J. Afr. Ass. Physiol. Sci. 8 (1): 8-15 July 2020

Journal of African Association of Physiological Sciences

Official Publication of the African Association of Physiological Sciences
http://www.jaaps.aapsnet.org

Research Article

TxA_2 Receptor Antagonist (SQ29548) attenuates Endothelium-Independent recovery from Thromboxane A_2 Contraction of Isolated Rabbit Aorta

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Keywords:
Thromboxane A_2, mimetic, receptor antagonist, smooth muscle, endothelium, nitric oxide

ABSTRACT

Background: Thromboxane A_2 (TxA_2) is a potent constrictor of blood vessels and has been implicated in the pathogenesis of some cardiovascular diseases. This study examined an intervention that can be used to displace the tight binding of thromboxane mimetic, U46619 to the thromboxane (TP) receptors thereby bringing about a faster relaxation. We hypothesized that the prolonged contraction of U46619 stimulated VSM could be due, in some part, to a tight binding between U46619 and the TP receptor, leading to continual activation of the receptor. **Methods:** Aortic rings were obtained from euthanized rabbit (n = 28) and placed in an organ bath system with temperature maintained at 37^{O}C and contractile responses to the thromboxane mimetic, U46619 (0.5 µM) measured. Following a maximum contraction, SQ29548 was added, and the vessels allowed to relax to baseline. We then investigated whether the displacement of U46619 and faster relaxation brought about by SQ29548 was dependent on the release of NO from the endothelium. **Results:** Following treatment with SQ29548 (3.0 µM) the aortic vessels relaxed at a significantly higher rate (0.23 ± 0.04 g/min) compared to the vehicle-treated vessels (0.03 ± 0.01 g/min) ($P < 0.0001$). Vessels treated with an inhibitor of NO production (L-NAME) or vessels where the endothelium was mechanically removed showed the same response to rate of relaxation as vessels treated with the vehicle or vessels in which the endothelium was not denuded ($P = 0.93$ and $P = 0.38$). **Conclusion:** Focusing on strategies to speed up relaxation of a contracted vessel adds to the significance of this work. Results from our experiments suggest that administration of SQ29548 may be useful in relaxing a vessel that is already contracted by TxA_2, for example, a vessel in spasm and also in an atherosclerotic vessel in which NO the relaxing factor in vascular smooth muscle has been affected.

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INTRODUCTION

Thromboxane A_2 (TxA_2) is one of the endogenously occurring vascular smooth muscle contracting and platelet activating substances (Orr *et al.*, 1993; Leung *et al.*, 2010; Wacker *et al.*, 2012). It also induces contraction of smooth muscles in the airways of the lungs (Liu *et al.*, 1997). Thromboxane A_2 (TxA_2) has been implicated in some cardiovascular abnormalities, such as hypertension (Davi and Patrono, 2007 and Mckenzie *et al.*, 2009). It produces its contractile properties by binding to TP receptor. The TP receptor is a protein that is encoded by the *$TBXA_2R$* gene in humans and it is one among the five classes of prostanoid receptors (Devillier and Bessard, 1997). It was the first eicosanoid receptor cloned (Rolin *et al.*, 2006), deriving its name from the endogenous ligand thromboxane A_2 and it is G-protein-coupled (McKenzie *et al.*, 2009 and Marcio *et al.*, 2011).

One of the important physiological/pathophysiological actions of T_XA_2 is platelet activation, leading to platelet shape change, aggregation and secretion, which promote thrombus formation (Navarro-Nunez *et al.*, 2007). Thrombus formation mediated by TxA_2– induced platelet aggregation and vasoconstriction sometimes causes acute myocardial infarction and cerebral infarction, which are prevented by inhibition of TxA_2 synthesis and/or use of TP antagonists (Nakahata, 2008 and Davi *et al.*, 2012). One stable TP antagonist,

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SQ29548 is a synthetic compound that binds to, but does not activate TP receptor and thereby inhibits its activation by activating ligands. (Ogletree *et al.,* 1985). An earlier study (Ogletree *et al.,* 1998) has demonstrated an antagonistic effect of SQ29548 on TP receptor. Other studies have also reported that dysfunction of the local, humoral, and neurogenic regulation of the molecular pathways involved in thoracic aorta vascular smooth muscle contraction, has been shown to contribute to numerous pathologies, including hypertension and arterial stiffness (Lindesay *et al*., 2010, Leloup *et al*., 2015, Leloup *et al*., 2019). The imbalance between the prostanoids pathway and NO pathway has also been attributed to the development of some cardiovascular diseases (Davi *et al.,* 2012 and Silva *et al*., 2017).

In this study, we focused on a pharmacological intervention that could attenuate the rate of recovery from U46619 induced vascular smooth muscle contraction. We hypothesized that the prolonged contraction of U46619 stimulated VSM could be due, in some part, to a tight binding between U46619 and the TP receptor, leading to sustained activation of the receptor. By dissociating U46619 from the TP receptor using a receptor antagonist, the VSM would conceivably have a faster rate of recovery from sustained contraction induced by U46619 in both endothelium-intact (E+) and endothelium-denuded (E-) vessels. A TP agonist produced by the vascular endothelium, especially under pathological conditions can cause endothelium dependent contraction and thereby contribute to endothelial dysfunction observed in diseases such as hypertension and diabetes (Feletou *et al.,* 2010). TP antagonists are therefore of vital importance in counteracting these effects. SQ29548 is a synthetic compound that binds to, but does not activate TP receptor and thereby inhibits its activation by activating ligands. It is an antagonist that binds specifically to the TP receptor and blocks TxA_2 and U46619 from binding (Ogletree *et al.,* 1985 and Krauss *et al.,* 1996). Without the continual stimulation of the intracellular events that lead to calcium release and smooth muscle contraction, the vessels should relax more quickly. The binding affinity (K_i) of SQ29548 to the TP receptor in human platelet membranes is 7.34 nm, while the K_i of U46619 to the TP receptor in human platelet membranes is 17.7 nm (Hedberg *et al.,* 1988). Given this, the concentration of SQ29548 used (3.0 µM) should be sufficient to dissociate U46619 (0.5 µM) from the receptor and block the effects of the TxA_2 mimetic.

METHODS

New Zealand white rabbits (average wt. 3.75 kg, n= 28) of both sexes were euthanized according to Institutional Animal Care and Use Committee (IACUC) Protocol 42–02 of the University of Kansas, USA. Following euthanasia of the animal, the thorax was opened and a segment of the descending trunk of the aorta was excised, placed in a physiological salt solution. The aortic rings were cleaned of excess connective tissue and cut into four or six ring segments of 2-3 mm each and then suspended in organ baths (Radnoti, Monrovia CA, USA). The organ bath (25 ml) contained physiological buffer solution maintained at 37 ^oC that was aerated with a gas mixture of 95% O_2 and 5% CO_2. The aortic rings were suspended from a stationary hook within the organ bath and connected to a force transducer that recorded the tension generated by the vessels. Tension was calibrated prior to each experiment with a known force (5 g). The contractions were recorded with the use of the data acquisition software power lab (AD Instruments, Colorado Springs, CO, USA). The aortic vessels were allowed to equilibrate for 60 minutes in the physiological salt solution at a tension of approximately 2 g. With the exception of exposure to high KCl (60 mM), all drugs were added directly to physiological salt solution of the organ bath. Prior to the initial treatment with high KCl, the tension on the vessels was maintained at 2 g in order to provide a stable tonic baseline tension to the aortic rings. Following treatment with either high KCl or U46619, sufficient time was allowed so that the tension of vessels returned back to the baseline value of 2 g. Two series of experiments were carried out:

Series 1: Experiments were designed to examine the relaxation of intact vessels following contraction to the thromboxane A_2 mimetic (U46619). After equilibration with the physiological salt solution, the tension on the vessel was adjusted to 2 g and the vessels were exposed to a solution containing high KCl (60 mM) to establish a baseline contraction. The organ bath was then drained of all fluid and replaced with physiological salt solution. Following relaxation of the vessels to baseline, they were stimulated with the thromboxane A_2 mimetic U46619 (0.5 µM). Following a steady maximal contraction, SQ29548 or a vehicle (DI H_2O) was added to the respective organ baths and the ensuing relaxation of vessels to baseline tension recorded.

Series 2a: A second series of experiments was designed to examine the response of U46619 relaxation following treatment with the TxA2 receptor antagonist (SQ29548 3.0 uM) in the presence (E+) and absence (E-) of the endothelium. Denudation of the endothelium was achieved with forceps and Q-tips. After the mechanical removal of the endothelium, the vessels were equilibrated in physiological salt solution for 60 min

after which the physiological salt was replaced with a physiological buffer solution containing 60 mM KCl and the contractile response to high KCl solution was recorded. To evaluate the involvement of TxA_2 mimetic, TxA2 receptor antagonist and the endothelium, at the peak of the contractile response to U46619 the aortic rings were exposed to SQ29548 (3.0 μM), TxA2 receptor antagonist , until the vessels reached the base line. SQ 29548 relaxation was calculated as a difference in rate of relaxation per minute. In order to verify the efficacy of removal of the endothelium or treatment with the NO synthase inhibitor the vessels were then allowed to return to base line tension, after which they were contracted by PE (1.0 μM) and then relaxed by Ach (1.0 μM).

Series 2b: The final series of experiments were designed to examine if the inhibition of NO synthesis altered the effect of SQ29548 on the relaxation phase following a TxA_2 induced contraction. NO synthesis was inhibited in the endothelium using L-NAME, a specific inhibitor of nitric oxide synthesis. After the initial incubation of the blood vessel rings in L- NAME (100 μM) for 30 min, U46619 (0.5 μM) was then added to the buffer solution and the vessels were then allowed to contract to a maximum level. At the peak of the contractile response to U46619, the aortic rings were exposed to SQ29548 so as to evaluate the association of TxA_2 mimetic, TxA_2 receptor antagonist and production of NO. After the vessels reached the baseline, the solution containing SQ29548 was replaced with physiological salt solution. SQ29548 relaxation was calculated as a difference in rate of relaxation per minute.

Administration of PE, followed by Ach, was used to test the efficacy of L-NAME with regards to inhibition of NO synthesis. After all vessels had relaxed to 2 g, the vessels were treated with final high KCl and allowed to maximally contract.

Statistical analyses

Data were collected by using chart for Windows (ADInstruments). The contraction in response to the initial high KCl (60 mM) was designated arbitrarily as 100% and all subsequent contractions were compared to this contraction. This procedure allowed us to standardize data from vessel segments of different sizes and reactivity. Data were expressed as mean ± standard error of mean (SEM). Unpaired t-tests were also used to compare the differences in the contraction and relaxation of aortic vessels in the presence and absence of the endothelium. SigmaStat 3.5 for Windows (Systat Inc., San Rafael, CA) was used for the data analyses and graphs were drawn using Microsoft Excel 2016. One-way analysis of variance (ANOVA) was performed to determine the statistical significance between the relative strength of contraction of drug treated versus control vessels. A value of $P < 0.05$ was considered statistically significant.

Drugs and solutions

The composition of physiological buffer was (mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, and dextrose 11, and pH was adjusted to 7.4 to mimic physiological conditions.

The composition of 60 mM KCl physiological buffer solution was (mM): NaCl 63.1, KCl 60, $CaCl_2$ 2.5, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, and dextrose 11.

U46619 (9,11-dideoxy-9α,11α-methanoepoxy-prosta-5Z,13E-dien-1-oic acid), L – NAME (N^{ω}-nitro-L-arginine methyl ester hydrochloride) and TP antagonist SQ 29548 ([1S-[1α,2α(Z),3α,4α]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid) were purchased from Cayman Chemical (Ann Arbor, MI, USA). PE (L-Phenylephrine hydrochloride) and Ach (Acetylcholine hydrochloride) were purchased from Sigma-Aldrich (St Louis, MO, USA). U46619 was dissolved in 100% ethanol and further diluted with normal saline to a concentration of 5μg/mL. L–NAME 20 mg was dissolved in 20 ml DMSO to a final concentration of 100 μM on the day of experiment. SQ29548 and ACh were both dissolved in deionized (DI) H_2O to a final concentration of 3.0 μM and 10 μM, respectively. PE was also dissolved in DI H_2O to a final concentration of 10 μM /ml

RESULTS

Representative tracings from aortic vessels exposed to various treatments and drug interventions are shown in Figs. 1-3. In Fig. 1, treatment with a strong depolarizing agent (high KCl) leads to constriction of the vessel. The vessel relaxes when the high KCl is drained from the organ bath and replaced with physiological salt solution (termed 'wash' on the x-axis).

A stronger contraction occurs when the vessel is treated with the TxA2 mimetic, U46619. However, when the buffer containing U46619 is removed and replaced with physiological salt solution, the vessel remains contracted and the time to baseline relaxation is significantly lengthened compared to the high KCl treatment (Fig.1 panel A). Quantifying the rate of relaxation of the vessels was accomplished by assuming a linear decrease in force from the initial removal of U-46619 from the bath to the return to baseline tension (2 g). This procedure is illustrated in Fig. 1, panel A. The rate of relaxation for this aortic vessel was calculated as 0.045

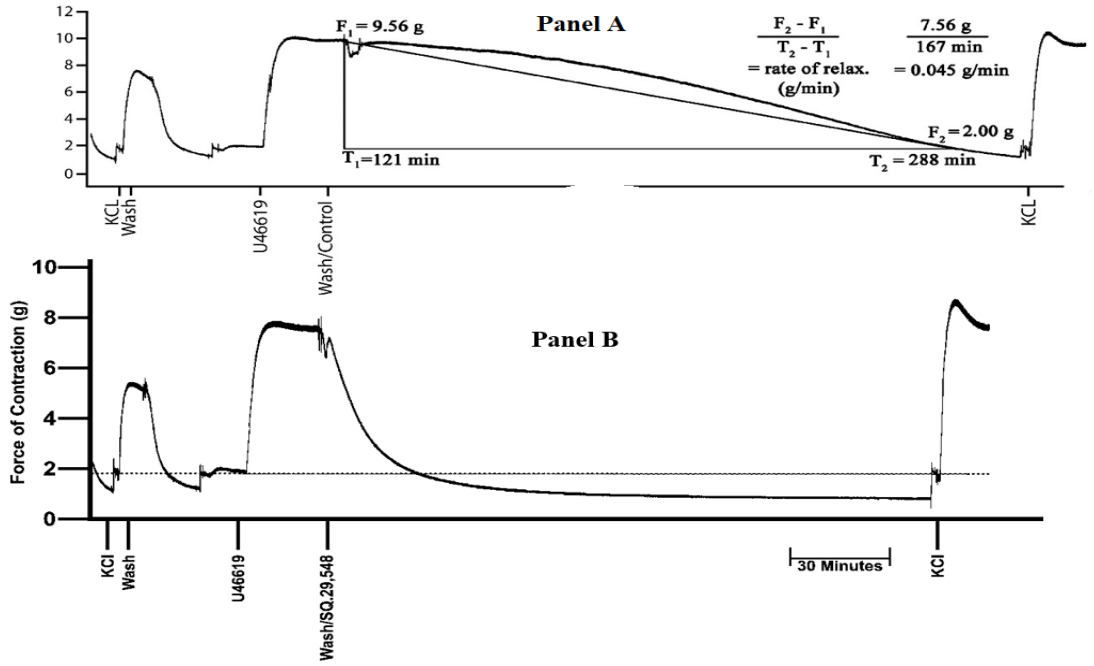

Fig.1: Panel A, A linear line was extrapolated from the point at which the vessel was treated with U46619 to the point at which the vessel reached 2 g of tension. A rate of relaxation was found by dividing the change in force (F) by the change in time (T). Panel B: Vessel treated with SQ29548 following treatment with U46619 (n= 6)

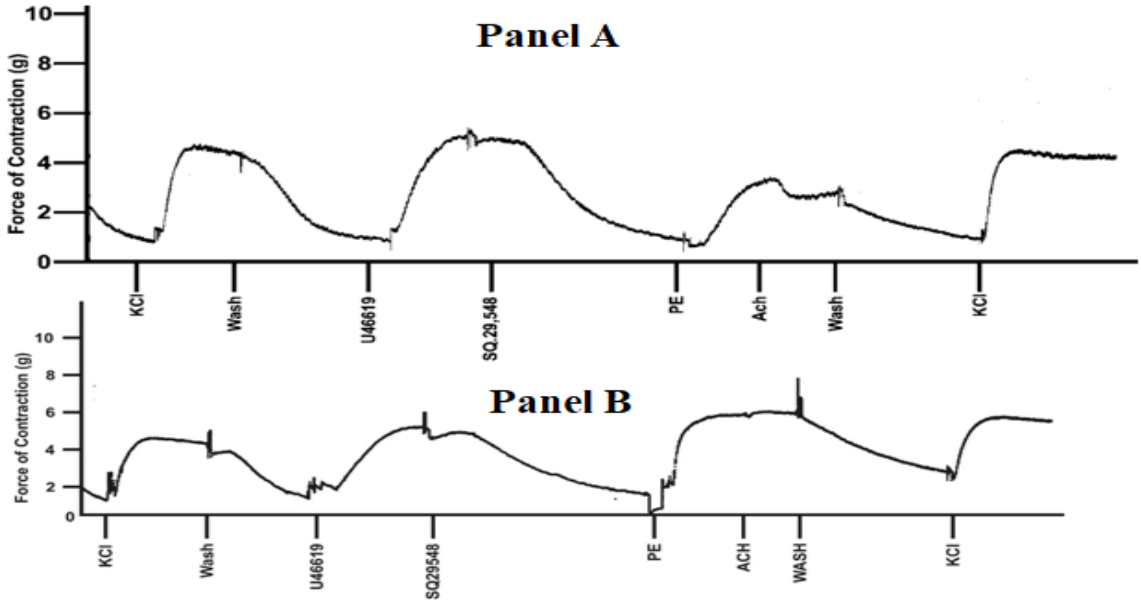

Fig. 2: Panel A, Relaxation responses induced by SQ29548 in isolated aortic vessels pre-contracted with U46619. Tracings are from endothelium-intact vessel, control E+ . Panel B, Endothelium denuded E- vessel (n = 11)

g/min. This procedure accounts for any differences in the steady state maximal contraction by U-46619 between vessel segments.

Aortic vessels treated with SQ29548 relaxed at a rate of 0.23 g/min (± 0.04) (Fig. 1 panel B and Fig. 4), while untreated vessels (control) relaxed at a slower rate of 0.03 g/min (±0.01) (Fig. 1 panel A and Fig 4) (P<0.0001: Fig. 4).

The absence of NO in the endothelium had no effect on the actions of SQ29548. The response of U46619 was sensitive to the binding of the receptor antagonist SQ29548 inhibition both in denuded aortic rings (Fig. 3; panel B) and in L-NAME treated aortic rings (Fig. 2;

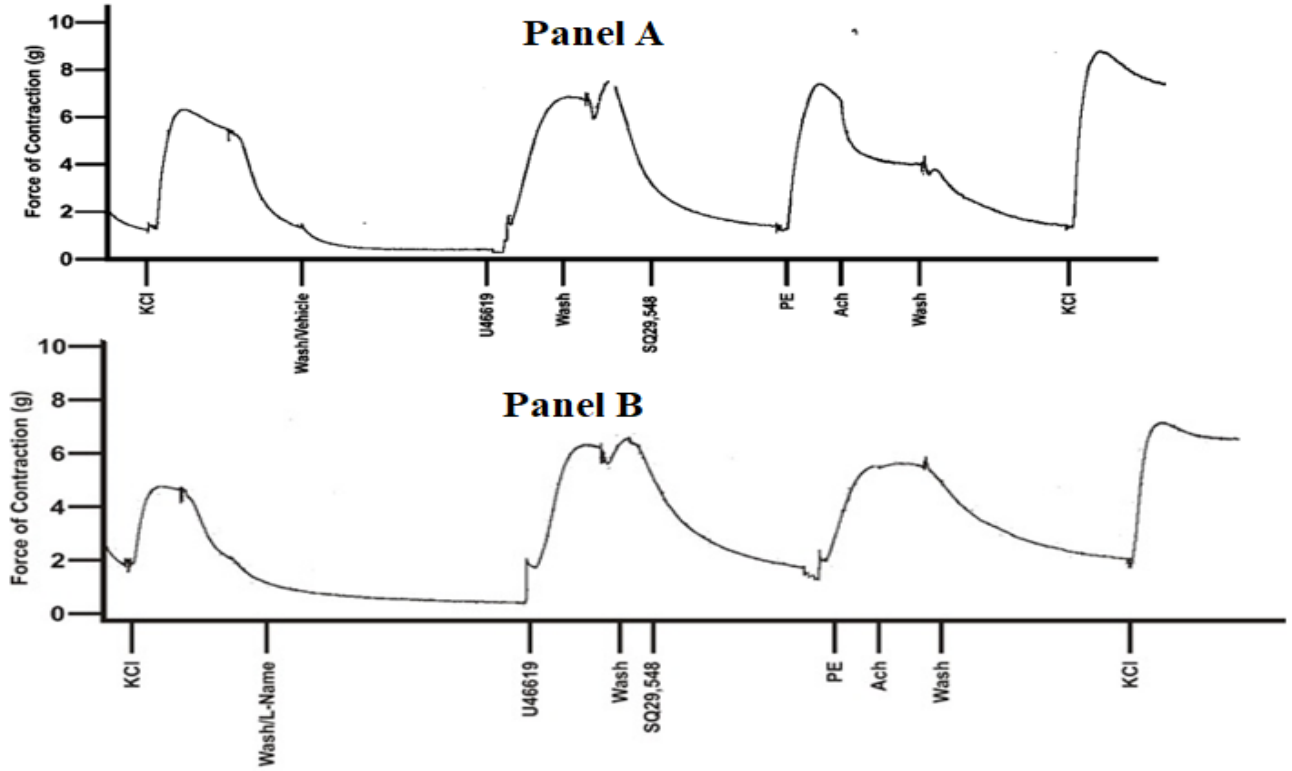

Fig. 3: Panel A, Tracings are from DMSO treated (vehicle for L-NAME) control E+; Panel B, L-NAME treated E- (n=11)

panel B). Relaxation caused by SQ29548 was not dependent on the presence or absence of endogenous NO, so there was no significant difference in the rate of relaxation between E+ and E- vessels both in endothelium-denuded vessels (P=0.38) and L-NAME treated aortic rings (P=0.93) (Fig. 5).

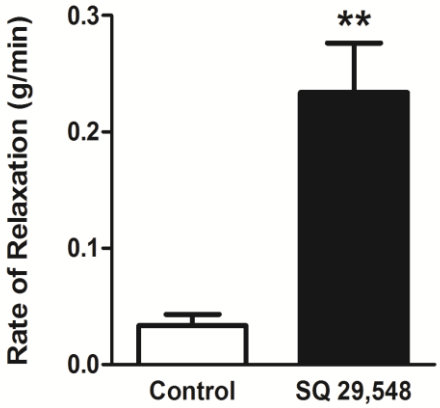

Fig. 4: The relaxation rate of treated (SQ29543= 3.0 µM) and untreated (control) vessels following treatment with U46619 (0.5 µM; n= 6).

When SQ29548 is administered, the final KCl contractions were not affected (Fig. 6; panel A). In both forceps denuded endothelium and L-NAME treated aortic rings, U46619, PE and the final KCl contractions were not attenuated in E+ vessels and E- vessels (Fig. 6; panel B and C).

DISCUSSION

Vascular smooth muscle cells (VSMCs) stimulated with U46619 remain contracted longer than VSMCs treated with other vasoconstrictive agents such as PE and high KCl (Neppl *et al.*, 2009 and Danborno *et al.*, 2018).

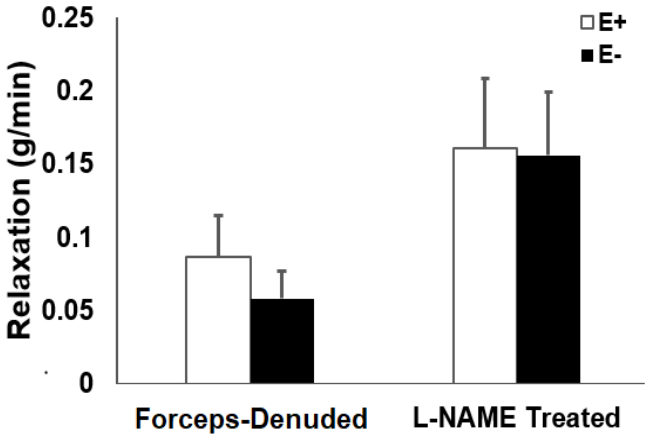

Fig. 5: The relaxation rate of forceps denuded vessels and L-NAME treated vessels following treatment with U46619 (0.5 µM). SQ29548 (3.0 µM) relaxation did not show significant difference (NS) between E+ and E- in both forceps denuded and L-NAME treated vessels (n= 22).

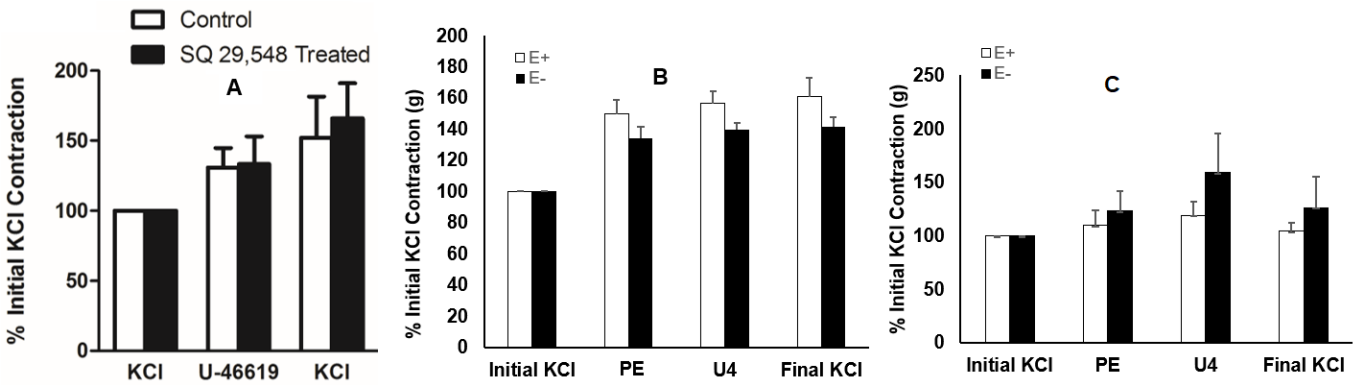

Fig. 6: The relative strength of contraction of vessels treated with KCl, U46619 and PE. Treatment with SQ29548 was given after exposure to the initial KCl, U46619 and PE and before exposure to the final KCl. All data are compared against the initial KCl contraction. A, B and C (respectively) denote endothelium intact (n= 6), endothelium denuded (n = 11) and L-NAME treated endothelium (n=11).

In our previous paper (Danborno *et al.*, 2018), we reported that inhibition of the Rho-kinase pathway with the Rho-kinase inhibitor Y-27632 shortens the relaxation phase of U46619 treated VSM. In these series of experiments, aortic vessels stimulated with U46619, also relaxed at a faster rate when treated with SQ29548 compared to the untreated vessels, $P< 0.0001$ (Fig. 4). This observation was also reported by Ogletree *et al.* (1985) and Krauss *et al.* (1996). Our data support the hypothesis that the use of the TP receptor antagonist, SQ29548, would be an effective method for increasing the rate of relaxation of TxA_2 mimetic-stimulated VSM. Moreover, because the use of the receptor antagonist SQ29548 should prevent U46619 from binding to the receptor, our data suggests that persistent binding of U46619 to the TP receptor is at least partially responsible for the prolonged contraction of U46619-stimulated VSM.

Endogenous NO is the main relaxing factor in vascular endothelium. However, in situations where the endothelium can no longer produce NO due to pathological conditions, there is need for finding ways to bring about relaxation after an induced contraction by TxA_2 which is usually persistent. It is pertinent to find an antagonist that can not only be selective and efficient, but also safe. This led us to hypothesize that NO aids the faster relaxation brought about by SQ29548. In order to test our hypothesis, in a separate set of experiments, the endothelial effect was removed either by using forceps to denude the endothelium (Liu *et al.*, 2009), or using L-NAME to inhibit the synthesis of NO by the endothelium (Kopincova *et al.*, 2012 and Danborno *et al.*, 2018). Following treatment with U46619, our most interesting finding was that, no significant differences was observed in the rate of recovery between endothelium-intact vessels (E+) and endothelium-denuded or L-NAME treated vessels (E-) (Fig. 5). This suggest that the elimination of NO did not affect the actions of SQ29548 in displacing the binding of U46619 from the TP receptors and bringing about a faster relaxation.

The vessels were subjected to KCl contractions so as to give reference to the other contractile agents. High KCl is a strong depolarizing agent while PE is an exogenous drug that stimulates the α-1 adrenergic receptor and contracts smooth muscle with a cellular transduction mechanism that is similar to TxA_2 (Somlyo and Somlyo 2000 and Fukata *et al.*, 2001). The depolarization in VSMCs caused by high KCl induces L-type voltage-dependent Ca^{2+} channels to open on the surface of vascular smooth muscle cells, allowing extracellular Ca^{2+} to enter the cell. Resulting increases in intracellular Ca^{2+} concentration can activate other second messengers such as protein kinase C (Woodsome *et al.*, 2006). We should point out however that when vessels were treated with high KCl in these experiments, the vessels relaxed quickly after KCl was removed from the organ bath. Interestingly, we observed that the strength of the final KCl contractions were higher than the initial KCl contractions, this suggest that not only were the vessels active throughout the period of the experiment but also that SQ29548 did not have effect on the contractile actions of KCl in VSM. Further work is needed to define the mechanism for these observations.

CONCLUSION

Cardiovascular diseases result in more deaths worldwide than any other single factor. Because of this, it is important to investigate mechanisms that may prevent its detrimental outcome. Thromboxane A_2 has been implicated as a factor leading to some cardiovascular disease, its attenuation should be investigated as a possible way to reduce heart disease-related deaths. This study presented evidence that agents capable of attenuating the rate of recovery from TxA_2 sustained

contraction could have beneficial effects in the management of cardiovascular diseases.

ACKNOWLEDGEMENTS

We are grateful to Prof. Barnabas Danborno of the Department of Anatomy, Faculty of Basic Medical Sciences, Ahmadu Bello University, Zaria for data analyses and Mr. Joseph Bamidele of Ndahi Printing Press, Zaria for improving the LabChart tracings.

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