µM), BH₄ (100Mm), and their combination for 45 minutes, rings were pre-contracted by ET₁ (0.01µM). Endothelium-dependent relaxations were determined by application of ACh (from 10⁻⁹ to 5* 10⁻⁴ M). The relaxations are expressed in percent of pre-contractile tone.

2.6: Statistical analysis

All results are presented as means ± standard error of the mean (SEM). Statistical analysis was carried out by GraphPad Prism (Version 7), the concentration-response curves of BQ-123 and ACh were fitted nonlinearly. Two-way analysis of variance (ANOVA) was applied to know the difference, followed by Sidak multiple comparison tests as an individual mean comparison, and Tukey-test as the potency difference (pD₂) and maximum response (Emax) comparison between groups with each other. Values were considered to be statistically significant at P < 0.05.

3. RESULTS

The result showed that pre-incubation of aortic rings with LA, BH₄, and the combination of LA and BH₄ significantly improved the relaxation that was evoked by BQ-123 and ACh.

**Table 1:** The maximum response (Emax) and the potency difference (pD₂) to BQ-123 in rat aortic rings

| Groups     | N  | Emax %KCL | pD₂    |
|------------|----|-----------|--------|
| Control    | 7  | 21.64 ± 2.892 | -8.156 ± 0.100 |
| LA         | 6  | 18.57 ± 2.854 | -8.797 ± 0.1277*** |
| BH₄        | 6  | 14.89 ± 1.786 | -8.512 ± 0.06505 |
| LA+BH₄     | 6  | 10.51 ± 1.967* | -8.9214 ± 0.07798*** |

The studied groups were compared with each other (ANOVA was applied with Tukey test). *, **, *** represents statistical differences at P<0.05, P<0.01 P<0.001 versus the corresponding control group, and □ Significant differences between groups vs BH₄ group at p < 0.05.

3.1: The effect of NO precursor on vascular response to BQ-123

To find out the role of NO precursor in vascular response to BQ-123, LA was used. Figure (fig.) 1A showed that LA noticeably magnified the relaxant effect of BQ-123 with a significant change in BQ-123 potency, however, BQ-123 efficacy remained significantly unchanged table 1.
3.2: The effect of BH$_4$ on vascular response to BQ-123

To investigate the effect of NOS cofactor, BH$_4$ was applied. The results showed that the altitude of the vascular response to BQ-123 was significantly unchanged in the presence of BH$_4$.

![Graph A](image1.png)

**Figure 1.** Concentration-response curves showing relaxation induced by BQ-123 in rat thoracic aortic rings under control condition and after pre-incubation with (A) LA, (B) BH$_4$, and (C) the combination of LA and BH$_4$. The relaxations are expressed in per cent of the pre-contracted tone induced by 0.01 µM ET-1. The asterisks; *, **, *** represents statistical differences at *P<0.05, *P<0.01, *P<0.001 versus the corresponding control group.

3.3: The combined effect of LA and BH$_4$ on vascular response to BQ-123

The data analysis of the present study showed a synergistic role of LA and BH$_4$ in altering the vascular reactivity of BQ-123 to left. As it can be seen in table 1 LA and BH$_4$ in the combination showed the significant difference in BQ123 potency and efficacy. Furthermore, combination of LA and BH$_4$ has a greater impact than each substance alone.

**Table 2:** The maximum response (E$_{max}$) and the potency difference (pD$_2$) to ACh in rat aortic rings.

| Groups      | N | E$_{max}$ (%KCL) | pD$_2$     |
|-------------|---|------------------|------------|
| Control     | 7 | 36.83 ± 1.732    | -6.867 ± 0.07907 |
| LA          | 5 | 12.99 ± 1.932*** | -7.087 ± 0.06988 |
| BH$_4$      | 5 | 15.25 ± 2.071*** | -6.913 ± 0.073 |
| LA+BH$_4$   | 5 | 2.761 ± 2.154*** | -7.132 ± 0.07023 |

The studied groups were compared with each other (ANOVA was applied with Tukey test). *** represent statistical differences at *P<0.001 versus the corresponding control group, # Significant differences between groups vs LA group at *P < 0.05, and ¤¤ Significant differences between groups vs BH$_4$ group at *P < 0.01.

3.4: The effect of NO precursor on vascular response to Ach

The present study showed that using LA as NO precursor significantly increased ACh efficacy while ACh potency did not change as shown in fig. 2 and table 2.

3.5: The effect of BH$_4$ on vascular response to Ach

To scrutinize the effects of BH$_4$ on ACh vascular actions and the possible role of eNOS, BH$_4$ (100µM) was used. The result showed that BH$_4$ noticeably magnified the vasorelaxant response to ACh with significant changes in efficacy although, ACh potency remained significantly unchanged (table 2).
3.6: The combined effect of LA and BH$_4$ on vascular response to Ach

To determine the influence of LA and BH$_4$ in combination on the vascular response of aortic rings to Ach. The Ach reactivity following after 45 min of LA and BH$_4$ pre-incubation and ET-1 pre contraction was significantly elevated the maximum response significantly rise (table 2), While Ach potency was stable.

Figure 2. Concentration-response curves showing relaxation induced by Ach in rat thoracic aortic rings under control condition and after pre-incubation with (A) LA, (B) BH$_4$, and (C) the combination of LA and BH$_4$. The relaxations are expressed in per cent of the pre-contracted tone induced by 0.01 µM ET-1. The asterisks; *, **, *** represents statistical differences at P<0.05, P<0.01 P<0.001 versus the corresponding control group.

4. DISCUSSION

The major findings of this study illustrated that LA and BH$_4$ significantly increased the relaxation effect of both BQ-123 and Ach. The current results demonstrate that pre-incubation of ET-1 in contact aortic rings cause activation of the ET-1A receptor stimulates phospholipase C to IP$_3$ and DAG from phosphatidylinositol 4,5-bisphosphate. IP$_3$ induces Ca$^{2+}$ outflow from intracellular stores in the sarcoplasmic reticulum. Furthermore, the ET-1A receptor acts on nonselective plasmalemmal Ca$^{2+}$ channels causing Ca$^{2+}$ input from the extracellular space. Consequently, increased concentrations of Ca$^{2+}$ leads to the contraction of VSMCs. The activated ET-1A receptor also stimulates cell growth. Production of DAG activates protein kinase C, which increases VSMCs contraction (Hynynen and Khalil, 2006, Lima et al., 2010, Khalil, 2011). Aortic rings that pre-contracted by ET-1 followed by BQ-123 as dose-response curves (DRC) caused the attenuation of vascular reactivity of ET-1.

From the result of this study, it could be concluded that BQ-123 produces blood vessel dilation via blocking ET1A receptor and subsequently preventing calcium release from the sarcoplasmic reticulum and entering calcium to the cell (Callera et al., 2003). Briyal, who is a researcher, detected that ET-1 induces the synthesis of superoxide anion through ET-1A receptors that cause lipid peroxidation (Briyal et al., 2011). BQ-123 pre-incubation in arteries and veins significantly block the increased production of superoxide in ET-1-induced oxidative stress (Cerrato et al., 2012). Another possible mechanism by which BQ-123 causes vascular relaxation is that BQ-123 associated with a significant increase in the concentration of total glutathione and superoxide dismutase (SOD) activities (Briyal et al., 2011). Over all, it can be concluded that ET-1A receptor blockade will improve the function of the aorta (Tirapelli et al., 2008). Blockade of ET-1A receptor allows ET-1B receptor to release more NO in the endothelial cells and hence, vasorelaxation.
Besides the aforementioned effect of BQ-123, as obtained from the present result, pre-incubation of LA increased the potency of BQ-123. In addition, LA significantly shifted BQ-123 DRC to left. LA is only substrate in the biosynthesis of NO, and NO plays an important role in the diverse physiological process including vasorelaxation through cyclic guanosine monophosphate (cGMP) pathway. In addition, BH4 pre-incubation did not change in DRC of BQ-123 compared to such changes occurred by LA pre-incubation.

The present investigation showed that pre-incubation of rat aortic rings with LA and BH4 in combination caused the marked increase in BQ-123 efficacy and potency. Schreiber et al. (2017) recorded that LA is the main substrate in the production of NO. Binding BH4 exert allosteric action to stabilize the active dimeric form of eNOS (Shinozaki et al., 2000) and increases the enzymatic turnover of LA. Jiang et al. (2000) demonstrated that combined pre-incubation of LA and BH4 induced a pronounced enhancement of ACh-induced relaxation.

Several mechanisms have been suggested to explain how ACh produces relaxation in rat aortic rings. ACh exerts a direct effect on vascular tone by binding to muscarinic receptors present on vascular endothelium, and hence, the activation of eNOS and prostaglandin production (Kellogg et al., 2005) and subsequently NO mediates relaxation through activation of potassium channels (Salihi et al., 2016). The current results showed that LA pre-incubation increased the efficacy of ACh significantly as shown in table 2 (fig.2A), because by the activity of eNOS change to NO and L-citrulline, as previously we mentioned that increasing of NO increase vasorelaxant effect of ACh (Tratsiakovich et al., 2013a). In the same manner, pre-incubation of BH4 increased ACh maximum response because of its cofactor for NO production and protective eNOS as an active dimer as shown in fig.2B as previously reported by Jiang et al. (2000). Interestingly, pre-incubation of LA and BH4 in combination in high significant shifted the DRC of ACh to the left (fig.2C), and this finding indicates that LA and BH4 in combination has a greater effect on ACh relaxation than each substance alone that is co-coordinated with Gunnett et al. (2005).

5. CONCLUSIONS

The present results demonstrated that pre-incubation of LA and BH4 alone and in their combination shift the BQ-123 DRC to left. It can be suggested that the above agents reduce the ET-1A activity. These effects are markedly changed by LA and BH4 combination then each substance alone. The results also indicated that the relaxation response curve of ACh that depends on NO, and prostacyclin pathways increased by LA and BH4 in combination. In summary, LA and BH4 may offer some pharmacological tools to modulate the ET-1A receptor activity and treat cardiovascular disease beyond pulmonary arterial hypertension.

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Conflict of Interest

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

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