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## Vasorelaxation of goat ruminal artery is mediated by endothelium independent NO-SGC-CGMP pathway

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### Abstract

The present study was designed to study the relative potency of NA in inducing vasoconstriction in endothelium intact goat ruminal artery and to study endothelium dependent and independent mechanisms of nitric oxide mediated vasorelaxation of the artery. The isolated ruminal arterial rings of goat were perfused in organ bath and isometric contraction was studied. (1) Noradrenalin (NA), 10 $\mu$ M caused a potent vasotonic response with mean net plateau tension of 5.09 $\pm$ 0.41g, n=31 obtained at 600 $\pm$ 32.6sec, n=31. Endothelium removal did not affect the vasoconstriction of the artery by noradrenaline. (2) Acetylcholine (ACh) produced very nominal relaxation (EC<sub>50</sub>= 28.2 $\mu$ M, E<sub>max</sub>= 70.66 $\pm$ 4.91) whereas sodiumnitropruside (SNP) produced significant relaxation (EC<sub>50</sub>=2.35 $\mu$ M, E<sub>max</sub>= 38.46 $\pm$ 3.91). (3) Vasorelaxation by ACh and SNP were unaffected in endothelium denuded arteries. (4) L-Arginine and L-NAME preincubation found to have no significant effect on the ACh and SNP induced relaxation. But ODQ potently blocked ACh as well as SNP induced relaxation by 16.24% and 52.73% respectively. (5) Indomethacin blocked the ACh relaxation by 16.78% and SNP relaxation by 15.66%. These observations concluded that NA is a potent vasoconstrictor in goat ruminal artery. SNP is more potent relaxing agent and ACh is a poor relaxing agent in this artery. Endothelial NO has insignificant role in vasorelaxation of this artery. Vasorelaxation of this artery by nitric oxide may involve an endothelium independent NO-sGC-cGMP pathway.

### 1. INTRODUCTION

Since the late 1980s, NO and carbon monoxide (CO) have been demonstrated to be gasotransmitters with a variety of vital functions including vasorelaxation, suppression of cell proliferation, inhibition of platelet aggregation, and so on. Recently hydrogen sulfide (H<sub>2</sub>S) has been suggested to be another gasotransmitter<sup>1</sup>. NO targets the soluble guanylyl cyclase (sGC) located in the smooth muscle cells and binds to its haem moiety leading to intracellular accumulation of the second messenger molecule cGMP, which in turn regulates numerous physiological events such as vessel tone and neurotransmission.

Although NO/sGC/cGMP/PKG pathway has been generally suggested to mediate the vasomotor actions of NO in vasculature<sup>2,3</sup>, this cyclic nucleotide may not be a universal effector of NO across all vascular beds<sup>4,5</sup>. For instance, it has been reported for aorta<sup>6</sup>, colonic smooth muscle<sup>7</sup> and lung vasculature<sup>8</sup> that vasorelaxation to NO may be mediated by activating K<sup>+</sup> channel and for skin<sup>9</sup> and eyes<sup>10</sup> too perhaps depend on prostaglandins, for neuronal excitability<sup>11</sup> and airway smooth muscle<sup>12,13,14</sup> by nitrosylation of cysteine-thiol groups, by activation of SERCA and consequent activation of store operated Ca<sup>2+</sup> influx in rabbit aortic smooth muscle<sup>15,16,17</sup>. Interactions between K<sup>+</sup> channels and prostaglandins<sup>18,19,20,21</sup> is reported and between c-GMP, prostaglandin and K<sup>+</sup> channel is also documented<sup>22,23</sup>. So the mechanisms that mediate vasomotor effects of NO in one type of vasculature may not necessarily apply to other vascular beds.

It is reported that exogenous NO can diminish the ruminal contractions, while endogenous NO is not involved in the regulatory mechanism of basal tone and regular phasic contractions of the rumen in healthy sheep<sup>24</sup>. Therefore, we have conducted the present study as an attempt to explore the endothelium dependent and independent mechanisms underlying the vasorelaxation of goat ruminal artery to nitric oxide.

## 2. MATERIALS AND METHODS

### 2.1 Materials

Noradrenaline (NA) and Acetylcholine (ACh) were purchased from Sigma, USA. Sodium nitropruside (SNP) was purchased from LOBACHemie, India. Indomethacin, N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1one (ODQ) were purchased from Cayman chemical, USA. L-arginine was purchased from HiMedia, India. All the solutions were prepared freshly before each experiment. All the solutions were prepared in deionized water except ODQ which was dissolved in dimethyl sulfoxide (DMSO) so similar control experiments were carried out with these solvents under same condition of the experiments.

### 2.2 Preparation of isolated rings

The right ruminal artery was traced from main celiac artery supplying to the right ventral and dorsal sac of rumen. Ruminal artery (4-5 cm long) was carefully dissected out from the rumen wall towards the anterior end before its bifurcation, as per anatomical description of Wesley and Alvin, 1969<sup>25</sup>. Ruminal artery collected in Modified Krebs–Hanseleit solution (MKHS) from freshly slaughtered goat was removed and dissected from surrounding fat and connective tissues and cut into uniform rings of 2.5mm length. The arterial rings were prepared carefully so that the endothelium was not damaged. Rings were mounted between two hooks attached to an isometric force transducer sensitive to 5mg-25g (Model: MLT 0201, AD instruments, Australia) and kept in a thermostatically controlled organ bath (37±0.5°C) of 20mL capacity (Panlab S.I, Spain), containing MKHS (P<sup>H</sup> 7.4) continuously bubbled with carbogen (5%CO<sub>2</sub> and 95%O<sub>2</sub>) and tension was recorded using 8/32 power lab data acquisition system (AD Instruments, Australia) with Labchat6 pro software. MKHS contained the following (in mM): 118.0 NaCl; 4.7 KCl; 2.5 CaCl<sub>2</sub> .2H<sub>2</sub>O; 1.2 MgSO<sub>4</sub>.7H<sub>2</sub>O; 1.2 KH<sub>2</sub>PO<sub>4</sub>; 11.9 NaHCO<sub>3</sub> and 11.1glucose. The vascular rings were kept under resting tension of 2g and allowed 90min for equilibration with continuous washing with Krebs solution in every 15 min interval before starting the experiment<sup>26</sup>.

### 2.3 Vascular response study

After the equilibration period, endothelium intact vascular rings perfused in PSS, pre contracted with sub maximal concentration of NA, 10µM were relaxed with ACh (0.1-100µM) and SNP (1µM-100µM) in a cumulative manner with 0.5 log unit increment for ACh and 1 log unit increment for SNP respectively, in presence or absence of L-NAME,100µM, L-Arginine,100µM, ODQ,10µM and indomethacin, 10µM pre incubated for 30min. Vasodilatation effect were expressed as the % of maximal response considering plateau tension as 100%.

### 2.4 Statistical analysis

All values were expressed as mean ± standard error of mean (SEM) of measurements in 'n' experiments. The relaxant effect were expressed as the percent response (percentage reduction of the maximum contraction induced by NA, 10µM considering plateau tension as 100%). Sensitivity (expressed as  $pD'_2 = -\log(B) + [E_{max}/E_{Bmax}] - 1$ ) and maximal relaxation ( $E_{max}$  or  $E_{Bmax}$ ) to agonists or antagonist, respectively was determined for each ring by fitting individual concentration-response data to a non-linear sigmoidal regression curve and interpolating in Graphpad prism software (Graphpad Prism5, GraphPad Software Inc, San Diego, CA, U.S.A.) and the data were analyzed using one-way analysis of variance (ANOVA) for significant differences between two groups. A level of  $p < 0.05$  was accepted as statistically significant.

## 3. RESULTS

### 3.1 Potency of NA in inducing contraction of goat ruminal artery

By exposing the isolated artery rings to NA in the dose range of (0.1-100µM), the threshold concentration for NA induced contraction were found to be 0.3 µM and concentration for maximal response ( $E_{max}$ ) were observed at 30µM (Tracing 1). The EC<sub>50</sub> for NA induced contraction presented with 95% CL (10.1 µM) confirms that NA has potent vasotonic effect on goat ruminal artery. The sub maximal concentration of NA (10µM) in goat ruminal artery elicited a sustained contraction consisting of a rapid initial phase followed by a slow sustained contraction. The mean time to peak and peak tension of fast phasic contraction induced by NA were 57.67±3.83 sec, n=31 and 3.07±0.33g, n=31, respectively. The mean net plateau tension 5.09±0.41g, n=31 of the slow phase of the NA induced sustained contraction was obtained at 600±32.6 sec, n=31.

**3.2 Effect of ACh and SNP on vasoconstriction evoked by NA in goat ruminal artery rings**

SNP(1nM-100µM) was found to have the least EC<sub>50</sub> value (Tracing 3) followed by and ACh (0.1-100µM) (Tracing 2) respectively on inducing vasorelaxation of goat ruminal arterial rings on NA(10µM) induced sustained contraction suggests that SNP is the more potent vasorelaxing agent (EC<sub>50</sub>= 2.35 µM) than ACh (EC<sub>50</sub>= 28.19 µM) on goat ruminal artery (Fig1).

**3.3 Effect of endothelium**

There was no significant alteration in the CRC of NA in this artery after removal of endothelium (Table-1, Fig 2). The vasorelaxation effect to ACh and SNP was not significantly affected in endothelium denuded rings (Table-2, Fig 3 & 4).

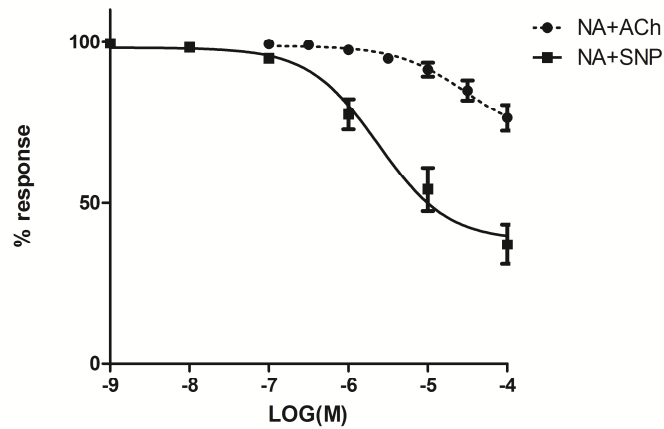


Figure 1 : Vasorelaxation of endothelium intact GRA by ACh (0.1µM-100µM) and SNP (1nM-100µM) in NA, 10 µM induced contraction.

Table 1 : P<sub>2</sub><sup>D</sup> and EC50 value of concentration response curves of NA, (0.1-100µM) induced contraction in endothelium intact and endothelium denuded goat ruminal artery.

	NA	
	P <sub>2</sub> <sup>D</sup>	EC <sub>50</sub>
+ endothelium, n=6	4.99±0.04	10.16 µM
- endothelium, n=6	5.01±0.04 <sup>ns</sup>	9.59 µM

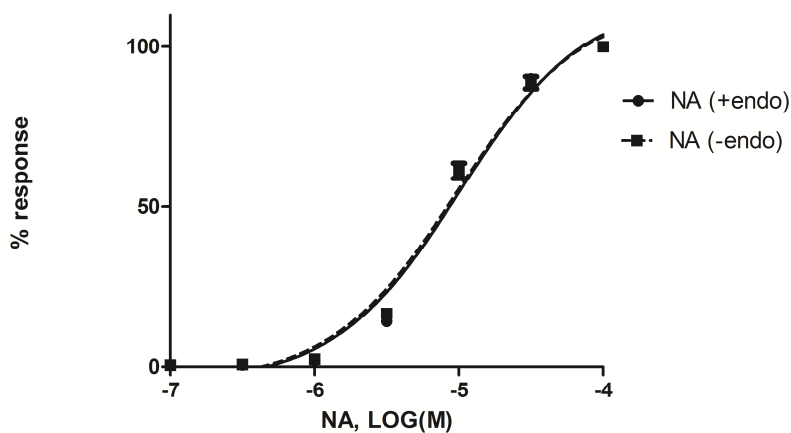


Figure 2 : Concentration Response curve (CRC) of NA, (0.1-100µM) induced contraction in endothelium intact and endothelium denuded rings.

### 3.3 Effect of L-Arginine, L-NAME and ODQ on ACh induced relaxation

L-Arginine, 100 $\mu$ M produced no significant increase in vasorelaxation ( $E_{B_{max}} = 77.81 \pm 2.85\%$ ) and L-NAME, 100 $\mu$ M did not block significantly ( $E_{B_{max}} = 73.29 \pm 8.14\%$ ) the ACh (0.1-100 $\mu$ M) induced relaxation ( $E_{max} = 70.66 \pm 4.91$ ). Whereas ODQ, 10  $\mu$ M considerably blocked the relaxation ( $E_{B_{max}} = 86.90 \pm 5.31\%$ ) (Fig 5; Table 3).

### 3.4 Effect of L-Arginine, L-NAME and ODQ on SNP induced relaxation

Neither L-Arginine, 100 $\mu$ M increased the relaxation ( $E_{B_{max}} = 21.04 \pm 0.75\%$ ) nor L-NAME, 100 $\mu$ M has blocked ( $E_{B_{max}} = 25.54 \pm 4.26\%$ ) the SNP (1 $\eta$ M-100 $\mu$ M) induced relaxation ( $E_{max} = 38.46 \pm 3.91\%$ ) significantly. Only ODQ, 10 $\mu$ M blocked the relaxation ( $E_{B_{max}} = 80.37 \pm 2.81\%$ ) significantly (Fig 6; Table 3).

### 3.5 Effect of indomethacin on ACh and SNP induced relaxation

Indomethacin blocked the effect of ACh and SNP to a significant extent (Table 3, Fig 7 & 8).

**Table 2 : ACh (0.1 $\mu$ M-100 $\mu$ M) and SNP (1 $\eta$ M-100 $\mu$ M) induced relaxation in endothelium intact and denuded rings of goat ruminal artery precontracted with noradrenalin (NA), 10  $\mu$ M.**

	ACh		SNP	
	$P^{D_2}$	$E_{max}$	$P^{D_2}$	$E_{max}$
+ endothelium	4.55 $\pm$ 0.22, n=9	70.66 $\pm$ 4.91, n=9	5.63 $\pm$ 0.15, n=9	38.46 $\pm$ 3.91, n=9
- endothelium	5.11 $\pm$ 0.16 <sup>ns</sup> , n=6	75.26 $\pm$ 2.19 <sup>ns</sup> , n=6	5.70 $\pm$ 0.07 <sup>ns</sup> , n=8	36.46 $\pm$ 1.93 <sup>ns</sup> , n=8

**Table 3 :  $P^{D_2}/P^{D'_2}$  and  $E_{max}/E_{B_{max}}$  value of ACh (0.1 $\mu$ M-100 $\mu$ M) and SNP (1 $\eta$ M-100 $\mu$ M) induced relaxation of goat ruminal artery in presence of L-Arginine, L-NAME, ODQ, Indomethacin.**

	Control, n=9	L-Arg, n=6	L-NAME, n=6	ODQ, n=6	Indomethacin, n=6
<b>ACh</b>					
$P^{D_2}/P^{D'_2}$	4.55 $\pm$ 0.22	5.65 $\pm$ 0.37 <sup>ns</sup>	4.80 $\pm$ 0.41 <sup>ns</sup>	4.64 $\pm$ 0.43 <sup>ns</sup>	5.06 <sup>ns</sup> $\pm$ 0.69
$E_{max}/E_{B_{max}}$	70.66 $\pm$ 4.91	77.81 $\pm$ 2.85 <sup>ns</sup>	73.29 $\pm$ 8.14 <sup>ns</sup>	86.90 $\pm$ 5.30 <sup>ns</sup>	87.44 <sup>ns</sup> $\pm$ 4.49
<b>SNP</b>					
$P^{D_2}/P^{D'_2}$	5.62 $\pm$ 0.15	5.82 $\pm$ 0.08 <sup>ns</sup>	5.77 $\pm$ 0.14 <sup>ns</sup>	4.75 $\pm$ 2.81 <sup>ns</sup>	5.89 <sup>ns</sup> $\pm$ 0.21
$E_{max}/E_{B_{max}}$	38.46 $\pm$ 3.91	27.88 $\pm$ 2.33 <sup>ns</sup>	25.54 $\pm$ 4.26 <sup>ns</sup>	80.37 $\pm$ 0.67 <sup>**</sup>	54.12 <sup>ns</sup> $\pm$ 3.83

ns = not significant ( $p > 0.05$ ), \*\*\* =  $p < 0.001$ , \*\* =  $p < 0.01$ , \* =  $p < 0.05$ . Values are mean $\pm$ SEM, n= no of experiments.  $P^{D_2}$  = Negative logarithm of concentrations of agonist producing 50% of the maximal response.  $P^{D'_2}$  = Negative logarithm of concentrations of agonist producing 50% of the maximal response in presence of antagonist.  $E_{max}$  = maximal response by the agonist.  $E_{B_{max}}$  = maximal response by the agonist in presence of antagonist.  $P^{D_2}/P^{D'_2}$  and  $E_{max}/E_{B_{max}}$  value were determined by linear regression analysis using GraphPad Prism 5.0 software.

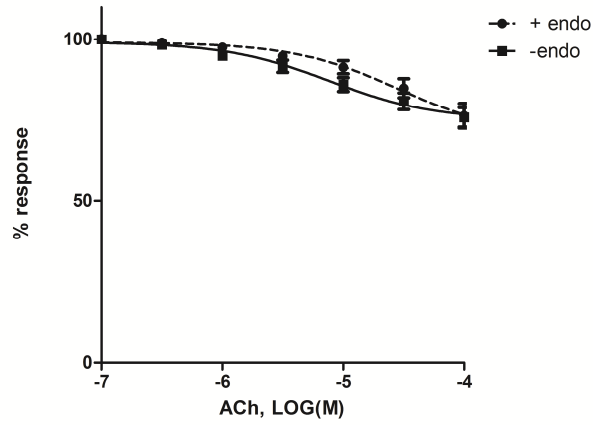


Figure 3 : ACh (0.1µM-100µM) relaxation in endothelium intact and denuded arterial rings

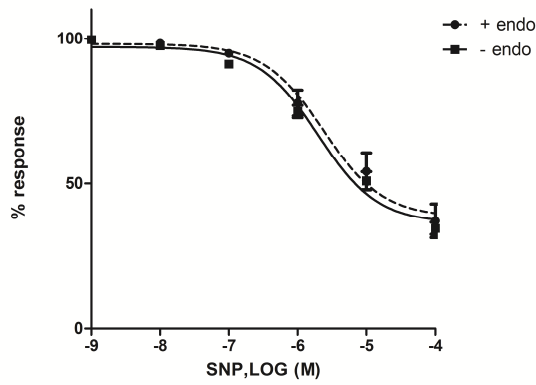


Figure 4 SNP (1nM-100µM) relaxation in endothelium intact and denuded arterial rings

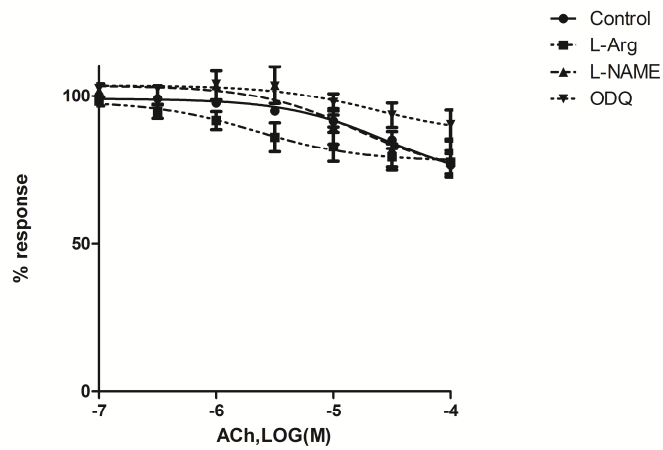


Figure 5 vasorelaxation by ACh,(0.1µM-100µM) in presence of L-arginine (100µM), L-NAME (100µM), ODQ(10 µM).

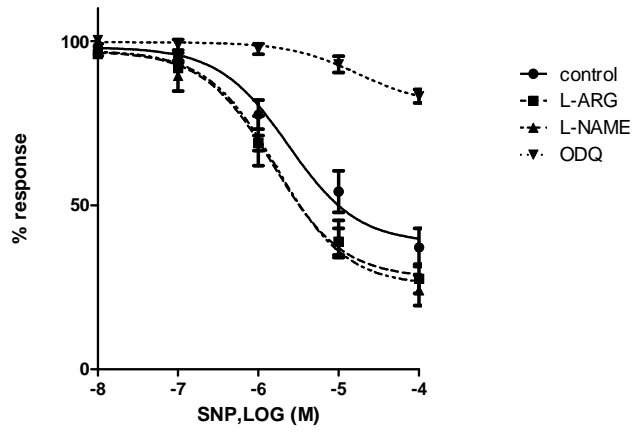


Figure 6 vasorelaxation by SNP (1 nM-100 μM) in presence of L-arginine (100 μM), L-NAME (100 μM), ODQ (10 μM).

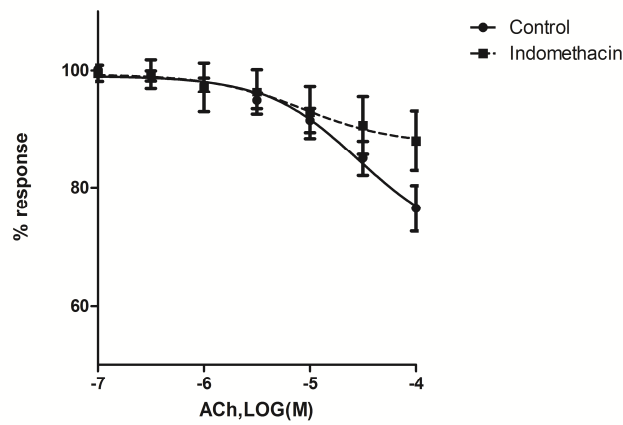


Figure 7 ACh (0.1 μM-100 μM) relaxation in presence of indomethacin (10 μM).

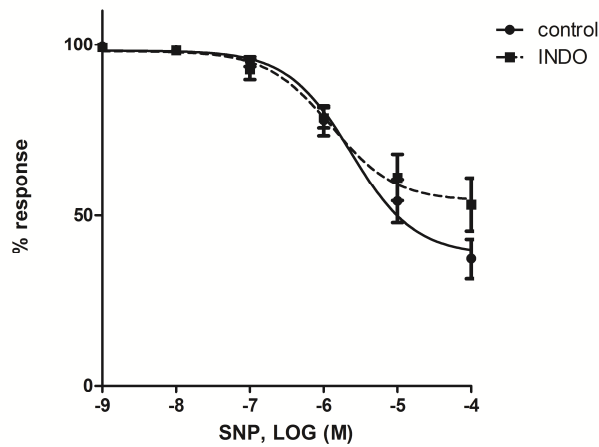
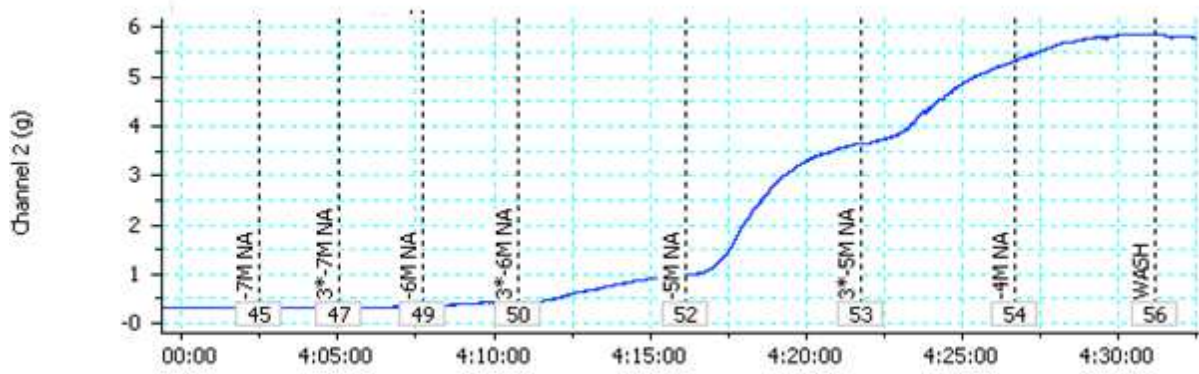
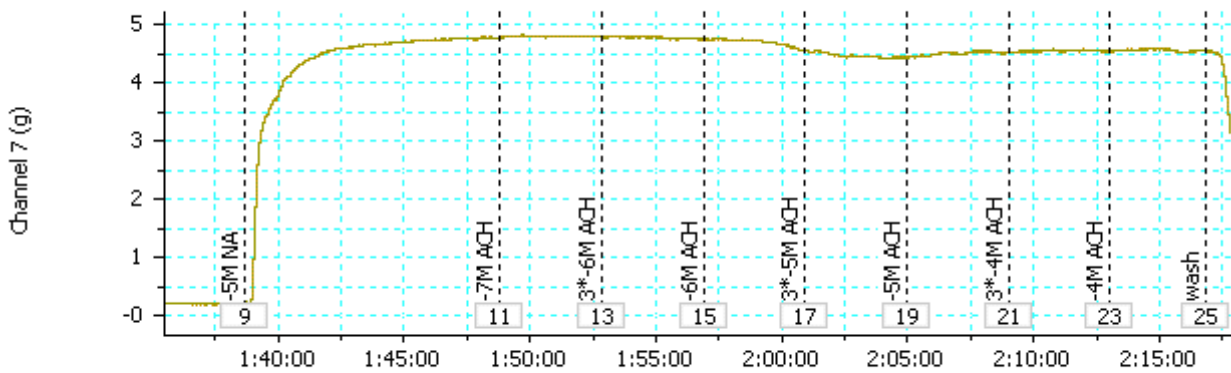


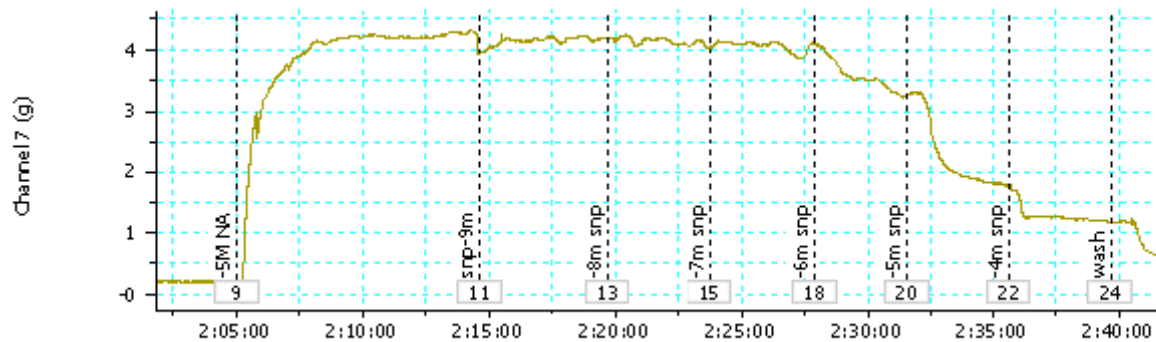
Figure 8 SNP (1 nM-100 μM) relaxation in presence of indomethacin (10 μM).



**Tracing 1** NA(0.1-100µM) induced concentration dependent contractile response in endothelium intact goat ruminal artery rings.



**Tracing 2** ACh(0.1-100µM) induced relaxation.



**Tracing 3** SNP(1µM-100µM) induced relaxation.

#### 4. DISCUSSION

In the present study the isolated arterial rings were exposed to 0.1-100 µM of NA in a concentration dependent manner with 0.5 log unit increment which is repeated after 45min in the same arterial ring with endothelium intact. No significant difference between the two CRCs (concentrations related contractile curves) indicates potent vasotonic effect of NA in goat ruminal artery (Tracing 1). There was no effect of endothelium on the vasotonic effect of NA on this artery (Fig-2, Table -1). ACh induced relaxation is endothelium dependent, NO is the involved

EDRF has been confirmed and reported in many species<sup>27,28</sup>. However no relaxation by ACh has also been reported in several vascular preparations including the human umbilical vessels<sup>29</sup> and bovine intrapulmonary veins<sup>30</sup>. In order to investigate the role of endothelium in NO mediated vasorelaxation in GRA, the endothelium intact arterial rings contracted with submaximal concentration of NA, 10 $\mu$ M were relaxed with ACh (0.1-100 $\mu$ M) in a dose dependent manner, surprisingly acetylcholine was found to have very insignificant role in vasorelaxation of this artery ( $E_{max} = 70.66 \pm 4.91$ ,  $EC_{50} 28.2 \mu$ M) (Fig 1) which was remain unaffected after removal of the endothelium ( $E_{max} = 75.26 \pm 2.12$ ) (Table 2, Fig 3). Whereas this artery was relaxed significantly by SNP (1 $\eta$ M-100 $\mu$ M) (Fig 1) which is an exogenous NO donor and does not involve EDRF and it was verified by repeating the similar experiment in endothelium denuded artery which show no significant alteration (Table 2, Fig 4) suggesting endothelial release of NO has no significant role in vasorelaxation of GRA, NO mediated vasorelaxation in GRA rather more endothelium independent.

Pre-incubation with L-Arginine, which generates NO and L-citrulline by NOS (Nitric oxide synthase) in mammalian cells found to have no significant effect on the ACh and SNP induced vasorelaxation on NA induced contraction (Table 3, Fig 5 & 6) and no significant blockade of neither ACh nor SNP induced relaxation by L-NAME, an eNOS blocker (Table 3, Fig 5 & 6), strengthen the hypothesis that endogenous NO synthesis has a very minor effect in vasorelaxation of this artery.

We observed that ODQ (10 $\mu$ M) potently blocked both ACh and SNP relaxation ( $E_{Bmax} = 86.90 \pm 5.31\%$ ,  $80.37 \pm 2.81\%$ ,  $91.04 \pm 8.39\%$  respectively) (Table 3, Fig 5 & 6). ODQ (1H[1,2,4] oxadiazolo [4,3,-a] quinoxalin-1-one) is a novel and selective inhibitor of sGC (which activates c-GMP synthesis). ODQ may be inhibiting ACh relaxation by blockade of c-GMP and may affect exogenous NO donor mediated vasorelaxation by inhibiting their reductive bioactivation via the cytochrome P-450 enzyme system<sup>31</sup>. In order to confirm the relevance of this discussion with sGC-cGMP pathway, similar experiments conducted in arteries pre-incubated with indomethacin, 10 $\mu$ M found to have potent blockade in the relaxing effect of both ACh and SNP (Table-3, Fig 7 & 8) which strengthened the hypothesis of synthesis of cGMP in vasorelaxation process of this artery.

## 5. CONCLUSION

In conclusion it can be stated that NA is a potent vasoconstrictor of goat ruminal artery. SNP is a more potent relaxing agent compared to ACh in NA induced contraction in this artery. The small relaxation of this artery to ACh which was not affected by removal of endothelium may be explained as because of some mechanism involving c-GMP synthesis as ODQ and indomethacin blocked ACh effect to a significant extent, however other pathways may not be ruled out and need further investigation. But eNOS mediated endogenous NO has very minor involvement in vasorelaxation of this artery as evident by no significant effect by L-arginine and L-NAME on ACh. The NO mediated vasorelaxation in this artery may primarily involve endothelium independent NO-sGC-cGMP pathways which may be due to activation of the heme moiety of the sGC which activates cGMP synthesis, as evident by the potent blockade of SNP effect by ODQ and indomethacin.

## 6. ACKNOWLEDGEMENT

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