

Research Article

The effect of a Rho-kinase inhibitor (Y-27632) on the relaxation of thromboxane a₂ mimetic (U-46619) induced contraction in the presence and absence of the endothelium

A.M. Danborn^{1,2}, P.L Monnet^{2,3} and J.A. Orr²

Departments of ¹Physiology, ²Molecular Biosciences, ¹Faculty of Basic Medical Sciences, Bingham University, Karu, Nigeria, ²University of Kansas, Lawrence KS, 66045 and ³Truman Medical Center, University of Missouri-Kansas City, Department of OBGYN, 2301 Holmes Street, Kansas City, MO 64108, USA

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ABSTRACT

Background: These experiments investigated mechanisms responsible for the slow relaxation of aortic rings following contraction elicited by the thromboxane mimetic, U46619. In this study we hypothesized that blocking only the Rho-kinase pathway with the Rho-kinase inhibitor (Y-27632) will shorten the extended relaxation time induced by U46619 and that production of NO contributes to the relaxation of blood vessels treated with the Rho kinase inhibitor. **Methodology:** Aortic rings were obtained from a euthanized rabbit and placed in heated organ baths and contractile responses to the thromboxane mimetic, U46619 measured. After removal of the endothelium or inhibition of NO production, we subjected the U46619 induced vessels to the Rho kinase inhibitor (Y-27632), so as to determine if Y-27632 causes relaxation of U46619 induced vessels by acting through NO, which is the relaxing factor in the endothelium. Vessels exposed to U46619 relaxed at a significantly slower rate compared to other agonists such as phenylephrine (1.0 μM) or high KCl solutions (60 μM). **Result:** Inhibition of Rho-kinase significantly reduced the relaxation time; i.e., the rate of relaxation was higher (0.13 ± 0.07 g/min) in the presence of a Rho kinase inhibitor (Y-27632; 1 μM) compared to vehicle-treated vessels (0.02 ± 0.01 g/min) (P < 0.05). We then investigated whether the enhanced rate of relaxation following inhibition of Rho kinase was dependent on the release of NO from the endothelium. Vessels treated with an inhibitor of NO production (L-NAME) or vessels where the endothelium was mechanically removed showed the same response to inhibition of Rho-kinase as vessels treated with the vehicle of L-NAME or vessels in which the endothelium was not denuded. **Conclusion:** We conclude that Rho kinase plays an essential role in sustaining the contractile phase of vessels treated with the Tx_{A2} mimetic U46619. The faster rate of relaxation of vessels following inhibition of Rho-kinase does not involve release of NO from the endothelium.

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Introduction

Thromboxane A₂ (TXA₂) is an arachidonic acid (AA) metabolite with a half-life of about 30 seconds. It is known to stimulate both platelet aggregation and smooth muscle contraction (Orr *et al.*, 1993; Leung *et al.*, 2010; Wacker *et al.*, 2012). The TP receptor (thromboxane receptor) within the plasma membrane of smooth muscle is a G-protein coupled receptor (Hirata *et al.*, 1991; Liu *et al.*, 2009) that, when stimulated, the

contraction of vascular smooth muscle (VSM) (De Godoy and Rattan 2011; Fu *et al.*, 1998; Fukata *et al.*, 2001). RhoA is a member of small GTP-binding proteins, which activates a serine/threonine protein kinase, Rho Kinase or ROCK. Although ROCK I and ROCK II are both expressed in smooth muscle cells (SMCs), it is well established that ROCK II is mostly involved in SMC contraction which is based on inhibition of the 20-kDa myosin light chains (MLC₂₀) dephosphorylating myosin light chain phosphatase (MLCP) which results in inhibition of MLCP via phosphorylation of CP1-17 at threonine-38 (Thr³⁸) residue (p-CP1-17) thereby sustaining the contractile

*Address for correspondence:

Email angeladanborn@yahoo.com

Tel: +234-8060038527

phase and delaying the relaxation of VSM (Kureisha *et al.*, 1997; Hartshorne *et al.*, 1998; Koyama *et al.*, 2000; Sakurada *et al.*, 2001; Smolock *et al.*, 2007; Wang, 2009).

Endothelial dysfunction can be defined broadly as an imbalance between endothelium derived relaxing factors (EDRF), such as nitric oxide (NO) and endothelium derived constricting factors (EDCF), it is a systemic pathological state of the endothelium (Iwatani *et al.*, 2008; Yao *et al.*, 2010). It can also be defined as an impairment of endothelium dependent vasorelaxation caused by a reduction or loss of nitric oxide (NO) bioactivity in the vessel wall (Cai and Harrison, 2000).

It is important to know the effect of thromboxane A₂ in diseases where the functions of the endothelium or the endothelium dependent relaxing factor (NO) are compromised and to determine if blocking the signal transduction pathway of TXA₂ would hasten the relaxation of the blood vessel, because TXA₂ is one of the most potent endogenous vasoconstrictors. Some earlier studies suggest that manipulation of RhoA/ROCK 2 activity through inhibiting its pathway with different inhibitors can prevent endothelial dysfunction (Yao *et al.*, 2010) and could represent a new therapeutic approach for various cardiovascular diseases (Satoh *et al.*, 2011). It has also been shown that Rho kinase is also important in regulating the production of NO in endothelial cells (Calvin *et al.*, 2009). But whether Y-27632 causes relaxation in U46619 induced contracted VSM through NO has not been fully determined. So in a case of an already existing endothelial dysfunction or reduced biosynthesis of NO, is there any significance in using the Rho kinase inhibitor to inhibit the RhoA/ROCK pathway in VSM? because this pathway is responsible for the maintained contractions induced by U46619 in VSM. Accordingly, this study was designed to determine if Y-27632 causes relaxation of U46619 induced vessels by acting through NO, which is the relaxing factor in the endothelium.

METHODS

New Zealand white rabbits (average wt. 3.75 kg, n= 38) of both sexes were anesthetized and 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 buffer solution and held overnight at 2–4 °C. Following overnight refrigeration, the vessels 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 warmed to 37 °C that was aerated with a gas mixture of 95% O₂ and 5% CO₂. The vessel segments were suspended from a stationary hook within the organ bath and connected to a force transducer that measured 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 vessels were allowed to equilibrate for 60–90 minutes in the physiological buffer solution at a tension of approximately 1 g. With the exception of exposure to high KCl (60 mM), all drugs were added directly to physiological buffer solution of the organ bath. Prior to the initial treatment with high KCl, the tension on the vessels was increased to 2 g in order to provide a stable tonic baseline tension to the vessels. 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. If the tension on a vessel fell below 2 g, the tension was manually increased in order to achieve the desired baseline tension (2 g) before administering the next contractile agent. However, in no case was the tension on a vessel lessened manually in order to attain that baseline value. Three series of experiments were carried out:

Series 1:

Experiments were designed to examine the relaxation of intact vessels following contraction in response to the thromboxane A₂ mimetic (U46619). After equilibration with the physiological buffer, the tension on the vessel was adjusted to 2 g and the vessels were treated with 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 buffer. Following relaxation of the vessels to a tension of 2 g, they were treated with the thromboxane A₂ mimetic U46619 (0.5 μM). Following a steady maximal contraction, approximately 5 to 10 min after adding U46619 to the organ baths, the organ baths were again drained of all fluid and replaced with physiological buffer. Immediately after refilling the baths with physiological buffer, Rho-kinase inhibitor Y-27632 (1.0 μM) or a vehicle (DI H₂O) was added to the respective organ baths and the ensuing relaxation of vessels to 2 g of tension recorded.

Series 2:

This series of experiments was designed to study the influence of the endothelium and NO on the relaxation

of vessels contracted with either the TXA₂ mimetic or phenylephrine (PE). Following the equilibration of the vessels in physiological buffer, the tension on the vessel was adjusted to 2 g and the vessels were treated with a solution containing high KCl (60 mM) to establish a baseline contraction. The endothelium was mechanically removed using forceps or NO synthesis was inhibited using L-NAME (100µM). For the L-NAME treated vessels, the vessels were incubated in L-NAME for 30 minutes, while the control vessels were incubated in the vehicle (DMSO) for the same period of time. PE (1.0 µM) and U46619 (0.5 µM) were administered separately, the aortic vessels were allowed to contract and then relax without any interference.

Series 3a:

A third series of experiments was designed to examine the response of U46619 relaxation following treatment with the Rho-kinase inhibitor (Y-27632 1.0µM) in the presence (E+) and absence (E-) of the endothelium denuded with forceps and Q-tips. After the mechanical removal of the endothelium, the vessels were equilibrated in regular buffer for 60-90 mins after which the regular buffer was replaced with a solution containing 60 mM KCl and the contractile response to KCl solution was recorded. 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 in the regular buffer, after which they were contracted by PE (1.0 µM) and then relaxed by Ach (1.0 µM). At the peak (maximal contraction) of the contractile response to U4 the solution containing U4 was then replaced with physiological buffer. To evaluate the involvement of TXA₂ mimetic, Rho-kinase pathway and the endothelium, the vessels were exposed to Y-27632 (1.0 µM), the Rho kinase inhibitor, until the vessels reached the base line. Y-27632 relaxation was calculated as a difference in rate of relaxation per minute.

Series 3b:

The final series of experiments was designed to examine if the inhibition of NO synthesis altered the effect of Y-27632 on the relaxation phase following a TXA₂ induced contraction. Nitric oxide 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 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 U4, the solution was replaced with regular

buffer and the vessels were exposed to Y-27632 so as to evaluate the association of TXA₂ mimetic, Rho-Kinase pathway and production of NO. After the vessels reached the baseline, the solution containing Y-27632 was replaced with regular buffer, and PE was used to contract the rings with or without endothelium and then relaxed by Ach. Y-27632 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 grams, 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 (60mM) was designated arbitrarily as a contraction equivalent to 100% and all subsequent contractions were compared to this KCl induced contraction. This procedure allowed us to standardize data from vessel segments of different sizes and reactivity. 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 2.0 for Windows (Systat Inc., San Rafael, CA) was used for the data analyses and graphs were drawn using standard spreadsheet software. A value of P < 0.05 was considered statistically significant. An analysis of variance (ANOVA) was performed to determine the statistical significance between the relative strength of contraction of drug treated versus control vessels.

Drugs and Solutions:

The composition of physiological buffer was (mM): NaCl 118.4, KCl 4.7, CaCl₂ 2.5, NaHCO₃ 25, H₂PO₄ 1.2, MgSO₄ 1.2, and dextrose 11, and pH was adjusted to 7.4 to mimic physiological conditions. The composition of 60 mM KCl was (mM): NaCl 63.1, KCl 60, CaCl₂ 2.5, NaHCO₃ 25, KH₂PO₄ 1.2, MgSO₄ 1.2, and dextrose 11.

U46619 (9,11-dideoxy-9 α ,11 α -methanoepoxy-prosta-5Z,13E-dien-1-oic acid) and L-NAME (N ω -nitro-L-arginine methyl ester hydrochloride) were both purchased from Cayman Chemical, Ann Arbor, MI. Y-27632 ((R)-(+)-trans-4-(1-Aminoethyl)-N-(4-Pyridyl)cyclohexanecarboxamide dihydrochloride monohydrate), PE (L-Phenylephrine hydrochloride) and Ach (Acetylcholine hydrochloride) were purchased from Sigma-Aldrich, St Louis, MO. 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

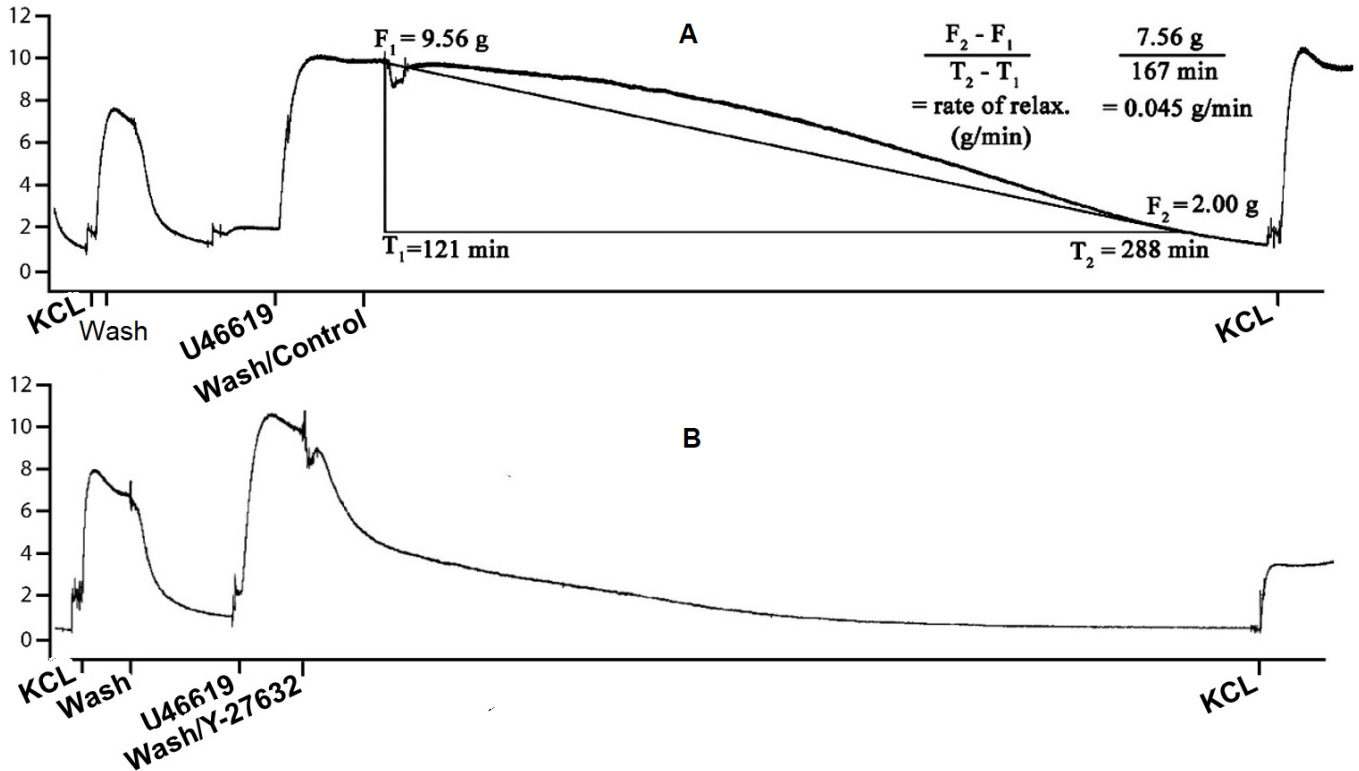


Fig.1: Panel A, A linear line was extrapolated from the point at which the vessel was treated with U46619 to the point at which the vessel reached 2 g of tension. A rate of relaxation was found by dividing the change in force (F) by the change in time (T). Panel B: Vessel treated with Y-27632 following treatment with U-46619 (n=7).

concentration of 100 μM on the day of experiment. Y-27632 and ACh were both dissolved in deionized (DI) H₂O to a final concentration of 1.0 μM and 10 μM , respectively. PE was also dissolved in DI H₂O to a final concentration of 10 $\mu\text{M}/\text{ml}$.

RESULTS

Representative traces from aortic vessels exposed to various treatments and drug interventions are shown in Figs. 1-4. 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 buffer (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 fresh physiological buffer, the vessel remains contracted and the time to relaxation (2 g) 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

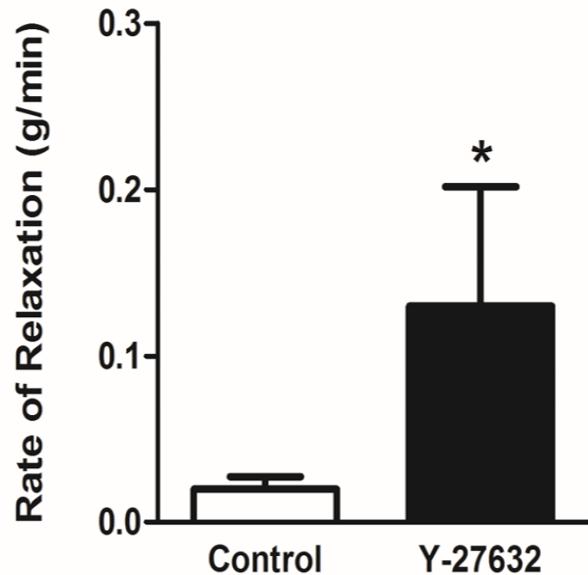


Fig. 2: Aortic vessels treated with Y-27632 (1.0 μM ; n=7).

Fig. 1, panel A. In this particular case, the rate of relaxation for this aortic vessel was calculated as 0.045 g/min. This procedure accounts for any differences in

the steady state maximal contraction by U-46619 between vessel segments.

Aortic vessels treated with Y-27632 relaxed at a rate of 0.13 g/min (± 0.07) (Fig.1 panel B and Fig. 5), while untreated vessels (control) relaxed at a slower rate of 0.02 g/min (± 0.01) (Fig.1 panel A and Fig. 2) ($P < 0.05$; Fig. 2).

Aortic vessels treated with U46619 had a significantly longer relaxation time than PE in both E+ (Fig.3 panel A) vessels (U4= 0.03g/min and PE= 0.27g/min) and in E- (Fig.3 panel B) vessels (U4= 0.02g/min and PE= 0.10g/min). Elimination of endogenous NO had a significant effect on the relaxation of vessels treated with U46619 and PE, NO intact vessels (Fig.3 panel A) relaxed faster when compared to vessels in which NO production was inhibited (Fig.3 panel B) in both U46619 treated aortic vessels ($P=0.044$) and PE treated vessels ($P= 0.030$).

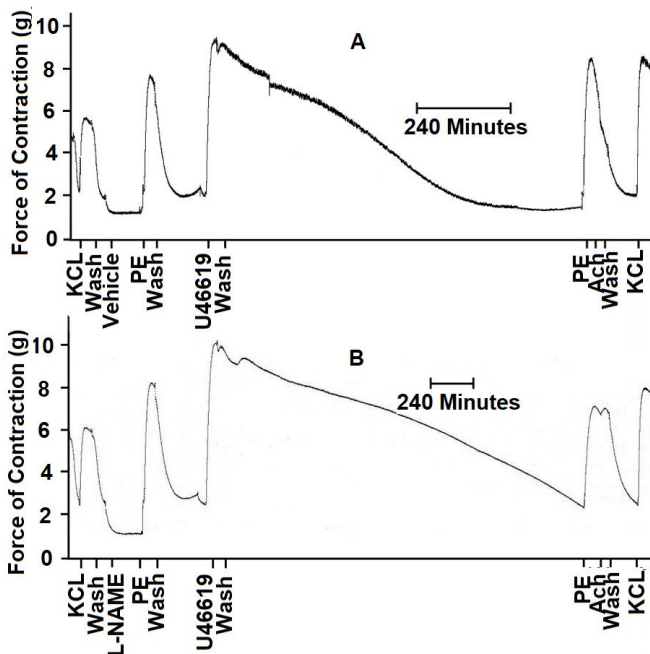


Fig. 3: Extended relaxation of U46619 and PE after incubation in DMSO (panel A, control) and L-NAME (panel B) . In A, aortic vessels were incubated in DMSO (vehicle for L-NAME) for 30min. (n=8)

However, the absence of NO in the endothelium had no effect on the actions of Y27632. The response of U46619 was sensitive to Rho-kinase inhibition both in denuded vessels (Fig. 4; panel B) and in L-NAME treated vessels (Fig. 4; panel D). Relaxation caused by Y-27632 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

forceps denuded vessels ($P=0.57$) and L-NAME treated vessels ($P=0.68$) (Fig. 5).

The presence and absence of NO was assessed for both forceps denuded and L-NAME treated vessels (Fig. 6). There was significant difference between vessels with intact and without NO synthesis for both forceps denuded vessels ($P = 0.001$) and L-NAME treated vessels ($P = 0.001$). This indicated that the physiological effect of NO was strongly inhibited. When Y-27632 is administered there is decrease in final KCl contractions (Fig. 7; panel A). In both forceps denuded endothelium and L-NAME treated vessels the final KCl contractions were attenuated in E+ vessels (Fig. 7; panel B and C).

DISCUSSION AND CONCLUSION

Following previously reported observations that Rho-kinase increases the contractile response of U46619 in pulmonary arteries (McKenzie et al., 2009) our first series of experiments were designed to determine if inhibition of Rho-kinase enhanced the rate of relaxation following a TXA2 induced contraction. Our results show that inhibition of the Rho kinase pathway with the Rho-kinase inhibitor Y-27632 shortens the relaxation phase of U46619-treated VSM. Aortic vessels stimulated with U-46619 relaxed at a faster rate when treated with Y-27632 compared to the untreated vessels. In the second series of experiments, elimination of endogenous NO slowed the U46619 and PE relaxation responses. Earlier McKenzie et al (McKenzie et al., 2009) reported that removal of endogenous NO increased tissue sensitivity and maximum response to U46619. In our experiments we observed a faster relaxation in tissues with intact endogenous NO, this then indicates that NO is important in speeding up relaxation in U46619 contracted tissues. This led us to hypothesize that NO which is a relaxing factor released by the endothelium is involved or aids the relaxing effect of Y-27632. In order to test our hypothesis, in a separate set of experiments the endothelium 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). The efficacy of these procedures were validated by observing the attenuation of the vasodilating effect of Ach on the aortic vessels (Fig. 6). Our results shows that Y-27632 speeds relaxation in U46619 contracted vessels in the presence of the endothelium (E+) and in the absence of the endothelium (E-) after the physiological actions of NO was inhibited (Fig. 5). This

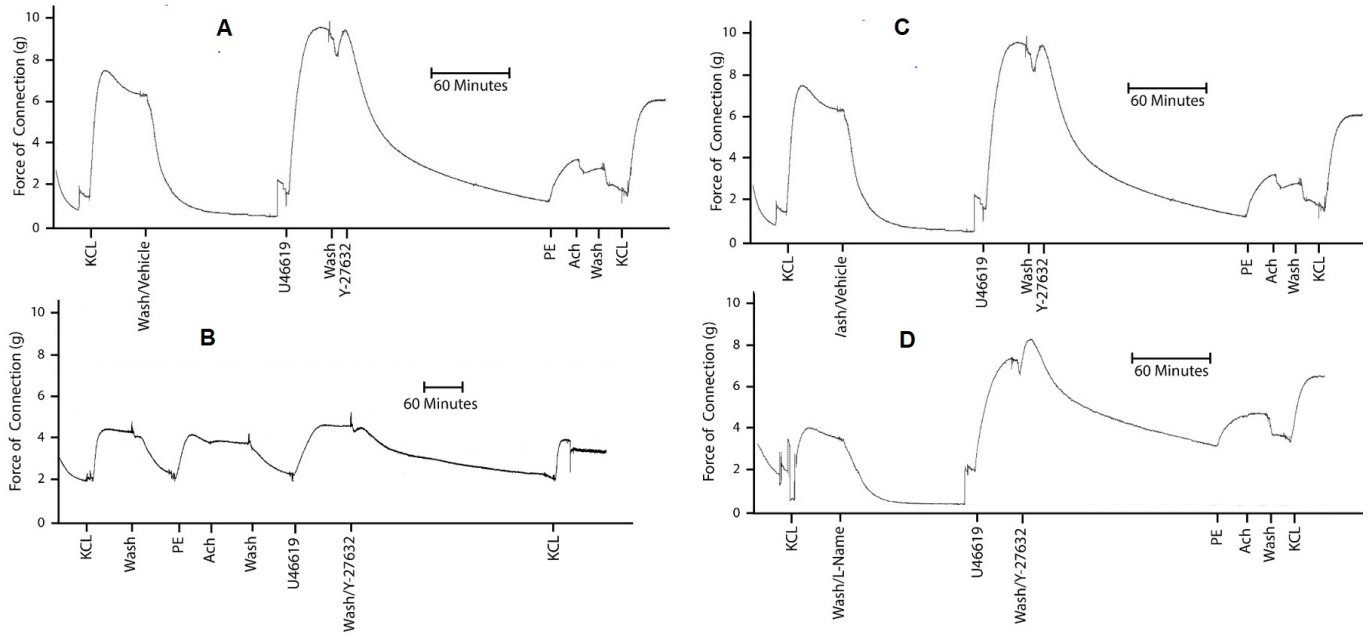


Fig. 4: Relaxation responses induced by Y-27632 in isolated aortic vessels pre-contracted with U46619. Tracings are from endothelium-intact (A; +E, n=10), endothelium-denuded (B; -E, n=10), DMSO treated (C; Control E+) and L-NAME treated (D; -E) aortic vessels. NO was eliminated by removal of the endothelium using forceps; aortic vessel was incubated in DMSO or L-NAME for 30 min before administration of U46619 and Y-27632 (n=11)

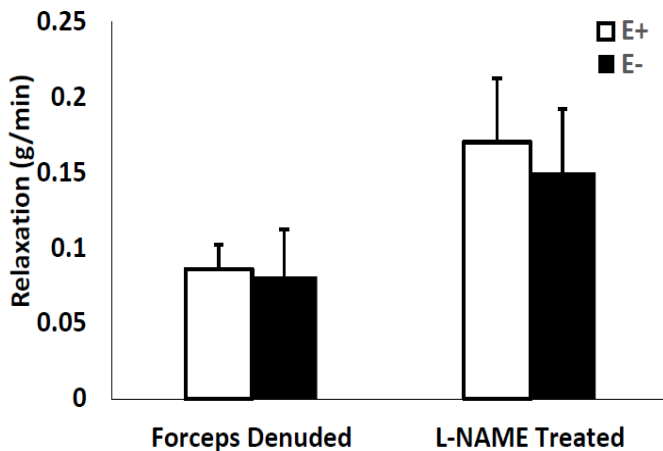


Fig. 5: The relaxation rate of Forceps denuded vessels and L-NAME treated vessels following treatment with U46619 (0.5 μ M). Y-27632 (1.0 μ M) relaxation did not show significant difference (NS) between E+ and E- in both Forceps denuded and L-NAME treated vessels (n= 21)

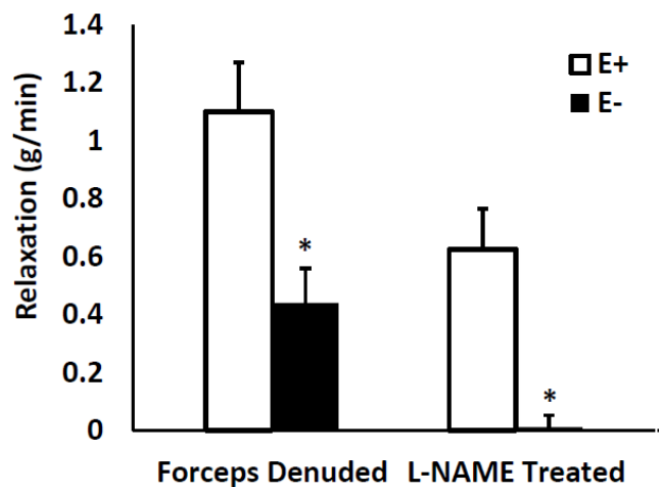


Fig. 6: Ach relaxation was significantly different between E+ and E- vessels (n= 31).

suggests that the absence of NO did not affect the actions of Y-27632 in speeding up relaxations in U46619 induced tissues. Previous studies have established that agents such as Y-27632 that bring about relaxation in VSMs can be of importance in lowering blood pressure, because increased contractility of VSMs results into increased peripheral vascular resistance and eventually an increase in blood

pressure and heart rate (De Godoy and Rattan, 2011; Asano and Nomura, 2003; Wirth, 2010). In our study the vasodilator effects of Y-27632 were found to be independent of the actions of NO. These observations suggest that Y-27632 endothelium-independent relaxation mechanism can be a tool to consider when considering treatment for TXA2 induced contractions in blood vessels.

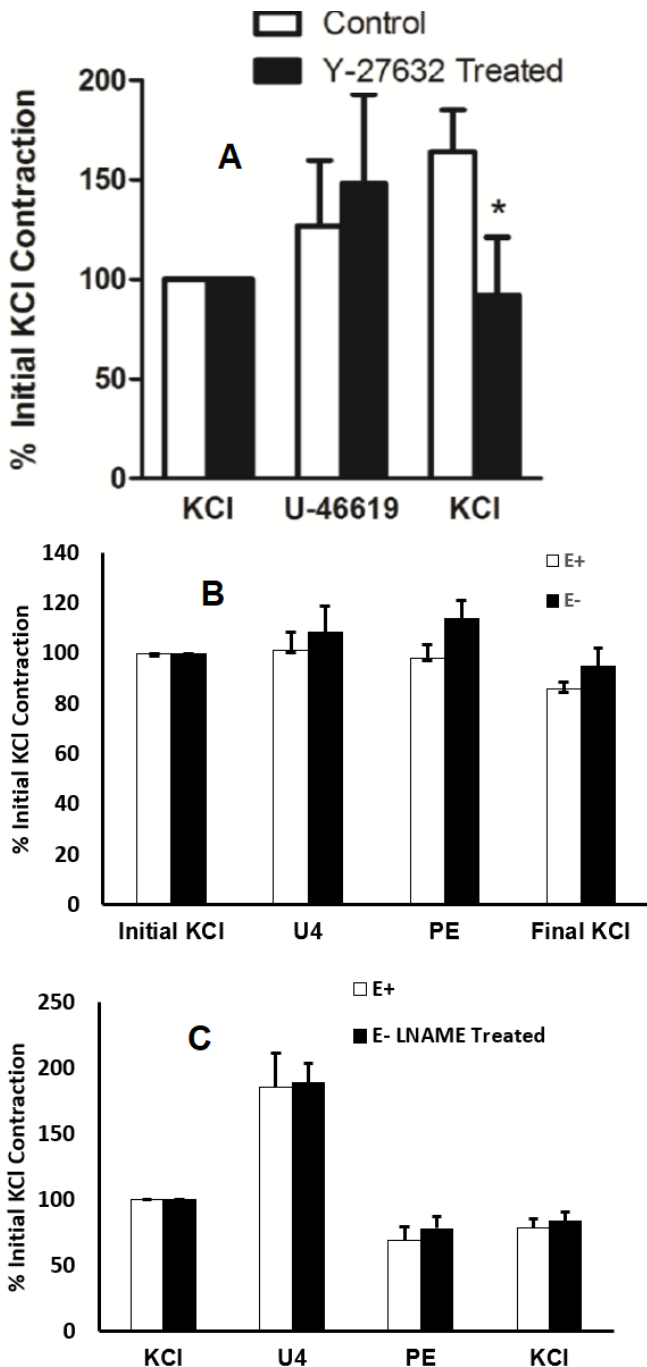


Fig. 7: The relative strength of contraction of vessels treated with KCl, U46619 and PE. Treatments with Y-27632 were given after exposure to the initial KCl, U46619 and PE and before exposure to the final KCl. All data are compared against the initial KCl contraction. A, B, and C (respectively) denote endothelium denuded (n=7), endothelium denuded (n=10) and L-NAME-treated endothelium-intact (n=11) aortic vessels.

The contractile response to U46619, PE and KCl were increased in vessels where synthesis of NO was inhibited (Fig. 7 Panel B and C) This is expected since

endogenous NO is the main relaxing factor found in the endothelium, so in its absence there is enhanced responses to vasoconstrictor agents (Furchgott and Zawadski, 1980; Moncada et al., 1991; Iwatani et al., 2008; Yao et al., 2010). The vessels were subjected to KCl activation at the beginning of the experiment and at the end of the experiment as a positive control, to ascertain that the vessel remained functional during the course of the experiment. KCl is a contractile stimulus that allows for the bypass of the GPCRs (G-Protein-Coupled-Receptors), it involves the activation of the voltage gated Ca⁺⁺ channels by depolarizing the membrane which ultimately allows the influx of Ca⁺⁺ into the cell without the involvement of GPCRs. Thereby it leads to contraction of normal physiological working vessels (Ratz et al., 2005). Unexpectedly, treatment of vessels with high KCl in the presence of Y-27632 resulted in a diminished response to high KCl compared to control vessels (Fig. 7 Panel A), suggesting the Rho-kinase pathway is activated when vessels are stimulated with high KCl. While the activation of the Rho kinase pathway by high KCl was initially an unexpected finding in our experiment, it has been previously reported in the literature (Sakurada et al., 2001). It appears that depolarization of VSMCs, which occurs following treatment of high KCl, can activate the Rho-kinase pathway in a voltage-dependent manner. The depolarization in VSMCs caused by high KCl induces L-type voltage-dependent Ca²⁺ channels to open on the surface of vascular smooth muscle cells, allowing extracellular Ca²⁺ to enter the cell. Resulting increases in intracellular Ca²⁺ concentration can activate other second messengers such as protein kinase C. Whether Ca²⁺ alone, Ca²⁺ coupled with depolarization, or the activation of other second messengers by Ca²⁺ can lead to the activation of Rho-kinase has not yet been established (Woodsome et al., 2006). Furthermore, we also observed that the strength of the final KCl contractions was lower than the initial KCl contractions, possibly this could mean that administration of U46619 is responsible for this decrease in response to KCl contraction. Further work is needed to define the mechanism for these observations. Earlier studies (Ratz et al., 2005) reported that Y-27632 inhibits KCl induced smooth muscle contractions. Our results also support a reduction in the strength of contraction of KCl induced vessels after administration of Y-27632 (Fig. 7). The endothelium plays a very important physiological role in relaxation of vascular smooth muscle. In pathological conditions where there is attenuation of its function, it is pertinent to find ways to compensate its functions especially in diseases such as atherosclerosis,

where the integrity of the endothelium has been compromised.

In conclusion, our results show that even though NO is pertinent in relaxing VSM that has been pre-contracted with both PE and U46619, the vaso-relaxation induced by Y-27632 occurs through an endothelium independent mechanism that does not involve the release of endogenous NO. Y-27632 may be useful in causing relaxation of TXA₂ induced contractions when NO production is impaired during the development of arteriosclerosis and other endothelium related cardiovascular diseases.

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