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INTERACTION BETWEEN ANGIOTENSIN II RECEPTORS AND α-ADRENOCEPTORS IN THE MURINE VASCULAR SYSTEM

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Summary

The mouse major conductive (aorta and carotid and superior mesenteric arteries) and small resistance arteries (first branch mesenteric artery) have a multiple population of adrenoceptor (ARs) and angiotensin II (Ang II) receptors capable of initiating contraction or relaxation. This thesis uses pharmacological methods to describe responses mediated by these receptors and to explore interactions between them.

On mouse aorta Ang II had a dual effect that could be best observed in the presence of elevated tone (by 5HT), initially causing contraction at lower concentrations, followed by a slow relaxant effect that became dominant over time or at higher concentrations. The contraction was attenuated by Losartan and the relaxation by PD123319 (AT_1 and AT_2 antagonists, respectively) indicating physiologically opposing actions of AT_1 and AT_2 receptors. The relaxation was abolished by L-NAME or endothelium removal, revealing a larger contraction to Ang II. This indicates an AT_1 action to contract vascular smooth muscle directly and an AT_2 action on endothelium to release nitric oxide.

The potential influence of Ang II on the effects of noradrenaline was studied. First the interaction of the relaxant (endothelial) effects of the two agents was explored by testing the effect of a "relaxant" concentration of Ang II (30nM) against the effects of UK14304, an α_2-AR agonist, serving as a surrogate for noradrenaline in order to avoid activation of other adrenoceptors. This revealed no synergism or other significant interaction, which contrasted with a strong interaction between Ang II and the contractile effects of α_2-AR activation in other blood vessels.

Ang II was then tested against the contractile effects of noradrenaline, applied as a cumulative concentration response curve. Preincubation with Ang II (30nM) significantly reduced the contractile response to NA ($p<0.0001$): this effect was

enhanced by losartan and blocked by PD123319. Thus the major influence of Ang II upon noradrenaline's actions is an AT_2-mediated attenuation that becomes greater if AT_1 receptors are blocked.

In both carotid and superior (main) mesenteric arteries the contractile effect of Ang II was dominant. In first branch mesenteric arteries the main effect of angiotensin II was relaxation; this was reversed to contraction by L-NAME suggesting that it was of endothelial origin. The balance of smooth muscle contractile (AT_1) and endothelial relaxant (AT_2) –mediated responses, thus varies amongst arteries.

A fluorescent derivative of Ang II, Rhodamine-Angiotensin II-Human (Rho-Ang II-H), was used to visualise angiotensin receptors on dissociated arterial cells and intact vessels, employing confocal microscopy. Losartan and PD123319 were used as competitor ligands to identify the receptor subtypes that were labelled by the fluorescent compound. This provided evidence for the presence of both AT receptor subtypes on both smooth muscle and endothelial cells. This was accomplished on both aorta and main (superior) mesenteric arteries.

In conclusion, mouse arterial endothelium has AT_2 that promote the release of nitric oxide, detectable as smooth muscle relaxation and vascular smooth muscle has contractile AT_1. This shows that the previously demonstrated dual, opposing actions of angiotensin II are due to receptors situated on different cell types. There was also, however, evidence for the presence of both receptor types on both smooth muscle and endothelial cells.

Next, the α_2-AR-mediated relaxation response was studied. A vasodepressor response to α_2-AR agonists has been shown on the blood pressure of conscious mice and has been studied in vitro in other species, though not in mice; the subtypes of receptor involved are not well characterised due to the relative lack of specificity of test drugs.

The opportunity was taken to study its pharmacology in the mouse so that further experiments could be performed on genetically modified mice with "knockouts" of one of the α_2-ARs.

UK14304-mediated vasodilator responses were studied on wire myograph-mounted mouse aorta, carotid, main mesenteric and first branch mesenteric arteries with a view to determining cells involved, mechanisms of action and subtypes of α_2 adrenoceptors (α_2-AR).

In aorta, carotid and main mesenteric arteries, in the presence of induced tone, UK14304 produced a concentration-related vasodilatation that was abolished by rauwolscine, L-NAME or endothelium removal indicating that endothelial α_2 adrenoceptors can release nitric oxide. In the first branch mesenteric artery rauwolscine and endothelium removal were effective but L-NAME was ineffective at blocking the response.

In the $\alpha_{2A/D}$-adrenoceptor knockout mouse and the D79N mouse, a functional knockout of the $\alpha_{2A/D}$-adrenoceptor, aorta and carotid arteries the relaxant effects of UK14304 were lost indicating the involvement of the $\alpha_{2A/D}$-adrenoceptor in these arteries. However, in these knockouts responses persisted in the main and first branch mesenteric arteries.

UK14304 could also contract aorta: a small contraction occurred at high concentrations, was enhanced by L-NAME and was absent in the α_{1D}-adrenoceptor knockout mouse indicating activation of the α_{1D}-adrenoceptor. There was no evidence in any artery tested of a contractile α_2-adrenoceptor-mediated response.

The visualization, on aortic endothelial cells, of rauwolscine-sensitive binding of a fluorescent ligand, QAPB, provided direct evidence for the presence of α_2-adrenoceptors on the endothelium.

The studies of endothelial α_2-adrenoceptor, showed overall that in mouse major conducting arteries an $\alpha_{2A/D}$-adrenoceptor promotes the release of nitric oxide, detectable as smooth muscle relaxation and which can be directly visualised. Main and first branch mesenteric arteries also have an endothelium-mediated relaxation via α_2-adrenoceptors but the subtype involved is not the $\alpha_{2A/D}$-adrenoceptor and, therefore, must be either the α_{2B}-adrenoceptor or the α_{2C}-adrenoceptor. Endothelium-mediated relaxation to α_2-AR activation in the first branch mesenteric artery, in contrast to the other vessels, does not involve nitric oxide; this is consistent with other agents in rat small mesenteric arteries, though not Ang II in the mouse (see above).

In the course of investigating the endothelium-mediated effects of Ang II and adrenoceptor agonists several issues arose which were further pursued in relation to a greater diversity of response than was initially anticipated. These included the nature of the contractions to i) L-NAME and ii) UK14304 and the variation of responses to adrenoceptors and AT receptors with age.

L-NAME (0.1mM) caused a contractile response in aorta that was inhibited by BMY7378 (0.1μM) by approximately 60%. In the α_{1D}-AR Knockout mouse this contractile effect was much smaller than in the wild type and was not sensitive to BMY7378 (0.1μM) but was reduced by approximately 35% by 5MU (0.1μM). This susceptibility to α-blockers would be consistent with the established proposal that α-adrenoceptors can be constitutively active. We now hypothesise that this spontaneous contraction is normally held back by constitutive release of nitric oxide and that L-NAME removes the influence of nitric oxide. The constitutive activity has previously been associated with α_{1D}-AR and uncovering it required manipulation of extracellular calcium. This new evidence indicates additional involvement of constitutively active α_{1A}-AR and suggests that it is there normally, at least under in vitro conditions, but is held back by inhibitory agents.

The relaxant response to UK14304 (1μM) was lost after L-NAME (0.1mM) (see above). In these circumstances UK14304 produced a contraction, additive to that of L-NAME, that was absent in the α_{1D}-AR Knockout, providing further evidence that its contractile action was via α_{1D}-AR. UK14304 acted as a partial agonist of α_{1D}-AR, causing weak contraction in high concentration that was absents in the α_{1D}-KO and antagonising α_{1D}-mediated contraction to phenylephrine in both intact and denuded vessels. In the presence of tone UK14304 produced relaxation that was absent in D79N or removal of the endothelium. Thus UK14304 contracts smooth muscle directly via α_{1D}-AR and relaxes smooth muscle via an endothelial effect indirectly.

The possible variations in involvement of different adrenoceptor and AT receptor subtypes at different ages and strains were studied. In aorta and superior mesenteric arteries, noradrenaline (NA) and phenylephrine (PE) produced responses related to age and strain. Comparisons of young D79N with WT showed reduced contractile responses to PE, suggesting reduced functionality of α_1-ARs in D79N. Laser scanning confocal microscopy showed that QAPB-binding intensity was reduced in the presence of in both control and BMY7378. This suggests a regulation of α_1-AR dependent on functional α_2-AR. The AT_2 mediated relaxation response to angiotensin II in young mice disappeared with age. Conversely, α_2-AR mediated relaxation was greater in older mice. This shows a remarkable age switch in the vasodilator influence of the renin-angiotensin II and adrenergic systems, in mouse major conductive arteries, in favour of adrenergic.

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Image 3-19: BMY7378 (0.1μM) {α_{1D}-antagonist} and 5MU (0.1μM) {α_{1A}-antagonist} and rauwolscine (0.1μM) {α2-AR antagonist} on α_{2A}-Knockout mouse aorta, stained with QAPB (0.1μM) (n=3).

Image 3-19: Control-four months α_{1B}-KO mouse superior mesenteric artery QAPB-binding presented in control.

Image 3-20: Four months wild type mouse superior (main) mesenteric artery, stained with QAPB (0.1μM).

Image 3-21: Unstained and control α_{2A}-Knockout mouse superior mesenteric artery.

Image 3-22: BMY7378 (0.1μM) {α_{1D}-antagonist} and 5MU (0.1μM) {α_{1A}-antagonist} or/and rauwolscine (0.1μM) on α_{2A}-Knockout superior mesenteric artery, stained with QAPB (0.1μM).

Image 3-23: Unstained α_{2A}-Knockout mouse superior mesenteric artery. Internal Elastic Lamina (IEL) autofluorescence (n=3).

Image 3-24: Control-four months α_{2A}-Knockout mouse superior mesenteric artery QAPB-binding present in the endothelial cells (n=3).

Image 3-25: BMY7378 (0.1μM) {α_{1D}-antagonist} and 5MU (0.1μM) {α_{1A}-antagonist}, on α_{2A}-Knockout superior mesenteric artery, stained with QAPB (0.1μM).

Image 3-26: Only rauwolscine (0.1μM) {α_2-AR antagonist}, on α_{2A}-Knockout superior mesenteric artery, stained by QAPB (0.1μM) (n=3).

Image 3-27: BMY7378 (0.1μM) {α_{1D}-antagonist} and 5MU (0.1μM) {α_{1A}-antagonist} and rauwolscine (0.1μM) {α_2-AR antagonist} on α_{2A}-Knockout superior mesenteric artery, stained with QAPB (0.1μM) (n=3).

Chapter Four

Fig. 4-1: Comparison between CCRC to in four months wild type mouse aorta (n=7).

Fig.4-2: comparison between cumulative concentration response to UK14304 in four months wild type in presence and absence of BMY7378 (0.1μM) in mouse aorta (n=5).

Fig. 4-3: Comparison between four months wild type (control) and α_{1D}-Knockout mouse aorta in cumulative response to UK14304 (n=7) on top of U19 pre-constriction.

Fig 4-4: Preconstricted, CCRC to UK14304 in four months wild type {intact and denuded} (n=5), Nashville D79N (n=4), α_{1D}-KO (n=7) and α_{2A}-AR Knockout (n=4) mouse aorta. Each point represents mean ± standard error.

Fig. 4-5: Effect of cumulative concentration of UK14304 on four months wild type (n=5) and Nashville D79N (n=4) mouse aorta.

Fig 4-6: Effect of UK14304 (1μM), prazosin (0.1μM) and BMY7378 (0.1μM) to shift CCRC to phenylephrine in four months wild type {intact and denuded} mouse aorta. In denuded aorta UK14304 could produce pre-constriction response due to lack of the endothelium.

Fig. 4-7: *Trace*; Using L-NAME (0.1mM) revealed the presence of constitutively active α_{1D}-AR in four months wild type aorta.

Fig. 4-8: *Trace*; Four months α_{1D}-Knockout mouse aorta. Using L-NAME (0.1μM) revealed the presence of constitutively active α_{1A}-AR.

Fig. 4-9: *Trace*; *High magnification*, Four months α_{1D}-Knockout mouse aorta. Using L-NAME (0.1μM) revealed the presence of constitutively active α_{1A}-AR.

Chapter Five

Fig.5-1: Comparison between CCRC response in four and fourteen months wild type mouse aorta to phenylephrine (n=7).

Fig. 5-2: Comparison between CCRC response in four and fourteen months wild type mouse aorta to noradrenaline (n=7).

Fig. 5-3: CCRC to noradrenaline and phenylephrine in four months wild type mouse aorta (n=7).

Fig. 5-4: CCRC to noradrenaline and phenylephrine in fourteen months wild type mouse aorta (n=6).

Fig. 5-5: Comparison between CCRC response in four and fourteen months wild type superior mesenteric artery to phenylephrine (n=6).

Fig. 5-6: Comparison between CCRC response in four and fourteen months wild type superior mesenteric artery to noradrenaline (n=6).

Fig. 5-7: CCRC to noradrenaline and phenylephrine in four months wild type mouse superior mesenteric artery (n=6).

Fig. 5-8: CCRC to noradrenaline and phenylephrine in fourteen months wild type mouse superior mesenteric artery (n=4).

Fig. 5-9: Comparison between four and fourteen months wild type mouse aorta in response to CCRC to UK14304 (n=5).

Fig. 5-10: Comparison between four and fourteen months wild type mouse superior mesenteric artery in response to UK14304 cumulatively (n=6).

Fig. 5-11: Comparison between four and fourteen months wild type mouse aorta in response to angiotensin II cumulatively.

Fig. 5-12: Comparison between four and fourteen months wild type mouse superior mesenteric artery in response to angiotensin II cumulatively (n=6).

Fig. 5-13: Comparison between response to CCRC to phenylephrine in wild type, D79N and α_{2A}-KO mouse aorta (n=7).

Fig. 5-14: Comparison between response to CCRC to phenylephrine in wild type and α_{2A}-KO carotid artery (n=4).

Fig. 5-15: Comparison between CCRC to phenylephrine in wild type and a_{2A}-KO superior mesenteric artery (n=6).

Fig. 5-16: Comparison between young & old wild type and α_{1D}-KO mouse aorta in response to UK14304 cumulatively on top of U46619 pre-constriction (n=7).

Fig. 5-17: Comparison between young & old α_{1D}-KO mouse aorta in response to UK14304 cumulatively on top of U46619 preconstriction (n=7).

Fig.5-18: comparison between old WT and α_{1D}-KO mouse aorta in response to UK14304 cumulatively on top of U46619 preconstriction (n=7).

Chapter five images

Image 5-1: Four months wild type (WT) and D79N mouse aorta smooth muscle cells which treated by both of losartan (10μM) and BMY7378 (0.1μM), then stained with rhodamine-angiotensin II-human (50nM) and QAPB (0.1μM).

Image 5-2: Four months WT and D79N mouse aorta smooth muscle cells which only stained with QAPB (0.1μM).

Final Conclusion images

Images FC-1: Four months wild type mouse aorta treated with losartan (10μM), rauwolscine (1μM) + BMY7378 (1μM) then stained with QAPB (0.1μM) and rhodamin-angiotensin II (50nM). Mosaicism related to myoendothelial connections.

Images FC-2: Young rat mesenteric artery endothelial cells, which connected to smooth muscle cells through the Internal Elastic Lamina (IEL) fenestrations.

Abbreviations

5HT	5-Hydroxytryptamin
A	Aorta
ACH	acetylcholine
ADP	adenosine 5'-diphosphate
Ang II	angiotensin II
Ang-(1-7)	amino-terminal heptapeptide fragment
Ang II-F	angiotensin II-Fluorescein
ATP	adenosine 5'-triphosphate
B_{Max}	maximum specific binding, expressed in fmol of radioligand/mg protein
AT-R	Angiotensin II -Receptors
BODIPY	4,4-Difluoro-4Bora-Fluoresent Dihydropyridine
BMY7378	8-[2-4(2-Methoxyphenyl)-1-Piperazin-8-azasprio[4,5]decane-7,9-dione-dihydrochloride
C	Carotid artery
Ca^{2+}	Calcium
$[Ca^{2+}]_i$	Intacellular calcium
CCR C	Cumulative Concentration Response Curve
CRC	Concentration Response Curve
Cyclic AMP/cAMP	adenosine-3', : 5'cyclic monophosphate
Cyclic GMP/cGMP	guanosine-3' : 5'cyclic monophosphate
CDNA	single stranded DNA
CI	confidence interval
cpm	counts per minute
CRC	concentration response curve
DAG	diacylglycerol
DR	dose retio
DMSO	dimethylsulfoxid [(CH3)2SO]
EC_{50}/pEC_{50}	The molar concentration of an agonist that produces 50% of the maximum response of that agonist/negative logarithm to base 10 of EC_{50}
EDRF	Endothelium Derived Relaxing Factor
EDTA	ethlenediaminotetra acetic Acid
EEL	External Elastic Lamina
Endo	Endothelial cell
eNOS	endothelial NOS
FC	Final conclusion
FM	First branch mesenteric artery
g	gravity
GDP	guanosine diphosphate
GIRK K^+	G Protein-gated Invardly Rectifying K^+ channel (A type of ion channel which found in Heart and CNS)
G protein	GTP-dependent regulatory proteins
GPCR	G protein coupled receptor
GRK	G protein receptor coupled kinase
GTP	guanosine triphosphate
IEL	Internal Elastic Lamina
iNOS	inducible NOS

Ins(1,4,5) P^3 or IP3	inositol (1,4,5,) triphosphate
Kb	kilobase
K_B/pK_B	equilibrium dissociation constant of an antagonist/ negative Logarithm to base 10 of K_B
K_D	concentration of radioligand which occupies 50% of receptors at equilibrium
kDa	kilo Daltons
K_i /p K_I	concentration of competitor that will bind to 50% of receptors in the absence of radioligand or any other competitor at equilibrium/ negative logarithm to base 10 of K_i (K_i = EC50/1+ [Ligand]/K_d)
KO	knockout / α_{1D}-knockout
K+	Potassium
KCl	Potassium chloride
L-NAME	N-nitro-L-arginine Methyl Ester
Lo	Losartan
NA	Noradrenaline
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
PE	Phenylephrine
PD123319	S-[+]-1-[(4-dimethylamino]-3-methylphenyl) methyl] -5-[diphenylacetyl] -4,5,6,7-tetrahydro-1H-Imidazo [4,5,-C] Pyridine-6 carboxylic acide
PG	prostaglandin
PI	Propidium Iodide (FW= 668.4)
QAPB	Quinazolinyl piperazine borate-dipyrromethene
Rho-Ang II-H	Rhodamine-Angiotensin II (Human)
Rauw	Rauwolscine
SEM	Standard Error of the Mean
SM	Superior mesenteric artery
SphA	Saphenous artery
SWT	Swiss Wild Type
U46619	9,11-dideoxy-11α, 9α-epoxymethanoprostaglandin $F_{2\alpha}$
UK14304	5-Bromo-6-(2-imidazolin-2-ylamino) quinoxaline

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Declaration

The work presented in this thesis is entirely my own, with the exception of Figure 1-2, which kindly donated by Jude S. Morton, Figures 2-15 and 3-24 which belong to Dr. C. J. Daly, Figures 2-17, 2-18 and 5HT potential synergism graphs in definition which belong to Ali Zeeshan and FC-2 images that belong to Dr. Jose Maria Gonzales.

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3. McGrath JC, Pediani JD, Macmillan J., Mackenzie J., Deighan C., Woolhead A., McGrory SP., McBride M., Ali Z., **Malekzadeh-Shafaroudi M.,** Cotecchia S., Arribas SM., Vila E., Briones A., Perez D., Mullins J., Tsujimoto G & Daly CJ. (2002). Adventitial cells are identified as the major location of vascular alpha1B-adrenoceptors and may drive vascular remodelling. *Br. J. Pharmacol.*, **137** (Suppl); 21P (Abstract)

4. **Shafaroudi M. M.,** Daly C. J. & McGrath J. C., (2003) "Analysis of alpha2-adrenoceptor- and angiotensin-mediated responses in mouse mesenteric artery."

Physiological society, Young Physiologist Symposium. Coventry University, 25th & 26th September/2003. (Oral Presentation).

5. McBride M. J., **Shafaroudi M. M.,** Daly C. J., and McGrath J. C., (2004) "A study of α2-adrenoceptor-mediated vasodilatation in mesenteric resistance arteries from transgenic mouse." The Physiological Society Abstracts., 28th to 31st March/2004, University of Glasgow (smooth muscles), Page: 101/PC35.

Definitions

Different types of antagonism

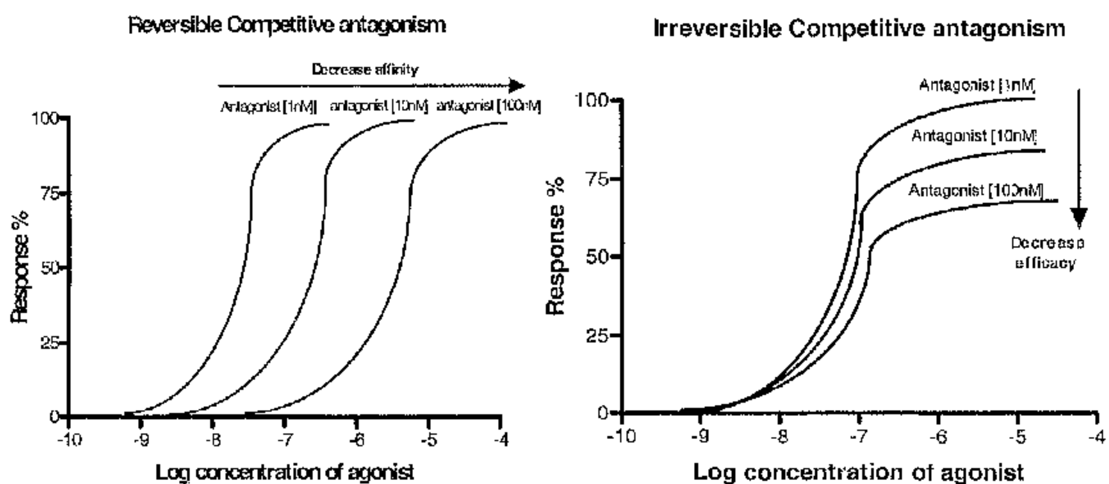
1) Competitive (Reversible or Irreversible): A competitive antagonist affects on agonist by acting at the same point of receptor chain.

The term *antagonist* refers to any drug that will block, or partially block, a response. When investigating an antagonist the first thing to check is whether the antagonism is surmountable by increasing the concentration of agonist. The next thing to ask is whether the antagonism is reversible. After washing away antagonist, does agonist regain response? If an antagonist is surmountable and reversible, it is likely to be competitive

A good example of this type of antagonism is Tubocurarine on response to Acetylcholine at the motor end-plates of skeletal muscle.

a) In reversible competitive antagonism, antagonist can shift CCRC to agonist to the right. However, the maximum response to agonist doesn't change.

b) In irreversible competitive antagonism, Antagonist does not shift the CCRC to agonist to the right (no changes along X-axis). However, the maximum response to



agonist will decrease.

A competitive antagonist binds reversibly to the same receptor as the agonist. A dose-response curve performed in the presence of a fixed concentration of antagonist will be shifted to the right, with the same maximum response and (generally) the same shape.

Gaddum derived the equation that describes receptor occupancy by agonist in the presence of a competitive antagonist. The agonist is drug A. Its concentration is [A] and its dissociation constant is Ka. The antagonist is called drug B, so its concentration is [B] and dissociation constant is Kb. If the two drugs compete for the same receptors, fractional occupancy by agonist (f) equals:

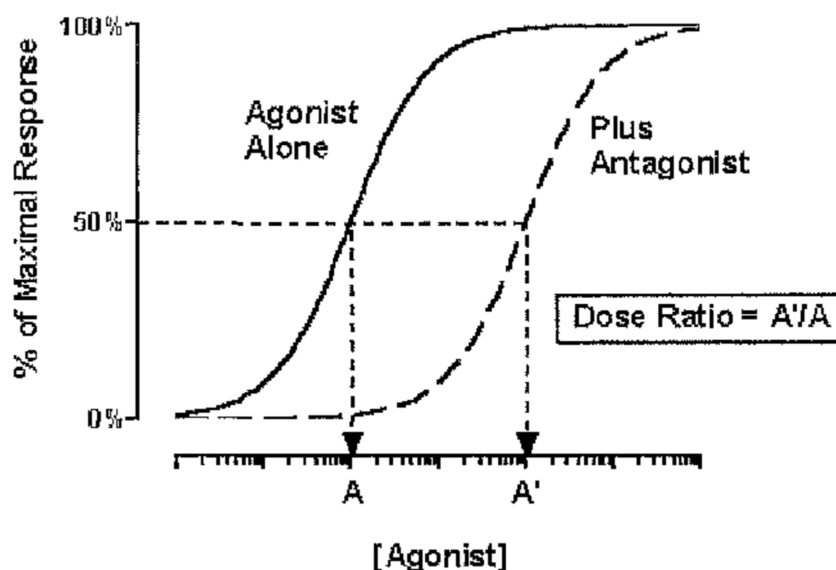
$$f = \frac{[A]}{[A] + K_a\left(1 + {[B]}/{Kb}\right)}$$

The presence of antagonist increases the EC50 by a factor equal to 1+[B]/Kb. This is called the dose-ratio.

You don't have to know the relationship between agonist occupancy and response for the equation above to be useful in analyzing dose response curves. You don't have to know what fraction of the receptors is occupied at the EC50 (and it doesn't have to be 50%). Whatever that occupancy, you'll get the same occupancy (and thus the same response) in the presence of antagonist when the agonist concentration is multiplied by the dose-ratio.

The graph below illustrates this point. If concentration A of agonist gives a certain response in the absence of antagonist, but concentration A' is needed to achieve the same response in the presence of a certain concentration of antagonist, then the dose-ratio equals A'/A. You'll get a different dose ratio if you use a different concentration of antagonist.

If the two curves are parallel, you can assess the dose-ratio at any point. However, you'll get the most accurate results by calculating the dose-ratio as the EC50 in the presence of antagonist divided by the EC50 in the absence of antagonist. The figure below shows the calculation of dose ratio.



2) Non-competitive antagonism: A Non-competitive antagonist affects on agonist by acting at some other point of receptor chain which different from agonist binding area.

3) Physiological / Functional antagonism: An antagonist acts on separate cells, physiological system or another second messenger inside, the effector to antagonise the agonist effect.

4) Pharmacokinetic antagonism: A pharmacokinetic antagonist reduces the concentration of agonist at its site of action by effecting on the pharmacokinetic of the agonist. For example Phenobarbitols can increase hepatic metabolism of Warfarin.

5) Chemical antagonism: A chemical antagonist chemically react with the agonist itself, the agonist is changing chemically and its activity as an agonist will loose. For example; mercury (Hg^{2+}) can combines with the sulphydryl groups of enzyme and co-enzyme and inactive them.

Schild (Competitive test) Plot:

Schild analysis is used to determine the nature of antagonists to its receptor. Schild plots also give information on the potency of competitive antagonists and can hint at the presence of multiple binding sites. The Schild equation is;

Schild Equation:

(From curvefit.com. Copyright 1999 by GraphPad Software, Inc. All Rights Reserved.)

(conc. ratio - 1) = (antagonist conc.) / K_B

If the antagonist is competitive, the dose ratio equals one plus the ratio of the concentration of antagonist divided by its Kd for the receptor. (The dissociation constant of the antagonist is sometimes called Kb and sometimes called Kd)

$$\text{Dose ratio} = 1 + \frac{[B]}{K_b} = 1 + \frac{[\text{Antagonist}]}{K_d}$$

A simple rearrangement gives:

$$\text{Dose ratio} - 1 = \frac{[\text{Antagonist}]}{K_d}$$
$$\log(\text{dose ratio} - 1) = \log([\text{Antagonist}]) - \log(K_d)$$

If you perform experiments with several concentrations of antagonist, you can create a graph with log(antagonist) on the X-axis and log(dose ratio -1) on the Y-axis. If the antagonist is competitive, you expect a slope of 1.0 and the X-intercept and Y-intercept will both equal the Kd of the antagonist.

(conc. ratio - 1) = (antagonist conc.) / K_B

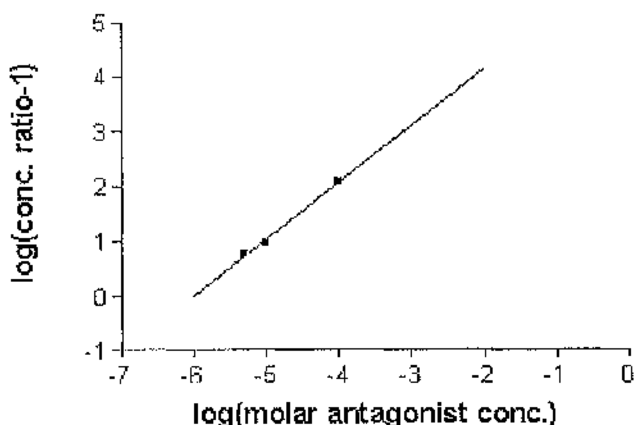
Conc. ratio = Conc. of agonist producing a defined response in the presence of an antagonist, divided by the concentration producing the same response in the absence of the antagonist. So antagonist EC_{50} / agonist EC_{50}.

K_B = dissociation equilibrium constant for the antagonist

From the Schild plot a value known as the pA_2 can be found. The pA_2 is the negative logarithm of the concentration of antagonist, which would produce a 2-fold shift in the concentration response curve for an agonist, and is a logarithmic measure of the potency of an antagonist.

The pA scale is useful as it performs as an empirical measure of antagonist potency, which theoretically could characterise activity, specificity, and time-action relationships. This scale allowed scientists to present findings empirically instead of describing their results as "very sensitive". The pA_2 is calculated by extrapolating the value on the x-axis when y=0

So in this example the pA_2 is 6.



The slope of the Schild plot gives information about the nature of the antagonist i.e. whether or not it is competitive binding and information on the cooperativity. The steepness of slope depends upon both the equilibration time and the degree of antagonism.

When the slope of the Schild plot is not 1 a number of possibilities arise: (1) the antagonist is not competitive, (2) a multimolecular interaction between drugs and receptors is being observed, (3) equilibrium conditions have not been attained in the experimental procedure.

The third condition is important as dynamic equilibrium in contrast to true equilibrium conditions may distort the nature of the antagonism; i.e. a competitive antagonist could

appear to be non-competitive. This could occur in isolated tissues, which possess uptake and/or degradative mechanisms for either the agonist or antagonist.

When the Schild slope is equal to 1 this indicates that the antagonism is competitive and reversible. It also indicates that the agonist is acting at a single receptor subtype, and that the tissue has no uptake mechanism for the agonist. It can also be concluded that the antagonist causes a parallel rightward shift of the log agonist concentration response curve with no loss of maximal response.

Synergy:

The application of two or more agonists results in a response that is much larger than the sum of the individual responses of the agonists alone.

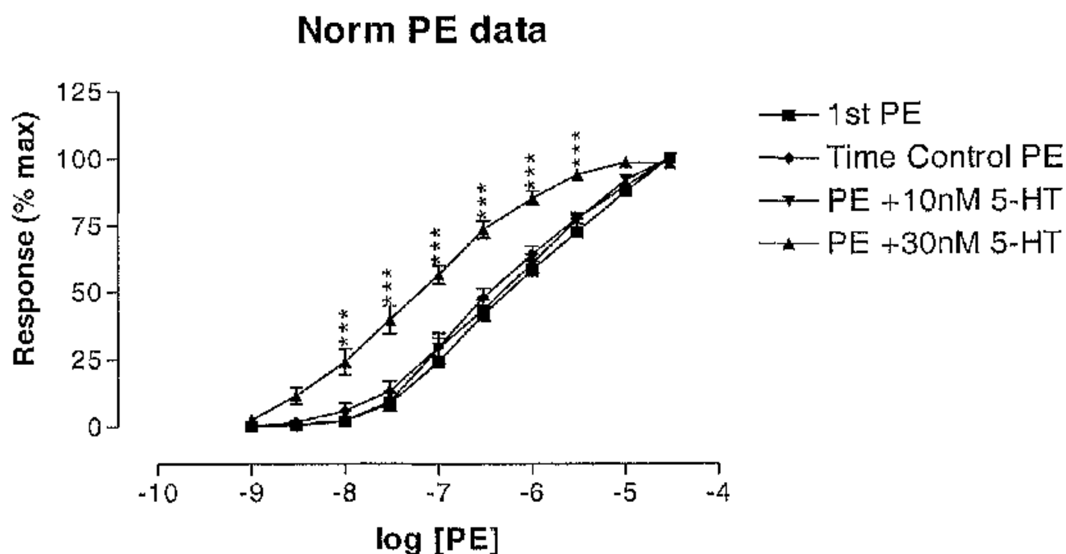
Stupecky *et al.* (1986) performed study in the rabbit aorta using concentrations of agonists that gave a 0.1g response and combined them. Response size varied from 0.5g to 2.7g.

Also did various dose response curves and described two types of synergy originally described by Draskoczy & Trendelenburg (1968) and later by Asano & Hidaka (1980)

Potentiation synergism: Presence of a synergist shifts dose response curve to the left in parallel fashion

Threshold synergism: Presence of a synergist shifts dose response curve to the left at threshold but converges with control curve: considered to be an additive effect. (Stupecky *et al.* termed this Threshold synergism)

Potentiation synergism: Phenylephrine Synergy with 5-HT



(Ali Zeeshan results on PE Synergy with 5HT):

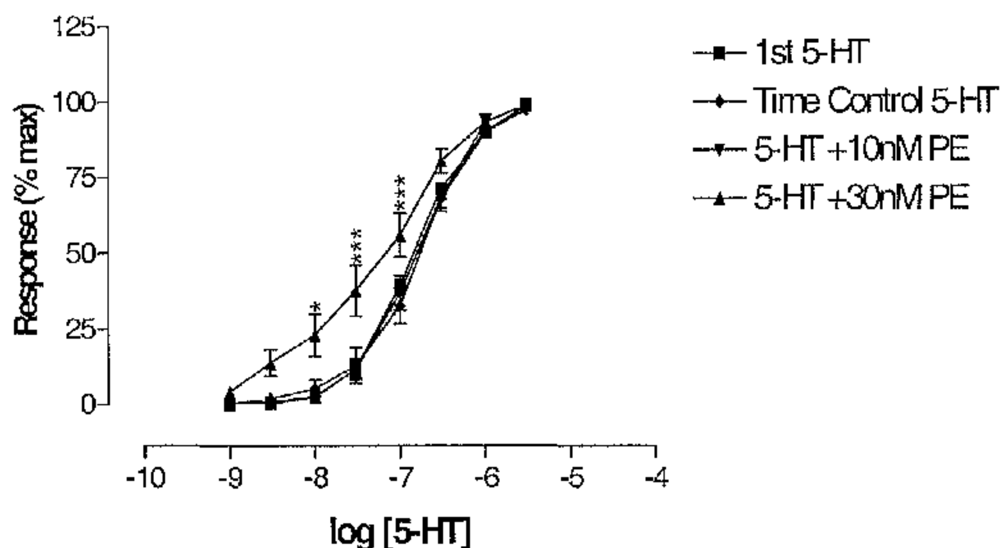
*** PE response driven primarily via α_{1D}-ARs-Minor role of α_{1B}-ARs in contraction**

*** 5-HT response mainly mediated via 5-HT$_{2A}$ receptors**

In the SWT mouse aorta the 5-HT response is partially α_{1D}-AR mediated: minor role

*** Adding 5-HT involves the recruitment of a 'new' component to the PE response here**

Threshold synergism: 5-HT Synergy with Phenylephrine



(Ali Zeeshan results on 5HT Synergy with Phenylephrine):

- **Synergy only using 30nM PE, no synergy observed with 10nM PE**
- **Curve shifts to left but converges**

Formulas:

A) Formula for calculation of concentration related to molecular weight (MW of FW), amount weighted (AW) and Volume (V) of dissolved drug.

IF:

V= Volume (ml), AW= Amount Weighted (mg)

Conc.= Concentration required (Mol), MW= Molecular Weight (g)

So:

V = AW / (Conc. * MW)

B) Formula for changing in concentration related to volume.

IF:

M1= concentration drug1 (Mol/Litter or Molar), V1= volume of drug 1,

M2= concentration of drug2 (Mol/Litter or Molar), V2= volume of drug2

So:

M1 V1= M2 V2

Amira and Imaris Software definitions

Calibration: A 2D series of images has both an XY resolution and a Z speacing. The XY resolution defines the number of Pixels per micron for a given set of conditions (i.e., objective power, wavelength etc.). The Z spacing describes the distance between XY planes in the axial (optical axis) dimension. For accurate measurement and effective visualisation, this geometry must be maintained when constructing iso-surfaces or 3D volumes.

Orthoslice: Orthoslicing is a mean of re-sectioning an image volume in a user defined axes. Typically this enables viewing in the X-Z or Y-Z plane. However, some software packages allow non-orthogonal slicing which enables an image volume to be viewed from any angle, plane or viewpoint.

Iso-Surface: An Iso-surface is formed by creating triangles or other geometric shapes, the points of which are located at areas or equal intensity. Thus, an Iso-surface is a map of intensity values which can be assigned different colours, transparencies and textures such that multiple surfaces can be more easily visualised.

Voltex: This module is used to render a 2D series of images into a 3D volume for visualisation. Rendering can be achieved by a variety of algorithms. Regardless of rendering method, generally each 3D Pixel (Voxel) is assigned both a colour and transparency value.

Deconvolution: Deconvolution provides the same exclusion of out of focus blur at a given point as confocal microscopy does, but the mechanism is mathematical processing by computer. In its best case, having been given the size of each pixel and information about the particulars of the optics, an algorithm excludes out of focus blur that is not from light scattering in the plane of focus and reassigns scattered light that should be in the plane of focus to its proper location.

Chapter 1

Literature review and introduction

1-1. Introduction:

Part of the study of the circulation system in vertebrates is focused on the vascular system. This part includes arteries, veins and lymphatic vessels. There are different type of arteries and veins in the bodies of vertebrates. These vessels have a major role in maintenance of blood pressure through the vascular smooth muscle in their media, and different other types of cells in intima and adventitia. The main system controlling blood pressure in the body is the sympathetic nervous system. At the end of Axons of the sympathetic system there are many variscosities, which contain noradrenaline in vesicles that can cause contraction in smooth muscle cells (SMC) of vessels. But also there are some hormones and other factors, which can act on Smooth muscle cells. One of them is the renin–angiotensin system. These neurotransmitters and chemical substances act on some complex molecules on cell membrane, which are called receptors. Receptors mediate some effects in cells by using second messengers. Many receptors exist on the cell membrane of vascular smooth muscle, including adrenergic receptors and angiotensin II receptors. There is also some interaction between these receptors. Understanding these interactions between receptors, vascular morphology, and modification which occur in pathological conditions in different parts of the vascular structure is useful for treatment of hypertension. Several methods will be used for study of vessels, particularly arteries, including ***wire and perfusion myography, Laser Scanning Confocal Microscopy (LSCM), Fluorescent Microscopy, Immunofluorescent Microscopy, Electron Microscopy (SEM & TEM), Light Microscopy and Histochemistry (using different staining and ligands).*** The aim of this literature review is to review vascular structure, particularly arteries and some current methods for study of them in normal and pathological conditions, study of receptors especially adrenergic and angiotensin II receptors and interaction between them, and definition of the role of these interaction in remodelling of vascular structure, methods

for localisation of receptors intracellular or on the cell membrane, and methods for tracing of these receptors during desensitisation or when any other changes occur in cell structure.

1-2. Arterial Morphology:

Resistance arteries may be defined as pre-arteriolar vessels with a luminal diameter of less than 500μm in human (Bloom & Fawcett 1968). However the lumen diameter is showed different size among animals. (i.e. in cat is less than 300μm) (Johnson 1962). In common with larger arteries, resistance arteries comprise an outer *tunica adventitia*, a central *tunica media* and an inner *tunica intima*.

The tunica adventitia is composed of connective tissue, which contains both elastin and collagen as well as other bodies such as *fibroblasts*, *macrophages* and *schwann cells*. This is also the layer of the blood vessel, which contains the nerves associated with sympathetic inervation (Lee *et al.,* 1983). These nerves are bundles of primarily adrenergic axons and do not penetrate into the media layer. Axons close to the adventitial-medial layer are found in the numbers of between 1 and 11 per bundle whereas remote outer areas of the adventitial-medial border contain bundles of axons up to 50 in number. A majority of these nerves are unmyelinated and surrounded by schwann cells. Each of the adrenergic axons contained in the adventitial layer have *variscosities* which, for example in the guinea pig submucosal artery, are less than 3 μm. Contained with these variscosities there can be anything up to 500 vesicles of 100 nm in diameter containing a number of neurotransmitters but predominantly noradrenaline. As a result of stimulation of these nerves the neurotransmitter from the vesicles is released upon which random diffusion takes place towards receptors located on the surface of the vascular smooth muscle cells.

The tunica media of resistance arteries is composed of vascular smooth muscle cells bound by an internal elastic lamina. In small resistance-sized arteries the external elastic lamina in fragmented or absent (Carlson *et al.*, 1982).

The smooth muscle cells are arranged circumferentially around the vessel diameter with an angular set up of less than 2°, although it has been reported that smooth muscle cells in the vascular wall can have pitch angles of +/- 10° or more (Gattone *et al.*, 1986) (Miller *et al.*, 1987) (Walmsley *et al.*, 1982) (Walmsley *et al.*, 1983) (Mulvany *et al.*, 1978) (Lee *et al.*, 1983).

The number of layers of smooth muscle within the blood vessel wall is directly proportional to the diameter of vessel (Lee *et al.*, 1983). In vessels with a diameter of around 300 μm there are approximately six layer of smooth muscle in the media whereas there may only be a monolayer of vascular smooth muscle cells in the media layer of arteries with a diameter of between 30 to 50μm (Miller *et al.*, 1987). The tunica media comprises approximately 70-80% of the vessel wall. This does not alter with decreasing lumen diameter.

The third and last layer of a resistance artery is the tunica intima. This layer comprises of a monolayer of endothelial cells orientated in the direction of the long axis of the artery and in the direction of blood flow. These cells have the approximate dimensions of 30-50μm length, 10-20μm width and 2μm thickness (Carlson *et al.*, 1982). This layer is bounding by a sheath of elastin known as the internal elastic lamina.

The endothelial layer plays an important role in the blood vessel's ability to modulate arterial tone. This is due to the ability of these cells to release substances known as endothelial derived relaxing factors, arguably the most important being *Nitric Oxide.*

This substance is released when stimulation acts on the surface of the endothelial cells. This may be due to mechanical factors such as sheer stress across the endothelial surface caused by viscosity and flow or to chemical stimulus by agonists or other agents. Examples of endothelium dependent agonists include *acetylcholine*, *bradykinin*, *angiotensin II* and a range of *prostaglandins*.

Within the internal elastic lamina there are small holes known as fenestrations, these allow nutrients and other substances to pass through the blood vessel wall from blood to the tissues and from tissues to the blood. Frequently endothelial cells in the tunica intima protrude through these holes and make direct physical contact with the smooth muscle cells in the tunica media (Carlson *et al.*, 1982) (Smeda *et al.*, 1988). Although the endothelial cells form a monolayer they are not packed tightly into the internal area of the artery but are loosely spaced and connected by elastin fibers and collagen fibrils. This allows the movement of substances across the vessel wall and determines *endothelial permeability* an important factor in vascular physiology.

1-3. Smooth Muscle Cell Contraction Mechanism:

In striated muscle sliding of one set of filamentous proteins (*myosin*) related to another (*actin*) leads to contraction. In skeletal muscle and cardiac muscle *troponin* and *tropomyosin* regulate the degree of interaction between *actin* and *myosin* and therefore the degree of contraction. Ca^{2+} binding to *troponin* regulates this activity. However, the regulation of smooth muscle contraction has completely different mechanism.

In smooth muscle cells myofibrils (bundles of *myosin* and *actin*) are arranged in a spiral formation, the origin and insertion on the membrane is marked by a dense body. Thus on contraction, the smooth muscle cell may twists as it shortens. Smooth muscle cell has no *troponin* and the role of *tropomyosin* is unknown.

In all muscles G-actin forms α-helical filaments, associated with F-actin is *tropomyosin*, a rod shaped protein. The *myosin* filament consists of two main parts.

1-Light meromyosin (LMM) a coiled coil α-helix

2-Heavy meromyosin (HMM) consists of HMM S1 and HMM S2.

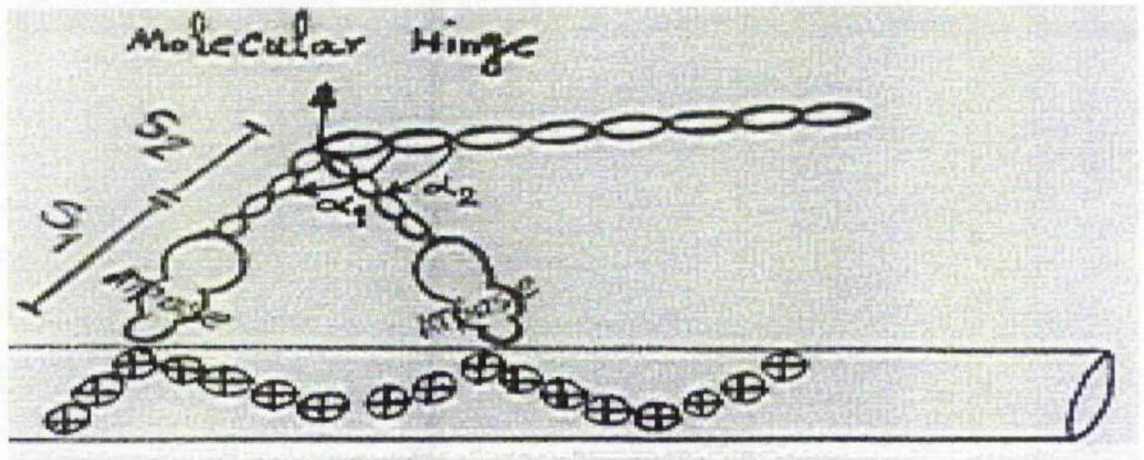
HMM S1 divided into three sections:

a) 50 KD actin binding site

b) 25 KD ATPase activity site

c) 20 KD light chain attach

HMM S_1 is a globular protein that can bind to a particular site on actin. There is a molecular hinge between HMM S_1 and HMM S_2 and this movement cause the relative sliding of *myosin* with respect to *actin*.



[Contractile filaments diagram in skeletal muscle cells]

Bound to each globular head is an additional proteins termed myosin light chains, These proteins are thought to play a major role in the regulation of contraction in smooth muscle. Each globular head contains two types of light chain (LC_1 and LC_2) each with a molecular weight of approximately 20000 KD. In general, smooth muscle has more actin than striated muscle and less myosin. {1 mole of myosin / 4 mole of actin VS 1 mole of myosin / 40 mole of actin}. Despite these differences the maximal force generated by smooth muscle per cross sectional area is very close to that generated by striated muscle.

1-The time a cross bridge spends attached to actin in smooth muscle is approximately 500 msec. However, in striated muscle this is only 10 –20 msec.

2-This phenomenon is also reflected in the speed of contraction of smooth muscle that is much slower than striated muscle.

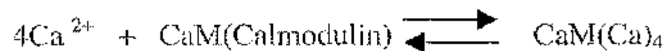
3-Therefore the rate of breakdown of ATP is again less.

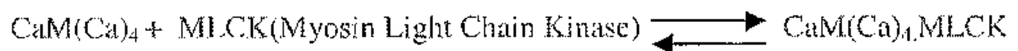
4-Thus force production from smooth muscle is much more economical than comparable level of force in striated muscle but at the cost of slowness of response.

1-4. Regulation of contraction:

a) Thick filament regulation:

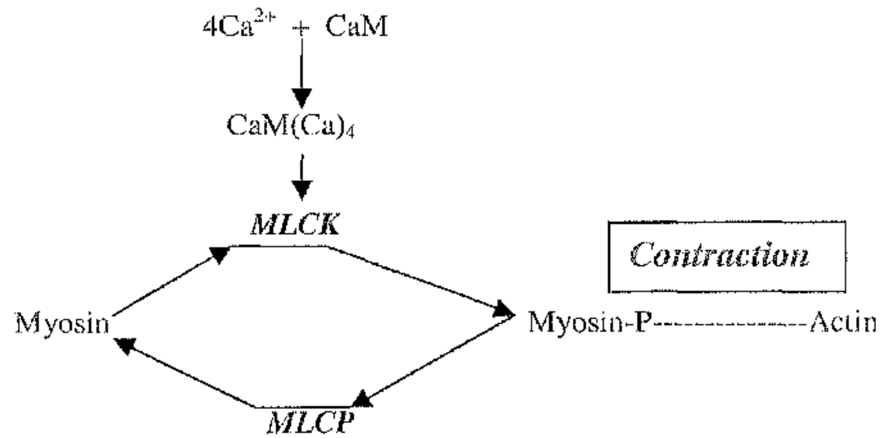
In smooth muscle myosin-based regulation is thought to be the main mechanism for regulation. As with other muscles [Ca^{2+}] at rest is equal to 100-200nM but, to initiate of contraction [Ca^{2+}]=1μM. The main Ca^{2+} sensitive protein in smooth muscle is *calmodulin*





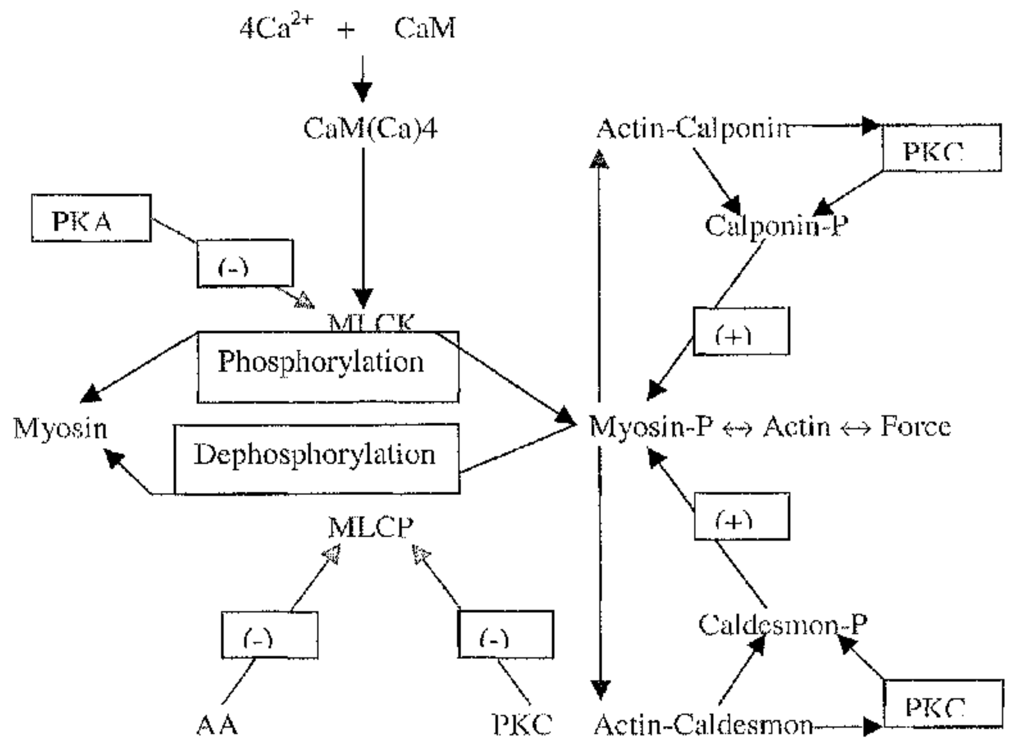
[Inactive] *[Active]*

MLCK is an enzyme that catalyses the phosphorylation of myosin light chain–1 (LC-1) in this situation. Myosin can interact with actin and generate force (and breakdown ATP). Relaxation would only occur if LC-1 on myosin dephosphorylated. Myosin Light Chain Phosphatase (MLCP) is the enzyme responsible for the dephosphorylation of LC-1 on myosin.

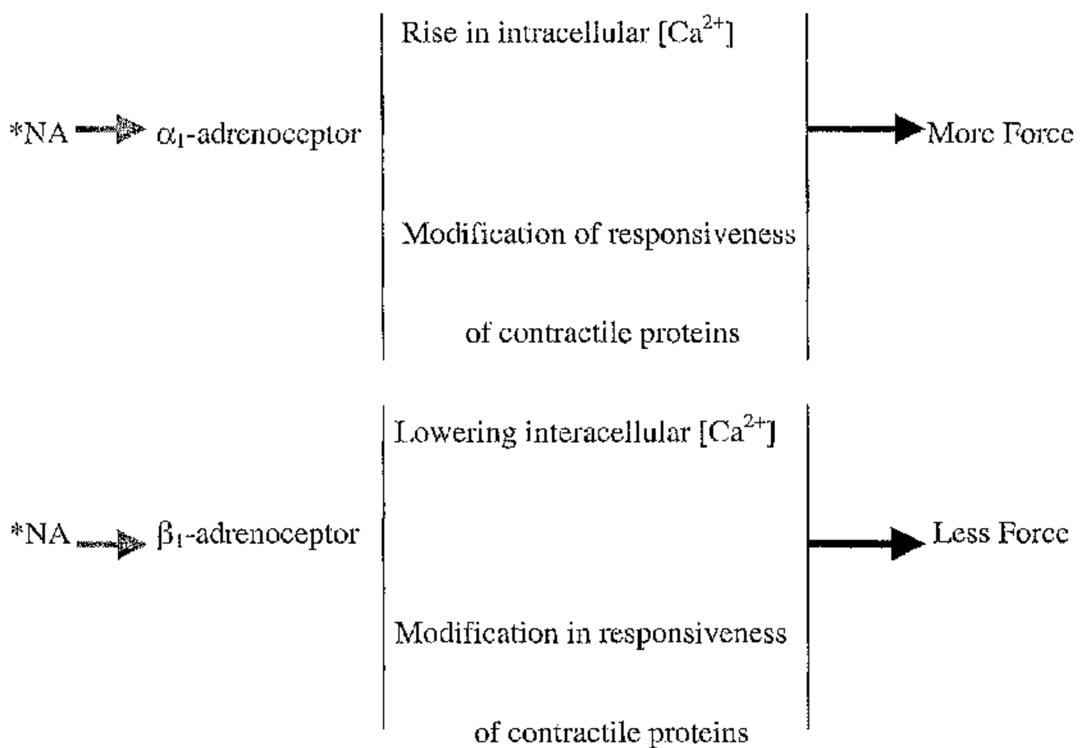


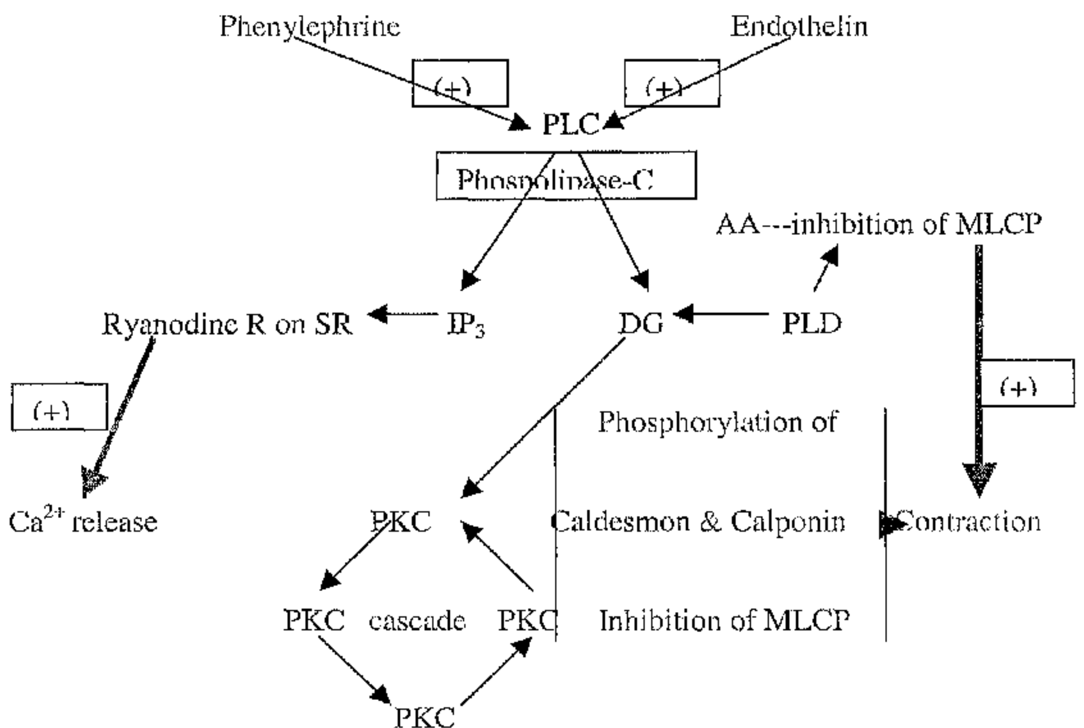
b) Thin Filament Regulation:

This regulation is due to troponin-like protein called caldesmon and calponin. Both of them bind to actin and inhibit actin-myosin interaction. However, the phosphorylated form of caldesmon or calponin has a very low affinity for actin.



Agonist induced changes in the responsiveness of contractile protein to Ca^{2+}:





Phenylephrine can stimulate PKC without a rise in intracellular [Ca^{2+}]. This effect may have a role in the maintenance of basal tone in smooth muscle.

c) cAMP & cGMP affects on smooth muscle lead to Relaxation:

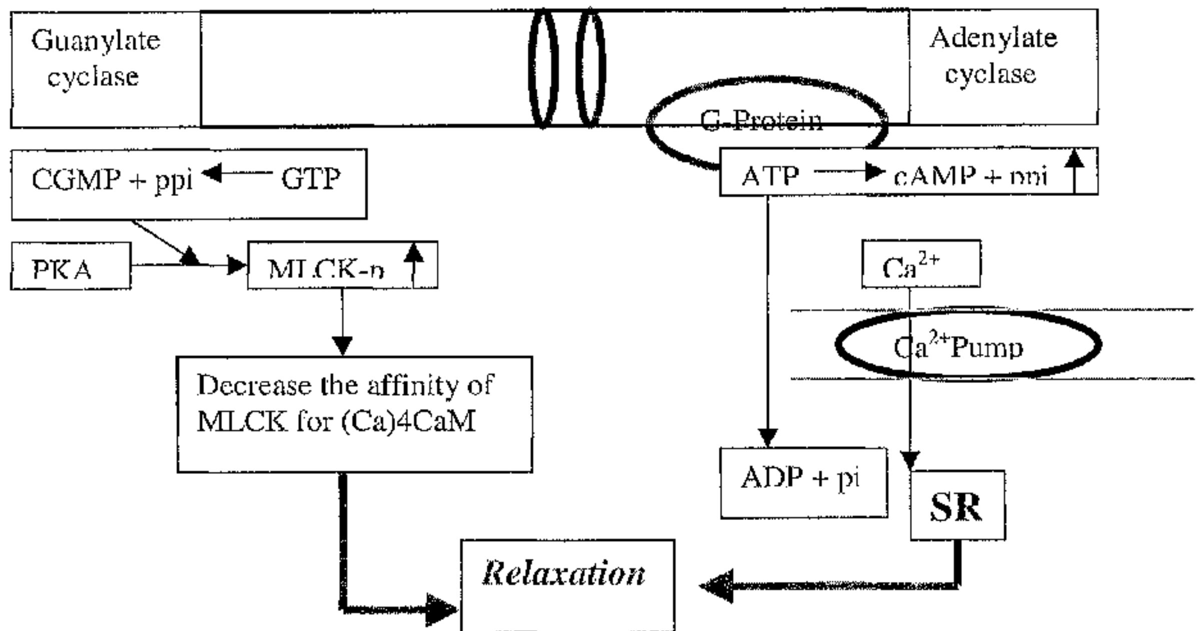
Atrial natriuretic factor

& NO (Nitric Oxid)

Noradrenaline

↓

B_1-adrenoceptor



1-5. Renin-Angiotensin-Aldosterone mechanism:

The juxtaglomerular apparatus:

The juxtaglomerular apparatus (JGA) is a specialisation of the glomerular afferent arteriole and the distal convoluted tubule of the corresponding nephron and is involved in the regulation of systemic blood pressure via the renin-angiotensin-aldosterone mechanism.

The juxtaglomerular apparatus is made up of three components, the macula densa of distal convoluted tubule, renin-secreting juxtaglomerular cells of afferent arteriole and extraglomerular mesangial cells.

Macula densa:

On returning to the cortex from renal medulla, the ascending thick limb of the loop of henle becomes the first part of the distal convoluted tubule and comes to lie in the angle between the afferent and efferent arterioles at the vascular pole of the glomerulus. The macula densa is an area of closely packed, specialised cells lining the distal convoluted tubule where it abuts onto the glomerular vascular pole. Compared with other DCT lining cells, the cells of macula densa are taller and have larger more prominent nuclei, which are situated towards the luminal surface. The basal cytoplasm is crammed with mitochondria. The basement membrane between the macula and underlying cells is extremely thin. The cells of the macula densa are thought to be sensitive to the concentration of sodium ions (or chloride ion) in the fluid within the DCT; decrease in systemic blood pressure results in decreased production of glomerular filtrate and hence decreased concentration of sodium ions in the distal tubular fluid.

Juxtaglomerular cells:

Juxtaglomerular cells (J) are specialized smooth muscle cells of the wall of the afferent arteriole forming a cluster around it just before it enters the glomerulus. Juxtaglomerular cell cytoplasm contains immature and mature membrane-bound granules of the enzyme renin. Juxtaglomerular cells show characteristics of protein-secreting cells, including an abundant rough endoplasmic reticulum, a highly developed Golgi complex, and secretory granules measuring approximately 10 –14nm in diameter.

Extraglomerular mesangial cells:

Also called goormaghtigh cells or lacis (L) or polkissen cells, these cells form a conical mass, the apex of which is continuous with the mesangium of the glomerulus: laterally it is bounded by the afferent and efferent arterioles and its base situated onto the macula densa. The lacis cells are flat and elongated with extensive fine cytoplasmic processes extending from their ends and surrounded by a network (lacis) of mesangial material. Despite their central location in the JGA, the function of the extraglomerular mesangial cells remains obscure. They have previously been attributed the function of secretion of the hormone erythropoietin which stimulates red cell production in the bone marrow.

Role of the Juxtaglomerular Apparatus in control of blood pressure:

The Juxtaglomerular apparatus is believed to act both as a baroreceptor and chemoreceptor controlling systemic blood pressure by the secretion of renin by the juxtaglomerular cells.

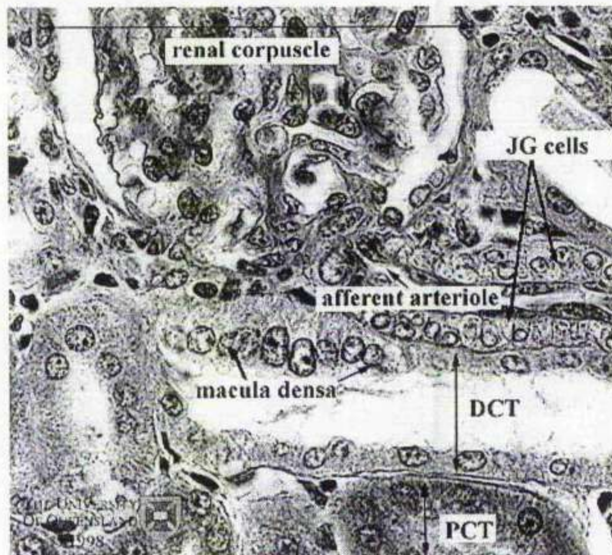
The juxtaglomerular cells are suitably placed to monitor systemic blood pressure, with a fall in blood pressure, resulting in renin secretion. Reduction in blood pressure results in

reduced glomerular filtration and consequently a lower concentration of sodium ions in the DCT. Acting as chemoreceptors, the cells of the macula densa in some way then promote renin secretion.

Renin diffuses into the bloodstream, catalysing the conversion of angiotensinogen, an alpha2-globulin synthesized by the liver, into decapeptide angiotensin I. In the lungs, angiotensin I change to angiotensin II by angiotensin Converting Enzyme (ACE) which is a potent vasoconstrictor. Angiotensin II formation in rodent vessels is almost totally ACE-dependent (Okumishi *et al.*, 1993). However, Animal studies have identified ACE-independent angiotensin II generation in a variety of species and preparations: chymostatin-sensitive Ang II generation (CAGE) has been observed in canine mesenteric arteries and monkey pulmonary and mesenteric arteries (Okunishi *et al.*, 1984) (Mangipane *et al*, 1994). In human myocardial tissue, angiotensin II generation is mediated by both ACE and chymase, with the latter pathway predominant (Urata *et al.*, 1990). Vascular ACE-independent Ang II generation has also been observed in adventitial cells. Thus, 60% of the Ang-I mediated contraction in human gastroepiploic arteries is ACE-independent (Bund *et al.*, 1989). Angiotensin II raises blood pressure in two ways:

1- Constriction of peripheral blood vessels and release of aldosterone from the adrenal gland cortex.
2- Aldosterone promotes the reabsorption of sodium ions and therefore water from the DCT and collecting tubes, by stimulating ADH release from parsnevrosa of hypophesis, thus expanding the plasma volume and hence increasing blood pressure

(Burkitt *et al.*, 1996) (Jeffrey 2000) (Junqueira *et al.*, 1998).



Mammalian Juxtaglomerular Apparatus H&E staining.

Angiotensin II properties:

Angiotensin II is well known to cause potent increases in systemic and local blood pressure via its vasoconstrictive effect, to influence renal tubules to retain sodium and water, and to release aldosterone from adrenal gland. Angiotensin II may play a central role not only in the aetiology of hypertension but also in the pathophysiology of cardiac hypertrophy and remodelling, heart failure, vascular thickening, atherosclerosis and glomerulosclerosis in humans. Angiotensin II directly causes cell growth, regulates the gene expression of various bioactive substances {vasoactive hormones, growth factors, extracellular matrix components (ECM), cytokines and so on}, and activates multiple intracellular signalling cascades {tyrosine kinases, mitogen-activated protein (MAP), kinase cascades, various transcription factors, and so on}. Angiotensin II may directly cause cardiovascular and renal diseases, independent of its blood pressure-elevating effect (Kim *et al.*, 2000).

1-6. Classification of vascular smooth muscle cell surface receptors:

There are three different groups of receptors, which situated on the cell membrane of smooth muscle cells.

1) G-protein coupled receptors:

This type of receptors acts where slower events are modulated at slow synapses or for hormones. (seconds or minutes)

-Acetylcholine (Muscarinic Receptors) {M_2 in heart and M_3 vascular smooth muscle cells}

-Adrenaline and Noradrenaline ($\alpha1$, $\alpha2$, $\beta1$, $\beta2$-receprors)

-Serotonin (5HT-receptors)

-Glucagon

-Angiotensin II (AT_1, AT_2-receptors)

-Most other small amines and peptides.

2) Ion-channel receptors:

Ligand-gated receptors ion channels act at fast synapses, where rapid onset and rapid termination are necessary for function. (Millisecond)

-Acetylcholine {ligand-gated ion channels include nAChR (nicotinic acetylcholine receptor) channel.

-Purinergic receptors (P 2x at nerve muscle junction)

3) Kinase receptors:

-Transforming growth factor-beta superfamily.

1-7. Classification and biomedical characteristics of angiotensin II receptors:

The existence of two subtypes of Ang II receptors, including AT_1 and AT_2. In rat and mice, AT_1 receptors consists of two subtypes, AT_{1a} and AT_{1b} which have 94% homology with regard to amino acids sequence and have similar pharmacological properties and tissue distribution patterns.

AT_1 Receptors:

The AT_1 receptor is a member of the seven transmembrane-spanning, G protein-coupled receptor family; binds to heterotrimeric G proteins which mediates virtually a majority of the known physiological actions of Ang II in cardiovascular, renal, neuronal, endocrine, hepatic, and other target cells. These actions include the regulation of arterial blood pressure, electrolyte and water balance, increase in cardiac contractility, facilitation of catecholamine release from nerve ending, thirst, hormone secretion, and renal function (De. Gasparo *et al.*, 2000). The AT_1 receptor also induced growth response particularly after birth. In addition recent accumulating in vivo and in vitro evidence supports the notion that Ang II, mediated by AT_1 receptor, may participate directly in the pathogenesis of various cardiovascular and renal diseases (Ohkubo *et al.*, 1997).

AT_1 receptors can cause intracellular signalling cascades via activation of PLC, PLA_2, PLD, adenylyl cyclase and ion channels such as L-type and T-type voltage-sensitive calcium channels lead to several intracellular signalling pathway such as Ca^{2+}, Ip_3, cAMP and DG. This can extend into the nucleus, which regulate gene transcription and the expression of proteins that control growth responses and cell proliferation in several Ang II target tissues.

Human AT_1 receptor gene is mapped to chromosome 3 and AT_{1a} and AT_{1b} receptor genes in rat are mapped to chromosomes 17 and 2, respectively.

Numerous selective and potent nonpeptide AT_1 receptor antagonists have been developed, such as losartan, candesartan, valsartan, irbesartan, eprosartan, telmisartan, tasosartan, and in recent years, several of these compounds, including losartan, candesartan, and valsartan have been in use clinically for treatment of hypertension (Bauer and Reams 1995) (Johnston 1995) (Pitt and Konstam 1998).

AT_2 Receptors:

The AT_2 receptor belongs to GPCR Superfamily which its gene is localised as a single copy on X chromosome. There is no evidence for subtypes of the AT_2 receptor (Kim and Iwao 2000).

The AT_2 receptor is ubiquitously expressed in developing foetal tissues, suggesting a possible role of this receptor in feotal development and organ morphogenesis. In contrast, AT_2 receptor expression rapidly decreases after birth, and in the adult, expression of this receptor is high mainly to the uterus, ovary, certain brain nuclei, and adrenal medulla (Kim and Iwao 2000). Ang II actions via AT_2 receptor paradoxically decreased blood pressure and produced a negative chronotropic effects (Masaki *et al.*, 1998) (Tsutsui *et al.*, 1999). In contrast to the hypotension and impaired vascular responses observed in AT_1-deficient mice, knockout of the AT_2 receptor leads to

elevation of blood pressure and increased vascular sensitivity to Ang II (Hein *et al.*, 1995). This has suggested that the AT_2 receptor may exert a protective action in blood pressure regulation by counteracting AT_1 receptor function (Tanaka *et al.*, 1999).

AT_2-dependent dilation represented 20% to 39% of flow-induced dilation in mesenteric resistance arteries in rat, and AT_2-receptor mRNA was found in mesenteric resistance arteries. Thus, resistance arterial tone was modulated in situ by locally produced angiotensin II, which might participate in flow-induced dilation through endothelial AT_2 receptor activation of Nitric Oxide release. Angiotensin II in the presence of losartan (selective AT_1 antagonist) increases the diameter of rat mesenteric artery, an effect that can be inhibited by PD123319 (AT_2 antagonist). PD123319 or losartan had no effect after Nitric Oxide synthesis blockade or after endothelial disruption. (Matrougui *et al.*, 1999).

Robert *et al.,* have reported that angiotensin II (200 pmol/kg per minute IV) alone could increased blood pressure from a control of 112±3 to 168±7 mm Hg (P<0.0001) and losartan (30 mg/kg) alone decreased blood pressure to 89±7 mm Hg (P< 0.0001 from control) in anesthetized rats. Angiotensin II administered together with losartan decreased blood pressure further to 71±4 mm Hg (P<0.0001 from control and losartan alone). AT_2 receptor antagonist PD123319 completely blocked the hypotensive response to losartan combined with angiotensin II (P= NS from control). In another study in conscious rats (n=5 per group), CGP-42112A (CGP, selective AT_2 receptor agonist) (70 μg/kg pre minute) also decreased blood pressure in AT_1 Blocked conscious rats. Blood pressure decreased from 119±3 mm Hg to 86±6 mm Hg during 3 days of valsartan alone, (P< 0.00001) and decreased further to 65± 7 mm Hg (P< 0.001 from daily VAL alone) with 7 days of CGP in presence of valsartan. They strongly suggested that the AT_2 receptor induces a systemic vasodilator response mediated by Nitric Oxide

that counterbalances the vasoconstrictor action of angiotensin II at the AT_1 receptor (Robert *et al.*, 2001). Vascular media thickness in peripheral resistance arteries was also increased in AT_2 blocked rats rather than AT_1 blocked animals (Levy *et al.*, 1996) (Cao *et al.*, 1999).

In uterine myometrium the AT_2 receptor is expressed under non-pregnant conditions and declines during pregnancy, but returns to non-pregnant level after parturition (de Gasparo *et al.*, 1994).

Selective AT_2 receptor antagonists include PD123177, PD123319, CGP42112, L-162, 686, L-162, 638, EXP801 and CGP42112A (Kim and Iwao 2000). Among these antagonists PD123319 has provided evidence for a variety of functions of the AT_2 receptor in several different type of cells and tissues including cardiovascular, renal, adrenal, central nervous as well as mesenchymal systems.

The AT_2 receptor seems to open a delayed rectifier K^+ channel at least in hypo-thalamic neuronal tissues (Kang, *et al.*, 1994 and 1995) to close a T-type Ca^{2+} channel (Buisson, *et al.* 1992 and 1995) to suppress tissue and cellular growth (Nakajima *et al.*, 1995) (Meffert *et al.*, 1996) to induced neuronal cell differentiation (Laflamme *et al.*, 1996) (Gallinat *et al.*, 1997) (Gendron *et al.*, 1999) and support apoptosis (Yamada *et al.*, 1996) (Chamoux *et al.*, 1999) (Gallinat *et al.*, 1999). In addition, the AT_2 receptor may exert hypotensive effects (Scheuer and Perrone 1993).

Hisophysiology of Angiotensin II receptors

It has been extensively documented that the renin-angiotensin system is a major factor in the regulation of cardiovascular homeostasis, including blood pressure, mineral balance and tissue remodelling (Weber 1998) (Fig. 1-1). Angiotensin II mediates its biological actions by binding with two major subtypes of receptors, which have been identified and cloned as AT_1 (AT_{1A} and AT_{1B}) and AT_2 (De. Gasparo *et al.,* 2000) (Griendling *et al.,* 1996). These receptors are differently localised and have different functions. Angiotensin II mediates the effects of the renin–angiotensin system by stimulating the AT_1 receptor, to produce vasoconstriction and proliferation, and the AT_2 receptor, to produce vasodilatation, anti-proliferation and promote apoptosis (Anti-remodelling receptor) (Horiuchi *et al.,* 1999). The AT_1 receptor, which is prominent in adult tissues, also stimulates cell growth (Matsukada and Ichikawa 1997). Angiotensin II receptors have been localised in vascular beds, heart, nervous system, kidneys and adrenal glands. However, there are different anatomical distributions of these receptors as well as differences in signalling pathways and function. In the foetal period, AT_2 receptor is the dominant subtype, although this situation rapidly reverses after birth to AT_1 being the dominant subtype in adult (Matsubara 1998) (Horiuchi *et al,* 1999) (Xoriuch *et al.,* 1999). Therefore, there is lower expression of AT_2 in adult tissues except in uterus, ovary and adrenal medulla and some parts of brain (Zhuo *et al.,* 1995). Since 1998, there has been increasing evidence suggesting that the AT_2 receptor may play a greater role, particularly in vasodilator and antigrowth effects as well as downregulating the AT_1 receptor (Matsubara 1998) (Horiuchi *et al.,* 1999) (De. Gasparo *et al.,* 2000) (Unger 1999). For example: AT_2 receptor plays its role in pressure natriuresis and opposing the antinatriuretic effects of AT_1 receptor, so that PD123319 (a

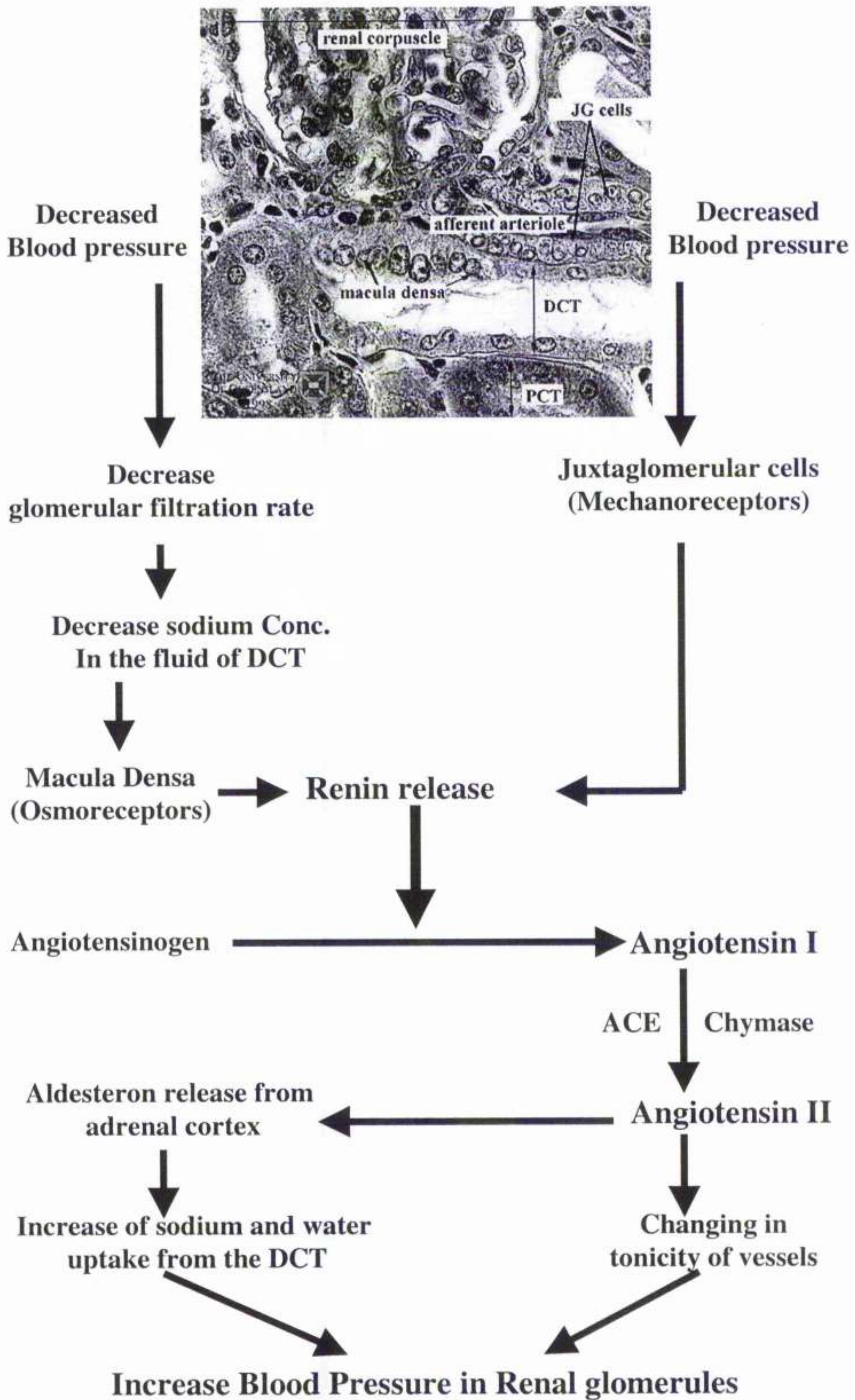


Fig. 1-1: Regulation of Renin secretion from juxtaglomerular apparatus and some of its effects.

selective AT_2 antagonist) decreases urinary sodium excretion in renal hypertensive rat while valsartan or losartan exert the opposite effect (Siragy and Carey 1999). Meanwhile, the beneficial effect of ACE inhibitors on tissue remodelling appear to be independent, at least in part, of their effects on blood pressure (Linz *et al.,* 1995). In this respect, angiotensin II can either be generate in kidney and subsequently released in the circulation activating different plasma membrane receptors or it can be produced in different tissues to exert its effects at the place of production (Danser and Schalekamp 1995).

In vasculature Chang and Lotti have reported the presence of AT_2 receptors comprising approximately 40% of AT receptors in rat aorta (Chang and Lotti, 1991). Also autoradiography provided some evidence for AT_2 in kidney vasculature (Zhuo *et al.,* 1995 and 1997) (Matsubara 1998) (Miyata *et al.,* 1999). Thus, AT_2 is dominant in adventitia of the human renal artery and interlobar arteries (Goldfarb *et al.,* 1994) (Zhuo *et al.,* 1997). AT_2 receptors are also present in endothelial and vascular smooth muscle cells (SMCs) in small resistance arteries such as coronary arteries (Akishita *et al.,* 2000).

Due to their role in regulation of cell proliferation, we expect AT_2 receptor expression to increase in inflammed and injured areas. There is some evidence that confirms this theory. For example, AT_2 receptor expression increases in skin during wound healing (Kimura *et al.,* 1992) (Viswanathan and Saavedra 1992). Likewise, an inflammatory cuff model caused over-expression of AT_2 in Media/neointima of mouse femoral artery (Akishita *et al.,* 2000).

Both receptor subtypes (AT_1 and AT_2) exist in the heart of most animals studied. AT_2 is the minor subtype and is expressed at low levels in adult rat cardiomyocytes (Bushe *et*

al., 2000). However, AT_2 expression level increased in both absolute terms and relative to AT_1 receptors in hypertrophied rat and failing hamster heart (Lopez *et al.*, 1994) (Ohkubo *et al.*, 1997). Moreover, myocardial infarction increased expression of AT_2 receptors from a basal state of 10% up to 50% during the first week after rat heart attack (Busch *et al.*, 2000).

Although animal studies indicate that the AT_1 is dominant in adult hearts, the AT_2 location has a prominent in human heart. In both normal non-infarcted and hypertrophied human hearts, there is a predominance of AT_2 receptor binding sites in the cardiac myocytes (Brink *et al.*, 1996).

In human heart, autoradiography and immunohistochemical studies have generally shown an increase in the ratio AT_2/AT_1. The AT_2 receptor is mainly localised on fibroblasts at the site of fibrosis. However in immunohistochemical studies AT_2 was confined on myocytes in arterial tissue obtained from coronary artery bypass graft surgery patients (Brink *et al.*, 1996) (Tsutsmi *et al.*, 1998) (Wharton *et al.*, 1998) (Matsumoto *et al.*, 2000).

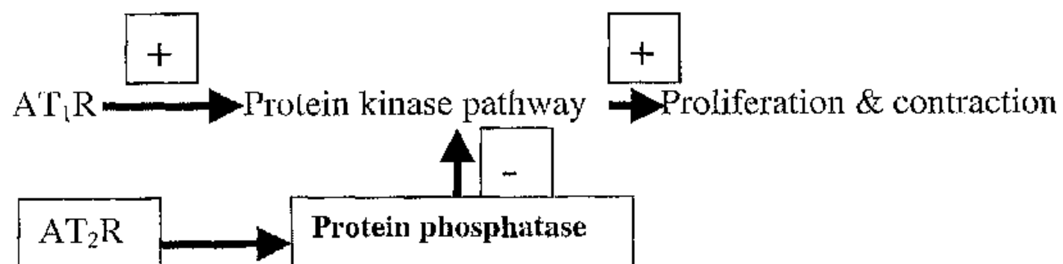
There is growing evidence for intracellular actions of angiotensin II not related to activation of classical plasma membrane receptors. Brailou et al. have reported effect of intracellular angiotensin II on rat aorta contraction, independent of activation of plasma membrane angiotensin II receptors (Brailoiu *et al.*, 1999).

Intracellular angiotensin II was reported to increase cytosolic [Ca^{2+}] in vascular smooth muscle cells (Haller *et al.*, 1996) and to affect L-type Ca^{2+} channels in a specific manner (Dimitropoulou *et al.*, 2001). Since the AT_1 and AT_2 receptors mediate opposing actions and they are both expressed on smooth muscle and endothelial cells, they are situated such that they can directly oppose each other's actions. Consequently the net action of

angiotensin II in a given tissue should depend on the ratio of AT_1 to AT_2 receptors. In order to, quantify the contribution of each receptor to the net action of angiotensin II, it is possible to use a mathematical model to predict that the changes in receptors populations will augment the switch from the positive growth effects of angiotensin II to the negative growth effects and vice versa (Nora, Elizabeth Hart 1999).

AT_1 and AT_2 signalling pathway:

It seems that the signalling transduction mechanism is different in the two family types of angiotensin II (AT_1 and AT_2) receptors. AT_2 coupling stimulates protein phosphatase (Protein Tyrosine Phosphatase or PTPase) which directly inhibits the protein kinase pathway associated with the AT_1 receptor (Horiuchi *et al.,* 1999). MAP-1 is one of the phosphatases involved in AT_2 receptor transduction (Fischer *et al.,* 1998).



Both AT_1 and AT_2 receptors also could increase cyclic GMP level in cultured bovine aortic endothelial cells, suggesting that AT_1 and AT_2 receptors have the same direction at least at the level of cGMP production Via Nitric Oxide dependent pathway (Wiemer *et al.,* 1996). A stimulatory effect of angiotensin II on cellular NO and cGMP level has also been reported in other aortic endothelial cell preparations (Pueyo *et al.,* 1998). Nitric Oxide can be produced by two alternative pathways; directly through AT_2 receptors and indirectly from effect on AT_2 receptors on bradykinin 2 receptors (B_2-R)

(Abadir *et al.*, 2003). Angiotensin II also could stimulate NO release directly in isolated blood vessels (Thorup *et al.*, 1998) (Thorup *et al.*, 1999).

There is also a direct link between AT_2 Receptor activation and bradykinin synthesis and release. Both endogenous and exogenous angiotensin II could stimulate an increase in cyclic GMP, which in turn could stimulate NO release indirectly via bradykinin 2 receptor (B_2-R). Thus infusion of Ang II in SHR (hypertensive model of Rat) could increase both AT_2 receptor and Bradykinin levels in renal artery. All of these effects were abolished by the AT_2 blocker (PD123319), B_2-R blocker (Hoe140 or icatibant), NOS blocker (L-NAME or L-NMMA) and also cyclooxygenase inhibitor indomethacin, suggesting that bradykinin 2 receptor modulates both internal prostaglandin E_2 and nitric oxide. (Siragy and Carey 1996) (Siragy and Carey 1997) (Siragy *et al.*, 1997) (Siragy and Carey 1999).

1-8. α-Adrenoceptors

α_1-Adrenoceptors:

The alpha 1 receptors that belong to GPCR superfamily are divided to alpha 1A/C (C8), alpha 1B (C5), alpha 1D (C20) and alpha 1L according to pharmacological classification. It has a single polypeptide chains, ranging from 429 to 561 amino acids in length (is very similar to that of rhodopsin 'The light -activated retinal receptor').

The α_{1B} subtype was considered to have low affinity for phentolamine and oxymetazoline, to be completely inactivated by CEC, to not require Ca^{2+} influx for signaling but rather to mobilize Ca^{2+} from intracellular stores, and to be expressed prototypically in rat liver, spleen, and cerebral cortex (Robert *et al.,* 1996).

The α_{1D}-AR subtype is expressed in variety of tissues, including conducting vascular smooth muscle, cerebral cortex, and probably rat lung. In rat aorta and iliac artery, it appears to be the predominant subtype mediating vasoconstriction

(Perrez *et al.,* 1991) (Piascik *et al.,* 1994) (Piascik *et al.,* 1995).

Stimulation of alpha adrenoreceptors (α-ARs) results in the activation of various effector enzymes including PLC, PLA_2, PLD as well as activation of Ca^{2+} channels, Na^+/H^+ and Na^+/Ca^{2+} exchangers and activation or inhibition of K^+ channels Additionally early and late response gene transcription. In most cells alpha-1 stimulation causes intracellular Ca^{2+} signalling, but can provide IP_3, DG, PKC pathway as an intracellular signalling (Robert *et al.,* 1996).

Blockade of L-type Ca^{2+} channels with dihydropyridines or removal of extra cellular Ca^{2+} was found to inhibit alpha 1A, but not alpha 1B-mediated contractile response in

isolated vascular smooth muscle or Ca^{2+} influx in hepatocytes. On the basis of such findings, it was suggested that α_{1A} gate Ca^{2+} influx through voltage-dependent Ca^{2+} channels, whereas α_{1B} mobilise intracellular Ca^{2+} via a PLC/IP_3 mechanism (Minneman *et al.*, 1988).

α_2-adrenoceptors

The Alpha 2-adrenoceptors belongs to GPCR receptor family. Three subtypes of α_2-adrenoceptors exist in human and animals (α_{2A}, α_{2B}, α_{2C}). In human they are located on the human chromosomes 10, 2 and 4, respectively (Byalund *et al.*, 1994).

The α_2-ARs are coupled to several signaling pathways, including activation of GIRK K^+ channels (Acetylcholine also can act via this type of channel), mitogen activited protein kinases (MAPK) and phospholipase D as well as inhibition of Ca^{2+} channels and adenylyl cyclase.

Despite the abundance of α_{2C}-AR in adult human heart in foetal human heart α_{2C} and α_{2A}-ARs has not been detected (Perala *et al.*, 1992). All the three subtypes of α_2-AR expression have been detected at the quantitative level in endocardium of adult human heart. However, frequency of α_{2C}-AR is greater than α_{2B}-AR (α_{2C}-AR expression is 30-fold less than α_{1A}-AR) and α_{2A}-AR, respectively ($\alpha_{2C} > \alpha_{2B} > \alpha_{2A}$) (Perala *et al.*, 1992 and Berkowitz *et al.*, 1994).

α_2-adrenoceptors were abundantly expressed in normal human foetal and adult tissues, including spleen, kidney, adrenal glands, plexus choroid, cerebral cortex, caudate nucleus and skin (Perala *et al.*, 1992).

In the mouse embryonic development, during midgestion (day 9-11), all three α_2-adrenoceptors subtypes are abundantly expressed in the embryonic and extraembryonic tissues, including placenta and yolk sac. The α_{2B}-AR is the major subtype that is expressed in the placenta. Mice lacking all three α_2-AR subtypes did not survive beyond day 11.5 of embryonic period due to a defect in formation of foetal blood vessels in yolk sac and placenta (Philipp *et al.*, 2002).

The α_{2A}-AR involves in presynaptic feedback inhibition of noradrenaline release, hypotension, anlgesia, sedation and inhibition of epileptic seizures (Brede *et al.*, 2004). The α_{2B}-AR involves in hypertension, placenta angiogenesis and analgesic effect of nitrous oxide (Brede *et al.*, 2004) and the α_{2C}-AR involves in feedback inhibition of adrenal catecholamine release, analgesic of moxonidine and modulation of behavior (Brede *et al.*, 2004).

1-9. Interaction between AT_1 & AT_2 and α_1 & α_2-adrenoceptors:

The vasoconstriction action of angiotensin II is subserved by AT_1 receptors. Angiotensin II receptors similar to the AT_{1B} subtype subserve enhancement of transmitter noradrenaline release (Cox and Story 1996).

Angiotensin II can increase the IC_{50} value of noradrenaline. The angiotensin AT_1 receptor antagonist, DUP753 (losartan), not only blocked this action but also decreased the IC_{50} value of noradrenaline. Activation of angiotensin II AT_1 receptors in the nucleus tractus solitarii may reduce the transduction of the α_2-adrenoceptors and thus the α_2-mediated vasodepressor responses (Firo *et al.*, 1994).

There is some evidence that angiotensin II can inhibit norepinephrine reuptake and increase its biosynthesis and responses mediated via both extrasynaptic alpha 2 and intrasynaptic alpha 1-adrenoceptors (Saxena 1992). AT_1 receptor blockade with losartan significantly reduced the angiotensin II induced increase in norepinephrine concentration (Teisman *et al.,* 2000).

In normal pregnancy angiotensin AT_2 receptors play a role in maintaining intrauterine blood flow for the fetus. When angiotensin II levels are elevated for a prolonged period this protective effect is lost partly because angiotensin AT_1 receptors are down regulated (McMullen *et al.,* 1999).

Several reports described the stimulatory effects of angiotensin II on release of noradrenaline from sympathetic nerve terminals and adrenaline from adrenal medullary cells (Zimmerman 1978) (Duckles 1981) (Boke and Malik 1983) (Maclean and Unger, 1986) and also it has been shown that angiotensin II increases noradrenaline activity on smooth muscle cells by inhibiting its reuptake into nerve terminals (Panisset and Bourdois 1968) (Khairallah 1972) (Day and Moore 1976). However, the synergism was dependent on calcium influx through nifedipine-sensitive calcium channels (L-type calcium channel). Similarly, noradrenaline induced amplification of the angiotensin II response. It has been shown also that noradrenaline induced a bigger response when smooth muscle cells had already been induced by angiotensin II in rabbit femoral artery by specific mechanisms which involved α_1-adrenoceptors (Prins *et al.,* 1992).

From observations in whole animals, it was initially suggested that postjunctional α_2-adrenoceptors were located extra-junctionally responding to circulating catecholamines (McGrath 1982). Responses to electrical field stimulation were virtually abolished by the selective α_1-adrenoceptor antagonist *prazosin* (0.1μM). In contrast, nerve-mediated responses were enhanced in the presence of both the selective α_2-adrenoceptor

antagonist *rauwolscine* (1μM) and *Ang II* (0.05μM). *Ang II* (0.05μM) was without effect on nerve-mediated responses in preparations previously exposed to *rauwolscine* (1μM). In contrast, responses in preparations previously exposed to *prazosin* (0.1μM) were markedly enhanced in the presence of *Ang II* (0.05μM). These enhanced responses observed in the presence of *prazosin* and *Ang II* were subsequently susceptible to *rauwolscine* (1μM). This clearly indicates that under normal in vitro experimental conditions, nerve-released NA acts upon postjunctional α_1-adrenoceptors to produce the functional contractile response. Under these conditions *rauwolscine* (1μM) enhanced nerve-mediated responses, an effect consistent with its well-documented action on prejunctional α_2-adrenoceptor mediated autoregulation (Stark 1987). *Ang II* also caused a potentiation of responses to nerve stimulation. While this may be due partly to an increase in the release of neuronal transmitter (Ziogas and Story 1987), a postjunctional site of action is implicated by the observation that this agent introduced a *prazosin-resistant* component of responses to sympathetic nerve stimulation as it does to the adrenoceptor agonists *UK-14304* (Dunn *et al.,* 1989). This *prazosin-resistant* response introduced by *Ang II* was abolished by *rauwolscine* at a concentration which has previously been shown to be selective for α_2-adrenoceptors in this preparation (Dunn *et al.,* 1989) and which, prior to exposure to *Ang II*, and enhanced responses to sympathetic nerve stimulation. This study therefore clearly demonstrates a role for postjunctional α_2-adrenoceptors in mediating the end-organ response of the distal saphenous artery to electrical field stimulation. Suggesting that potentiation by angiotensin II requires that α_2-adrenoceptors are available for activation (Dunn *et al.,* 1991).

Daniel Henrion *et al.,* in 1992 reported that angiotensin II amplifies arterial contractile response to noradrenaline without increasing Ca^{2+} influx. They studied rabbit facial artery segments mounted isometrically and measure the Ca^{2+} influx and net uptake in

response to noradrenaline. The contractile response to noradrenaline in the presence of angiotensin (synergism) was not associated with changes in noradrenaline- induced Ca^{2+} influx or net uptake. This amplification was prevented by pretreatment with either *Staurosprine (10nM)* or *Calphostin C (100nM)*, two inhibitors of Protein Kinase C. Hence, angiotensin II potentiation of noradrenaline-induced vascular tone occurs in the absence of changes in stimulated Ca^{2+} entry, and may be due to an increase in intracellular sensitivity to Ca^{2+}, possibly mediated by protein Kinase C. (Henrion *et al.*, 1992)

In 1991 Dunn, *et al,* reported that on isolated distal saphenous artery angiotensin II mediated synergism that allowed noradrenaline to act via postjunctional α_2-adrenoceptors. They reported that angiotensin II made responses to noradrenaline less susceptible to the antagonistic action of prazosin. This was particularly evident on the lower portion to the CCRC for noradrenaline. This evidence suggests that in the presence of angiotensin II, noradrenaline produce contractile responses by an action mediated through a prazosin-resistant adrenoceptor. After use of a receptor protection procedure involving the combination of rauwolscine (α_2-antagonist) and phenoxybenzamine (α_1-antagonist), no responses were observed to α-adrenoceptor agonists noradrenaline (α_1 and α_2 -agonist), phenylephrine (selective α_1-agonists) or UK14304 (selective α_2-agonist). However, in the presence of angiotensin II, concentration-dependent contraction was observed to each of these agonists (UK14304 > NA > Phenylephrine).

This phenomenon is consistent with that of an effect mediated through postjunctional α_2-adrenoceptors. The responses to noradrenaline, after the protection protocol, in the presence of angiotensin II, were susceptible to the selective α_2-adrenoceptor antagonist, rauwolscine (1μM), but resistant to the selective α_1-adrenoceptor antagonist, prazosin (0.1μM). Inducing a small degree of tone with a low concentration of the selective α_1-

adrenoceptor agonist, phenylephrine, markedly increases the threshold sensitivity to the selective α_2-adrenoceptor agonist UK14304, in a manner analogous to that seen with angiotensin II.

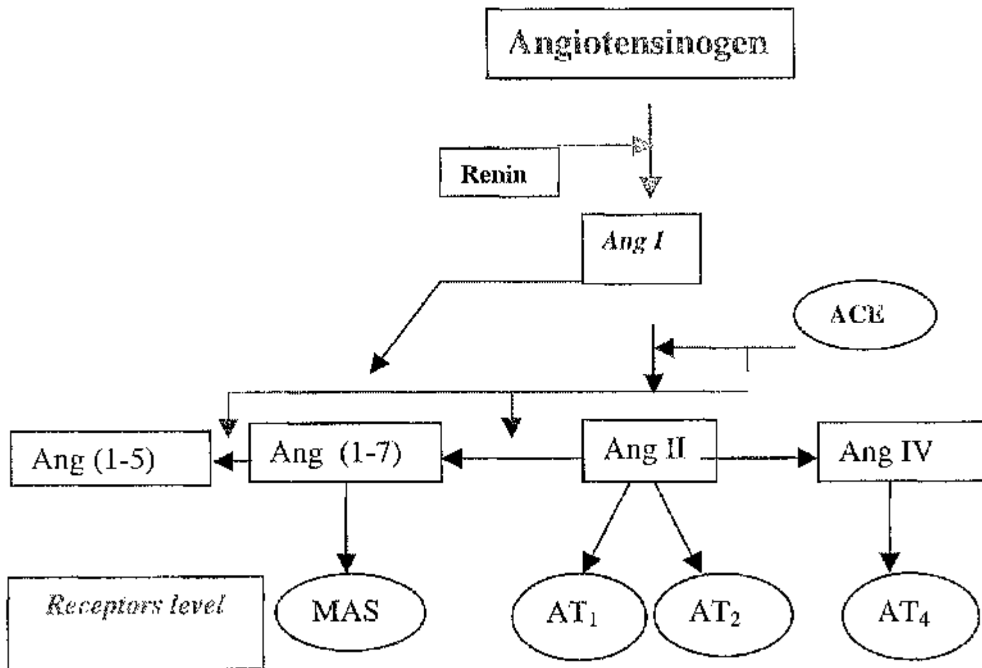
These above mentioned results indicate that responses mediated via postjunctional α_2-adrenoceptors in rabbit isolated distal saphenous artery are dependent upon a degree of vascular smooth muscle stimulation by an other receptor system. It is hypothesised that the contraction response to noradrenaline or phenylephrine is caused by stimulation of α_1-adrenoceptors, while after α_1-adrenoceptor blockade the necessary positive influence can be provided by stimulation of angiotensin II receptors (AT_1 receptor). It seems there is some interaction between α-adrenoceptor subtypes, (α_1 and α_2) and angiotensin II receptors in demonstrating prazosin-resistant, rauwolscine (α_2 antagonist)-or yohimbine (α_2 antagonist)-sensitive response in isolated blood vessels (Dunn *et al.,* 1991).

α_2-adrenergic receptors are known to have a critical role in regulating neurotransmitter release from sympathetice nerves and from adrenergic neurones in the central nervous system. There are at least three subtypes of α_2-adrenergic receptors, $\alpha_{2A/D}$, α_{2B}, α_{2C}. Both of $\alpha_{2A/D}$ and α_{2C}, are required for normal presynaptic control of transmitter release from sympathetic nerves in heart and central noradrenergic neurons. $\alpha_{2A/D}$-adrenergic receptors inhibit transmitter release at high stimulation frequencies, whereas the α_{2C}-subtype modulates neurotransmission at lower levels of nerve activity. Both low-and high-frequency regulation seem to be physiologically important, as mice lacking both of these receptors have elevated plasma noradrenaline concentrations and develop cardiac hypertrophy with decreased left ventricular contractility by four months of age (Hein *et al.*, 1999).

The contraction of aortic strips generated by angiotensin II in the mice was significantly greater in the AT_2 gene-deleted mice than the control, which was completely abolished

by AT_1 antagonist (losartan). The aortic content of AT_1 receptor was significantly increased in the AT_2 null mice (212 +/- 58.9 fmol/mg protein) compared with control (98.2 +/- 55.9 fmol/mg protein). While both AT_1 and AT_2 mRNAs were expressed in the aorta of the control mice, only AT_1 mRNA was expressed in the AT_2 Knockout mice. The expression of AT_1 mRNA in the AT_2 Knockout mice was significantly higher (1.5-fold) than that in the control. It seems AT_2 works against AT_1-mediated vascular action of angiotensin II through downregulation of AT_1 receptors by a crosstalk between these receptors by some as yet unknown mechanism (Tanaka *et al.*, 1999).

1-10. Angiotensin- (1-7) and (1-5):



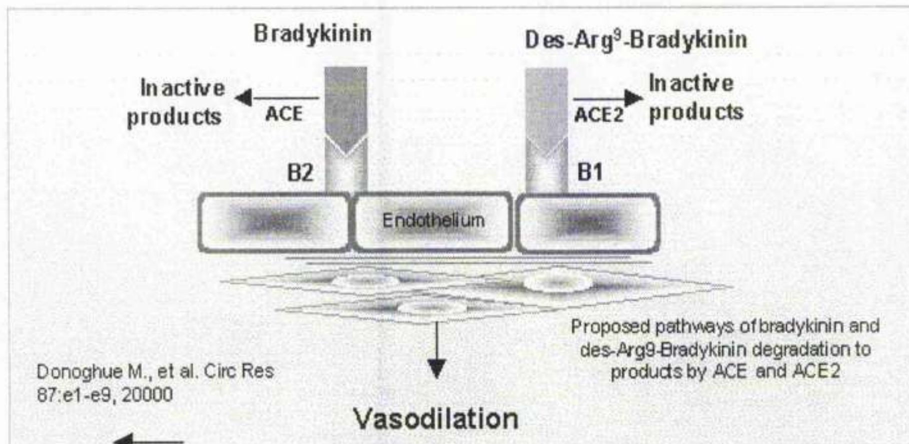
1-Laboratory studies have characterised the enzymatic pathways of formation of amino-terminal heptapeptide fragment, angiotensin- (1-7) or Ang (1-7). Two types of endopeptidase are forming Ang- (1-7) which implicated in the metabolism of vasodilator peptides such as bradykinin and atrial natriuretic factor (Carlos and Ferrario 2002).

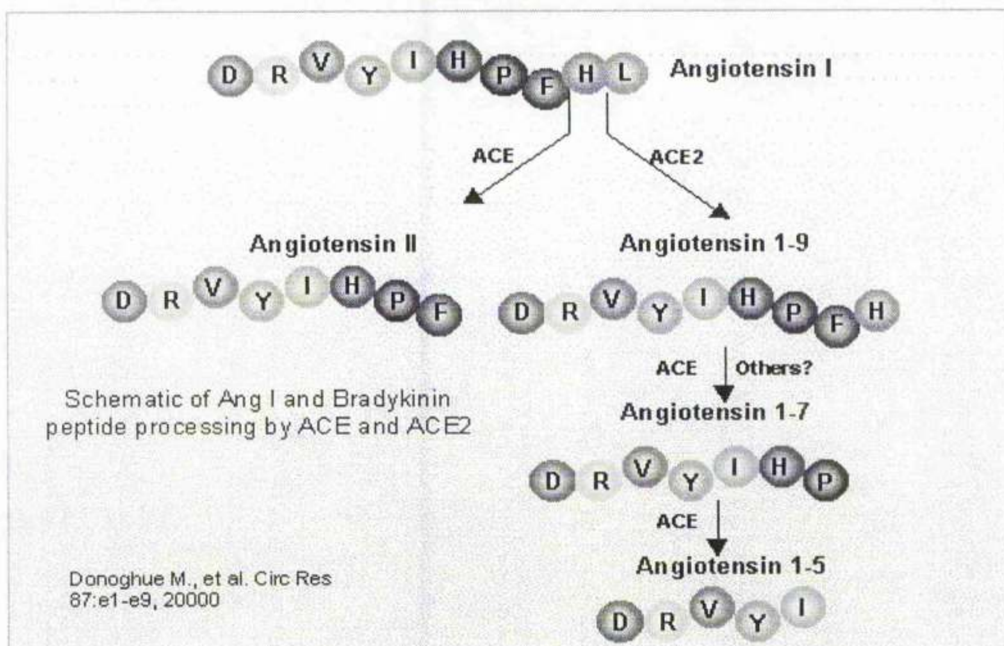
2-Ang (1-7) have vasodilator actions due to effect of it on Mas receptors. Activation of mas receptors could reduce calcium signal processing in rat cardiac myocytes ***(Walther et al., 2001).***

3-Angiotensin--(1-7), the amino-terminal angiotensin heptapeptide, is a biologically active peptide of the renin-angiotensin system that has both vasodilatory and antiproliferative activities that are opposite to the constrictive and proliferative effects of angiotensin II. Angiotensin- (1-7) can downregulate the AT_1 receptor in vascular

smooth muscle as an endogenous angiotensin II antagonist (Clark *et al.*, 2001) (Ueda *et al.*, 2000).

4-Inhibition of ACE blocks metabolism of angiotensin- (1-7) to angiotensin-(1-5) and can lead to elevation of angiotensin- (1-7) levels in plasma an tissue. In animal models, angiotensin- (1-7) itself causes or enhances vasodilatation and inhibits vascular contraction response to angiotensin II. Angiotensin- (1-5) inhibited plasma ACE activity with a potency equal to that of angiotensin I but had no effect on arterial contraction. Hence, both of angiotensin- (1-7) and (1-5) can inhibit ACE. However, only angiotensin- (1-7) can downregulate the AT_1 receptors (Roks *et al.*, 1999).





Schematic of Ang I and Bradykinin peptide processing by ACE and ACE2

1-11. Angiotensin II-mediated cardiovascular and renal diseases:

There are opposing actions of AT_1-Receptor (excitatory) and AT_2-Receptor (inhibitory) on cardiovascular system. Blockade of AT_1-R has emerged as an effective hypertension and heart failure treatment as well as ACE inhibition (Matsubara 1998 and 2001) (Horiuchi *et al.*, 1999).

Unlike ACE inhibitors, AT_1-R blockade increases circulating levels of Ang II that could provide more AT_2-R stimulation, which leads to additional complementary therapeutic benefit. PD123319 and Hoe 140 attenuated these effects. However, did not effect the losartan-induced fall in blood pressure (Dukel et al., 2003).

The progression of left ventricular hypertrophy and cardiac fibrosis in hypertensive heart disease is influenced by sex and age. However, angiotensin II converting enzyme inhibition has been shown to prevent progression of the disease in postmenopausal women (Grohe *et al.*, 1998).

Thus, there is apparently marked tissue heterogeneity, which is likely to reflect the balance of AT_1/AT_2 receptor expression in various tissues, including greater diversity effects of AT_2-R on cardiac hypertrophy than cardiac fibrosis, which reflects the greater AT_2-R expression in cardiac fibroblasts (Ohkubo *et al.*, 1997) (Tsutsumi *et al.*, 1998).

1-12. General materials and methods for study of receptors:

1) Isolated Organ Bath Studies with use of wire myography:

Wire myography is the most common method for study of vessels. An advantage of this method is the possibility for further histological studies of the fixed artery (Thybo *et al.*, 1995). Wire myography was introduced by Bevan and Osher in 1972, to study functional responses in resistance arteries. But, it was not until 1976 that Mulvany and Halpern first used wire myography to study the structure of small arteries (Mulvany *et al.*, 1976). This technique involves threading two 40-micron stainless steel or tungesten wires through small-dissected arteries of approximately 2mm. The two wires are held in position between two heads, one attached to a micrometer and the other one to a force transducer (Fig.1-2). This allows the tension and the internal circumference of the artery to be measured and controlled. The dimensions of the artery wall can be calculated by placing the myograph on to the stage of a microscope fitted with a 10X magnification water objective lens. This will allow measurement such as the wall to lumen ratio and the cross sectional area to be measured with an accuracy of 1μm. Other important parameters, that can be measured including the internal diameter and length of blood vessel, the mean wire thickness (t) and the mean distance between the inner edges of the wires (d). By using these obtained measurements the internal circumference (Ci) of an artery mounted on a wire myograph can be calculated using the following formula:

$$Ci = 2\pi R \quad , \qquad Ri = 2t + d / 2$$

Ri = internal Radius lumen of vessel , Ci = internal circumference

t = mean wire thickness

d = mean distance between inner edges of the wires

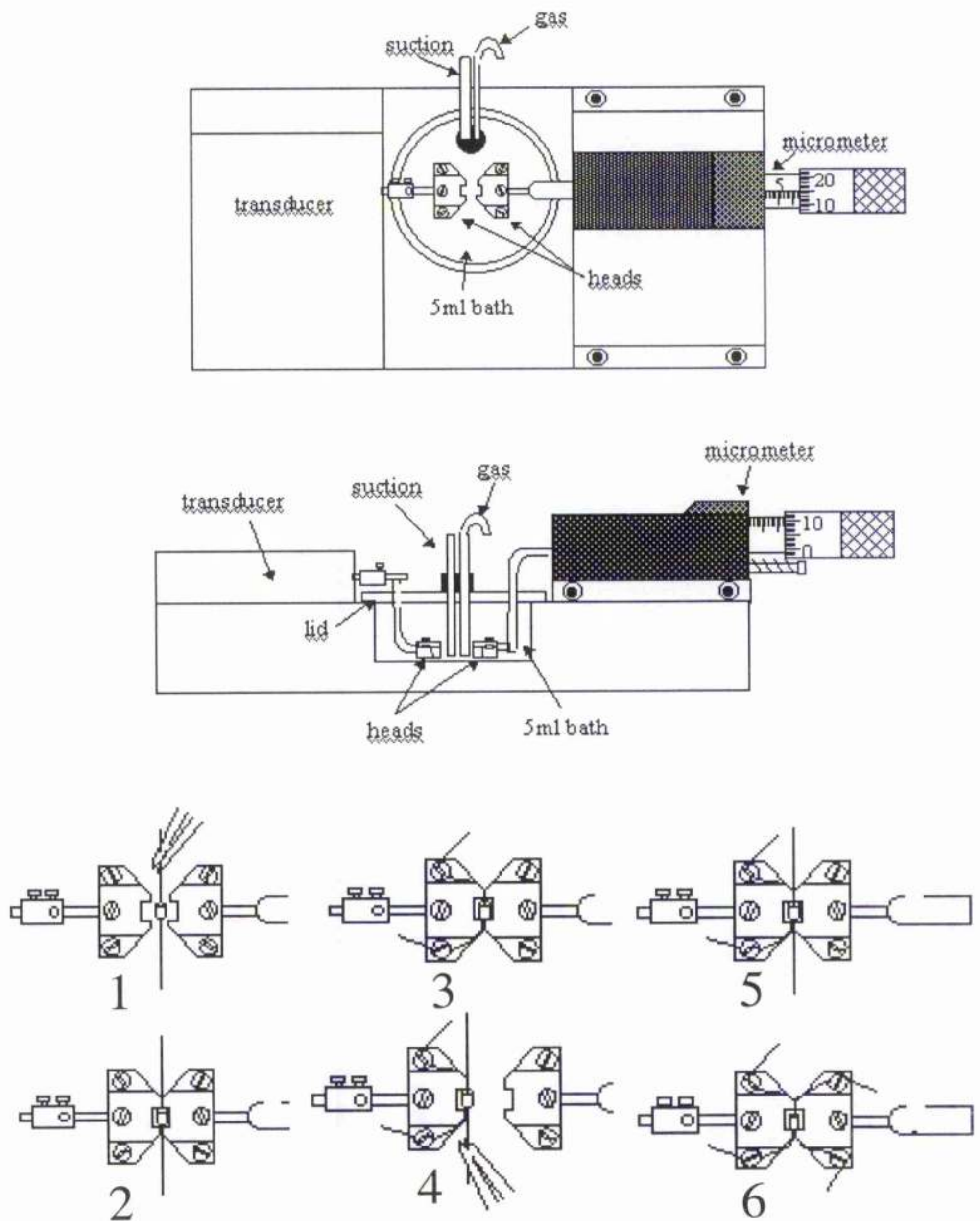
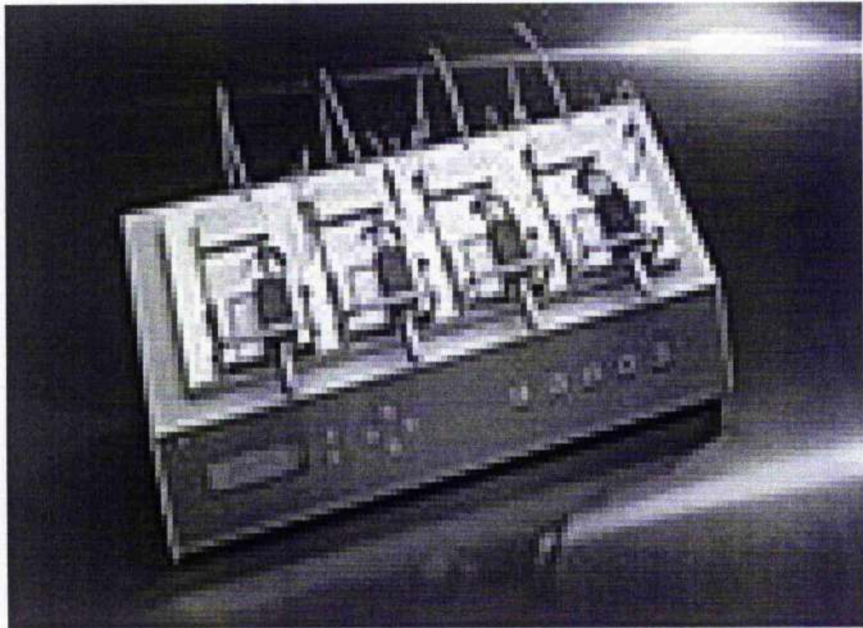


Fig. 1-2: Wire Myograph and vessel set up between micrometer and transducer.



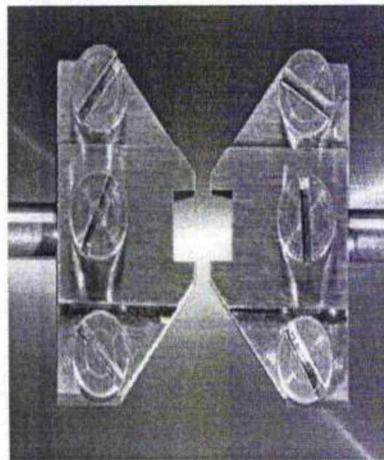


Fig. 1-3: Wire Myograph Model 600M

Then we can get the following formula: $Ci = \pi (2t + d)$

Male wild type or transgenic mice (aged between 4-6 months) were killed by C02 and the vessels (aorta, carotid artery, tail artery, mesenteric artery and....) removed. Connective tissue including adipose tissue was then removed and the samples dissected into rings. Tissues were mounted in warm Krebs solution (NaCl 118.4mM, KCl 4.7mM, $CaCl_2$ 2.5mM, KH_2PO_4 1.2mM, $MgSO_4$ 1.2mM, $NaHCO_3$ 25mM & glucose 11.1 mM bubbled with 95% O_2 5% CO_2 to pH 7.4) at 37°C in a multi-myograph (myo-interface, model 600M Version 2 and 610M Version 2.2, Aarhus) using 40 micron tungsten wires. An isometric force transducer (linseis L6512B, Belmont Instruments that calibrated for 1g force as sixty small boxes and Powerlab software Version 4.2.2 for windows 98 on a Pentium III computer which calibrated for 1g force as a 1 Volt) was used to measure force development (Fig. 1-2 and 1-3). The vessel rings were placed under appropriate initial tension and left to equilibration period for 30-45 minutes. Experiments had specific start-up protocols, designed for particular aims or hypothesis testing.

2) Perfusion Pressure myography:

Perfusion pressure myography was developed as a tool for studying the function of small artery preparations from normotensive and hypertensive rats. (Halpern *et al.*, 1978) This technique involves securing a resistance vessel to a fine glass cannula, which is filled with physiological salt solution (PSS). The vessel is secured at one or both ends with fine strands of silk teased from surgical suture thread. Any blood within the lumen of the vessel is gently flushed out before the second end of the vessel is secured to the other cannula or tied off with another strand of silk thread. A system of PSS filled tubing is attached to the cannula via a pressure transducer. The opposite end

of the tubing form the vessel is attached to either a servo-pump or a fluid reservoir, to control the pressure to which the vessel is exposed. Once mounting is complete the myograph is placed on to the stage of a microscope. A video camera is attached to the microscope, which allows images to be taken of the blood vessel under magnification. The signal from the camera is shown on a monitor so that the experimenter can visualise the vessel throughout the experiment. The signal is also fed into a video dimension analyser. This allows continuous measurement of the lumen diameter and wall thickness to be made. It also allows the experimenter to make measurements along the length of the vessel as the position of lines on the monitor can be manually adjusted using controls on the analyser box (Fig. 1-4). These measurements can be added together and a mean arterial wall thickness and lumen diameter can be calculated. Other measurements, which can be made using this myograph system, include arterial diameter and arterial length. To measure the arterial diameter the video camera is rotated until the image on the monitor is horizontal. The lines can then be placed on the inner margins of the wall and a value can be read from the video analyser display. Similarly the vessel length can be calculated by turning the camera until the vessel is vertical. The measurements made for length are between 2 natural landmarks in the vessel or between two lightly tied silk threads placed at opposite ends of the vessel. The natural landmarks are often isolated fat cells on the surface of the vessel or larger darker cells in the adventitial layer or the wall. This method of using video measurements is very precise as the measuring lines on the monitor can be calibrated using a calibrating grid placed on the microscope stage. The only disadvantage to video measurements is that it is very subjective and biased, as the experimenter has to decide where to measure in the arterial wall. Although it has this disadvantage, it has a high rate of reproducibility, approximately 5% of error was noted between observers looking at the same vessel. In one study a computerised diameter measuring system was used to make

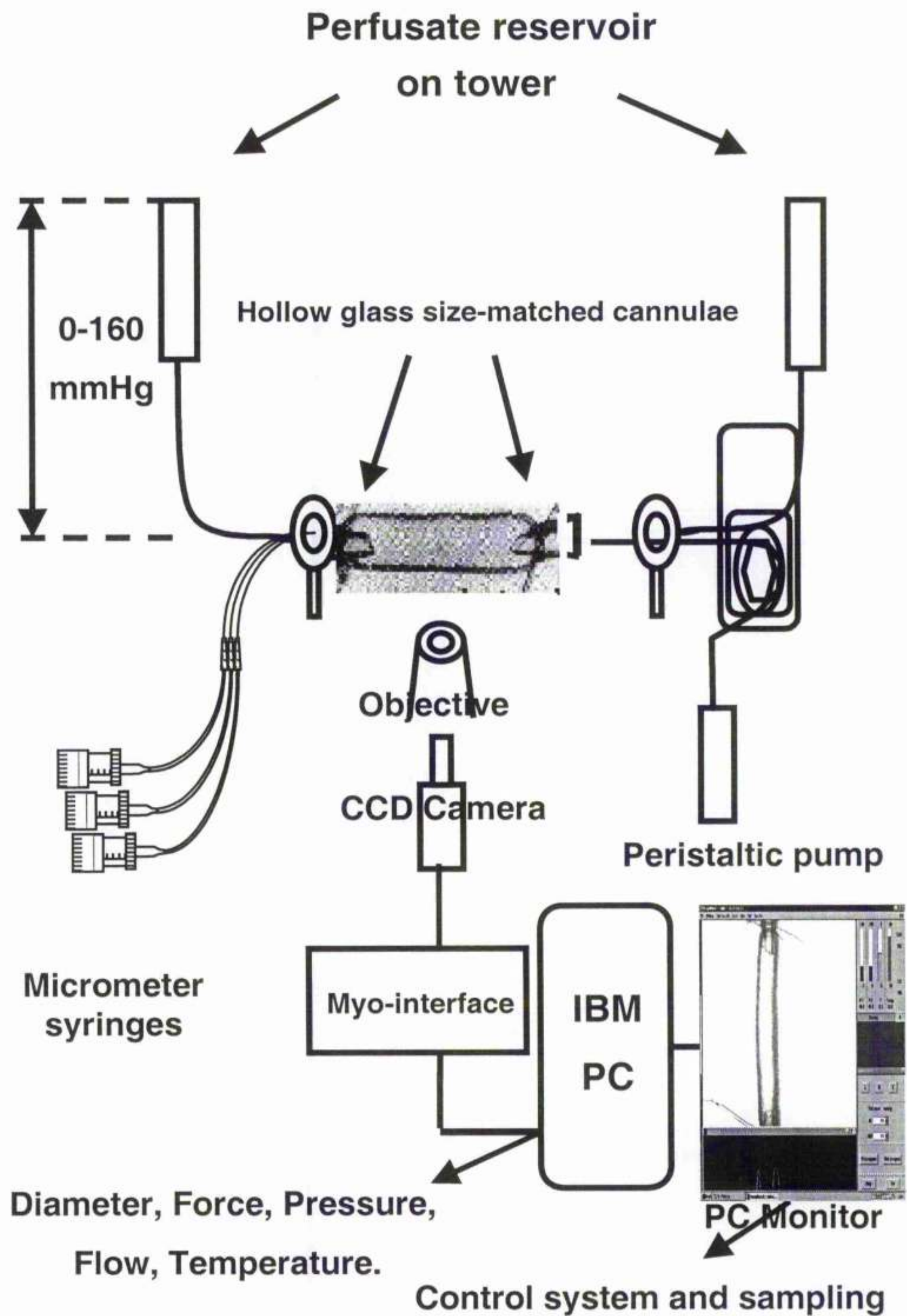


Fig. 1-4: Small Vessel Pressure/Perfusion Myography.

a comparison with video measurements. The results showed an excellent correlation between the diameter measurements and manual measurements. The regression coefficient was 0.992 showing that there are near similarities and validity between both methods (Lew *et al.*, 1992).

Once again this method can be combined with histological studies of the blood vessels and therefore a more detailed analysis of the cellular structure of the blood vessels wall can be made. Often fixatives are used to hold the vessel in the shape it takes under pressure. Once the vessel has been fixed it is embedded in paraffin wax or resin, sections are cut and stained allowing the experimenter to identify distinct cell types in the layers of the artery wall. Placing these sections over a morphometeric grid can make morphometeric measurements. Due to the fact that the pressure myograph offers more physiologically correct conditions for blood vessels, a more morphologically correct structure of the vessel wall is seen.

3) Laser Scanning Confocal Microscopy:

One of the most novel methods currently being used to study vascular morphology is the technique of combination of pressure myography and laser scanning confocal microscopy (LSCM) in order to visualise the component cells with in the vessel wall. Several methods have been described using confocal microscopy to study the vascular wall at the cellular level.

There are different types of laser scanning confocal microscopy:

1) Slit Laser Scanning Confocal Microscopy, which is more susceptible for quantitative measuring of fluorescent compound. It's more sensitive. However, Can not create clear specific images due to penetration of more waves whereas

interfering to each other into the sensor in order to localisation of receptors. So images from this type of microscope need to more deconvelution.

2) Pinhole Laser Scanning Confocal microscopy, which can create sharper and better images in order to localise receptors (Fig. 1-5).

The laser scanning confocal microscopy is using three types of common range of wavelength using laser bulbs {argon (445nm-488nm wavelength), Green Heeni (515-580nm wavelength) and Neon (580-680nm wavelength)}. For each of the wavelength range we need to use particular emission filter to delete non-excitation waves from sources.

a) Jeffrey Dickhout and Robert Lee described a method using mesenteric arteries from normotensive or hypertensive rats (Dickhout and Lee 1997).

The rats were anethetizesed with sodium pentobarbital and the mesenteric vessels were exposed and cleared of blood by perfusion with hanks basic salt solution (HBSS) containing 1 μmol/L sodium nitroprusside. The arteries were measured to ensure that an in vivo length was maintained after mounting on the pressure myograph. Once the blood is removed and the ties put in place for length measurements the arteries are transferred to the bath of the myograph where they are mounted as previously described on to glass cannula. The artery is then repressurized under 70 mmHg, which is required to expand the arteries to their in vivo length. The arteries were then fixed using a fixative containing 3.5% *formaldehyde*, 0.75% *gluteraldehyde* in 0.05 mol/L *phosphate buffer* which preserved the artery structure with little non-specific autofluorescence (normal fixatives contain 2.5% gluteraldehyde which gives high levels of unwanted autofluorescence). Then fixed vessels were washed repeatedly with Hanks Buffered salt solution (HBSS), which removed most of the fixative. Any traces of free aldehyde were removed by reduction with 1 mg/L sodium *borohydride* for 5 minutes. The tissues are then again washed with HBSS but this time it contains 0.1% *Triton X-100* (pH=8),

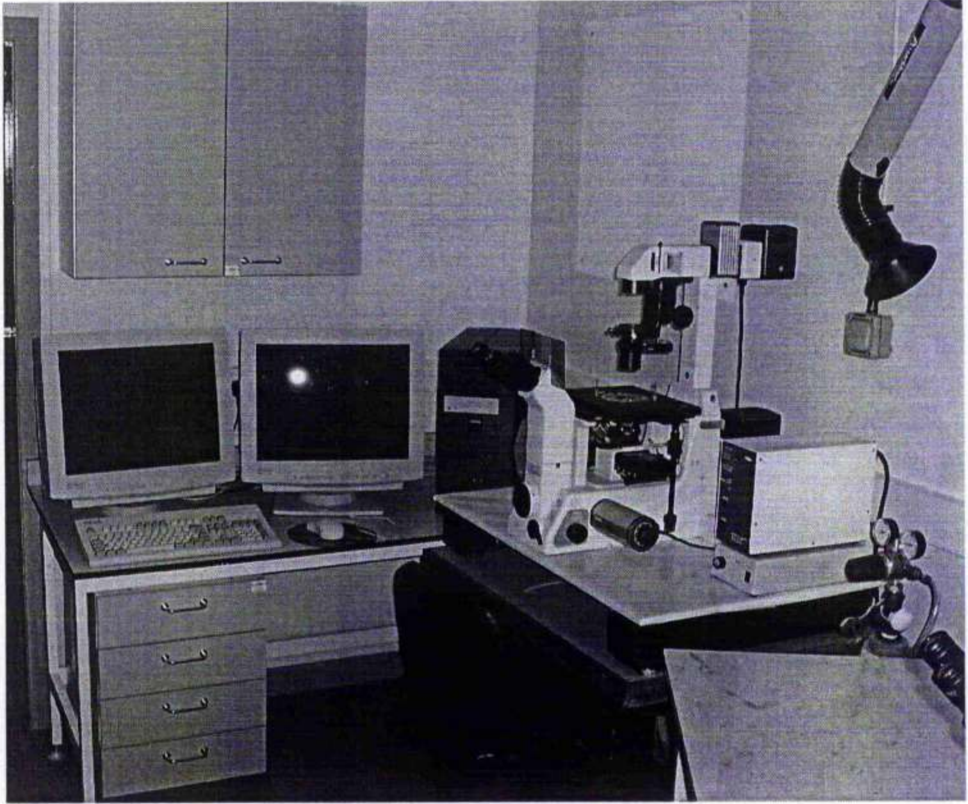


Fig. 1-5: Radiance 2100 Laser Scanning Confocal Microscope (LSCM) room (Lab440), West Medical Building, University of Glasgow.

which is used to permeabilise the cell membrane. Once the cell membrane is permeabilised the nuclear dye *ethidium bromide* in a reduced form was added to the bath. The arteries were stained for 20 minutes and the washed with HBSS to remove any excess stain. After washing the arteries were mounted in 1:1 glycerol/HBSS on a concave glass slid and exposed to ultraviolet light to redevelop the reduced ethidium bromide into its normal state (orange fluorescent characteristic). Then vessels were set up on the confocal microscope for imaging. Dickout and Lee used an Argon Ion Laser with emission lines at 488nm; this gave the best excitation of the dye with the lowest non-specific autofluorescence. The images were taken from random planes more than 10 μm above the artery. A computer aided tracing package was used to determine the area of each optical section in the medial layer, This allowed calculation of media volume to be made as a Cavalierian estimator of the volume:

$$\text{Volume} = \Sigma(\text{Area}) * \delta T$$

Volume = volume of the medil layer.

Area = area of medial layer found in each optical section.

δT = distance between each optical section.

b) Another study in which confocal microscopy is used to study morphological aspects of arteries was carried out by Arribas et al (Arribas *et al.*, 1997).

Mesenteric arteries were removed from WKY or SHR rats and mounted on either a wire or perfusion myograph as previously described. Measurements were made in both cases and values calculated for cross-sectional area, wall thickness, wall to lumen ratio, internal diameter and equivalent pressure.

In the perfusion pressure system the vessels were fixed with 10% formal saline and stored for further analysis on the confocal microscope. A comparison was also made in this study between live and fixed vessels. Arribas et al, used five arteries to compare

perfusion myography and Laser Scanning Confocal Microscopy (LSCM) measurements.

Arteries are mounted on the perfusion myograph and pressurised under 50mmHg. After an equilibration period (30 minutes) measurements were made using the dimension analyser. Once measurements were made the physiological salt solution (PSS) was replaced with PSS containing the vital dye Hoechst 33342 for 30 minutes. This dye was also perfused intraluminally for two minutes to enhance the staining of the endothelial layer. The myograph was then placed on the stage of the confocal microscope and measurements of the lumen were made. Following this the PSS was replaced with fixative and the vessels were fixed under 50mmHg pressure and measurements were again made using the LSCM and the video dimension analyser system. Vessels were prepared for imaging on the confocal microscope by placing the blood vessels on a glass slide and covering it with a coverslip attached to the slide by a thick layer of vacuum grease. This ensured that the coverslip did not press against the artery therefore minimising any changes, which may occur in the wall of the vessel if the coverslip lay directly on top of the artery.

Measurements of parameters could be make by using either data obtained from the VDU system or the laser scanning confocal microscope. These include:

Wall cross section area (CSA) = external CSA – internal CSA

External CSA = π (lumen / 2 + wall thickness)(lumen / 2 + wall thickness) Internal CSA = π (lumen / 2)(lumen / 2)

Total number of cells for each layer:

Cell number = number of nuclei per stack $\times$ number of stacks per vessel

Luminal circumference = $2\pi R = 2\pi$ (diameter / 2)

Luminal surface = $2\pi Rh$ = Luminal circumference $\times$ length of vessel

1-13. Differences between wire and perfusion pressure myography:

Wire and perfusion myography have been compared in a few studies. However, Arribas et al, have shown: larger lumen, smaller wall thickness and smaller wall to lumen ratio for perfusion compared with wire myography. These differences may lie in the theoretical assumptions necessary for the wire myography. For example physical differences such as the axial distension with increasing pressure that occurs in perfusion myography, but is not experienced on a wire myograph (Arribas *et al.*, 1997).

1-14. Difference between LSCM and Perfusion myography:

Arribas et al, found significantly larger wall thickness and Cross section area (CSA) measurements with Laser Scanning Confocal Microscopy (LSCM) under both live and fixed conditions, compared with the perfusion method. This was not due to a general calibration problem of the LSCM because lumen measurements were similar when taken with both methods, provided the condition was the same (live or fixed vessel). They suggested that the differences observed in wall thickness measurements between the two methods is due to an underestimation of wall thickness when measured with the perfusion myography VDA (video dimension analyser). Often adventitia is more translucent than the media, and it is difficult to detect when the vessel is brightly illuminated for optimal visualisation of lumen. It is likely that much of the adventitia and intima are not included in the wall measurement taken with the VDA. In addition, the terminology used in the literature to define "wall" measurements is confusing, reflecting the difficulty of clearly measuring different layers. Some studies use the term "wall thickness" others use media thickness and one established that "media" was clearly discernible but considered it "wall". Also it is considered that the images can be inadequate for providing an exact measurements of these layers because the outer limits

of adventitia and intima were not always clearly shown. Only the ability to distinguish between cell types with use of nuclear dyes allows visualisation and separate measurement within the different layers in the wall. Another important factor is the capacity of the different cell nuclei to capture the dye; this is always less for the endothelial nuclei, even when the dye is applied intraluminally (Arribas *et al.*, 1997).

4) Fluorescent ligands for study of receptors:

Ligands attach to receptive sites on receptors in proportion to the numbers of each. The basis of radioligand binding is that ligands bind to receptors long enough, on average, that sufficient will still be present after rapidly washing away unbound ligand to give a signal proportional to the amount of ligand-receptor complex. Theoretically, exactly the same principles could be applied to fluorescent ligands.

Many attempts have been made at conjugating fluorescent molecules to receptor ligands in the hope of identifying their binding sites. This was aimed mainly at the localisation of the receptors rather than studying their properties.

1-15. Advantages of Fluorescent ligands Compared with radioligands:

1-Fluorescence is relatively safe and inexpensive compared with tritiated or iodinated compounds.

2-Spatieal resolution is greatly enhanced compared with autoradiography or cell fractionation

3-Experiments can be performed at true equilibrium.

4-Fluorescent ligands can be displaced from their binding site by non-fluorescent ligands. Using image subtraction it is thus possible to identify the sites recognized by the non-fluorescent ligand.

5-Bleaching of certain fluorescent molecules (i.e. those which are only fluorescent when bound) can be used for fluorescent recovery after photo-bleaching type experiments. Once an area has been bleached the rate of recovery of fluorescence provides information on the association of the fluorescent ligand.

6-The signal does not degrade. Although photo-bleaching of individual molecules can occur, the Kinetic nature of binding replenishes the site with an undamaged ligand This also allows signal averaging, of radioligand decay.

7-Different fluorophores are available to suit particular experimental set-ups. This enables the use of multiple fluorescent ligands or co-localisation of ligands and antibodies.

8-Live or fixed tissue can be used.

9-Small amounts of tissue or single cells can be studied.

10-Immediate results are obtained.

1-16. Disadvantages of fluorescent ligands compared with Radioligands:

1-The large fluorescent group can affect the affinity of some (but not all) ligands.

2-The yield of a single fluorescent molecule may be different for different fluorophores and may be affected by the binding conditions. This complicates the calculation of a βmax.

3-Very sensitive detectors may be required for the low concentrations of fluorescent ligands, which are required to maintain specificity.

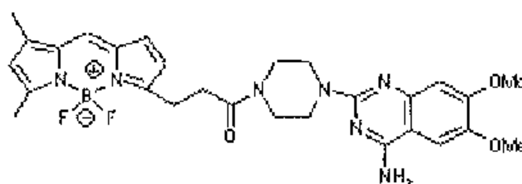
4-Bleaching will occur if the excitation source is too great or the ligand concentration too low. In some cases it may be difficult to find the right balance if the source light is not tunable.

5-Tissue autofluorescence can cause significant problems in some samples. Elastin is particularly problematic.

6-If fluorescent ligands are internalised in live tissue or cells they may be subject to degradation (McGrath *et al.*, 1996).

1-17. Fluorescent Ligands:

Cell surface G-protein-coupled receptors are notoriously difficult to localise accurately, even in fixed tissue using antibodies, due to the non-specificity caused by the high degree of conservation of sequence, or using radioligands due to the inherent low resolution of autoradiography. However, high affinity fluorescent ligands based on



"antagonist" drugs/ ligands could be used in a manner analogous to radioligands, but with much higher spatial resolution and in real-time on live tissue, if their concentration can be measured photometrically.

The structure above is a fluorescent alpha1-adrenoceptor antagonist based on prazosin. This particular form of **BODIPY** is excited at 488nm and emits above 515nm. The compound was obtained from molecular probe and is listed in their catalogue as **"BODIPY FL-prazosin"** but since it lacks the furan group which defines prazosin, as opposed to other compounds which share the quinazolinyl piperazine group, such as doxazosin, we refer to it by an acronym, **QAPB**, derived from its chemical name (quinazolinyl piperazine borate-dipyrromethene).

1-18. Discussion:

According to the World Health Organisation (WHO) dependent to the United Nations Organisation (UN) many people around the world have involved in cardiovascular diseases which control by multifactorial system. Although genetic background has a main role in cardiovascular disease,

However, many of changes in normal situation, which leads to pathological condition are acting via changing in receptors balance. A majority of these receptors are situated on both smooth muscle and endothelial cells of the vascular system. We often know their molecular structure and function. However, interaction between these receptors is much complicated. Identification of these interactions between receptors is very useful for defining mechanism in hypertension, vascular remodelling and cardiac dysfunction, which are involved in cardiovascular diseases. Scientists have described several methods for studies of the circulatory system, particularly the vascular system. However, the big problem is difference between in vivo and in vitro studies. We don't know exactly, whether this knowledge, which discovered *in vitro* actually applies *in vivo* or not. It is necessary to know this in clinical treatment of hypertension and cardiovascular diseases. Also distribution and expression of receptors are different in different species of animals. Scientists have made progress in this field of knowledge, However, there is still much work to be done. Most studies on the vascular system are based on work in animal models, such as dog, rabbit, rat and also mice. Recently, the use of transgenic animals, new drugs, fluorescent LSCM and better technology particularly using fast processor computers and new softwares have provided more details about the different types of receptors. However, scientists need to more evidence for interpretation of every aspect of hypertension, vascular remodelling mechanism and effect of adventita or intima in these processes.

Chapter two

Effects of angiotensin II on different mouse arteries.

In aorta AT_2 angiotensin II receptors can produce endothelium-mediated vasodilatation that can overcome AT_1 mediated contraction at high concentrations. This inhibitory effect is the major effect of Ang II against noradrenaline.

In both carotid and superior (main) mesenteric arteries the contractile effect of Ang II via AT_1 receptors is dominant.

In first branch mesenteric arteries relaxation via AT_2 receptors is the dominant response to Ang II.

2-1. Abstract:

1. Angiotensin II (Ang II) mediated vasodilator responses were studied on wire myograph-mounted mouse aorta with a view to determining contributions and sites of action of receptor subtypes and potential synergism between the angiotensin receptor and adrenoceptor families.

2. Ang II had a concentration-related dual effect in the presence of elevated tone (5HT), causing contraction at low concentrations and a relaxant effect at higher concentrations. losartan (Selective AT_1 antagonist) or PD123319 (Selective AT_2 antagonist) attenuated contraction and relaxation, respectively. This indicates a role for AT_1 and AT_2 receptors in Ang II mediated vasoconstriction and vasodilatation respectively. The relaxation was abolished by L-NAME or endothelial removal, revealing a larger contraction. This indicates an AT_1 action to contract vascular smooth muscle and an AT_2 action on endothelium to release nitric oxide.

3. Ang II-mediated relaxation had no detectable synergistic effect on UK14304-mediated relaxation in mouse aorta.

4. In Aorta pre-incubation with Ang II (30nM) significantly reduced the maximum response to NA ($p<0.0001$): this effect was increased by losartan and blocked by PD123319.

5. In both carotid and superior (main) mesenteric arteries the contractile effect of Ang II was dominant.

6. In first branch mesenteric arteries the main effect of angiotensin II was relaxation. This was reversed to contraction by L-NAME suggesting that it was of endothelial origin.

7. A fluorescent derivative of Ang II, Rhodamine-Ang II-H, was used to visualise angiotensin receptors on dissociated arterial cells and intact vessels, emplying confocal microscopy. Losartan and PD123319 were used as competitor ligands to identify the receptor subtypes that were labelled by the fluorescent compound. This provided evidence for the presence of both AT receptor subtypes on both smooth muscle and endothelial cells. This was accomplished on both aorta and main (superior) mesenteric arteries.

2-2. Introduction:

Studies of the mouse cardiovascular system have increased markedly in the past 2-3 years reflecting the development of genetically modified mouse strains, lacking (knockout) or over expressing (transgenic) individual receptor subtypes. These can be used to clarify the role of such receptors, for example where the pharmacology is complicated by multiple subtypes responding to the same agonist (Daly *et al.*, 2002).

Being the largest vessel available, the mouse aorta is of interest for pharmacological analysis. Its general pharmacological properties resemble that of the much studied rat aorta allowing its use to unravel the large database of vascular biology available from that and other species. For example contractile responses of mouse and rat aorta to noradrenaline are mediated by α_{1D}-adrenoceptors (Yamamoto & Koike 2001) (Tanoue *et al*, 2002) (Daly *et al.*, 2002) and both contract well to 5HT and appropriate prostaglandins (Russell & Watts 2000). However there are some reports of differences, one of which is a relatively smaller response to angiotensin II in the mouse (Russell & Watts, 2000).

The mouse vascular system has been shown to possess two opposing actions of angiotensin II, mediated by its two receptors. AT_1 receptors mediate vasoconstriction. There is an increased vascular reactivity to Ang II in AT_2 knockout mice. This suggests that AT_2 receptors mediate an effect that counteracts the effect via AT_1. This is believed to be at least partly due to an increased vascular AT_1 receptor expression, suggesting that AT_2 counteracts AT_1-mediated vascular action of Ang II through downregulation of AT_1 receptor by a crosstalk between these receptors by some as yet

unknown mechanisms (Tanaka *et al,* 1999; Akishita *et al,* 1999). Angiotensin II is reported to be weakly efficacious on mouse aorta compared with NA and phenylephrine. In a study of strips of mouse aorta, (Russell and Watts 2000) reported a weak AT_1 mediated vasoconstrictor effect of Ang II and a lack of any AT_2 mediated vasodilatation. This failure to find a direct vascular AT_2 mediated response is disappointing given the documentation on the role of Ang II in maintaining mouse blood pressure (Oliverio *et la.,* 1998) (Siragy *et al.,* 1999) and thus its utility as a model system for the human renin-angiotensin system: although as a conducting artery the aorta need not be involved in such a response.

The present study have uncovered and analysed the constrictor and relaxant effects of angiotensin II in the mouse aorta, using straightforward pharmacology and myograph-mounted aortic rings, showing that the contractile and relaxant actions are mediated by vascular smooth muscle AT_1 and endothelial AT_2, respectively. In order to demonstrate these actions on other vessels, e.g. carotid and superior mesenteric arteries. We also, tried to localise Ang II receptors in both smooth muscle and endothelial cells using fluorescently labelled Ang II detected by Laser Scanning Confocal Microscopy (LSCM) and selective antagonists. In order to clarify the location of these receptors, in relation to another major vasoactive family of GPCRs we used QAPB (Fluorescent Prazosin) which binds to adrenoceptors. In this way, we revealed the position of adrenoceptors compared with angiotensin receptors in mouse aorta and superior mesenteric artery.

We were also interested in possible synergism between α_2-AR and AT receptors. There are many reports of the presence of AT_1 receptors in different vessels which can amplify arterial contractile responses to NA in a synergistic fashion (Rat and rabbit) (Dunn *et al,* 1989) (Henrion *et al.,* 1992) (Gasparo *et al.,* 2000) (Matsusaka *et al.,*

1997). However, a synergistic link between Ang II receptors and α_2-ARs mediating vasodilatation seemed worth investigating in the mouse.

We used wire myograph-mounted aortic rings to ensure minimal damage to the vascular endothelium (relative to the damage with spiral strips). Previous studies used strips of aorta (Tanaka *et al.*, 1999) which may account for their failure to find endothelium-mediated relaxation to Ang II. It was the relative fragility of drug-induced relaxant responses in spiral strips that led Furchgott and Zawadski (1980) to discover endothelium derived relaxant factors.

The overall aim of the study was to establish the characterisation of AT_2-mediated vasodilatation in mouse aorta, and to investigate the presence of any functional interaction between this receptor and both AT_1 receptors and adrenoceptors.

2-3. Materials and Method:

Wire Myography:

The strains of mice employed were Swiss wild type (129/sv/C57BL6) and the $\alpha_{2A/D}$-adrenoceptor mutant D79N mouse (MacMillan *et al*, 1996, 1998), which had been back-crossed on to C57BL/6; this is a strain which loses an endothelium-mediated response mediated by another receptor (the $\alpha_{2A/D}$-adrenoceptor, see Chapter 3) and indeed the Ang II response acts as another control for that lost response. In the current chapter this simply shows that the Ang II responses are similar in another strain of mouse.

Male mice (aged 4 months) were killed by CO_2 inhalation and the descending thoracic aorta, carotid, main mesenteric and first branch mesenteric were removed, cleaned of connective tissue then dissected into rings (2-3mm in length).

Endothelium was removed, where appropriate, by gently rubbing the intimal surface. Tissues were then mounted in Kreb's solution (NaCl 118.4mM, KCl 4.7mM, $CaCl_2$ 2.5mM, KH_2PO_4 1.2mM, $MgSO_4$ 1.2mM, $NaHCO_3$ 25mM & glucose 11.1mM bubbled with 95% O_2 5% CO_2 to pH 7.4) at 37°C in a multi-myograph (myo-interface, model 600M,DMT, Aarhus) using 40μm stainless steel wires (Mulvany and Halpern. 1976, 1977). Aortic rings were placed under a resting tension of 1g; carotid artery 0.33 g; superior (Main) mesenteric artery and first branch mesenteric artery each 0.25g; and left to equilibrate for 30-45 minutes. Reproducible responses were obtained to 5HT (0.1μM), NA (0.1μM), phenylephrine (0.1μM), or U46619 (10nM), according to protocol, before commencing experiments. Tissues were tested with increasing cumulative concentrations of Ang II in 0.5 log unit increments from 1nM-1μM, added at four minute intervals (cumulative concentration response curve, CCRC)

At the plateau of contraction to noradrenaline (0.1 or 1μM), 5HT (0.1 or 1μM) or U46619 (0.01 or 0.1μM), Acetylcholine (1μM) was added to assess endothelial integrity. Criteria for functional endothelium was >50% and for denuded endothelium was < 5% relaxation. At the end of each experiment endothelium was re-checked using the same criteria and samples included only if meeting criteria at both times. All four arteries relaxed to Acetylcholine (1μM).

Tissues were washed at 5 minutes intervals following each experimental protocol and given a 60 minutes recovery period. Following the rest period, selective antagonists were added where appropriate for at least 30 minutes before construction of a second cumulative concentration response curve (CCRC).

b) **Visualisation of arterial angiotensin II receptors:**

Visualisation of angiotensin II receptors on endothelial cells in large arteries (aorta and main mesenteric artery) using fluorescent-angiotensin II microscopy presents technical challenges related to (i) the 3-dimensional nature of the endothelial layer and its close proximity to background fluorescence from elastin in the internal elastic lamina and (ii) the presence of both AT_1 and AT_2 that bind the ligand.

The first factor was overcome by the use of 3-dimensional confocal microscopy as summarised in "Results". The second factor was overcome by a strategy that eliminated the binding to either AT_1 or AT_2.

Rhodamine fluorophore can be specifically conjugated to human angiotensin II to make a fluorescent analogue of angiotensin II (Rhodamine-Ang II-Human) without altering the biological activity of the parent compound (Yaday *et al*, 2002). Rhodamine-Ang II-Human has affinity for both AT_1 and AT_2. However, it can cause downregulation of receptors that seems to be greater in extent with AT_1. A study on cultured bovine

adrenal cells (BAC), which express both receptor subtypes and PC12W and R3T3 cells, which express only AT_2 receptors, using the [^{3}H] uridine-thiouridine method, indicated that AT_1-binding sites decreased by more than 50% within the first three hours (AT_1 receptor internalisation), an effect proposed to result from agonist-induced internalisation and down regulation. The rhodamine-angiotensin II effectively binds to AT_1 receptors causing internalisation of clathrin coated vesicles by endocytosis. In contrast, AT_2-binding sites and mRNA remained stable within the first six hours of Ang II treatment, and AT_2-binding sites declined much more slowly with half-life of about sixteen hours (Ouali *et al.,* 1997). This indicates that rhodamine conjugated peptides can be used in ligand-receptor binding and also in ligand-receptor complex internalisation but that both issues must be taken into account.

Thus, we set out to create a protocol in which we could identify binding sites for fluorescent Ang II on smooth muscle and endothelial cells that could be proved to be to one or other subtype by eliminating the other type with the selective antagonists losartan and/or PD123319.

We wanted to avoid complex image analysis and to enable a simple yes or no decision on whether antagonists had been effective. We had expected to find AT_1 on smooth muscle and AT_2 on endothelium making the analysis simple. Unexpectedly we found evidence that there were AT_2 on smooth muscle cells and AT_1 on endothelium which complicated the analysis. We used the selective antagonists for AT_1 (losartan) and AT_2 (PD123329) at concentrations that we could show to be selective in these tissues with classical agonist/antagonist functional pharmacology. In these circumstances we visualised Rhodamine-Ang II-H binding at low concentrations to endothelial cells that could be completely removed by the antagonists. For localising angiotensin II receptors, it was useful to employ QAPB (Fluorescent–Prazosin) to identify the cells, since this ligand bound to all endothelial and dissociated vascular cells. QAPB has high

affinity for of α_1 –AR and lower affinity for α_2-AR (Fig. 2 -15 and 2 -16). BODIPY-Ang II (Rhodamine-Ang II) also revealed the same characters as normal angiotensin II which could appear contractile response via AT_1 (Fig. 2-17) and relaxation via AT_2 in presence of losartan (Fig. 2-18).

c) Laser Scanning Confocal Microscopy (LSCM):

Cell dissociation:

1) Male SWT or D79N mice (aged 4 months) were killed by CO_2 inhalation and the descending thoracic aorta or main mesenteric were removed, cleaned of connective tissue, dissected into rings (2-3 mm in length) and then their lumen was opened (any coagulated blood was removed from inside the arteries).

2) Following dissection, tissue were placed in Buffer One* (see appendix) and stored at 4 °C until dissociation. (We can change also the tissue from PSS to Buffer One three hours before dissociation. All the vessels were cut by blade to small pieces to increase the effectiveness of enzymes). *Buffers are listed in the Appendix.

3) Arteries were washed once in Buffer One (Spin at 13000 rpm, for one minute) and supernatant discarded (using electrical pipette).

4) Arteries were resuspended in 200μL of Buffer 2A (containing papain and dithioerythritol- see appendix) and incubated at 35 °C (optimum temperature for enzymes) for 30 minutes.

5) Buffer 2A (see appendix) was removed by centrifuging at 13000 rpm for one minute and arteries resuspended in 200μL of Buffer 2B (containing collagenase II and hyaluronidase-see appendix) for no longer than one minute {because collagenase has potential to destroy receptors situated on cell membranes of

SMCs, endothelial and fibroblast cells (dissociated cells)}. Dissociation of cells was accelerated using an electrical Pasteur pipette (lowest suctioning rate) inside a glass pipette, aspirated repeatedly (BSA, Bovine Serum Albumin which included in Buffers, prevents dissociated cells and tissues attaching to glass). We can observe dissociation by disappearance of tissue pieces inside the eppendorf.

6) Cells are centrifuged at 13000 rpm for one minute and supernatant discarded.

7) Cells are resuspended in 200 μL of Buffer Two (the volume of Buffer Two at this stage depends on the density of cells, which are dissociated in Buffer 2A & 2B and required).

8) A defined volume of Buffer Two which contain dissociated cells is transferred on 0.00 number coverslips inside special medium petri dishes and stored for at least 30 minutes at 4 °C in fridge.

9) After 30 minutes for settlement of dissociated cells and their on coverslips. We added antagonists (30 minutes before staining) or Fluorescent dyes (QAPB and Rhodamine Ang II-H) according to the volume of supernatant on coverslips.

10) Following incubation, without washing, dissociated arteries cells on a coverslip (0.00 Number) were inverted and placed in the sample well of a glass slide prepared using high vacuum grease.

Tissue Preparation:

2-3mm segments of aorta and Superior mesenteric artery from wild type and D79N mice were incubated for 60 minutes in QAPB (0.1μM) and Rhodamine-Ang II-H (50nM) at room temperature with or without losartan (10μM) and/or PD123319 (10μM), Following incubation, without washing, aortic and superior mesenteric artery

segments were cut open and placed endothelial side up in the sample well of a glass slide. The well containing the tissue and prior incubation medium was sealed with a glass coverslip (No:1.5).

Image Capture:

Serial optical sections were collected on a Biorad 1024 & Radiance 2100 confocal laser scanning microscope. The Excitation/Emission parameters used were 488/515nm for QAPB and 567/610nm for rhodamine angiotensin II (Rho-Ang II-H). In all experiments the laser power, gain and offset (contrast and brightness) were kept constant. The distance between optical sections was maintained at 1μm for each image stack. Tissues were visualised using a X40 oil immersion objective on which the numerical aperture is 1.00 and therefore optimal pinhole setting is 1.5. Image size was set to 512 x 512 pixels which equates to a field size of 289um x 289um. (Each 1μm is equal to 1.77 pixels).

Image Analysis:

3D volumes (image stacks) were transferred to either Metamorph (Universal Imaging, Version 4.2) or Imaris (Version 3.2) or Amira (TGS, Version 3.2) software packages for subsequent analysis and volume visualisation respectively. 3D volumes containing two channels of data are pseudocoloured green and red for QAPB & Rho-Ang II-H respectively. Where two channels co-localise and their intensities are roughly equivalent, the co-localised area is displayed in yellow. Spatial localisation of fluorescent signals was achieved using orthogonal viewing of the XY, XZ & YZ planes. 3D views were rendered using the Amira 'voltex' module.

Drugs:

All drugs were of analytical grade and were dissolved in either distilled water (H_20), ethanol or DMSO as indicated below. Noradrenaline dilution included 23uM EDTA to prevent oxidation.

noradrenaline (H_20), Phenylephrine (H_20), angiotensin II (H_20), Acetylcholine chloride (H_20), Losartan (DMSO) [MERCK USA], PD 123319 (H_20), 5HT (H_20), U46619 (ethanol), L-NAME (N-Nitro-L-Arginine Methyl Ester) (H_20), rauwolscine (H_20) [Sigma-Aldrich Co; Poole, UK]. UK14304 (DMSO),

Fluorescent compounds:

Fluorescent prazosin (QAPB) (DMSO) [Molecular Probes INC; EUGENE-USA], Fluorescin Conjugate angiotensin II (F-AII) (H2O) [Sigma-Aldrich Co: Gmbh, Germany], rhodamine-angiotensin II-human (Rho-Ang II-H) (H2O) [Phoenix Pharmaceuticals INC; Germany].

Statistics:

Values are means ± Standard error mean from n experiments. Difference between maximal contraction response to CCRC to agonist in presence and absence of drugs was compared with one-way ANOVA followed by Bonferroni's Multiple Comparison Test and two-tailed none-parametric (Mann-Whitney test) and unpaired t-test for different population and strains. Statistical and graphical analysis was carried out using Excel 97 and GraphPad Prism 3.00 for PC. Data used to plot the concentration response curves are the mean contraction induced at each concentration of the agonist.

2-4. Results:

Sub-maximal contraction to 5HT provided basis for observing contractile or relaxant effects in mouse aorta:

In the absence of other drugs, or of induced tone, angiotensin II produced weak or no contractions (not shown). The vessel contracted in a concentration-dependent manner to several other vasoconstrictors, including 5HT, NA, PE, UK14304 and U46619 (Figure 2-1). It was found that raising the tone submaximally with other agents allowed reproducible and consistent contractile or relaxant responses to be superimposed. With angiotensin II this could be achieved satisfactorily with phenylephrine, 5HT or U46619 but for the purposes of the present study, where α_1-adrenoceptor-mediated activation would be confounding, we employed 5HT or U46619 at submaximal concentrations (based on Fig. 2-1). Endothelial cell functional integrity was assessed using ACh (1μM) on top of the response to 5HT or U46619. ACh-induced relaxation was completely abolished by adding L-NAME (0.1mM) in intact aortic rings (not shown). In endothelium-denuded aortic rings, no relaxation was produced to ACh and, indeed, contraction was produced in denuded aortic rings due to direct activation of vascular smooth muscle.

In Aorta:

Dual effect of angiotensin II following pre-constriction with 5HT:

In four month old wild type mouse aorta preconstricted with 5HT (0.1μM), the short exposures to each concentration of Ang II during construction of a CCRC, produced contractions to low concentrations (Ang II; 1-30nM) whilst higher concentrations (0.1-1μM) produced relaxation. The maximum Ang II contractions represented a 10%

increase in 5HT tone and the maximal relaxation was approximately 5%. In the D79N strain relaxation attained higher values of approximately 10%. However, two-tailed none-parametrical t-test (Mann-Whitney test) revealed no significant difference (P value: 0.3829 > 0.05) between wild type and D79N (Figure 2-2).

The responses during a CCRC were consistent but were not well-maintained, making it obvious that the dual effects of Ang II coupled with some desensitisation, made analysis complex. In separate experiments a single concentration of Ang II (30nM) was employed in place of a cumulative concentration response curve, to avoid desensitisation and achieve equilibrium. During a CCRC this concentration had produced a contraction. When given as a single concentration it produced a short lived, small contraction followed by a slow relaxation that achieved an equilibrium of around 15% relaxation (of 5HT tone) in over 10-15 Minutes (n=18). This relaxation was lost in endothelium-denuded arteries (intact, relaxation of -14.9 ± 2.2 %; denuded, contraction of $+16.7 \pm 3.5$ %) (n=15) (Fig 2-3).

The effects of AT antagonists on CCRCs to Ang II

Losartan (AT_1 antagonist; 1μM, 30 minutes) abolished the contractile effect of Ang II in the CCRC. In contrast, there was no significant effect of losartan versus the relaxation response. PD123319 (AT_2 antagonist; 1μM, 30 minutes) abolished the relaxation component to Ang II leaving only the contractile component that now extended to higher concentration (Figure 2-4).

L-NAME (0.1mM) could completely blocked relaxation response to cumulative Ang II. However, In presence of both losartan (0.1μM) and L-NAME (0.1mM) adding Ang II cumulatively could produced bigger contraction (Fig. 2-5). This shows presence of AT_2-R in smooth muscle cells, which could co-operate with AT_1-R to provide greater contractile effect to cumulative concentration of Ang II (Fig. 2-6a and 2-6b-Traces).

Functional interaction between α-ARs and Ang II receptor subtypes:

Effects of Ang II on contraction to noradrenaline:

Tissue rings were incubated with Ang II at a concentration just subthreshold for relaxation of 5HT-induced tone in the CCRC, but which produced a slow relaxation when given as a single concentration (30nM; 10 minutes). In the absence of pre-established tone it produced no change in basal tone but it caused a reduction in a subsequent CCRC to noradrenaline compared with control (n=12). The combination of losartan (1μM) with Ang II further decreased the contractile effect of NA ($P<0.0001$, One way ANOVA followed by Bonferroni's Multiple Comparison Test) (Figure 2-7). In contrast, incubation with PD123319 (1μM) for 30 minutes could prevent the decrease in the contractile effect of NA. The difference between control and PD123319 was significant ($P< 0.05$, One way ANOVA- Bonferroni's post test) (Figure 2-7). In a time control experiment, three consecutive CCRC to NA did not show any alternation in sensitivity over (data not shown).

This is a potentially complicated experiment with the possibility of many subtypes of adrenoceptor and angiotensin receptor being activated. Nevertheless the outcome clearly shows that an AT_2-mediated relaxant effect of angiotensin II was its dominant action against noradrenaline-induced tone and that this could be enhanced by eliminating its AT_1 mediated contractile action or blocked by eliminating AT_2 with PD123319.

Interaction of vasorelaxant responses to Ang II and an α_2-AR agonist:

Synergism between relaxant responses to activation of adrenoceptors- and AT receptors was sought by comparing a single effective concentration of UK14304 (1μM) in the presence and absence of a single effective concentration of Ang II (30nM), on top of 5HT (0.1μM) preconstriction (Fig. 2-8). Relaxation to both Ang II (AT_2, as validated

above) and UK14304 (α_{2A}-AR, as demonstrated in Chapter 3) (Fig. 2-8) were blocked by L-NAME (100μM) (Fig. 2-5) (Fig. 3-9, see chapter three). This was carried out in both WT and Nashville D79N. The relaxant response to UK14304 was not significantly different in the presence and absence of Ang II (n=15) (Figure 2-8). Again, this is a potentially complicated scenario, with the possibility of many receptor subtypes being activated. However the concentrations of agonists were chosen to produce dominant endothelium-mediated relaxation. Thus, the failure to find any meaningful synergism contrasts with the dramatic synergism found between the contractile effects of α_2-AR and Ang II, suggesting that endothelial AT2 and α_2-AR receptors are working independently.

Response to angiotensin II in old mouse aorta:

In sixteen months old wild type mouse aorta studied under identical conditions only contraction with no relaxation was observed to either cumulative or single concentrations of Ang II (n=18) (Fig. 5-11, See chapter five).

Other arteries:

The mouse carotid, main mesenteric and first branch mesenteric arteries responded in a concentration-related manner to a range of vasoconstrictors. In general, of the drugs tested, U46619 was most potent and had the highest maximum, while noradrenaline, phenylephrine and 5HT produced responses of similar size and with similar potency (Figs. 2-9, 2-11, 2-13). A diversion from this was that phenylephrine was more potent than noradrenaline in the first branch mesenteric artery (Fig. 2-13).

On this basis U46619 (0.1μM) was chosen as the basis for studying CCRCs to Ang II. In both carotid and superior (main) mesenteric arteries the contractile effect of Ang II was dominant (Figs. 2-10 and 2-12). In first branch mesenteric arteries the main effect of angiotensin II was relaxation (Fig. 2-14). This was reversed to contraction by L-NAME (0.1mM) suggesting that it was of endothelial origin (Fig. 2-14).

Selectivity of Fluorescent Ligands:

The most important feature of a fluorescent ligand is that its potency and/or selectivity are comparable with the non-fluorescent ligand form. The fluorescent form of Prazosin (QAPB) has been extensively characterised and has been shown to exhibit approximately 10 fold lower affinity for α_1-adrenoceptors compared with the native ligand in functional and binding experiments (Daly et al, 1998; Mackenzie et al, 2000) (Fig. 2-15). In addition, the potency of QAPB at α_2-ARs is confirmed by its action on UK14304-mediated relaxation in α_{1D}-Knockout mouse aorta (Fig. 2-16 and 3-26-See chapter three).

Functional studies using Rhodamine (BODIPY)-Angiotensin II indicate that the fluorescent-angiotensin retained most of its agonist properties (Fig. 2-17 and 2-18).

Having gained confidence in the activity and selectivity of the fluorescent ligands, imaging studies can then beconducted.

Preliminary studies have shown that it is often not possible to displace a fluorescent ligand with a non-fluorescent competitor. This is presumably due to the slow dissociation rate of the fluorescent ligand. Furthermore, classical agonist/antagonist studies always rely on pre-incubation of the selective competitor prior to addition of the agonist.

In addition, if live tissue is used, and as anticipated receptor recycling is induced, then later application of a competitor drug will not have access to the full population of fluorescent ligand bound receptors. Therefore, a pre-incubation protocol seems more appropriate.

Visualisation of adrenoceptors on endothelial cells using fluorescence microscopy presents several technical challenges. The cells form a monolayer, on a convoluted surface caused by folds in the internal elastic lamina. The central parts of the cell

containing most of their material are also well spaced out. There is thus no single two dimensional plane with a high probability of locating cells. This is addressed by using Laser Scanning Confocal Microscopy (LSCM), allowing scanning in the z direction to locate cells. The images were obtained from the endothelial side of vessels opened longitudinally. The convolutions remain, however, so that the typical 2D image contains endothelium, the edges of the internal elastic lamina and smooth muscle cells are located beyond the lamina (Fig. 3-3, 3-4 and 3-5-See chapter three). The elastin of the lamina is autofluorescent over a broad range of wavelengths, so cannot be avoided. However, visualising autofluorescence at its longest emission wavelength produces an image of autofluorescence without revealing fluorescent-ligand (which has a narrower emission spectra). Thus, the "autofluorecence only" image can be subtracted from the image of autofluorescence plus ligand to show only the fluorescent-ligand. With this in mind, the endothelial cells binding QAPB can be seen attached to the folds of the IEL.

Pre-incubation of aortic segments with α_1-AR subtype selective drugs enabled isolation of the α_2-AR population. QAPB (0.1μM) bound to endothelial cells following pre-incubation with BMY7378 (α_{1D}-AR antagonist) and 5MU (α_{1A}-AR antagonist) in α_{1B}-Knockout mouse. This indicated the presence of endothelial α_2-ARs. This is further supported by the complete absence of QAPB-binding following pre-incubation with BMY7378, 5MU and rauwolscine (selective α_2-AR antagonist) in α_{1B}-Knockout mouse aorta and superior mesenteric arteries (Images: 3-13).

Fluorescent angiotensin II:

A similar pre-incubation protocol was used to examine the binding of Fluorescent Ang II to the smooth muscle and endothelial cells of mouse aorta.

Fluorescent Ang II binding showed an EC50= 4.2nM of the Rho-Ang II contraction (for angiotensin II EC50 was equal to 1.9nM) (Fig. 2-17) and relaxation response to losartan revealed EC50= 7nM for Rho-Ang II compared with 40nM for angiotensin II in young mouse aorta (Fig. 2-18).

Pre-incubation with both PD123319 (AT_2 selective antagonist) and losartan (AT_1 selective antagonist) eliminated fluorescent Ang II binding to endothelium. Binding of angiotensin can be blocked by a combination of AT receptor antagonists (second bottom row from image 2-4).

Visualisation results:

Incubation for one hour in fluorescent angiotensin II produced red fluorescent "staining" of cells in the endothelium and smooth muscle of aorta and superior mesenteric artery. This fluorescence could be prevented by prior and concomitant incubation with AT receptor antagonists. Simultaneous incubation with a fluorescent derivative of prazosin (QAPB) produced labelling of all cells (in a different colour - green) so that when AT receptors were blocked the cells could still be visualised. The reciprocal arrangement is used in the next chapter when the objective is to analyse α-adrenoceptors. In both arteries there was autofluorescence from the internal elastic lamina which helped orientation for location of endothelial cells since they are located directly on top of it (as viewed from the lumen). The autofluorescence has a broad spectrum and so shows up in both green and red channels.

The concepts are built up through a series of images. The first series deals with aorta, followed by another on superior mesenteric. In each case the experiment was repeated 5 or 6 times and the images are representative.

Aorta:

Image 2-1 shows a 3D Model of the aorta viewed from the intimal surface stained with fluorescent angiotensin (red). Autofluorescence of Internal Elastic Lamina (IEL) is recognisable as a green grooved surface and helps orientate the viewer. Both Smooth Muscle Cells (SMCs) and Endothelial (Endo) cells show up in red, set off against the green of the lamina and it is clear that the endothelial cells characteristically lie in the laminar grooves.

Image 2-2 shows the same 3D Model as in Image 2-1 viewed from intimal and adventitial sides. Internal External Elastic Lamina (IEL) & External Elastic Lamina (EEL) are both recognisable from green autofluorescence. The external lamina is less

dense and appears as loose network of fibres. The high degree of fluorescence on adventitial fibroblasts indicates a high level of AT receptor expression.Image 2-3 shows the same model, this time as a Black & White view from the intimal side. This time the layers in the z direction have been separated. The upper panel shows those z layers that constitute the internal elastic lamina, while the lower panel shows the layers that lie above and below this. The ovoid forms of their perinuclear staining show the endothelial cells lying above the lamina, arranged in its grooves. The elongated smooth muscle cells (SMCs) run at right angles to the grooves and are seen to run in and out of the field around the grooves.

Image 2-4 shows single planes, viewed from the lumen that cut through the ridges of the lamina with endothelium attached. This figure allows comparison of the localisation, on endothelial cells, of fluorescent angiotensin II (red) and a fluorescent prazosin analogue (QAPB, green). Dense "waves" of fluorescence are the autofluorescence of the ridges of elastic lamina immediately under the endothelium. Binding of angiotensin can be blocked by a combination of AT receptor antagonists. The "mosaic" nature of the endothelium is shown up by eliminating the AT_1 receptors and the major α-adrenoceptors: some cells show only Red and others only green; thus, the AT_2 and minor AR α_{1A}- & α_{1B}-ARs) are not present in every cell (for analysis of AR types see next chapter). Image 2-5 shows that different receptor types have different localisations. An individual endothelial cell is seen layered on an Internal Elastic Lamina groove. The data set is a 3D volume. 3 different 2D views are shown. The issue was treated with BMY7378 (0.1μM) and losartan (10μM) before staining by Rhodamine-Ang II-Human (0.1μM) and QAPB (0.1μM). The Green colour "granules" reveal α_1-AR (α_{1A} & α_{1B}) subtypes or α_2 -ARs and Red granules illustrate AT_2-Receptors inside the cell, around the nucleus (arrows). Image 2-6 shows that, when AT_1 receptors are eliminated, AT_2 receptors are seen to be intracellular. In this example individual smooth muscle cells,

dissociated from four months wild type aorta, are stained with QAPB (0.1μM, green) and Rhodamine-Ang II-H (0.1μM, red). This is an Amira" view which provides a "surface" connecting regions of equivalent fluorescence density. A first cell is shown in the upper row without other drugs. On this cell the two ligands are seen to be located in similar regions of the cell, particularly on the surface. A second cell from the same source, shown on the lower row, treated with losartan (10μM) for 30 minutes and then stained in the same way as control. The red channel shows a "shrunken"! image compared with the green channel and when the two are superimposed it can be seen that there are few AT-Receptors on the cell surface, where green α-AR now dominate. In the right hand image a translucent view shows that the red receptors lie "inside" the green ones, indicating an intracellular location for the AT_2-R. In image 2-7 a different view ("Imaris" software) shows the surface location of AT_1 and intracellular location of AT_2. The image in the first row illustrates that a majority of angiotensin II-Receptors are present on the cell membrane. In the lower row losartan (10μM) treated SMCs from the same source show that the membrane compartment containing angiotensin II-Receptors is reduced in volume with losartan treatment. This suggests that a majority of cell surface angiotensin II-receptors are AT_1-R and implies that the AT_2-R are dominantly localised inside the SMCs, particularly around the nucleus. Image 2-8 makes a further comparison of receptor location. The upper panels show that a majority of AT-Receptors (Red) are located around a convoluted nucleus with some present on the surface. However, α-ARs (Green) are dominant on cell membrane. The lower panel shows the predominantly surface location of AT_1 receptors when AT_2 binding is blocked with PD123319 (10μM).

Image 2-9 shows another view of the surface location of AT_1 alongside α-ARs that emphasises how the different receptors occupy their distinctive territories. Cells treated with PD123319 (10μM) show AT_1-Receptors localised to the cell surface while

Losartan (10μM) shows intracellular location of AT_2-Receptors. In image 2-10 losartan (10μM) and BMY7378 (0.1μM) are used to eliminate the AT_1 (previously shown to be on the cell surface) and α_{1D}-ARs (generally considered to be intracellular) leaving AT_2-Rand α_{1A}-AR or α_{1B}-AR. AT_2-Receptors are seen to be localised mostly inside the cell and $\alpha_{1A \text{ or } B}$-adrenoceptors are dominant on the cell membrane.

Superior Mesenteric Artery Image 2-11 shows the localisation of AT and AR on superior mesenteric artery. The internal elastic lamina has a different form, being thinner with regular holes known as "lamellae". In this vessel it is easy to see endothelial cells from their binding of fluorescent angiotensin II and QAPB. In this example the fluorescence is simply shown as a pseudocolour image in which fluorescence intensity is mapped to a palette of colours. Left and right lower images show these intensity maps for the Ang II (Left) and QAPB (Right) channels. SMCs and Endothelial Cells both show both receptor families. Image 2-12 illustrates a further localisation of receptors showing endothelial and smooth muscle cells. The AR show up particularly well in the SMCs. Image 2-13 shows a different view of the images in 2-12. The presence of angiotensin II-receptors and adrenoceptors is visible in both smooth muscle and endothelial cells in all the images but adrenoceptors are dominant. Image 2-14 makes a comparison of fluo angiotensin II binding to AT_1 or AT_2 receptors. In the first row AT1-Receptors are shown after 1hr with PD123319 (10μM) and in the second row AT_2-Receptors are shown after 1hr with losartan (10μM). The images compare location of AT_1 and AT_2-Receptors in the endothelial cells. AT_1-Receptors are predominantly situated on cell membrane and AT_2-Receptors are concentrated around the endothelial nucleus surrounded by adrenoceptors.

Chapter Two Graphs and LSCM Images

Effects of Angiotensin II on mouse arteries

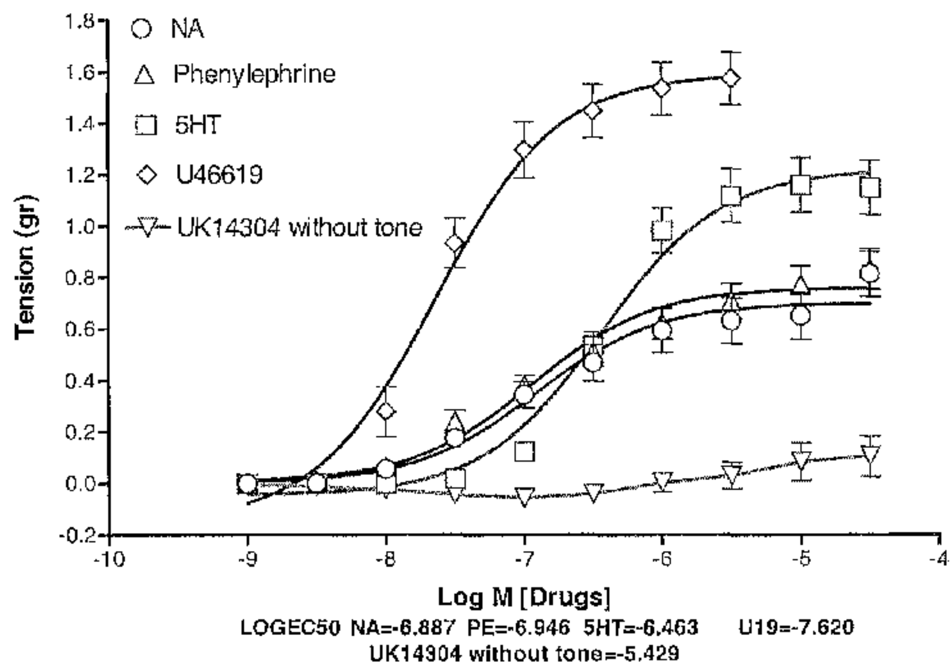


Fig. 2-1: Comparison between CCRC to noradrenaline (O), phenylephrine (△), 5HT (□), U46619 (◇) and UK14304 (▽) in four months wild type mouse aorta (n=7).

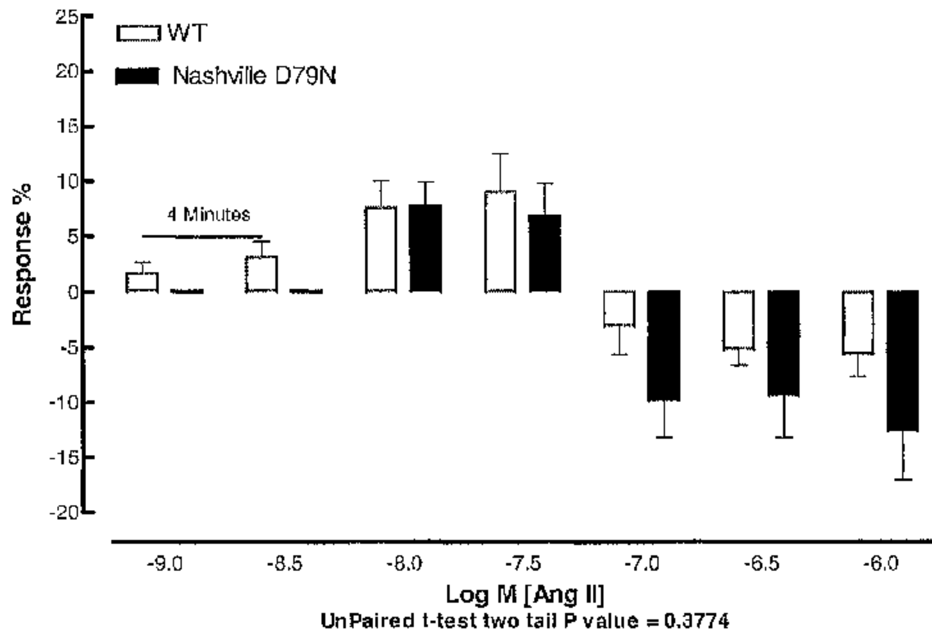


Fig 2-2: Cumulative concentration effect of angiotensin II on top of 5HT (0.1μM) preconstriction in four months wild type (n=18) and Nashville D79N (n=6) mouse aorta.

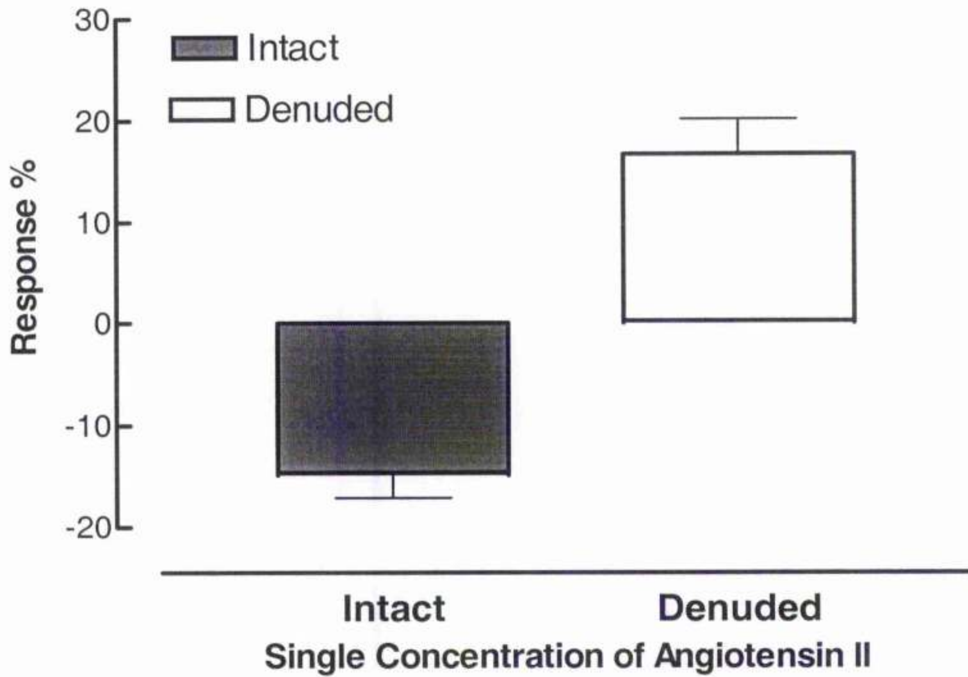


Fig. 2-3: Single concentration effects of angiotensin II (30nM) on intact and denuded four months mouse aorta on top of 5HT (0.1μM) (n=15).

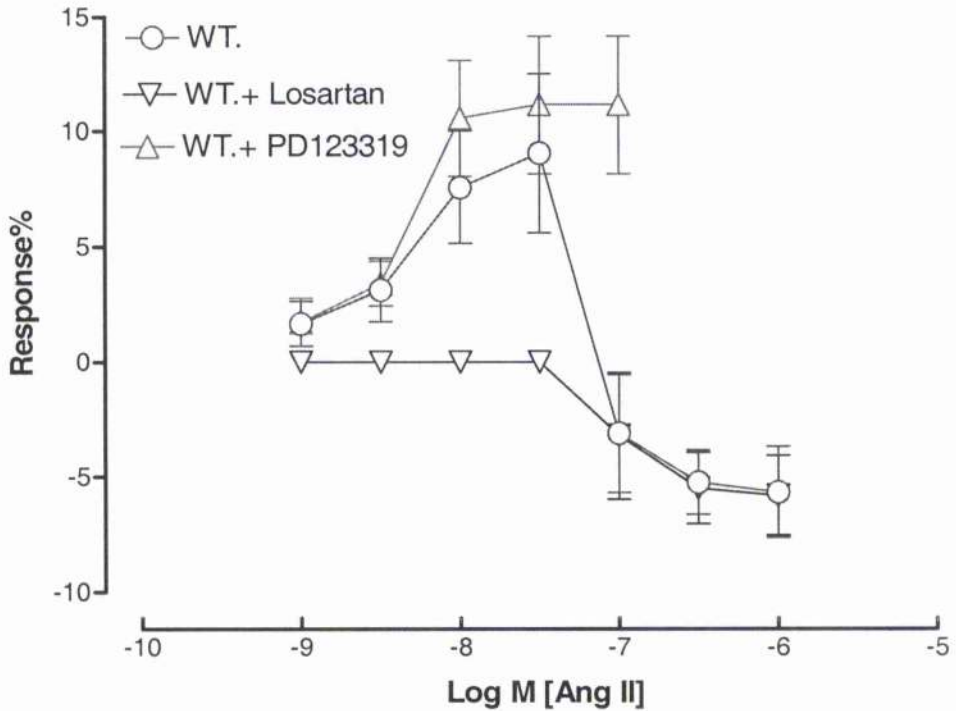


Fig. 2-4: Cumulative concentration effect of angiotensin II on top of 5HT (0.1μM) preconstriction with (losartan Δ or PD123319 ∇) or with out antagonists (O) in four months wild type mouse aorta (n=11).

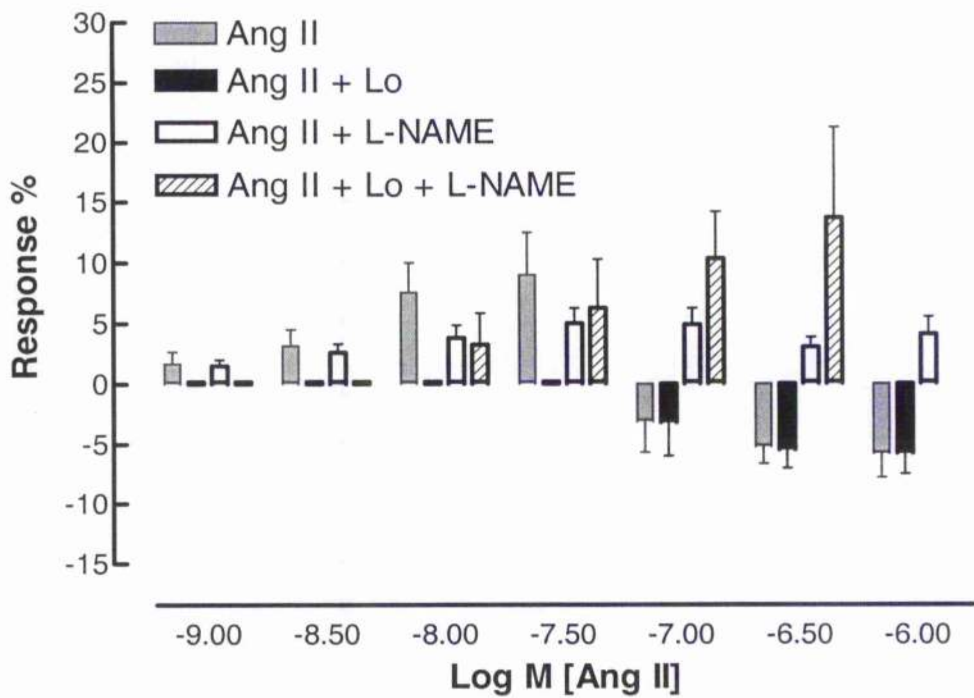


Fig. 2-5: Comparison between CCRC to angiotensin II in four months wild type aorta in presence of L-NAME (0.1mM)and losartan (1μM) on top of 5HT (0.1μM) preconstriction (n=18).

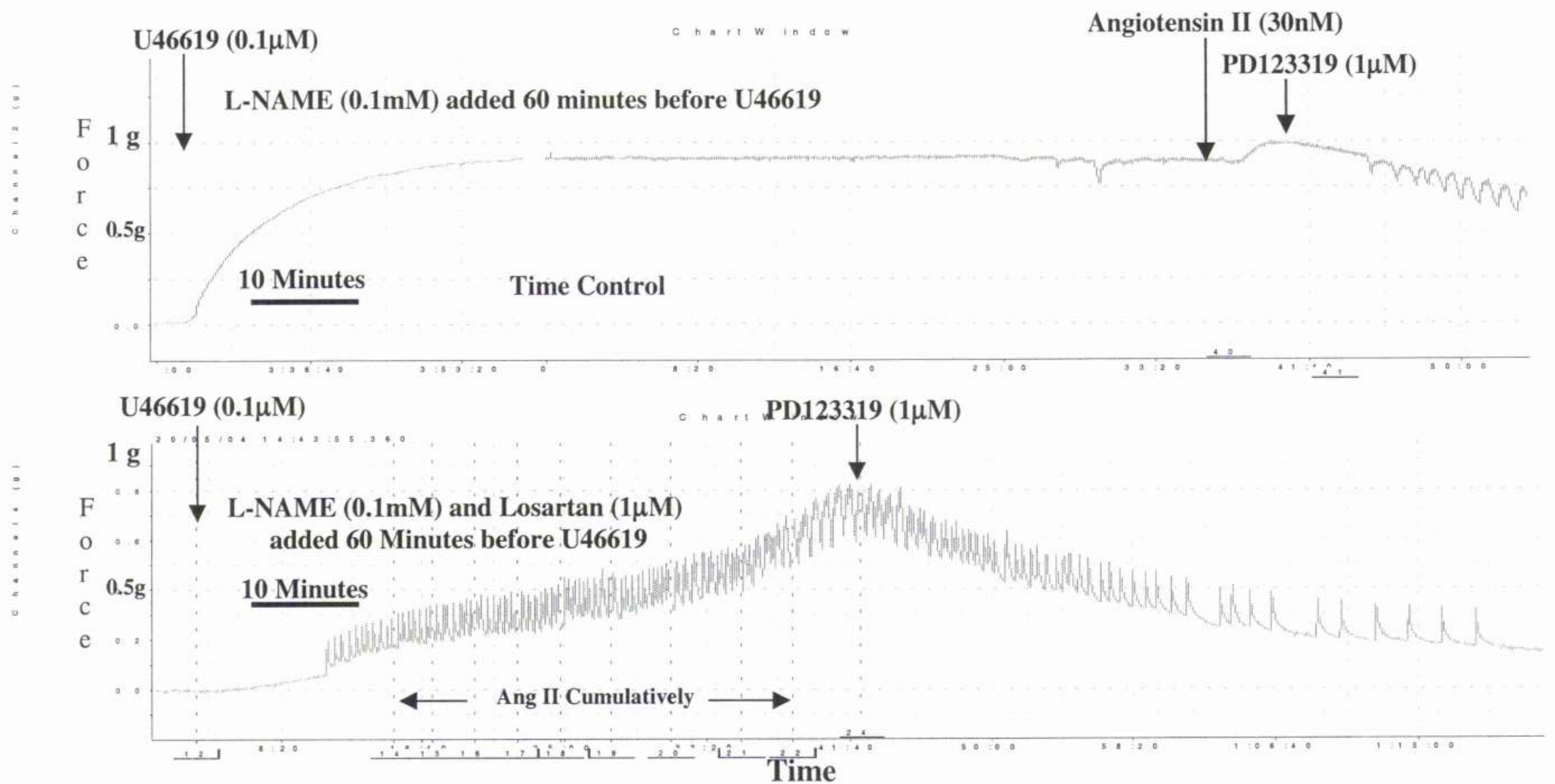


Fig. 2-6a: ***Young Mouse Aorta***-Effect of angiotensin II on top of U46619 tone in presence of losartan (1μM) and L-NAME (0.1mM) in four months (Young) mouse aorta. Ang II contractile responses were opposed by PD123319 (1μM). Presence of L-NAME (0.1mM) is necessity to neutralize endothelial AT_2-R relaxation response.

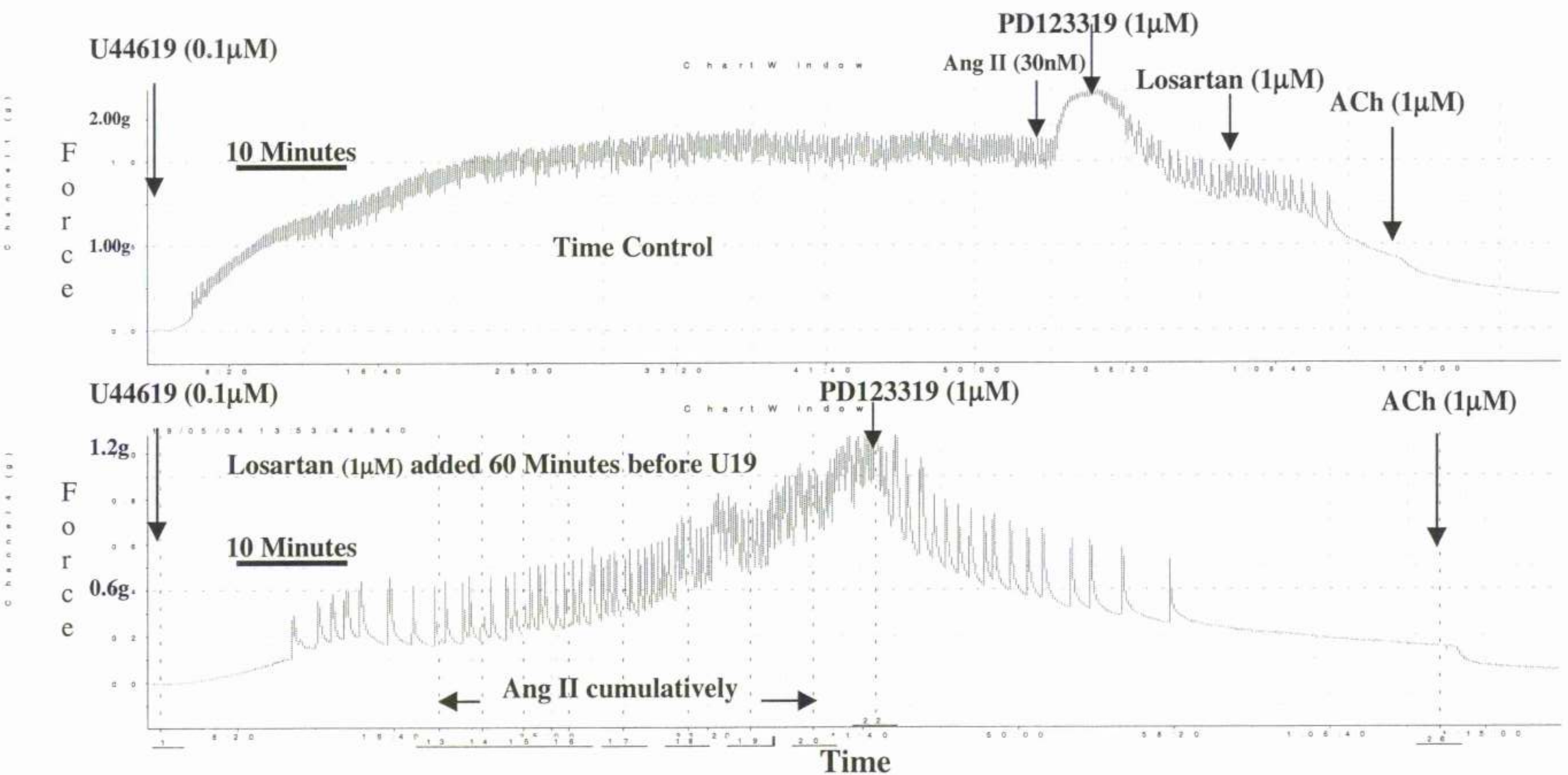
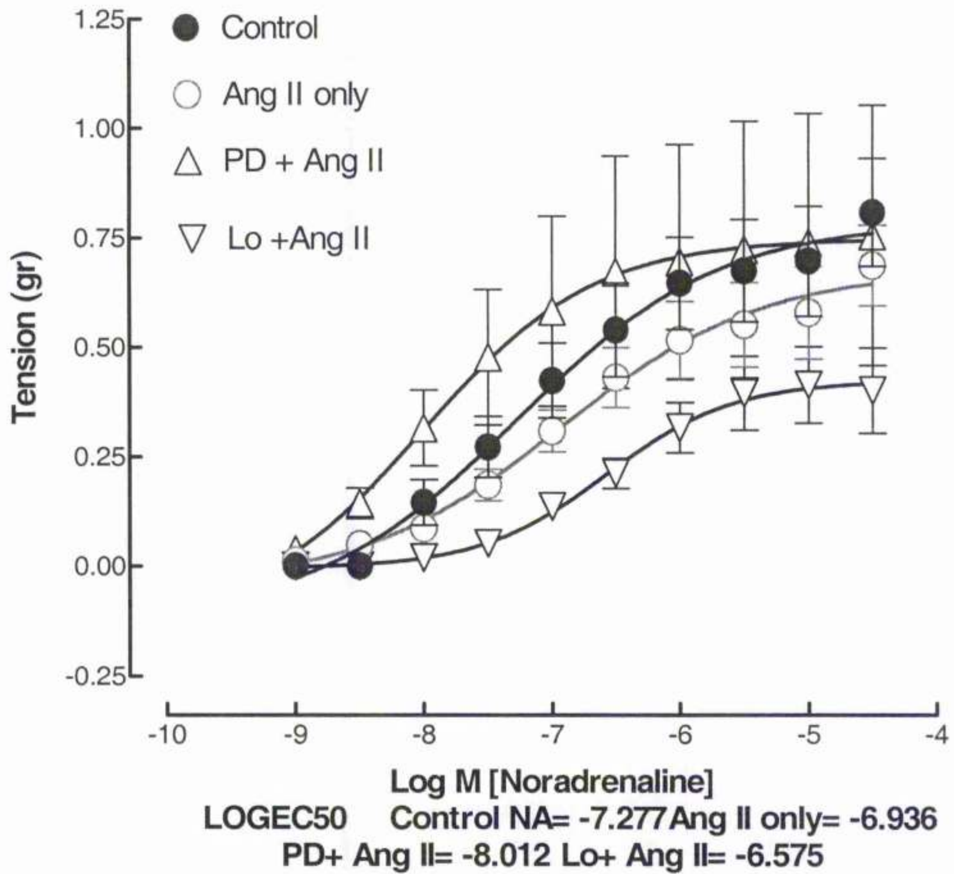


Fig. 2-6b: ***Old Mouse Aorta***-Effect of angiotensin II on top of U46619 tone in presence and absence of losartan in fifteen months (old) mouse aorta ($C57BL_6$) . Ang II contractile responses were opposed by PD123319 (1μM). However, ACh (1μM) could provide more relaxation in both of action and time control.



ANOVA P value < 0.0001

Bonferroni's Multiple Comparison Tes	P value
Control NA vs Ang II only	P > 0.05
Control NA vs PD+ Ang II	P < 0.05
Control NA vs Lo+Ang II	P < 0.001
Ang II only vs PD+ Ang II	P < 0.001
Ang II only vs Lo+Ang II	P < 0.001
PD+ Ang II vs Lo+Ang II	P < 0.001

Fig. 2-7: CCRC to noradrenaline in four months wild type mouse aorta in presence of Ang II (30nM) only, PD123319 (1μM) + Ang II (30nM) and losartan (1μM) + Ang II (30nM) compare with control (n=12).

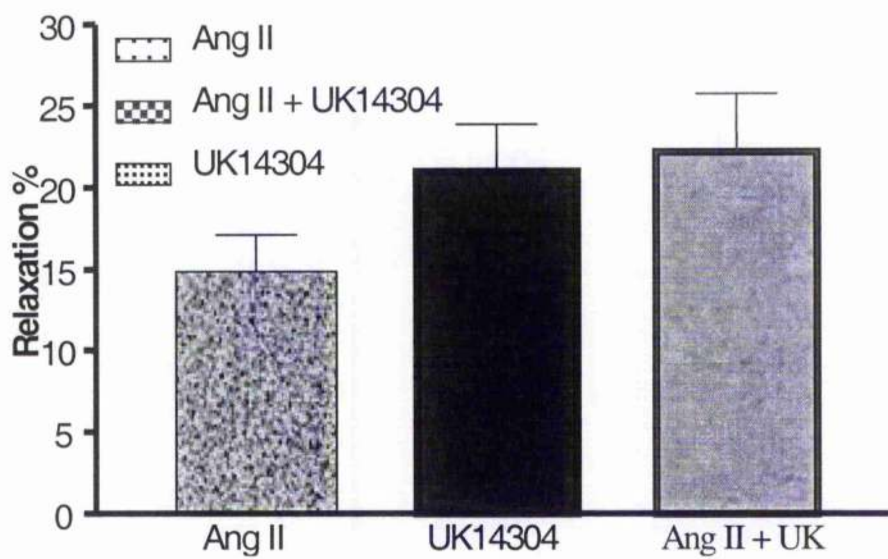


Fig. 2-8: Comparison between effect of angiotensin II and UK14304 lonely and together in four months wild type mouse aorta (n=15).

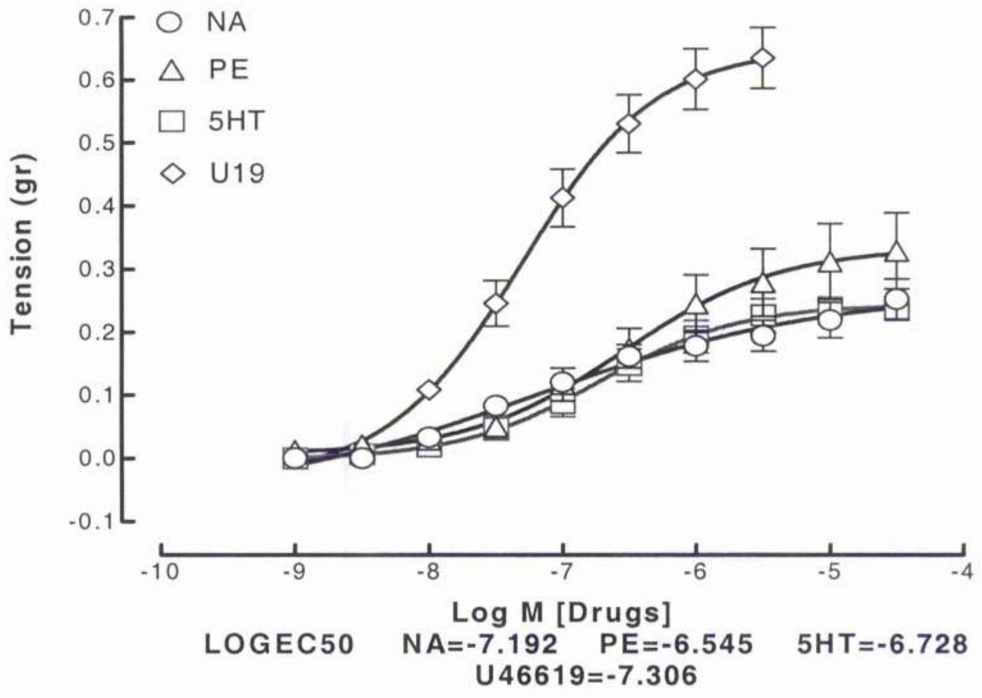


Fig. 2-9: CCRC in four months wild type mouse carotid artery (n=4).

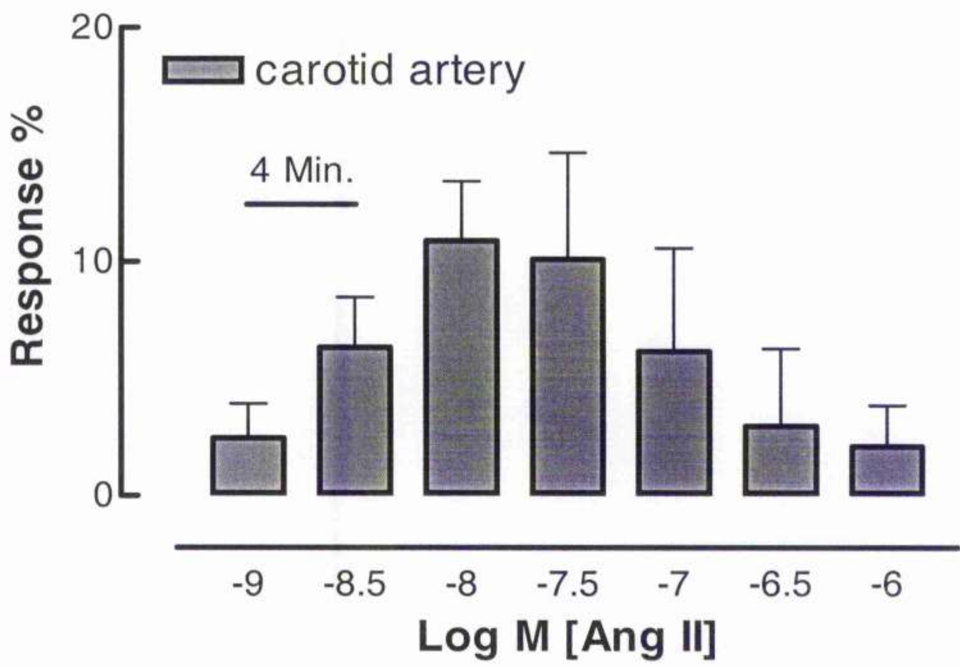


Fig. 2-10: CCRC to angiotensin II on top of U46619 (0.1μM) tone in four months wild type carotid artery (n=6).

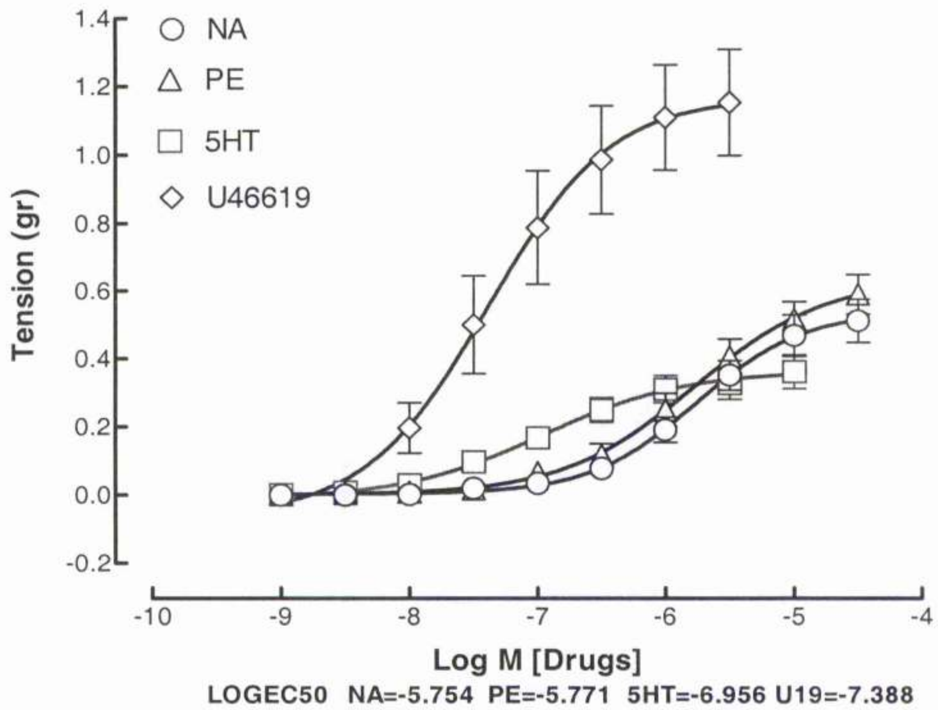


Fig. 2-11: CCRC to different agonists {noradrenaline (O), phenylephrine (△), 5HT (❑), U46619 (✧)} in four months wild type mouse superior mesenteric artery (n=6).

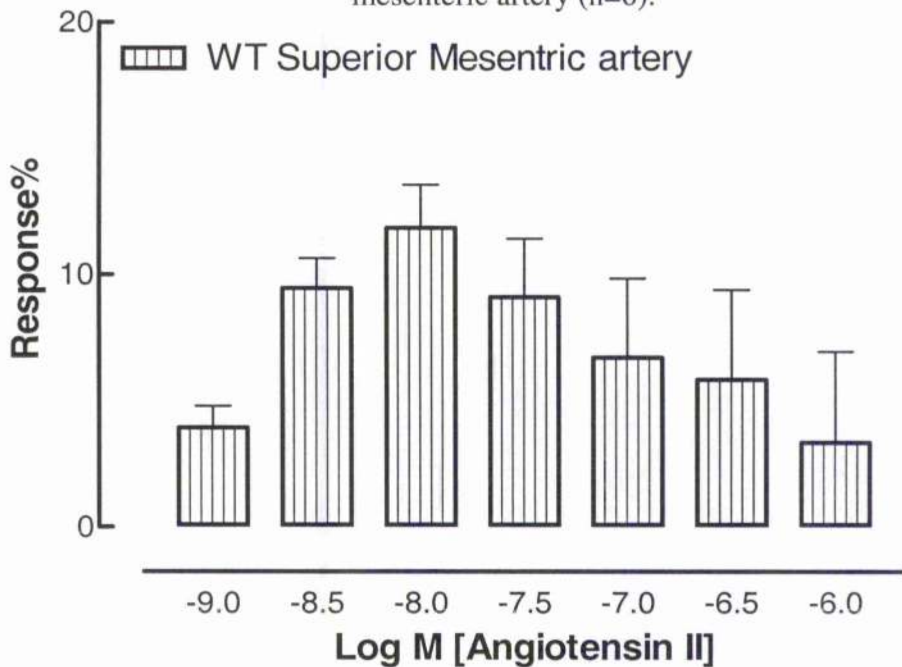


Fig. 2-12: CCRC to angiotensin II in four months wild type superior mesenteric artery on top of U46619 (0.1μM) tone (n=6).

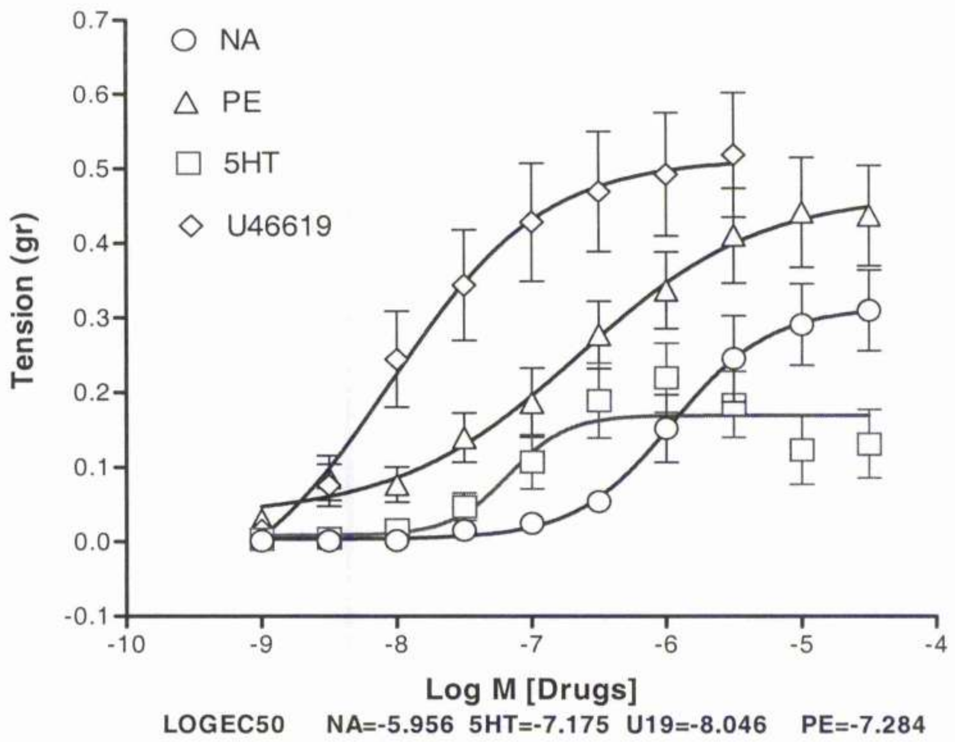


Fig. 2-13: CCRC to different agonists {noradrenaline (O), phenylephrine (△), 5HT (❑), U46619 (✧)} in four months wild type first branch of mouse mesenteric artery (n=4).

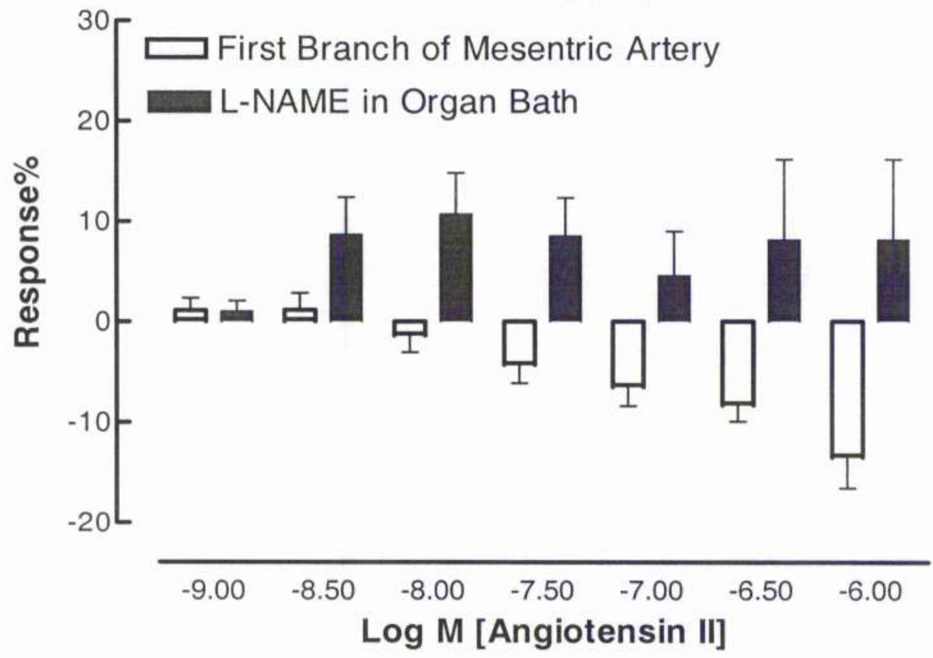


Fig. 2-14: CCRC to angiotensin II on top of U46619 (0.1μM) preconstriction in four months mouse first branch of mesenteric artery in presence and absence of L-NAME (0.1mM) (n=6).

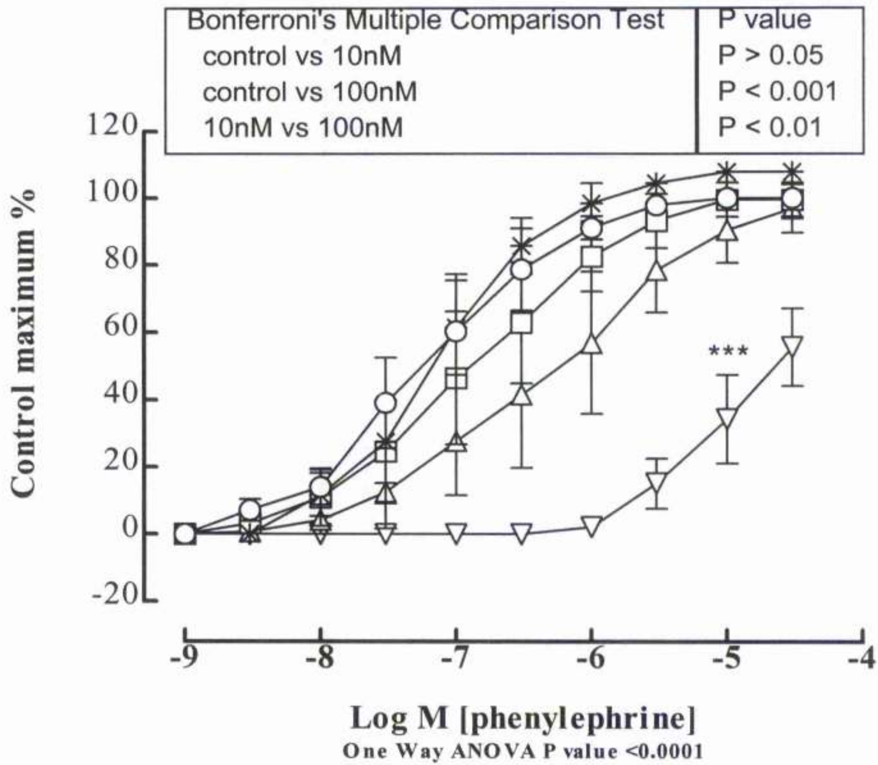


Fig. 2-15: QAPB antagonism on cumulative concentration response to Phenylephrine in young rat aorta. control (O), time control (*), 1nM (Φ), 10nM (≅) and 100nM (∇) of QAPB (graph from Dr. C. Daly & S. p. MacGrory) (n=6).

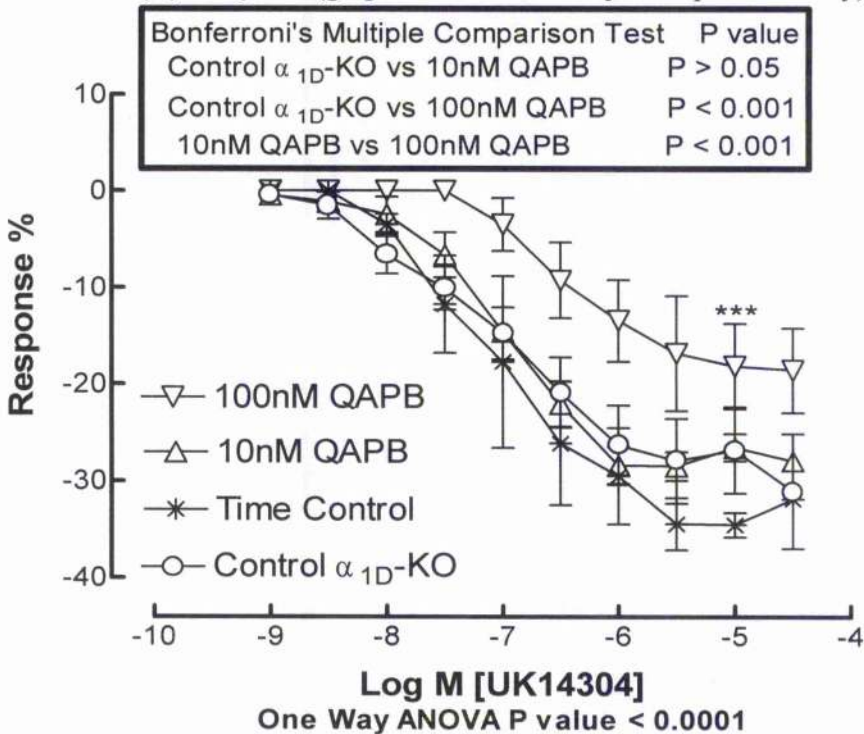


Fig 2-16: QAPB affinity on α_2-AR in α_{1D}-KO mouse aorta (n=5).

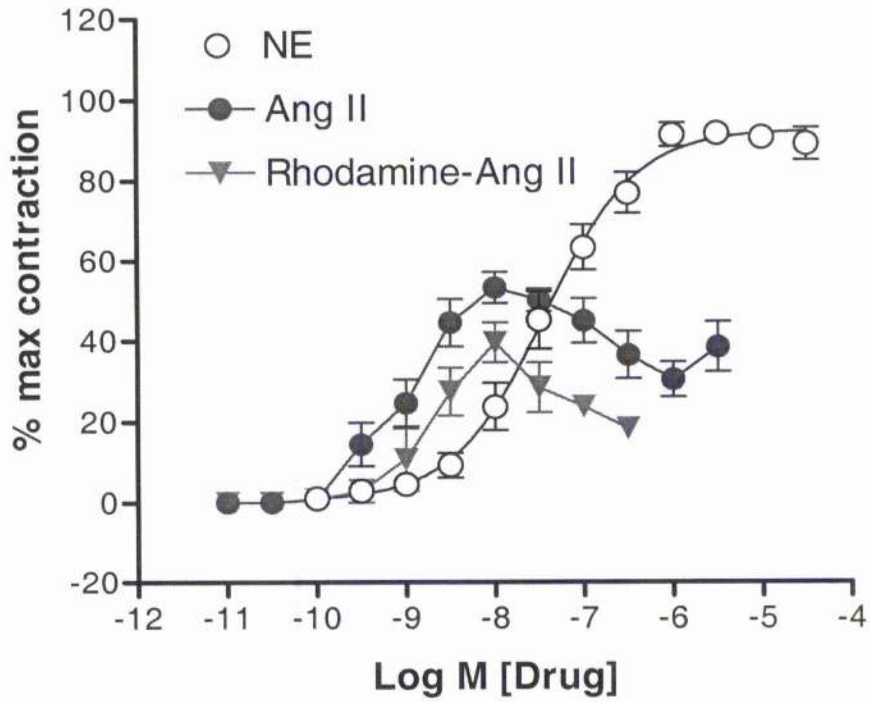


Fig. 2-17: Noradrenaline, angiotensin II & rhodamine-angiotensin II contractile responses in young mouse aorta (n=5){from Ali Zeeshan}.

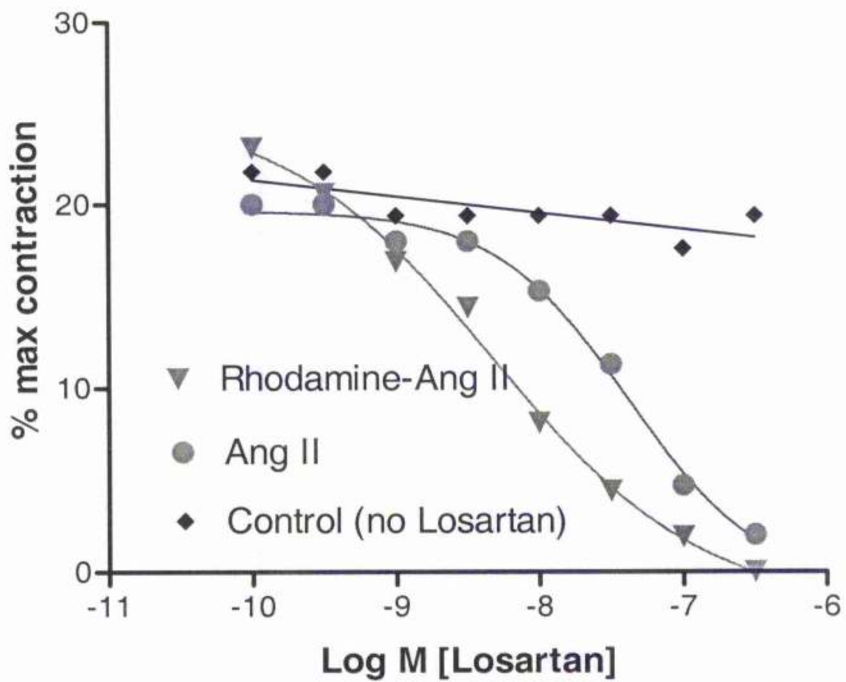
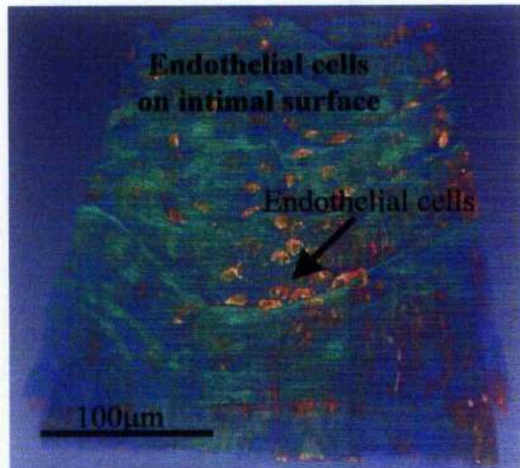


Fig. 2-18: Both angiotensin II and rhodamine (BODIPY)-angiotensin II could produce relaxation in presence of losartan in denuded young mouse aorta (n=2){from Ali Zeeshan}.



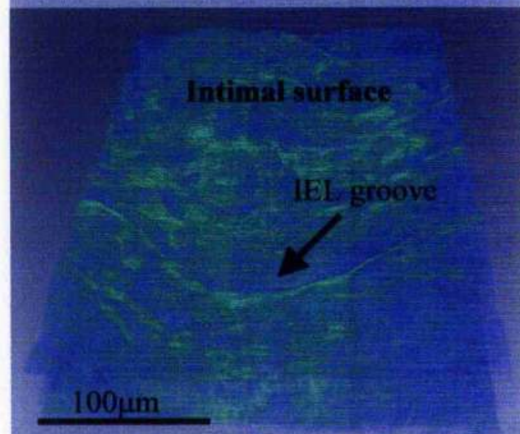


Image 2-1: 3D Model of aorta viewed from intimal surface stained with fluorescent angiotensin (red) and QAPB (green).

Four months wild type mouse aorta (Control) stained with Rho-Ang II-Human (50nM) {Excitation/Emission = 567nm/610nm}.

Binding to angiotensin II receptors shows as red. Autofluorescence of Internal Elastic Lamina (IEL) is recognisable as a green grooved surface and shows up on its own in the middle panel (QAPB Excitation/Emission 488nm/515nm). Both Smooth Muscle Cells (SMCs) and Endothelial (Endo) cells show up red in the lower panel, indicating the presence of AT receptors. The upper panel shows that endothelial cells characteristically lie in the grooves of the internal lamina.

Laser power: 50%, Iris: 1.5, Gain: 15, step: 0.5 µm, Speed: 500 imp, Objective: 40X Oil, Numerical Aperture: 1, Pixel * Lines: 512 X 512 (1 µm = 1.77 Pixel)

Images developed as a 3D model in Metamorph (version 4.2) and Amira (version 3.2) software.

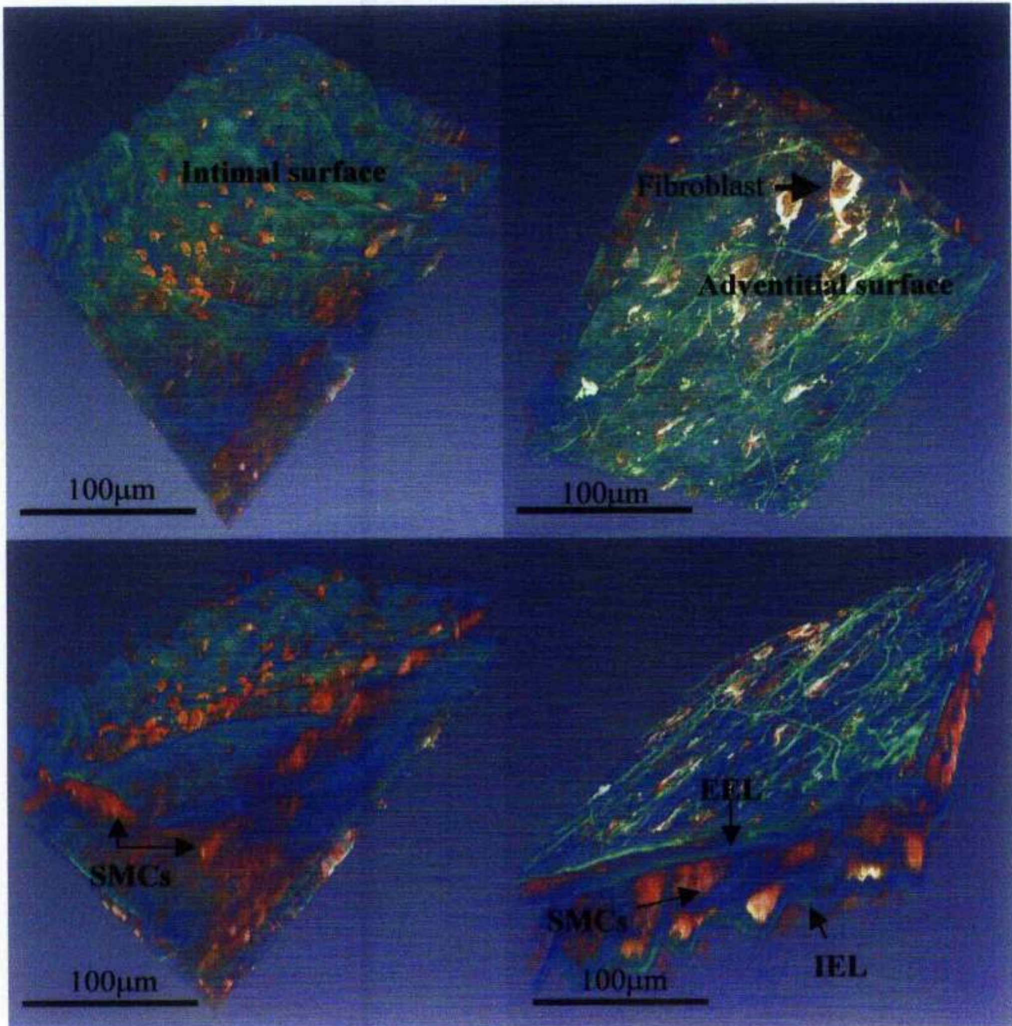


Image 2-2: The same 3D Model as in Image 2-1 shows views from intimal and adventitial sides. Internal External Elastic Lamina (IEL) & External Elastic Lamina (EEL) are recognisable from green autofluorescence. Notice the highly stained adventitial fibroblasts, which express high levels of AT receptors. Yellow colour is due to overlapping of Green and Red channels showing presence of both α-ARs and AT-Receptors in the same region.

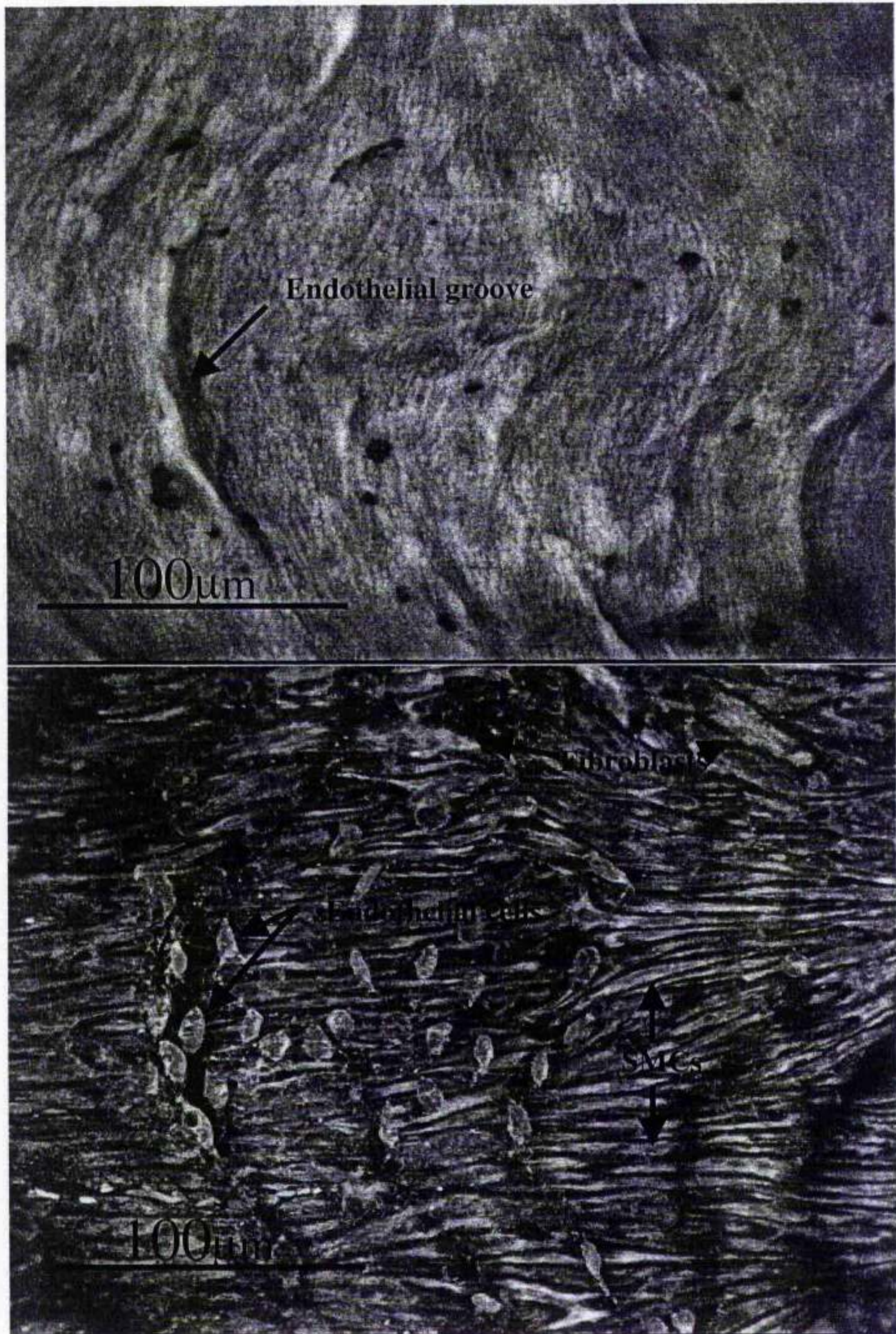


Image 2-3: Black & White view from the intimal side of the 3D Model from Image 2-1. The upper panel shows the layers which constitute the internal elastic lamina while the lower panel shows the layers that lie above and below this. The ovoid forms of their perinuclear staining show the endothelial cells lying above the lamina, arranged in its grooves. The elongated smooth muscle cells (SMCs) run at right angles to the grooves and are seen to run in and out of the field around the grooves.

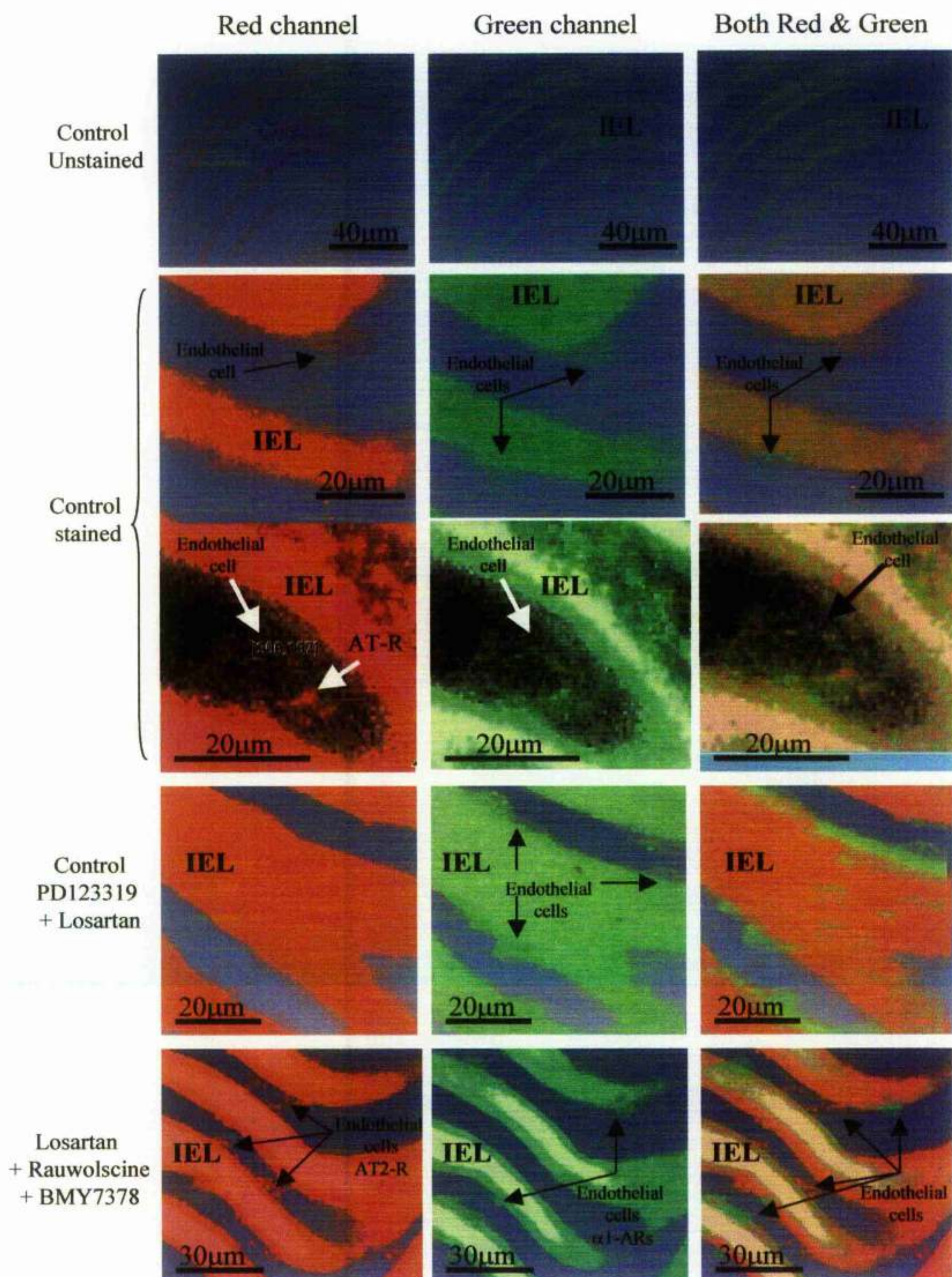


Image 2-4: Comparison of localisation on endothelial cells of fluorescent angiotensin II (red) and a fluorescent prazosin analogue (QAPB, green) in single sections. Dense waves of fluorescence are autofluorescence of the ridges of elastic lamina immediately under the endothelium. Binding of angiotensin can be blocked by a combination of AT receptor antagonists (second bottom row). The "mosaic" nature of the endothelium is shown up by eliminating the AT1 receptors and the major α-Adrenoceptors (bottom row): some cells show only Red and others only green; thus, the AT2 and minor AR (α_{1A}- & α_{1B}-ARs) are not present in every cell. Four Months wild type aorta. Losartan (10µM); Rauwolscine (1µM); BMY7378 (1µM). All except the top row are stained with Rhodamine-Ang II (50nM) for AT-Receptors (Red) and QAPB (0.1µM) for α-Adrenoceptors (Green) (n=6).

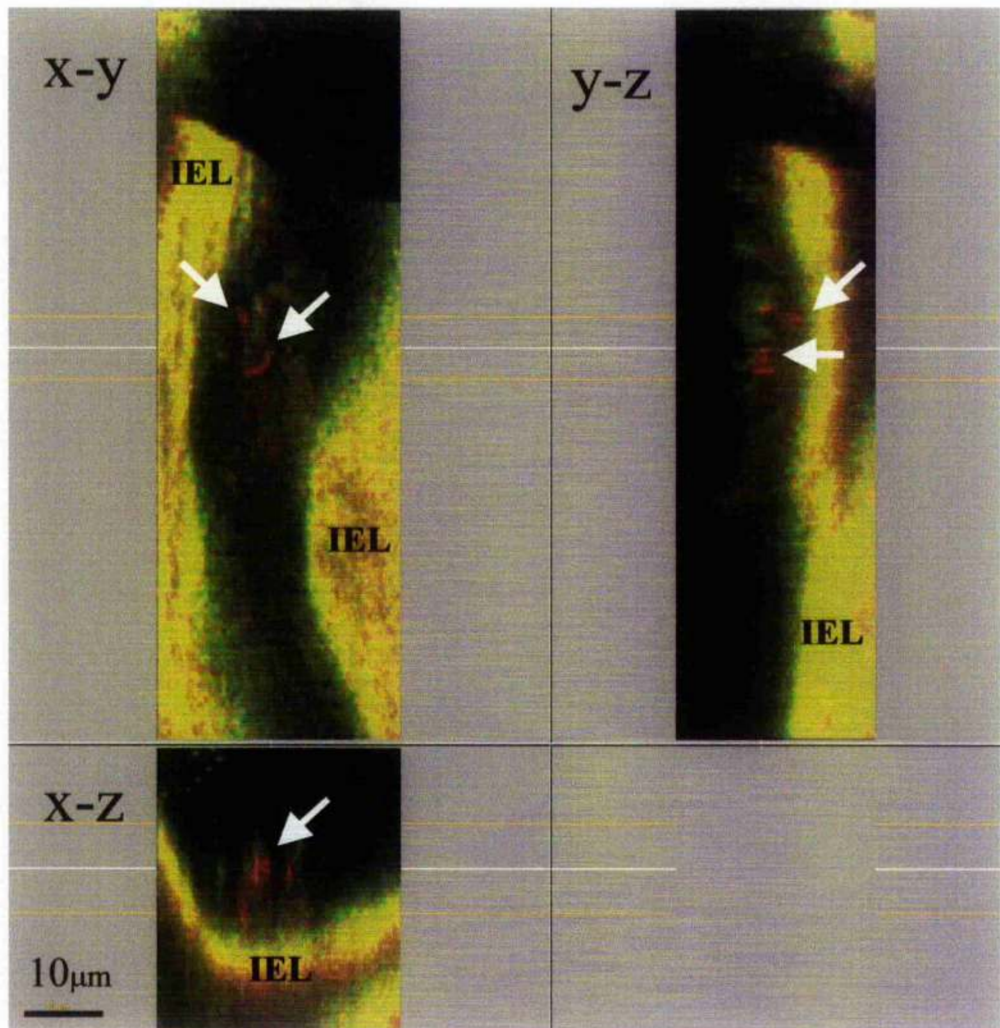


Image 2-5: An individual endothelial cell layered on an Internal Elastic Lamina groove: this shows different localisation of receptor types. 3 different 2D views are shown of a 3D volume. Tissue was treated with BMY7378 (0.1μM) and Losartan (10μM) before staining by Rhodamine-Ang II-Human (0.1μM) and QAPB (0.1μM). LSCM-Imaris analysis, on four months wild type (n=6).

The Green colour granules reveal **α 1-AR** *(*α_{1A} **&** α_{1B}*)* subtypes or **α 2-ARs** and Red granules illustrate **AT_2-Receptors** inside the cell, around the nucleus. (arrows).

Laser power: 50%, Iris: 1.5, Gain: 15, step: 0.5 μm, Speed: 500 imp, Objective: 40X Oil, Numerical Aperture: 1, Pixel * Lines: 512 X 512 (1μm = 1.77 Pixel)

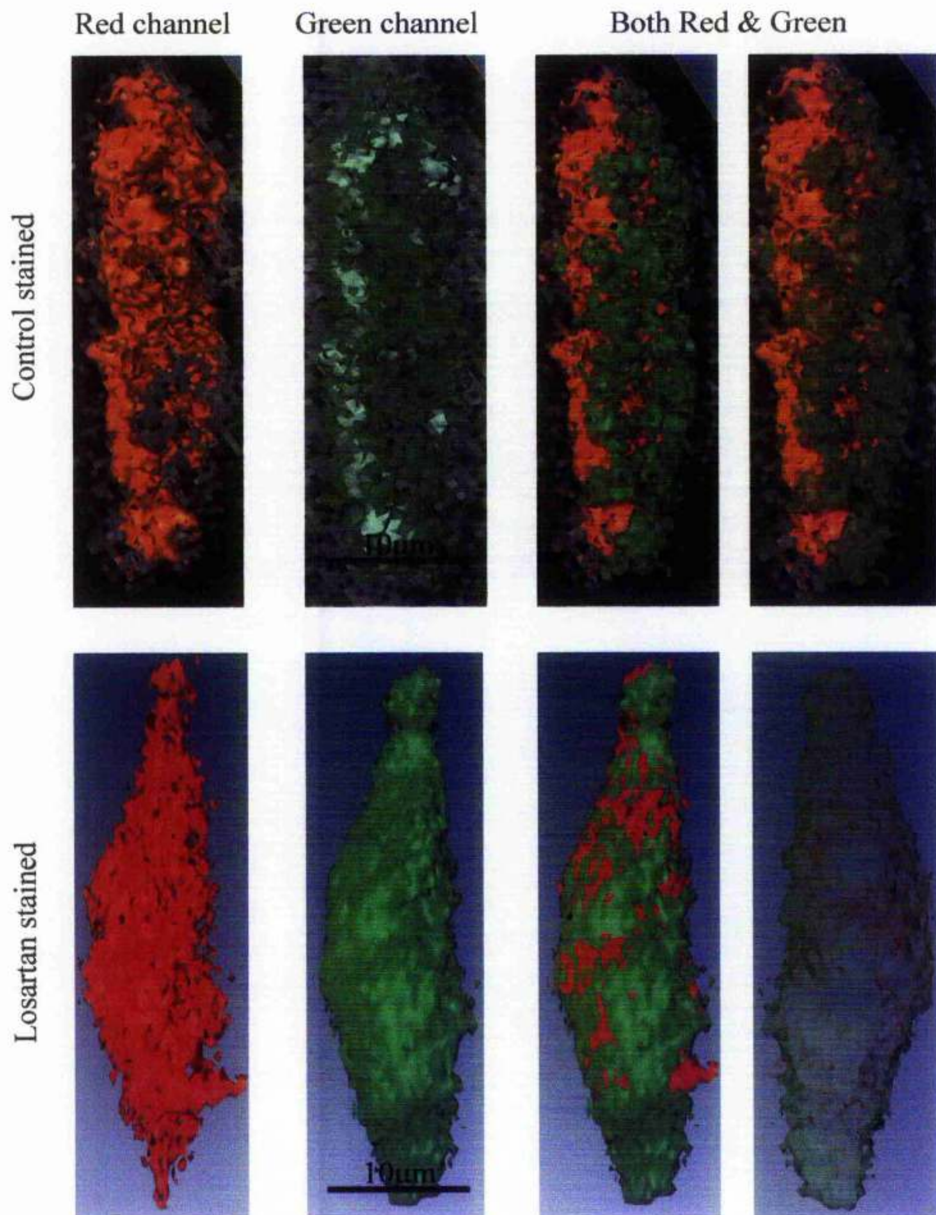
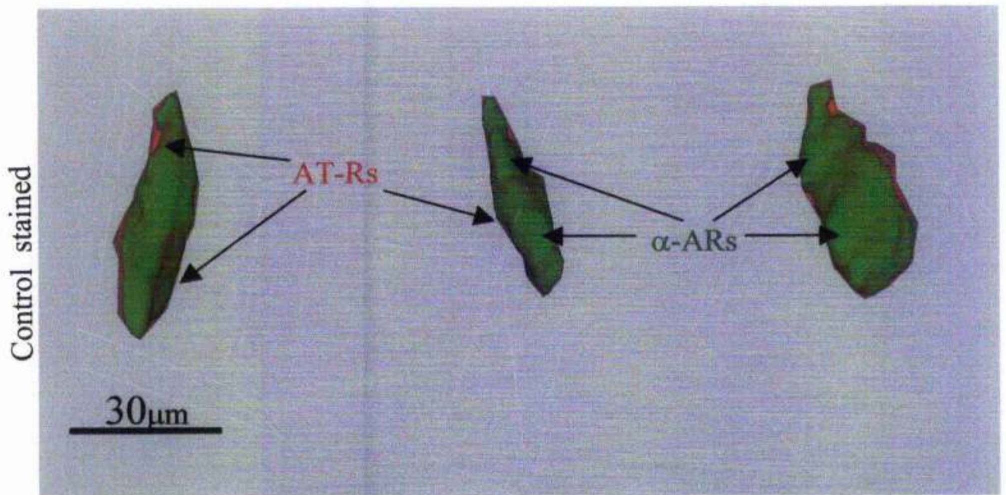


Image 2-6: When AT1 receptors are eliminated, AT2 receptors are seen to be intracellular. Individual smooth muscle cell, dissociated from four months wild type aorta, stained with QAPB (0.1µM, green) and Rhodamine-Ang II-H (0.1µM, red) . LSCM-"Amira" view (n=5).

First row: Without other drugs, the two ligands are seen to be located in similar regions of the cell.

Second row: The same source of smooth muscle cells treated with Losartan (10µM) for 30 minutes and then stained in the same way as control. When the two channels are viewed together the frequency of AT-Receptors is reduced on the cell surface and limited to subcellular levels. This shows that AT_2-R are located intracellularly.



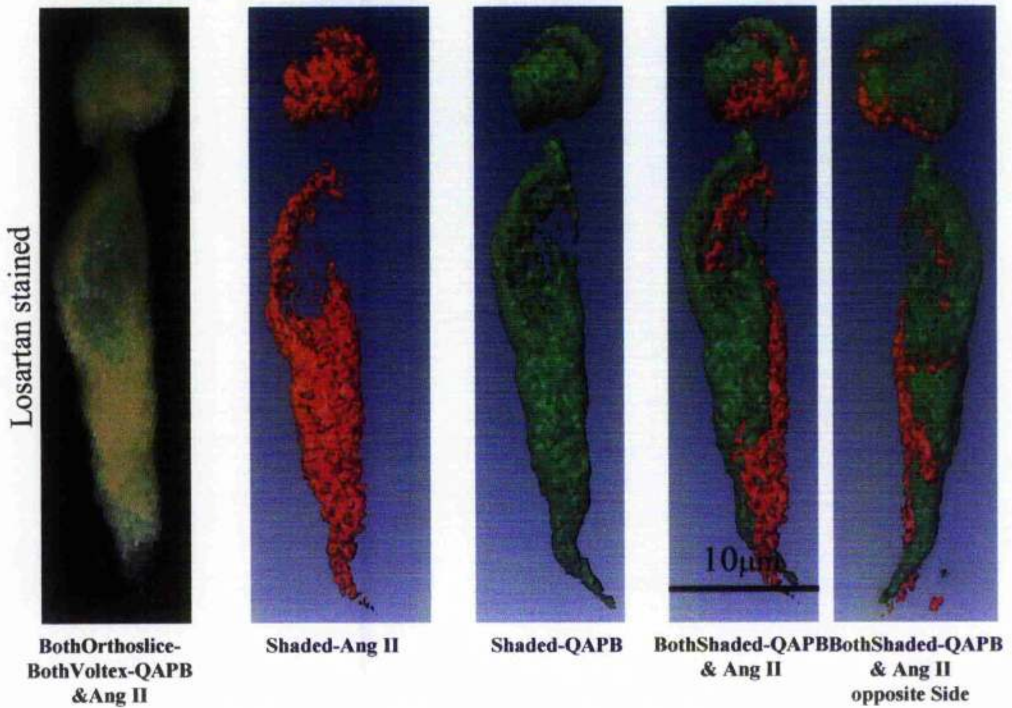


Image 2-7: A different view, showing surface location of AT1 and intracellular location of AT2. "Imaris" software. Dissociated smooth muscle cells treated as in Image 2-6. Green-QAPB (0.2μM) and Red-Rhodamine-Ang II-H (50nM) (n=5). **First row**: This images illustrates that a majority of Angiotensin II-Receptors are present on the cell membrane.

Second row: Losartan (10μM) treated SMCs from the same source show that the membrane compartment containing Angiotensin II-Receptors is reduced with Losartan treatment. This suggests that a majority of cell surface Angiotensin II-Receptors are AT_1-R and implies that the AT_2-R are dominantly localised inside the SMCs, particularly around the nucleus.

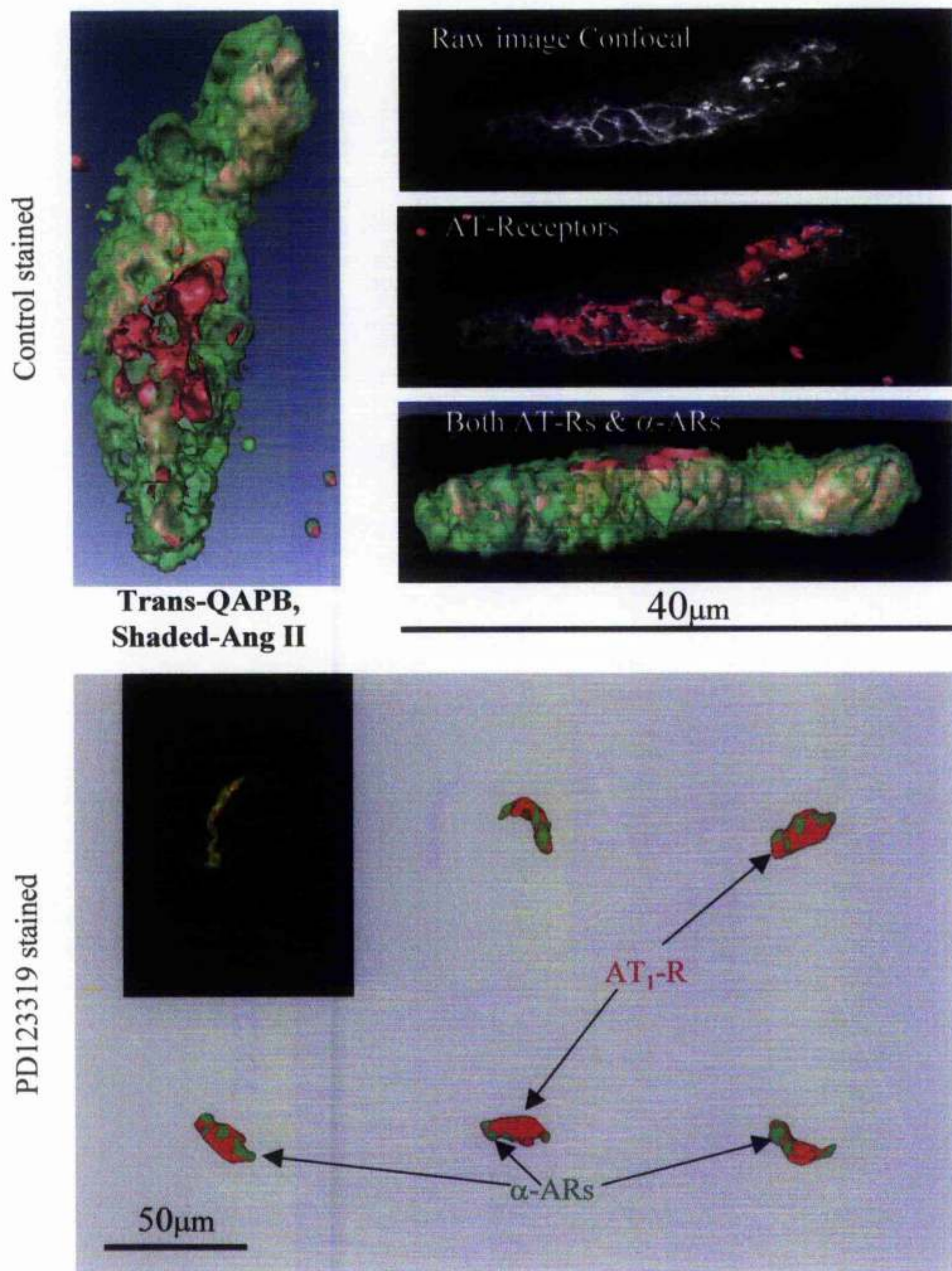


Image 2-8: Further comparison of receptor location. Lower panel shows the predominantly surface location of AT1 receptors when AT2 binding is blocked. Four months D79N Smooth Muscle Cell (SMCs) aorta was stained with Rho-Ang II-H (50nM) and QAPB (0.2μM) (n=5).

Upper collection: Control- A majority of AT-Receptors (Red) are located around the convoluted nucleus with some present on the surface. However, α-ARs (Green) are dominant on cell membrane.

Lower collection: The same source of SMCs treated with PD123319 (10μM) and then stained in the same way as control. AT_1-Receptors are dominant in cell membrane. However parts of the surface show α-ARs.

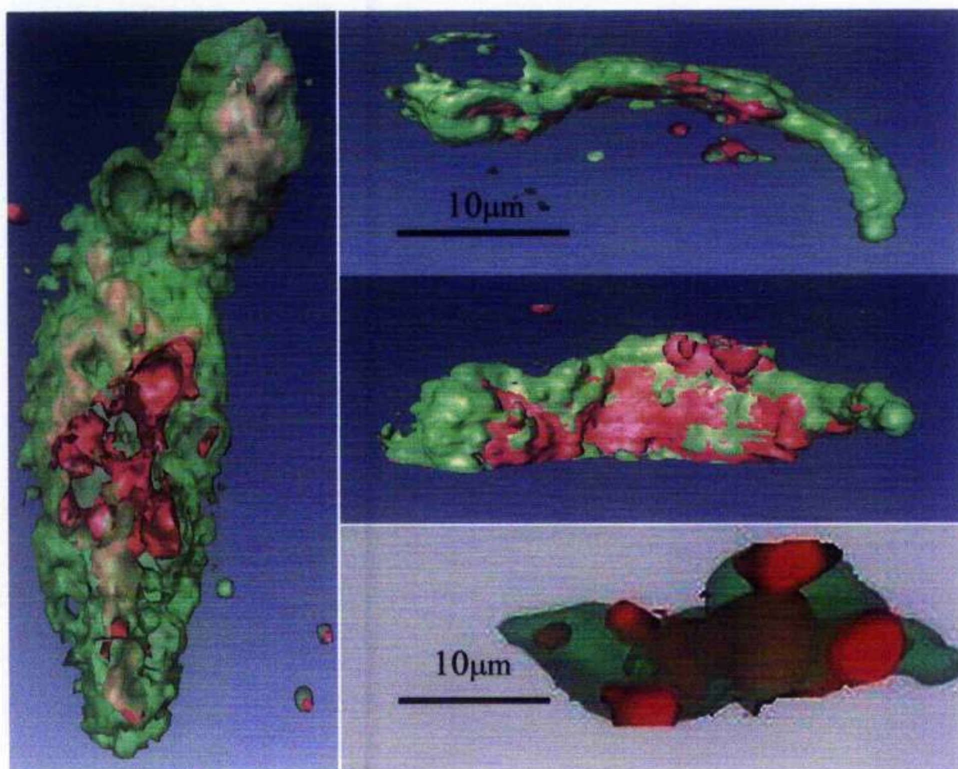


Image 2-9: Further demonstration of surface location of AT1. Dissociated cells from four months D79N mouse SMC aorta stained with Rho-Ang II-H (50nM) and QAPB (0.2µM). AT-Receptors stained Red and α-ARs stained Green (n=5).

Vertical image: Control

Horizontal images (Above and Middle): Treated with PD123319 (10µM) 30 minutes before staining. Red colour localised AT_1-Receptors.

Horizontal image (Lower): Treated with Losartan (10µM) 30 minutes before staining. Red colour localised AT_2-Receptors.

Both Green and Red channels **Green channel only** **Red channel only**

Image 2-10: Comparison of AT and AR location. Four months D79N mouse aorta endothelial cell treated by Losartan (10µM) and BMY7378 (0.1µM) for 1hr then stained with Rho-Ang II-Human (50nM) and QAPB (0.1µM). Green colour shows presence of α_{1A}-AR or α_{1B}-AR and red colour AT_2-R. AT_2-Receptors mostly localised inside the cell and adrenoceptors are dominant on the cell membrane (n=5).

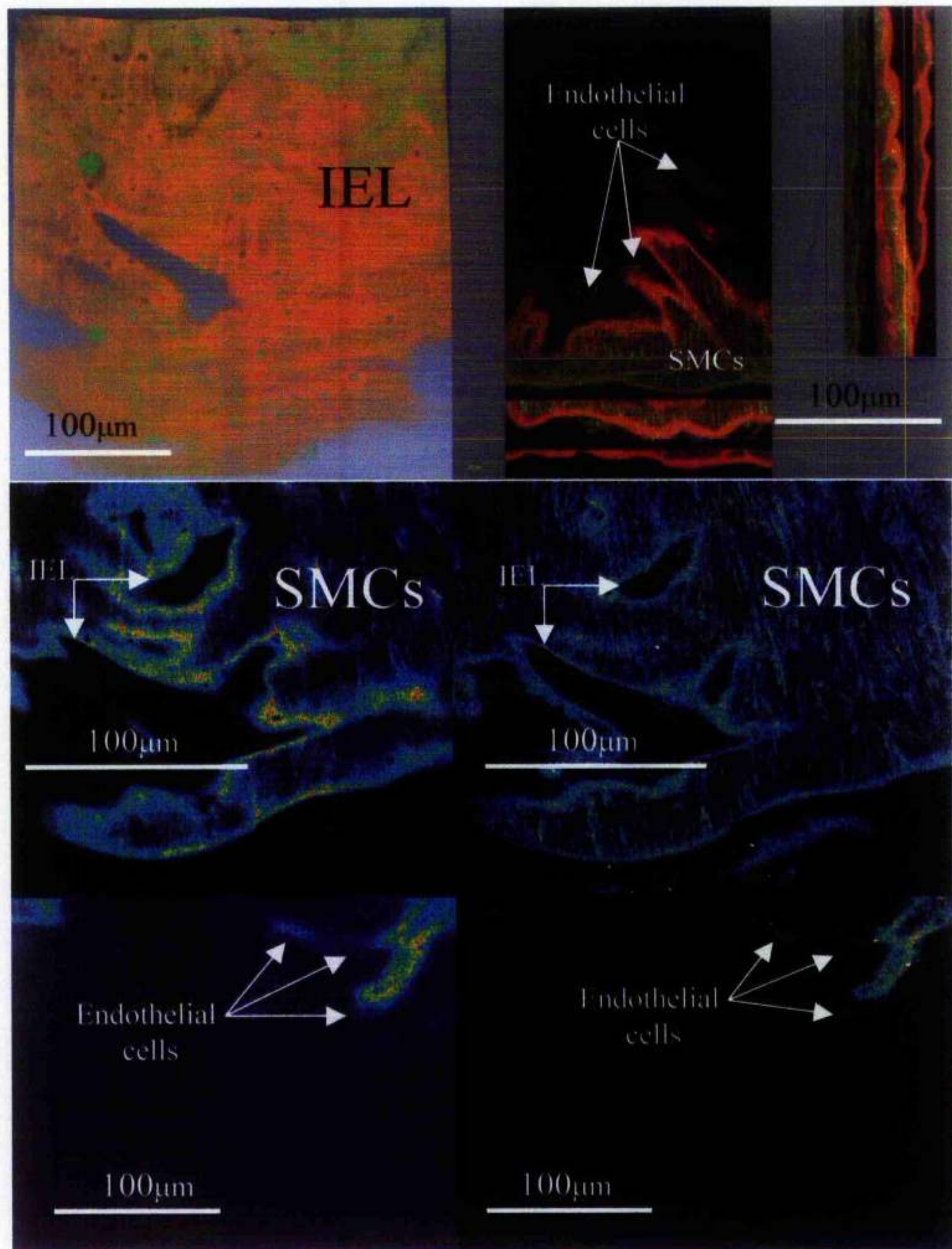


Image 2-11: Localisation of AT and AR on superior mesenteric artery. Control, four months wild type mouse. Rho-Ang II-H (50nM) and QAPB (0.1μM) (n=5).

Above-left-Amira: Both Voltex, Internal Elastic Lamina (IEL)

Above-right-Imaris: Both Channels, SMCs and endothelial cells

Below both sides–Metamorph: Two raw-pseuodocolour images from Ang II (Left) and QAPB (Right), SMCs and Endothelial Cells.

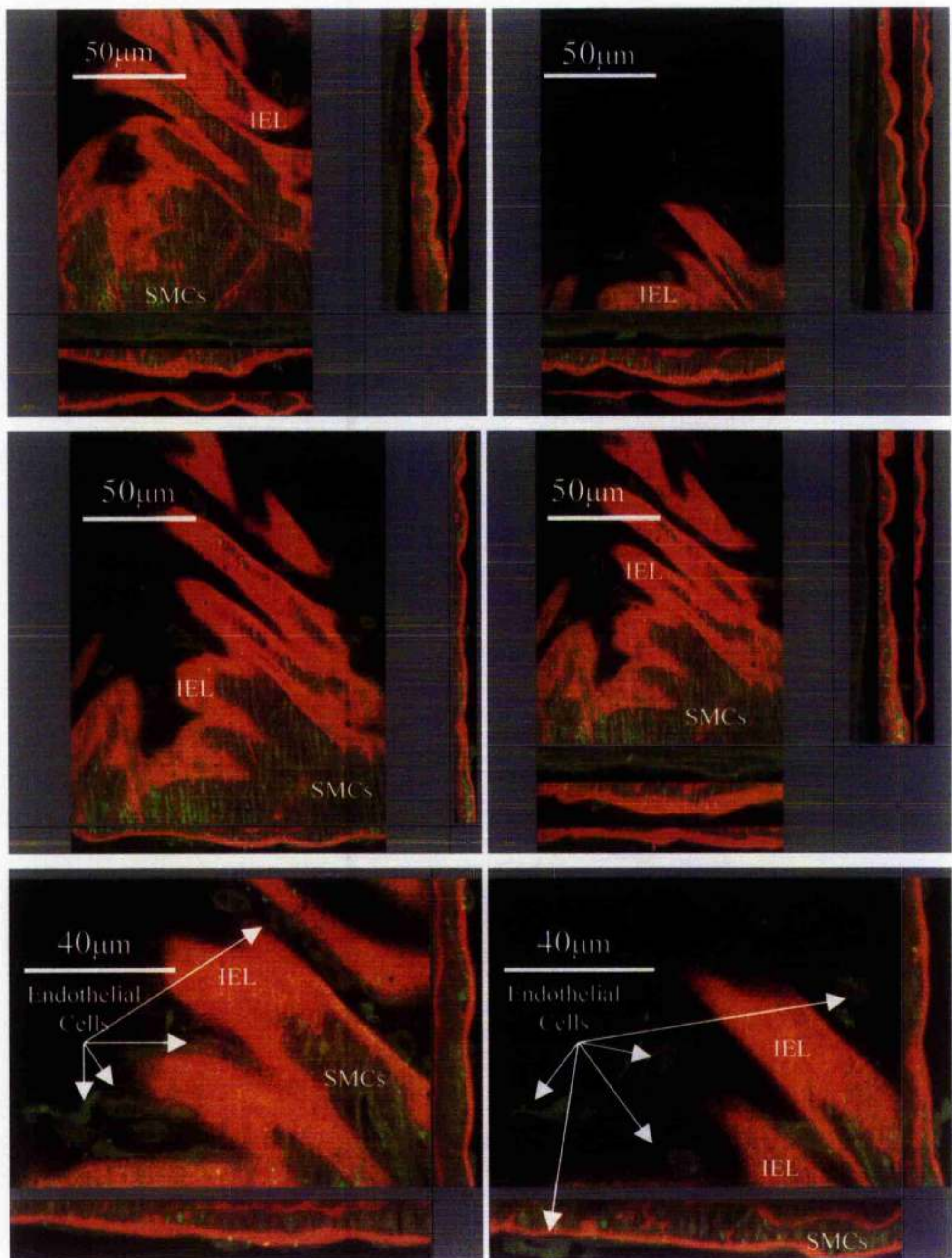


Image 2-12: Further localisation showing endothelial cells. Control-four months wild type, Imaris analysis mouse superior mesenteric artery stained with Rho-Ang II-H (50nM) and QAPB (0.1μM). Red for Ang II-Receptors and Green for α-ARs (n=5).

Laser power: 50%, Iris: 1.5, Gain: 15, step: 0.5 μm, Speed: 500 imp, Objective: 40X Oil, Numerical Aperture: 1, Pixel * Lines: 512 X 512 (1μm = 1.77 Pixel).

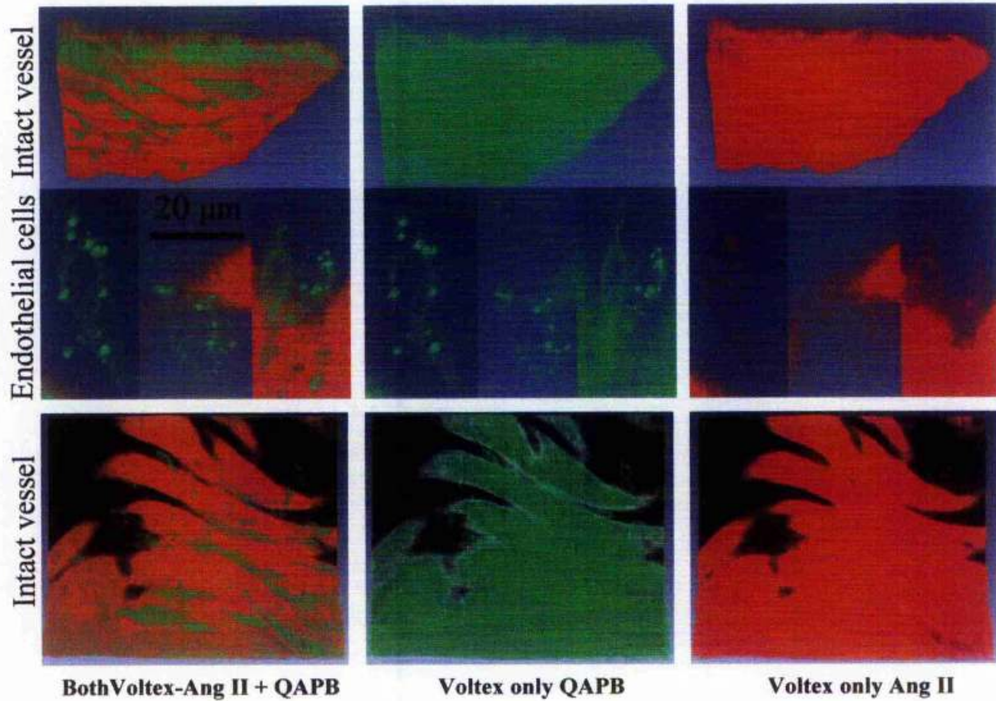


Image 2-13: Different views of image 2-12. Presence of Angiotensin II-Receptors and adrenoceptors is visible in both smooth muscle and endothelial cells in all the images but adrenoceptors are dominant (n=5).

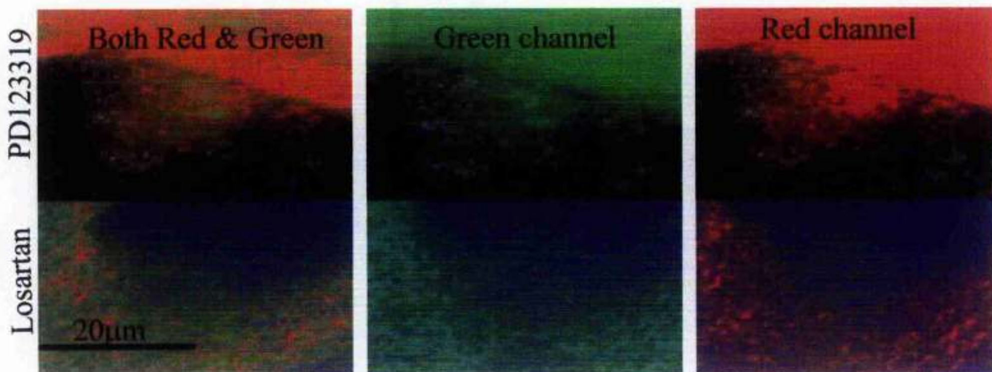


Image 2-14: Comparison of fluo angiotensin II binding to AT1 or AT2 receptors. Four months wild type mouse superior mesenteric artery, stained by Rho-Ang II-H (50nM) and QAPB (0.1µM) (n=5).

First row: AT_1-Receptors are shown after 1hr with PD123319 (10µM).

Second row: AT_2-Receptors are shown after 1hr with Losartan (10µM). The images compare location of AT_1 and AT_2-Receptors in the endothelial cells. AT_1-Receptor is predominantly situated on cell membrane, and AT_2-Receptor concentrated around the endothelial nucleus surrounded by adrenoceptors.

2-5. Discussion:

The AT responses were amenable to straightforward classical pharmacology. There was contraction of vascular smooth muscle via losartan-sensitive AT_1 receptors and indirect relaxation via NO released by endothelial, PD123319-sensitive AT_2 receptors. This confirms the general interpretation of Tanaka et al. (1999) using an AT_2 knockout mouse that loss of relaxation in the KO shows this subtype to be responsible for that action. However we do not have a sufficiently detailed analysis to say whether the AT_1 response was upregulated. Superficially, and taking into account our observations with noradrenaline, it would appear that the loss of AT_2 relaxation was sufficient to explain the larger AT_1-mediated contraction. Our data also shows that the standard AT subtype-selective antagonists distinguish the receptors well in this simple mouse preparation.

In conducting arteries and large veins from other species the contractile responses via α-AR and AT receptors show considerable synergism (Dunn *et al*, 1991). Establishing this in mouse vessels to enable further investigation of this phenomenon was one of our initial objectives. Both receptors, on their own, invoked weak contractile responses, as in vessels showing synergism. However, it soon emerged that the characteristic interaction was not synergism but negative interaction between excitatory and inhibitory effects. This showed up clearly in the interaction between noradrenaline and angiotensin II. Despite the potential for involvement of up to eleven receptors, the dominant interaction was between the noradrenaline's contractile response via α_{1D}-AR and angiotensin relaxant effect via endothelial AT_2. This is quite different from the synergism characteristically seen between contractile α_2-AR (of unknown subtype) and contractile AT_1. However we found no evidence of a contractile α_2-AR in mouse aorta. The contractile effect of UK14304 that is uncovered by blocking the endothelial α_2-AR-

mediated response has been shown, in a parallel study, to be mediated by α_{1D}-AR (See chapter three). The response was absent in the α_{1D}-AR KO mouse (Fig 3-2 & 3-3, see chapter three) and antagonised by selective α_{1D}-AR antagonist, BMY6378 (Fig 4-6 and 4-7) (Shafaroudi et al, 2002). Presence of AT_2 in small resistance arteries like rat first order mesenteric arteries was reported which could provide relaxation response in presence of losartan which was sensitive to bradykinine 2-Receptor blocker. So a proportion of relaxant effect of Ang II was due bradykinine-2 receptor in rat first branch mesenteric artery (Berthiaume *et al.,* 1997) which opposed by $\alpha_{1\ A/C}$ –AR contractile effect (Rokosh *et al.,* 2002) (Yamamoto *et al.,* 2001) However, angiotensin II could stimulate relaxation in absence of losartan up to 13% in mouse first branches of mesenteric arteries. This leads to rather obvious conclusion that the interaction between angiotensin and catecholamines will depend on the dominant receptor populations in the particular blood vessel. However, the dominant influence of the AT_2 endothelium-mediated response in mouse aorta and first branch mesenteric artery is new and unexpected.

Having failed to find a significant synergism between excitatory effects of adrenoceptors and angiotensin receptors we sought synergism between their inhibitory actions. Despite attempting a wide range of protocols including the one for which we present data this proved completely negative.

In conclusion, mouse aortic and endothelium has both smooth muscle AT_1 receptors that initiate contraction and AT_2 that promote the release of nitric oxide, detectable as smooth muscle relaxation. This provides a useful system for analysis of these receptors. The dominant catecholamine-angiotensin interaction is between contractile α_{1D}-AR and relaxant AT_2.

For mouse mesenteric arteries the dominant catecholamine-angiotensin interaction is between contractile α_{1A}-AR and relaxant AT_2.

These results have shown stronger presence of AT_2 receptor in small resistance arteries compared with large conductive arteries like aorta, carotid and superior mesenteric arteries. This, may make smaller arteries more susceptible for relaxation to angiotensin II and noradrenaline due to direct effect on endothelium AT_2 and α_2-AR stimulation respectively or indirect effect on pre-synaptic α_2-AR which is responsible for re-uptake of NA (See chapter three).

Chapter Three

Effect of UK14304 on different mouse arteries

Endothelial $\alpha_{2A/D}$-adrenoceptor-mediated vasodilatation in mouse aorta and carotid is lost in two $\alpha_{2A/D}$-adrenoceptor "knockout" models.

In superior and first branch mesenteric arteries an unknown α_2-AR is still responsible for relaxation via endothelium through nitric oxide release.

In first branch mesenteric artery (resistance and distributing arteries) Endothelium Derived Hyperpolarising Factor (EDHF) may also be involved in the relaxation response. Since, L-NAME could not block the relaxant effect of UK14304 completely.

3-1. Abstract:

1. UK14304-mediated vasodilator responses were studied on wire myograph-mounted mouse aorta, carotid, and mesenteric (main and first branch) arteries with a view to determining sites, mechanisms of action and subtypes of α_2 adrenoceptors (AR).

2. In all four arteries, in the presence of induced tone, UK14304 produced a concentration-related vasodilatation. This was abolished by L-NAME or endothelium removal and was reversed by rauwolscine, indicating that endothelial α_2 adrenoceptors can release nitric oxide.

3. In the $\alpha_{2A/D}$-adrenoceptor Knockout mouse and the Nashville D79N mouse, a functional knockout of the $\alpha_{2A/D}$-adrenoceptor, these relaxant effects of UK14304 were lost in aorta and carotid but remained in the two mesenteric arteries. This indicates the involvement of the $\alpha_{2A/D}$-adrenoceptor in aorta and carotid but of another subtype of α_2-adrenoceptor in the mesenteric arteries.

4. UK14304 could also contract aorta: a small contraction occurred at high concentrations, was enhanced by L-NAME and was absent in the α_{1D}-adrenoceptor knockout mouse indicating activation of the α_{1D}-adrenoceptor. There was no evidence in any of these four arteries of a contractile α_2-adrenoceptor-mediated response.

5. The visualisation on aortic endothelial cells of rauwolscine-sensitive binding of a fluorescent ligand, QAPB, provided direct evidence for the presence of α_2-adrenoceptors as well as α_{1B}-AR on the endothelium.

6. Reduction of QAPB binding by antagonists (rauwolscine and /or BMY7378) provided evidence for the presence of rauwolscine-sensitive α_2-adrenoceptors as well as BMY7378-senstive α_{1D}-adrenoceptors on wild type aortic and mesenteric artery

endothelial cells. In the presence of rauwolscine, 5MU and BMY7378 fluorescence in endothelial cells was still detectable in control mice. Under similar conditions in the α_{1B}-Knockout mouse no endothelial cells were detectable suggesting that the QAPB-binding in control mice under antagonists (rauwolscine + 5MU and BMY7378) was α_{1B}-AR in both of aorta and superior mesenteric artery. Comparing α_{2A}-Knockout and control images, a relatively good population of fluorescent cells after antagonists, also suggests the over-expression of α_{1B}-AR in α_{2A}-Knockout mouse. In aorta using Rhodamine-Ang II-H and QAPB revealed mosaicism in aorta endothelial cells.

7. In conclusion, the endothelium of mouse major conducting arteries has a $\alpha_{2A/D}$-adrenoceptor that promotes the release of nitric oxide, detectable as smooth muscle relaxation and which can be directly visualised. In mesenteric arteries responses are similar but another α_2-adrenoceptor is involved. Unexpectedly, evidence for endothelial α_{1D} and α_{1B}-AR was found in visualisation studies.

3-2. Introduction:

α_2-adrenoceptors can mediate vasodilatation through release of endothelium-derived relaxant factors (Cocks & Angus, 1983). Previously this had been demonstrated in a limited range of vessels and species, obscuring its general importance in cardiovascular control. However, recent observations of possible links between α_2-adrenoceptors and cardiovascular disease (Gavras *et al.,* 2001; Brede *et al.,* 2002; Bristow, 2003; Small *et al.,* 2002) make it timely to elucidate the mechanisms underlying vascular α_2-adrenoceptors. We now demonstrate α_2-adrenoceptor-mediated vasodilator responses in a range of mouse arteries. Using a combination of pharmacology, transgenic models and fluorescent ligand binding microscopy we have elucidated the site, mechanism and receptor subtypes involved.

The three α_2-adrenoceptor subtypes (α_{2A}, α_{2B}, α_{2C}) constitute one of the three sub-families (α_1, α_2, β,) of adrenoceptor through which catecholamines exert autonomic control (Bylund *et al.,* 1994). The direct vascular actions of the other two sub-families, β-adrenoceptors and α_1-adrenoceptors, have already been analysed in a range of mouse arteries (Chruscinski *et al.,* 2001; Daly *et al.,* 2002). The objectives of the present study were: first to establish the vasodilator phenotypes for α_2-adrenoceptors in the mouse, secondly to identify whether the $\alpha_{2A/D}$-adrenoceptor subtype is involved in either response by examining the mouse harbouring a "knockout" of this receptor (Altman *et al.,* 1999) and the Nashville D79N mouse, a functional knockout of the $\alpha_{2A/D}$-adrenoceptor (MacMillan *et al.,* 1996, 1998), and finally, to establish the involvement of the endothelium and nitric oxide.

α_2-adrenoceptors have several distinct roles in the control of the vascular system. They were first shown to modify the influence of the sympathetic nervous system on the

cardiovascular system by reducing sympathetic traffic through an effect in the central nervous system or by inhibiting the release of transmitters from sympathetic post-ganglionic nerve varicosities (Langer, 1981; Starke 2001). They were subsequently found to have two separate direct effects on blood vessels that can modify vascular tone: a vasopressor action (Docherty & McGrath, 1980) through activation of contraction of vascular smooth muscle (Demey & Vanhoutte, 1981; Wilson *et al.*, 1991) and vasodilatation through release of endothelium-derived relaxant factors (Cocks & Angus, 1983).

Distinguishing which of the three cloned subtypes of α_2-adrenoceptor is responsible for each of these actions has proven difficult by classical pharmacological methods due to lack of specificity of the relevant agonists and antagonists and the possible activation of more than one receptor in a particular test preparation. Targeted deletion or mutation of receptors might move this forward but has been applied, so far, mainly in *in vivo* experiments that cannot distinguish between indirect inhibition of the sympathetic nervous system and direct dilator effects on the blood vessels.

In mouse strains harbouring knockouts or mutations of the three α_2-adrenoceptors, blood pressure responses to α_2-adrenoceptor agonists such as clonidine, suggest that $\alpha_{2A/D}$- and α_{2B}-adrenoceptors are involved in vasopressor responses and that $\alpha_{2A/D}$-adrenoceptors mediate a vasodepressor response. The authors concluded that the pressor response involves the direct stimulation of vascular smooth muscle and that the depressor response is due to the effect in the CNS to reduce sympathetic output (Link *et al.*, 1996; MacMillan *et al.*, 1996). However the direct vascular actions of α_2-adrenoceptor activation have not been determined in the mouse. Analysis of the relevant subtypes remains at the level of classical pharmacology in genetically intractable species. This suggests that both responses may be mediated by the $\alpha_{2A/D}$-adrenoceptor, e.g. the endothelial α_2-adrenoceptor-mediated response in pig or rat

arteries (Bockman *et al.,* 1996) and the direct contraction of various veins mainly from rabbit, pig and dog (Guimaraes & Moura, 2001). However, such conclusions are controversial and, when knockout technology has been used in conjunction with pharmacology to understand the other adrenoceptor mechanisms, the full picture has been unexpectedly complex, always involving more than one receptor subtype, e.g. vascular α_1 and α_2-adrenoceptors (Chruscinski *et al.,* 2001), vascular α_{1A}-, α_{1B}- and α_{1D}-adrenoceptors (Daly *et al.,* 2002) or pre-junctional α_{2A}- and α_{2C}-adrenoceptors (Hein *et al.*, 1999).

If the mouse does possess endothelial α_2-adrenoceptors that mediate vasodilatation, this could be at least partly responsible for the "vasodepressor" effect of α_2-adrenoceptor-agonists, such as clonidine, on mouse blood pressure (Link *et al.,* 1996; MacMillan *et al.,* 1996). This aspect of α_2-adrenoceptor action is likely to be of physiological importance. There is evidence in the rat for a physiological role of nitric oxide released by α_2-adrenoceptors in protecting the kidney from excessive adrenergic vasoconstriction (Zou & Cowley, 2000). Such countervailing actions may be of great importance for the protection of specific functions of many vascular beds in the face of a generalised sympathetic activation in "fight or flight". It has also been proposed that α_2-adrenoceptors play an essential role in prevention of heart failure progression, an effect hypothesised to be attributable to pre-synaptic α_2-adrenoceptors (Brede *et al.,* 2002) but which could be attributable to receptors on endothelial or vascular smooth muscle cells.

There are two published studies of vascular α_2-adrenoceptors in mouse. First, in pressurised mouse tail artery antagonist studies suggest that the contractile response is mediated by $\alpha_{2A/D}$-adrenoceptors at 37^0C but additionally by α_{2C}-adrenoceptors at 28^0C (Chotani *et al.,* 2000). Secondly, Vandeputte & Docherty (2002) and Vandeputte et al,

(2003) hypothesised, on the basis of pharmacological analysis and knockouts of $\alpha_{2A/D}$-adrenoceptors and NOS-3, that the overall contractile response to noradrenaline results from the stimulation of multiple adrenoceptor subtypes. They reported that an α_2-adrenoceptor agonist, xylazine, caused vasodilatation that was absent in the NOS-3 KO mouse (Vandeputte *et al.,* 2003) but found that at least part of this response was present despite endothelial denudation (Vandeputte & Docherty, 2002). They proposed that there was a vasorelaxant effect due to activation of $\alpha_{2A/D}$-adrenoceptors, some, but not all, of which are endothelial.

Thus, there is no consensus for the α_2-adrenoceptor(s) responsible for direct vascular actions, constrictor or dilator. Yet both of these responses are potentially of considerable significance for the therapeutic use of α_2-adrenoceptor agonists and antagonists, e.g. in anaesthesia or antihypertensive therapy, and for the role of α_2-adrenoceptors in the physiological control of the cardiovascular system.

In the present study we have employed the major conducting arteries in which α_2-adrenoceptor-mediated vasodilatation and vasoconstriction have been found in other species (Guimareas & Moura, 2001) and use rings of arteries mounted on a wire myograph to ensure minimal damage to the vascular endothelium. Previous studies using strips of mouse aorta have not found evidence for α_2-adrenoceptor-mediated vasodilatation (Russell & Watts 2000; Tanaka *et al.,* 1999). It was the relative fragility of drug-induced relaxant responses in spiral strips that led Furchgott & Zawadski (1980) to discover endothelium-derived relaxant factors. Similarly, in the present study, preserving the endothelium uncovers the α_2-adrenoceptors in mouse arteries.

Four strains of "knockout" mouse were employed to simplify interpretation and reinforce the pharmacological analysis. The involvement of the $\alpha_{2A/D}$-adrenoceptor in vasodilator responses to agonist was analysed using the $\alpha_{2A/D}$-adrenoceptor KO mouse

(Altman *et al.*, 1999) and mice harbouring the dysfunctional D79N mutation of that receptor, which serves as a functional knockout (MacMillan *et al.*, 1996). Aortic contraction, even to "selective" α_{2A}-adrenoceptor agonists, was a potentially confounding factor; we demonstrated that this was via α_{1D}-adrenoceptors by demonstrating the absence of this response in the knockout of this receptor (Tanoue *et al.*, 2002). Finally we set out to make a direct visual demonstration of endothelial α_2-adrenoceptors using a fluorescent ligand. Proof of specificity of binding was complicated by the unexpected demonstration of endothelial α_1-adrenoceptors: this was overcome by using the knockout of the α_{1B}-adrenoceptor (Walkenbach *et al.*, 1992) (Dora *et al.*, 2000) (Cavalli *et al.*, 1997).

3-3. Materials and Method:

a) Myography:

Male mice (aged 4 months) were killed by CO_2 inhalation and the descending thoracic aorta, carotid, main mesenteric and first branch mesenteric were removed, cleaned of connective tissue then dissected into rings (2mm in length). The strains of mice employed were the $\alpha_{2A/D}$-adrenoceptor mutant Nashville D79N mouse (MacMillan *et al.,* 1996, 1998), which had been back-crossed on to C57BL/6; $\alpha_{2A/D}$-adrenoceptor knockout C57 Black mice ($\alpha_{2a/d}$-KO, Jackson Laboratories, Bar Harbour, Maine, U.S.A.) and the α_{1D}-adrenoceptor knockout (Tanoue et al, 2002), which is on a background of 129SV/C57BL6. We have a colony of 129SV/C57BL6 controls (Japanese wild type) for the α_{1D}-adrenoceptor knockout (Japanese Knockout) and have compared several aspects of adrenergic pharmacology between this and the C57BL/6 (Swiss wild type) without finding significant differences. Thus in this study we have used the 129SV/C57BL6 as control.

Endothelium was removed, where appropriate, by rubbing the intimal surface with a human hair. Tissues were then mounted in Kreb's solution (NaCl 118.4mM, KCl 4.7mM, $CaCl_2$ 2.5mM, KH_2PO_4 1.2mM, $MgSO_4$ 1.2mM, $NaHCO_3$ 25mM & glucose 11.1mM bubbled with 95% O_2 5% CO_2 to pH 7.4) at 37°C in a multi-myograph (myo-interface, model 600M and 610M, DMT, Aarhus) using 40μm stainless steel wires (Mulvany & Halpern, 1976, 1977). The data was transferred to a Powerlab (Version 4.2.2 for windows 98) installed on a Pentium three computer. Aortic rings were placed under a resting tension of 1g; Carotid artery 0.333 g; superior (Main) mesenteric artery and first branch mesenteric artery each 0.25g; and left to equilibrate for 30-45 minutes. Reproducible responses were obtained to 5HT (0.1μM), NA (0.1μM), phenylephrine

(0.1μM), or U46619 (10nM), according to protocol, before commencing experiments. Tissues were tested with increasing cumulative concentrations of UK14304 in 0.5 log unit increments over 1nM-30nM.

At the plateau of contraction to 5HT (0.1μM) or noradrenaline (0.1μM), acetylcholine (1μM) was added to assess endothelial integrity. Criteria for functional endothelium was >50% and for denuded endothelium was < 5% relaxation. At the end of each experiment endothelium was re-checked using the same criteria and samples included only if meeting criteria at both times.

All four arteries relaxed to acetylcholine (1μM). In all arteries L-NAME (100μM) could abolish relaxation responses to acetylcholine.

Tissues were washed at 5 minutes intervals following each experimental protocol and given a 60 minutes recovery period. Following the rest period, test drugs were added where appropriate for at least 30 minutes before construction of a second cumulative concentration response curve (CCRC).

b) Visualisation of endothelial α_2-adrenoceptors:

Visualisation of adrenoceptors on endothelial cells in large arteries using fluorescent-ligand microscopy presents technical challenges related to (i) the 3-dimensional nature of the endothelial layer and its close proximity to background fluorescence from elastin in the internal elastic lamina and (ii) the presence of α_1-adrenoceptors that bind the ligand.

The first factor was overcome by the use of 3-dimensional confocal microscopy as summarised in "Results".

The second factor was overcome by a strategy that eliminated the binding to α_1-adrenoceptors. The fluorescent analogue of the "prazosin" family, QAPB has affinity for both α_1- and α_2-adrenoceptors, though with higher affinity for α_1- (Daly *et al*, 1996). We set out to create a protocol in which we could identify fluorescent binding sites on endothelium that could be eliminated by rauwolscine. We wanted to avoid complex image analysis and to enable a simple yes or no decision on whether rauwolscine had been effective. The complications included the expected one that the most obvious binding of the ligand at the arterial endothelial-medial region would be to α_1-adrenoceptors on smooth muscle. We also discovered the unexpected issue that there were α_1-adrenoceptors on endothelium. We do not, however, wish to complicate our current objective by detailing this. For our present analysis we needed simply to eliminate it. After extensive consideration of drug combinations that would eliminate α_1-adrenoceptors while leaving α_2-adrenoceptors we found that a clear-cut situation was produced by employing aortae from α_{1B}-adrenoceptor-KO mice and using selective antagonists for α_{1A}-adrenoceptor (5MU) and α_{1D}-adrenoceptor (BMY7378). In these circumstances we visualised QAPB binding at low concentrations to endothelial cells that could be completely removed, as judged by independent blinded observers. QAPB (FL-Prazosin) had good affinity on both of α_1 and α_2-AR (Fig. 3 -24 and 3-25).

c) Laser Scanning Confocal Microscopy (LSCM):

Tissue preparation:

2-3mm segments of aorta from WT and α_{1B}-Knockout mice were incubated for 60 minutes in either QAPB (1μM) or Rhodamine-Ang II-H (50nM) in the presence or absence of BMY7378 (0.1μM) and /or rauwolscine (0.1-1μM) and /or losartan (10μM)

introduced 30 minutes prior to incubation with the fluo-ligand. Following incubation, without washing, aortic segments were cut open and placed endothelial side up in the sample well of a glass slide. The well containing the tissue and prior incubation media was sealed with a glass coverslip (No. 1.5 for confocal use).

Image capture:

Serial optical sections were collected on a Biorad 1024 & Radiance 2100 confocal laser scanning microscope. The Excitation/Emission parameters used were 488/515nm for QAPB and 567/610nm For Rhodamine-Angiotensin II-Human (Rho-Ang II-H). In all experiments the laser power, gain and offset (contrast and brightness) were kept constant. The distance between optical sections was maintained a 0.3 μm for each image stack. Tissues were visualised using a x40 oil immersion objective on which the numerical aperture is 1.00 and therefore optimal pinhole (Iris) setting is 1.5. Image size was set to 512 x 512 pixels which, equates to a field size of 289μm x 289μm.

Thus, each one micrometer (1μm) was equal to 1.77 pixel (512 ÷ 289 = 1.77).

Image analysis:

3D volumes (image stacks) were transferred to either MetaMorph (Universal Imaging, Version 4.2) or Amira (TGS, Version 3.2) and Imaris (Version 3.2) software packages for subsequent analysis and volume visualisation respectively. 3D volumes containing two channels of data are pseudocoloured green and red for QAPB & Rho-Ang II-H respectively. Where two channels co-localise and their intensities are

roughly equivalent, the co-localised area is displayed in yellow. Particular localisation of fluorescent signals was achieved using orthogonal viewing of the XY, XZ & YZ planes. 3D views were rendered using the Amira or Imaris 'voltex' module.

Drugs:

All drugs were of analytical grade and were dissolved in either distilled water (H_20), ethanol or DMSO as indicated below. Noradrenaline dilution included 23μM EDTA to prevent oxidation.

noradrenaline (H_2O), phenylephrine (H_2O), Acetylcholine chloride (H_2O), 5HT (H_2O), U46619 (ethanol), L-NAME (N-Nitro-L-Arginine Methyl Ester) (H_2O), rauwolscine (H_2O) [Sigma-Aldrich Co; Poole, UK], UK14304 (DMSO) [Pfizer, Sandwich, UK]. BMY 7378 (H_2O) [Sigma-Aldrich Co; Poole, UK], 5MeU (H_2O [Sigma-Aldrich Co; Poole, UK].

Statistics:

Values are means ± Standard error mean from n experiments. Differences between maximal contraction response to CCRC to agonist in presence and absence of drugs were compared with one-way and two way ANOVA followed by Bonferroni's post test and two-tailed unpaired and paired t-test. Statistical and graphical analysis was carried out using Excel 97 and GraphPad Prism 3.00 for PC. Data used to plot the concentration response curves are the mean contractions induced at each concentration of the agonist.

3-4. Results:

Sub-maximal contraction provided a basis for observing contractile or relaxant effects:

In aorta rings, in the absence of other drugs, or of induced tone, UK14304 produced either no response or weak contractions (aorta only) as previously described by Russell & Watts (2000) in aortic strips (Figure 3-1.). If the tone was raised submaximally with 5HT or U46619, in wild type mouse aorta, UK14304 produced a concentration-dependent relaxation of pre-constricted tone (Fig. 3-2 and 3-3). In aorta the contractile responses to high concentrations also became more apparent (Fig. 3-2, 3-3). Under similar conditions of raised tone the other three wild type arteries (Carotid and mesenteric arteries) showed only relaxation to UK14304 (Fig. 3-10, 3-12, 3-14, 3-16). This was of similar magnitude to other standard vasodilators but the concentration response curve had a shallower slope, e.g. carotid in (Fig. 3-10).

In endothelium-denuded rings, acetylcholine and UK14304 produced no relaxation and, indeed, produced contraction due to direct activation of vascular smooth muscle, as found in other species (results not shown).

Mechanism of action of UK14304:

Biphasic responses in aorta:

In aorta, the concentration/response curve was biphasic: the relaxation was at first concentration-related but reached a "Maximal" relaxation of 23% at 0.3 μM UK14304 (Figures 3-2, 3-3, 3-4), higher concentrations producing, progressively, a smaller relaxation then a contraction to beyond the initial pre-constricted tone (Figure 3-2, 3-3).

This might have been due to desensitisation but subsequent experiments showed that it was due to the onset of a countervailing contractile effect.

In aorta of $\alpha_{2A/D}$-KO and Nashville D79N mice, UK14304 caused only contraction (Figure 3-2, 3-4, 3-6 and 3-7). This indicates that the vasodilatation is due to activation of $\alpha_{2A/D}$-adrenoceptors and also that the upswing in the curve in the control mice is due to the onset of a contractile response rather than simply desensitisation. This contractile response of UK14304 was absent in the α_{1D}-adrenoceptor knockout mouse aorta indicating that the contractile effect is attributable to activation of the α_{1D}-adrenoceptor (Figure 3-2, 3-3, 3-4, 3-20, 3-21). Cumulative responses to UK14304 in D79N mouse aorta, carotid and superior (main) mesenteric arteries were summarised in figure 3-23.

The involvement of nitric oxide in the relaxant response was demonstrated in aorta by employing a single exposure (non-cumulative) to a moderately high concentration of UK14304 (1μM). This produced a small relaxation (~8%) that was converted to a contraction by L-NAME (0.1mM). In D79N aorta the same concentration of UK14304 produced a contractile response that was not significantly affected by L-NAME (Figure 3-9). This indicates that the $\alpha_{2A/D}$-adrenoceptor subtype is necessary for the production of nitric oxide by UK14304. The source of nitric oxide was identified as endothelium since UK14304 did not produce relaxation after endothelial denudation (Fig. 3-3). Rauwolscine 0.1μM produced a reversal of relaxation providing pharmacological proof that relaxation is mediated by α_2-adrenoceptors (Fig. 3-5, 3-6, 3-8 and 3-18 – traces from Powerlab).

Relaxant responses in carotid artery:

In carotid arteries the relaxant effects of UK14304 were antagonised by rauwolscine, were abolished by L-NAME and, as in the aorta, were absent in the $\alpha_{2A/D}$-KO and D79N mice (Figs. 3-10, 3-11). In the $\alpha_{2A/D}$-KO mouse, UK14304 produced a weak contraction.

Relaxant responses in mesenteric arteries:

UK14304 produced relaxation of preconstricted superior (main) mesenteric arteries and their first branch. In each case the response was susceptible to L-NAME and rauwolscine (Figs. 3-12, 3-13, 3-14, 3-15, 3-16, 3-17, 3-18) however, relaxant effect of UK14304 still present in the $\alpha_{2A/D}$-KO and D79N superior mesenteric arteries (Fig. 3-12). In superior mesenteric artery, comparison between the relaxation response to a single concentration of UK14304 (1μM) and maximum response to UK14304 cumulatively, revealed desensitisation in the cumulative protocol that reduced the maximum relaxant effect up to 45% (Fig. 3-15).

The relaxation response to single concentration of UK14304 in D79N was smaller than WT in superior mesenteric artery (n=7) (Fig. 3-19).

After damaging the endothelium the vasodilator response to UK14304 was replaced by a small concentration-related contraction in first branch mesenteric artery (Fig. 3-16). In first branch mesenteric artery, L-NAME could not remove 100% of UK14304 relaxant effect. However, endothelial damaging revealed a small contraction to cumulative concentrations of UK14304. This phenomenon may due to the presence of another

mechanism for relaxation via endothelial cells, which remains effective when nitric oxide generation is blocked by L-NAME. This additional relaxation mechanism in small resistance distributing arteries like mesenteric branches may make them more susceptible for relaxation rather than contraction (Fig. 2-14, 3-16 and 3-22) (see chapter two).

Visualisation of endothelial α_2-adrenoceptors: value of confocal

We obtained images in circumstances that eliminated binding to α_1-adrenoceptors to allow us to reveal the α_2- adrenoceptors of the endothelium.

Visualisation of adrenoceptors on endothelial cells using fluorescent-ligand microscopy presents technical challenges. The cells form a monolayer, on a convoluted surface caused by folds in the internal elastic lamina. The central parts of the cell containing most of their material are also well spaced out. There is thus no single two dimensional plane with a high probability of locating cells. This is addressed by using Laser Scanning Confocal Microscopy (LSCM), allowing scanning in the z direction to locate cells. The images were obtained from the endothelial side of vessels opened longitudinally. The convolutions remain, however, so that the typical 2D image contains endothelium, the edges of the internal elastic lamina and smooth muscle cells located beyond the lamina (images 3-3, 3-4 and 3-5). The elastin of the lamina is autofluorescent over a broad range of wavelengths, so cannot be avoided. With this in mind, the endothelial cells binding QAPB can be seen attached to the folds of the Internal Elastic Lamina (IEL). Pharmacological proof that these were α_2-adrenoceptors was then obtained by eliminating the fluorescence with rauwolscine.

Visualisation overview:

Chapters 2 and 3 of this thesis are, respectively, concerned with visualising angiotensin (AT) receptors and α_2-adrenoceptors. In each of these studies, in the control situation in the absence of antagonist drugs, both the fluorescent angiotensin and the fluorescent analogue of prazosin were capable of binding to every smooth muscle and endothelial cell. Thus, when selective antagonists were employed to determine the subtypes of each of the two receptors families by preventing binding, the ligand for the other family could be used to identify cells that would, otherwise, have been invisible.

Thus, in this Chapter, where the main target was receptors binding QAPB (Fluorescent Prazosin) (Fig. 3-24 and 3-25), some initial studies employed this together with Rhodamine-Angiotensin II-Human (Rho-Ang II-H). Wild type mouse aorta showed the presence of α-AR and angiotensin II receptors (AT-R) in both smooth muscle and endothelial cells (images 3-1, 3-2, 3-3, 3-5). This showed some interesting differences between the binding when circumstances were created to leave certain combinations of receptors. For example, when losartan blocked AT_1 receptors the predominant intracellular location of AT_2 receptors became apparent and this was shown up by the presence of QAPB binding on the cell surface (image 3-1, 3-3). Even more strikingly, on removal of the major binding sites for QAPB, α_2-AR and α_{1D}-AR, to reveal the minor α_{1A}-AR and α_{1B}-AR binding sites, it was found that these were present on only a minority of cells (as opposed to the ubiquitous presence of AT_2 receptors shown simultaneously – in the presence of losartan). This reveals a *mosaicism* among the endothelial cells.

Wild type mouse aorta

At the outset, we expected to find endothelial binding that was susceptible to rauwolscine and, thus classifiable as α_2-AR. We found good endothelial binding with QAPB alone (image 3-2). We employed BMY7378 to eliminate the major smooth muscle binding site (α_{1D}-AR) and found that endothelial binding was, as expected, still present (image 3-3). However, binding was still present in the presence of rauwolscine (images 3.6 and 3-7) leading us to hypothesis either that our antagonist regime was not as selective as we had expected or that there were α_{1A}-AR or α_{1B}-AR present on endothelium, for which the literature had not prepared us. Since we had no reliable α_{1B}-AR antagonist available we decided to pursue the concept that α_{1B}-AR might be present by employing the α_{1B}-KO mouse, since we did have access to α_{1A}-AR-selective antagonists.

Aorta of α_{1B}-AR Knockout

The aorta of the α_{1B}-AR Knockout mouse showed similar autofluorescence to controls (image 3-8) and good binding of QAPB to both smooth muscle and endothelium (image 3-9). After elimination of the α_{1D}-AR with BMY7378 good binding remained on endothelium and smooth muscle (image 3-10); the possibilities for binding were now reduced to α_{1A}-AR and α_2-AR. The addition of 5MU to BMY7378 still left binding on both cell types but caused a greater reduction of binding on smooth muscle as would be expected since all α_1-AR have now been eliminated (image 3-11). Rauwolscine on its own reduced endothelial binding to a greater extent than the α_1-AR antagonists providing a strong indication for the expected α_2-AR (image 3-11). Finally, the combination of the α_{1A}-AR and α_{1D}-AR antagonists with rauwolscine eliminated all endothelial binding (image 3-13) confirming that when all the α_1-AR subtypes are taken

out of consideration the endothelial binding of QAPB is rauwolscine-sensitive and, therefore, can be characterised as α_2-AR. This series of observations is summarised, using a 3D view in image 3-14.

Having accomplished this inadvertent diversion into the demonstration of endothelial α_{1B}-AR, we then used the α_{2A}-KO to consolidate the demonstration that there are endothelial α_2-AR binding sites and to test whether we could further characterise these as α_{2A}-AR as was suggested by the functional experiments.

Aorta of α_{2A}-AR Knockout

When unstained, aorta of the α_{2A}-KO showed the characteristic autofluorescence of the internal elastic lamina (image 3-15). As expected, since fewer α_{2A}-AR were anticipated, QAPB produced less staining of endothelial cells but, unexpectedly, also produced less staining of smooth muscle cells, where most receptors were expected to be α_1-AR (image 3-16); later experiments demonstrated that the responses via α_1-AR were attenuated in this knockout strain (see chapter five).

The effects of antagonists were consistent with expectations from the functional study. Rauwolscine, on its own, did not make much difference (image 3-17), in line with the expectation from functional experiments that this is the endothelial α_2-AR responsible for endothelium-mediated vasorelaxation. The combination of 5MU and BMY7378 (image 3-18) or their combination with rauwolscine (image 3-19) resulted in some reduction of endothelial binding but left definite endothelial staining, consistent with the residual α_{1B}-AR. Smooth muscle cells also showed some binding at this stage, again consistent with a low level of α_{1B}-AR expression.

Having established these correlations between functional responses and α-AR binding on aorta, we then moved on to superior mesenteric artery in which functional

experiments suggested that there was a functional endothelial α_2-AR but that it was not the α_{2A}-AR found in aorta. This artery is large enough to treat in the same way as aorta, opening the lumen without causing gross damage. We first employed the wild type and then α_{1B}-KO in which the situation is already somewhat simplified and later the α_{2A}-AR to see whether a different α_2-AR is present and survives this KO.

Superior mesenteric artery of wild type:

Comparison between control and rauwolscine treated intact tissues eliminates presence of α_1-ARs on endothelial cells of young mouse superior mesenteric artery (image: 3-20-second row). The combination of 5MU, BMY7378 and rauwolscine resulted in some reduction of endothelial binding, however, left definite endothelial staining, consistent with the residual α_{1B}-AR (image 3-20-third row).

Superior mesenteric artery of α_{1B}-AR Knockout

The unstained vessel showed autofluorescence from a thin but complete internal elastic lamina, which showed regular, characteristic lamellae (holes) of this vessel (image 3-21, upper). QAPB showed excellent binding on endothelial cells, which in this case, were easy to distinguish from the finer elastin (image 3-21, lower). Binding survived the presence of the combination of 5MU and BMY7378 (image 3-22, upper) but was eliminated in the additional presence of rauwolscine (image 3-22, lower). This is all consistent with an endothelial population of α_2-AR.

Superior mesenteric artery of α_{2A}-AR Knockout

Again autofluorescence was clearly detectable as a fine continuous lamina with holes (image 3-23). There was good binding to both smooth muscle and endothelial cells

(image 3-24) that was not greatly affected by 5MU and BMY7378 (image 3-25) but also survived rauwolscine (image 3-26) or the combination of the α_1-AR antagonists with rauwolscine (image 3-27). Thus, we cannot finally distinguish between the possibilities of α_2-ARs and α_{1B}-AR, since we do not have the combination that would be given by a double α_{2A}-AR Knockout and α_{1B}-AR Knockout.

Chapter Three Graphs and Images

Effects of UK14304 on different mice arteries

The graphs created in Prism (Version 3).
The Images created in Amira (Version 3.20),
Imaris (Version 3) and Metamorph (Version 4.2).

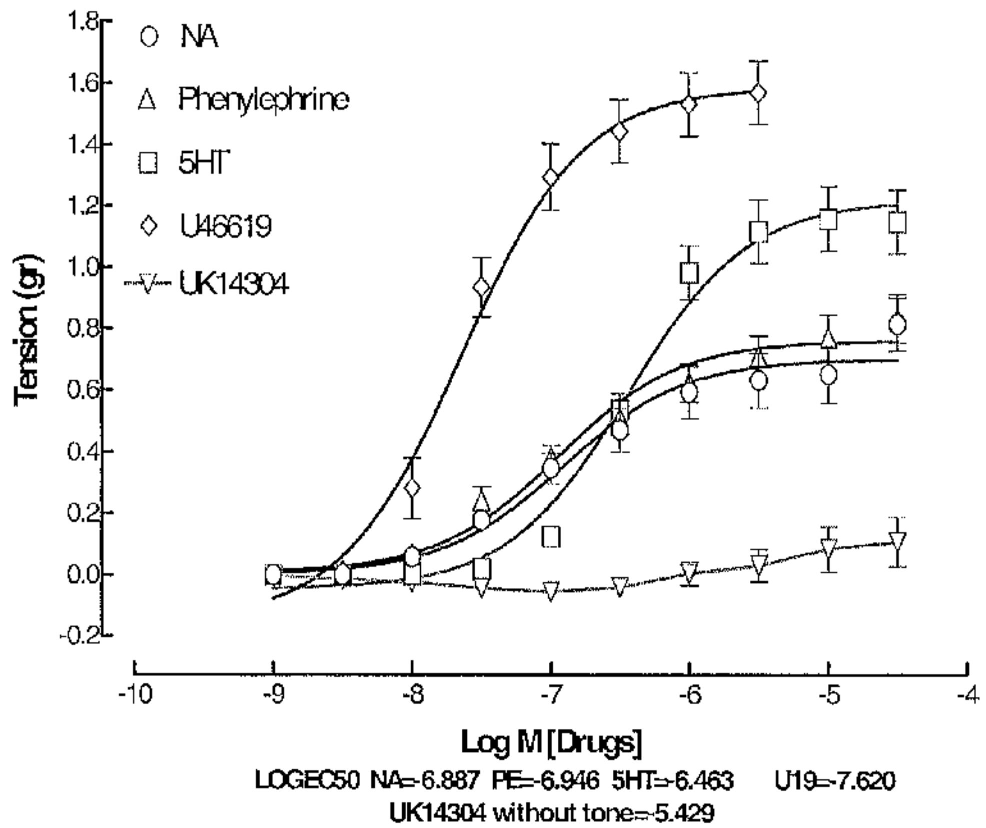


Fig. 3-1: Comparison between CCRC to noradrenaline (○), phenylephrine (△), 5HT (□), U46619 (◊) and UK14304 (▽) in four months wild type mouse aorta (n=7).

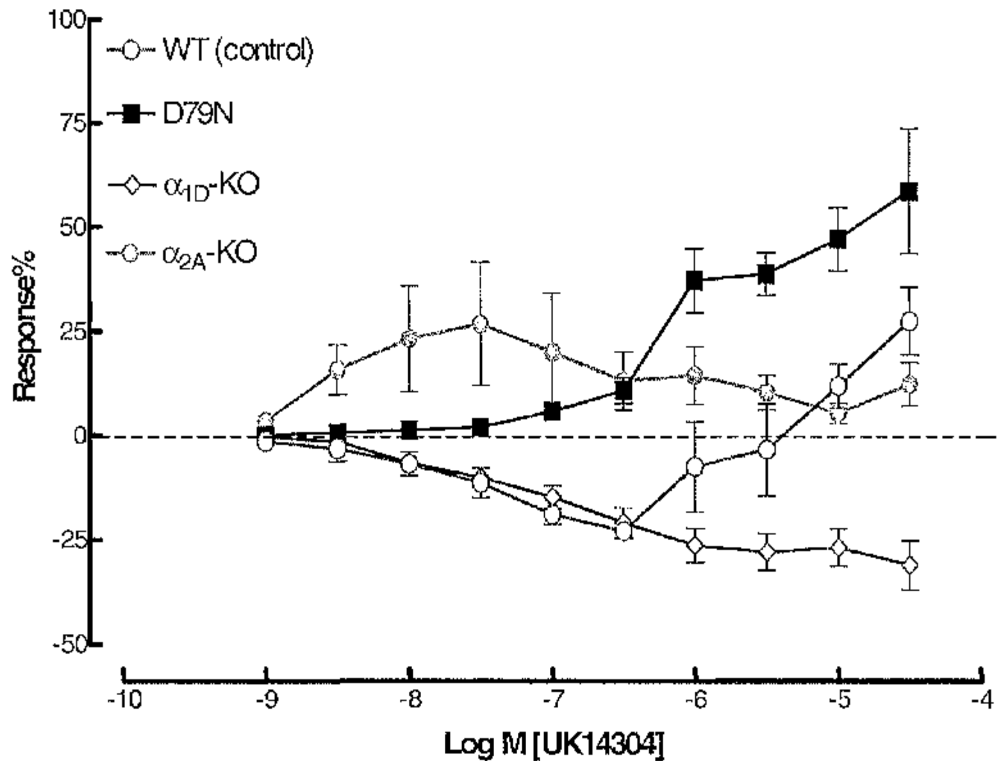


Fig. 3-2: Comparison between cumulative concentration response to UK14304 in wild type (Ó) (n=5), α_{1D}-KO (◊) (n=7), Nashville D79N (■) (n=4) and α_{2A}-KO (●) (n=4) mouse aorta.

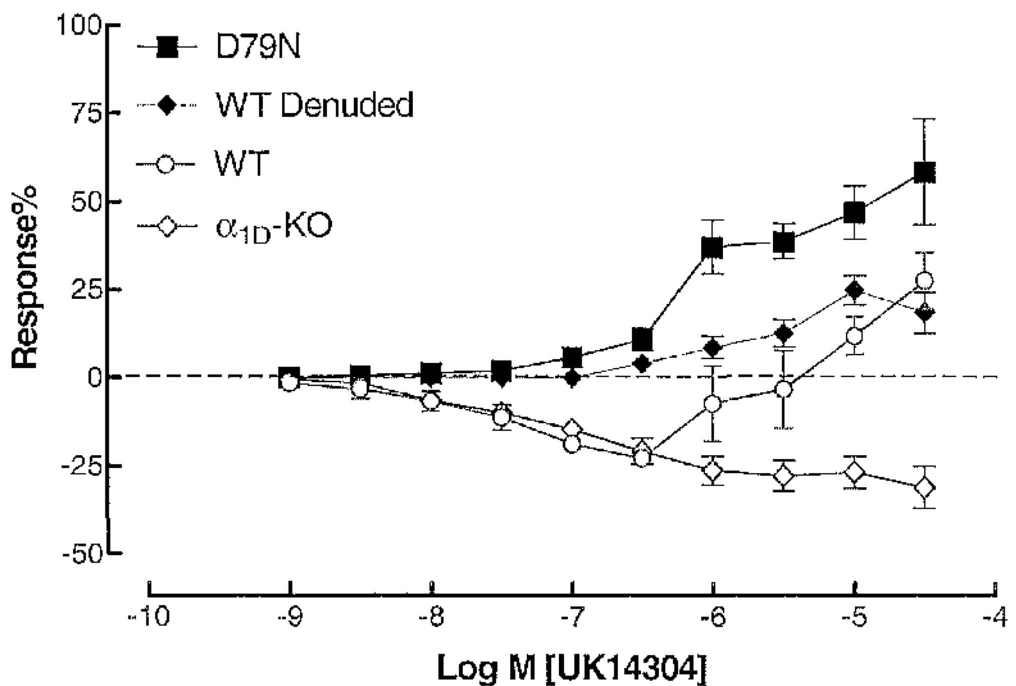


Fig 3-3: Preconstricted with U46619 (10nM), CCRC to UK14304 in four months wild type {intact (Ó) and denuded (◆)} (n=5), Nashville D79N (■) (n=4), α_{1D}-KO (◊) (n=7) mouse aorta.

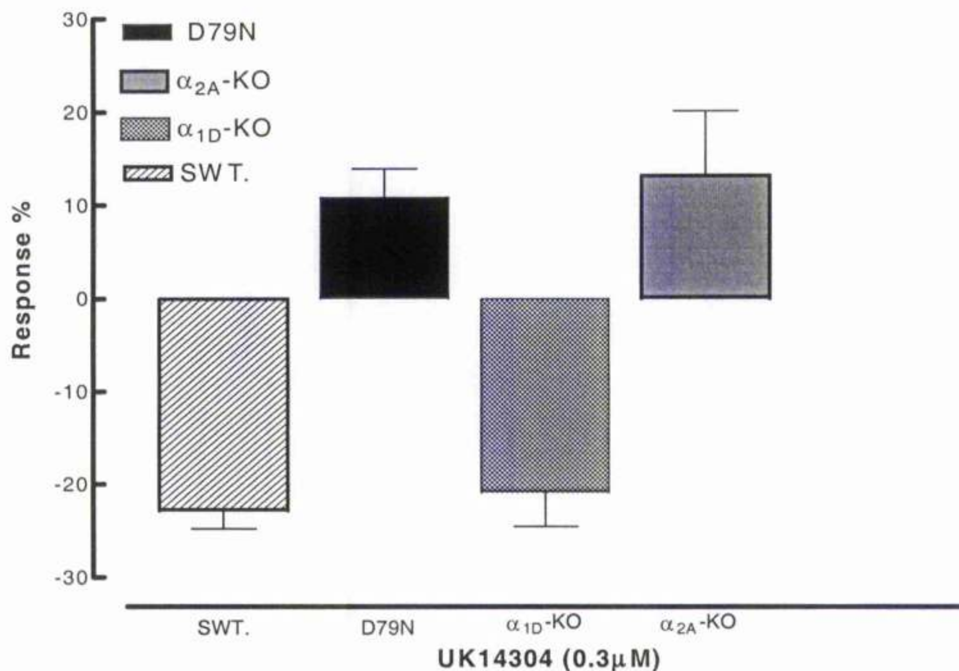


Fig. 3-4: Comparison between cumulative concentration response % to UK14304 at the point of (0.3μM) in four months male wild type (n=5), α_{1D}-KO (n=7), Nashville D79N (n=4) and α_{2A}-KO (n=4) mouse aorta.

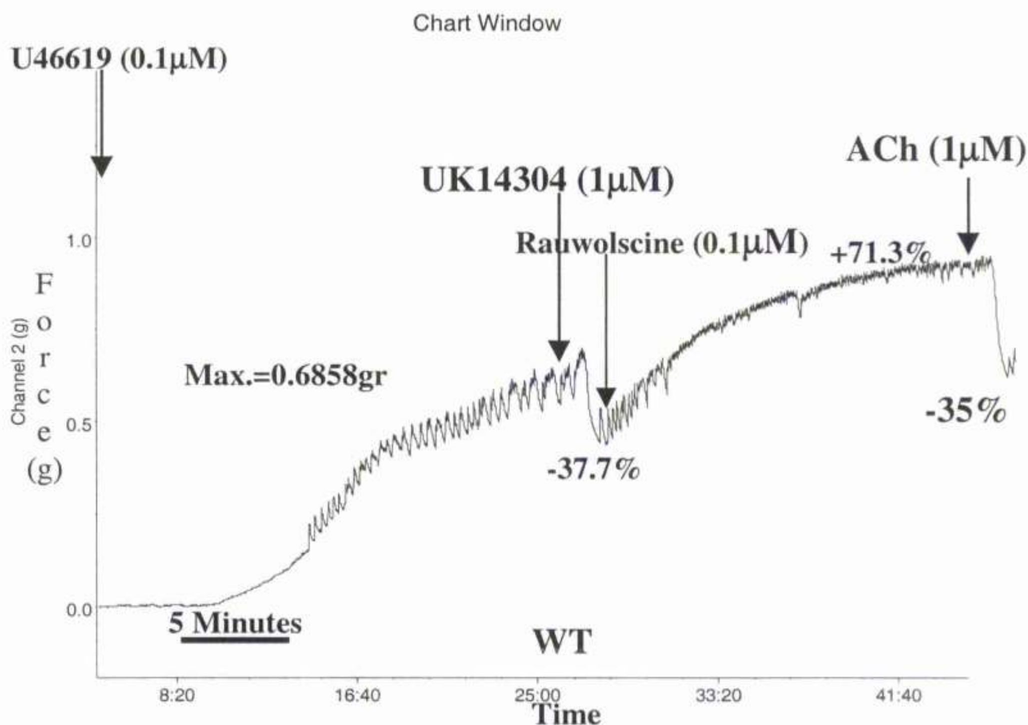


Fig. 3-5: *Trace*; *From Powerlab* (4.2)- Four months wild type mouse aorta. Effect of single concentration of UK14304 (1μM) on top of U46619 (0.1μM) preconstriction. Rauwolscine could reverse relaxation response to UK14304 completely.

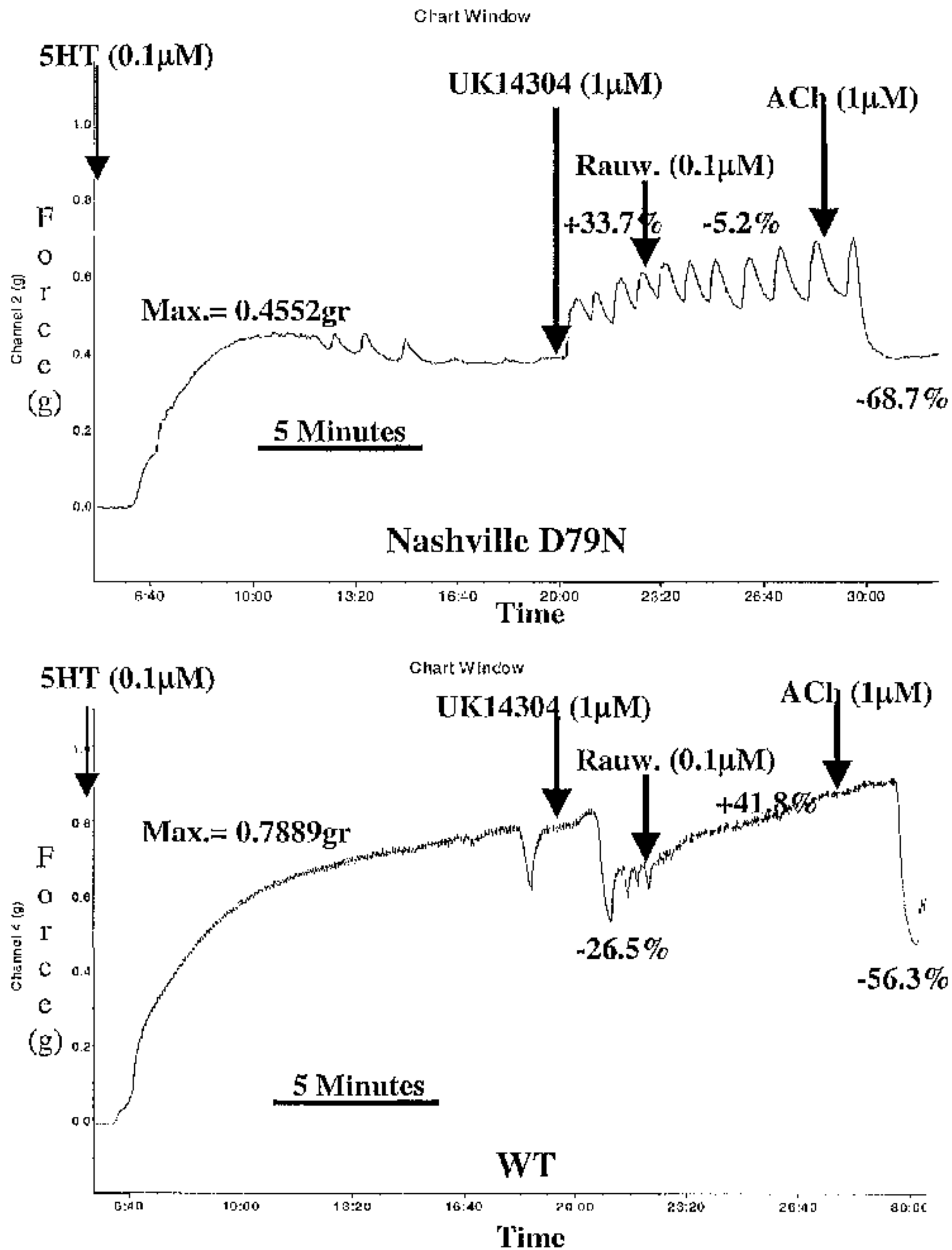


Fig. 3-6: *Trace;* comparison between wild type and Nashville D79N [functionally $\alpha_{2A/D}$-AR knockout mouse aorta. Single concentration of UK14304 (1μM) has opposite effect on two different strains. Therefore, revealing $\alpha_{2A/D}$-AR involvement in this relaxation response. Both the contractile and relaxant effect opposed by rauwolscine (0.1μM). Hence, these reveal presence of $\alpha_{2A/D}$-AR in endothelial side of mouse aorta.

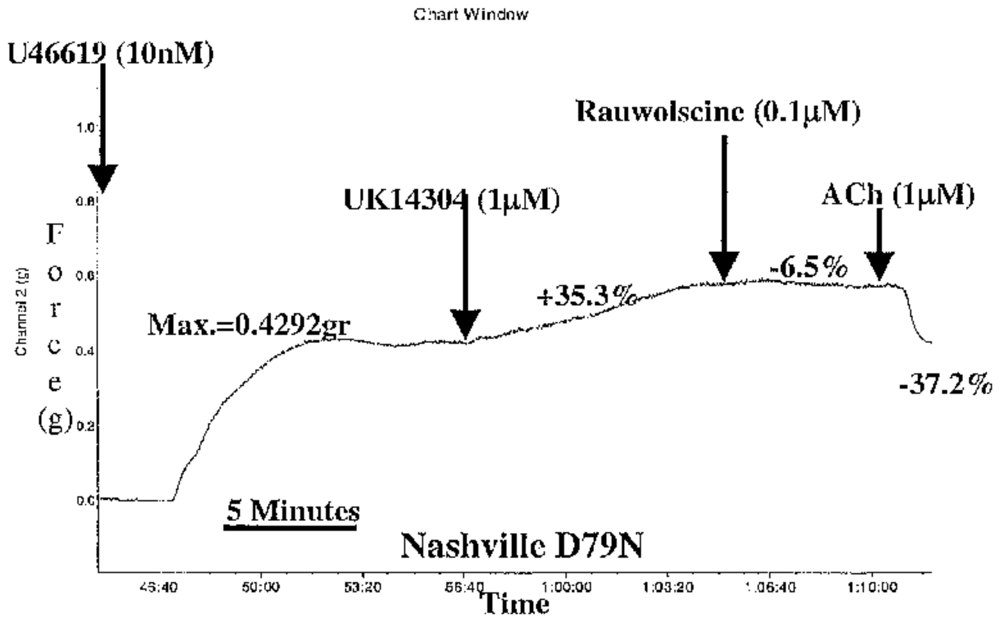


Fig. 3-7: *Trace*; *From Powerlab* (4.2.2)- Four months Nashville D79N aorta. Effects of UK14304 (1μM) and rauwolscine (0.1μM) on top of U46619 (10nM) preconstriction. UK14304 cause +35.3% contraction response in D79N {$\alpha_{2A/D}$-non-functional receptor} mouse aorta which antagonised by rauwolscine (-6.5%). This confirm presence of postjunctional other subtypes of α2-AR on smooth muscle cells of mouse aorta which are responsible for contraction.

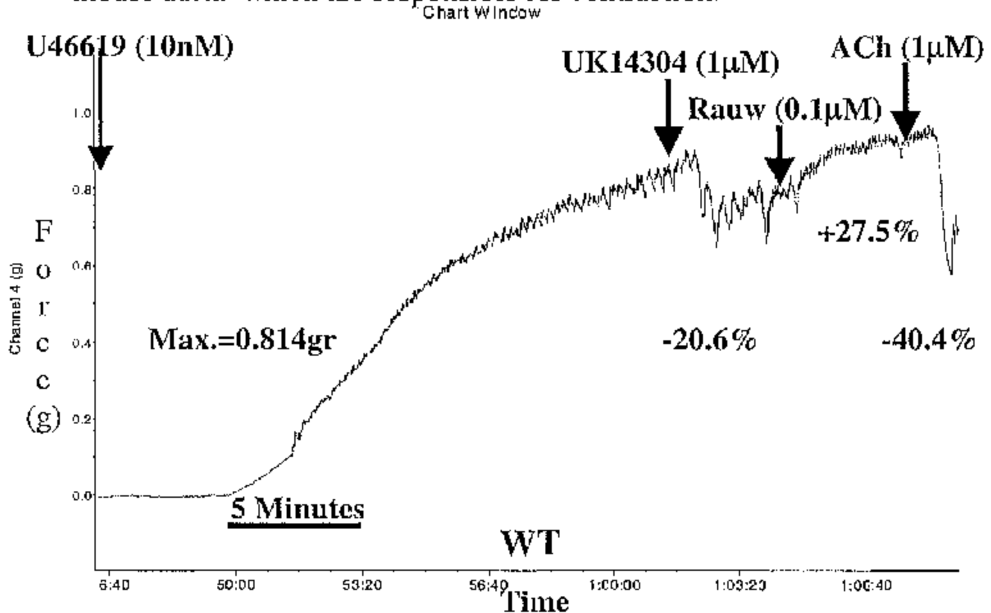


Fig. 3-8: *Trace*; *From Powerlab* (4.2.2)- Four months wild type mouse aorta. Effects of UK14304 (1μM) and rauwolscine (0.1μM) on top of U46619 (10nM) preconstriction. UK14304 cause -20.5% relaxation response in wild type mouse aorta which antagonised by rauwolscine (+27.5%). This confirm presence of $\alpha_{2A/D}$-AR on WT mouse aorta which is responsible for relaxation.

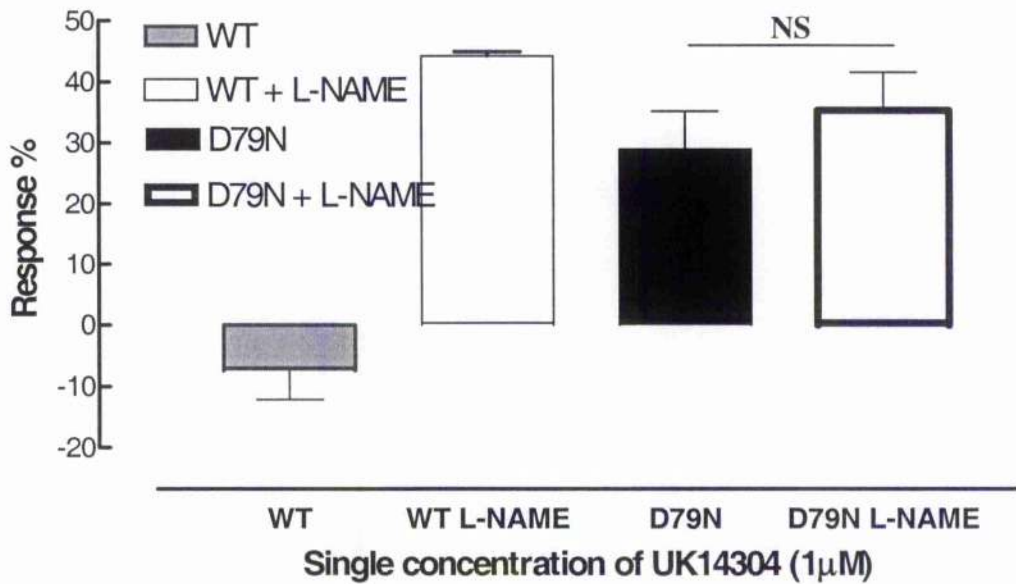
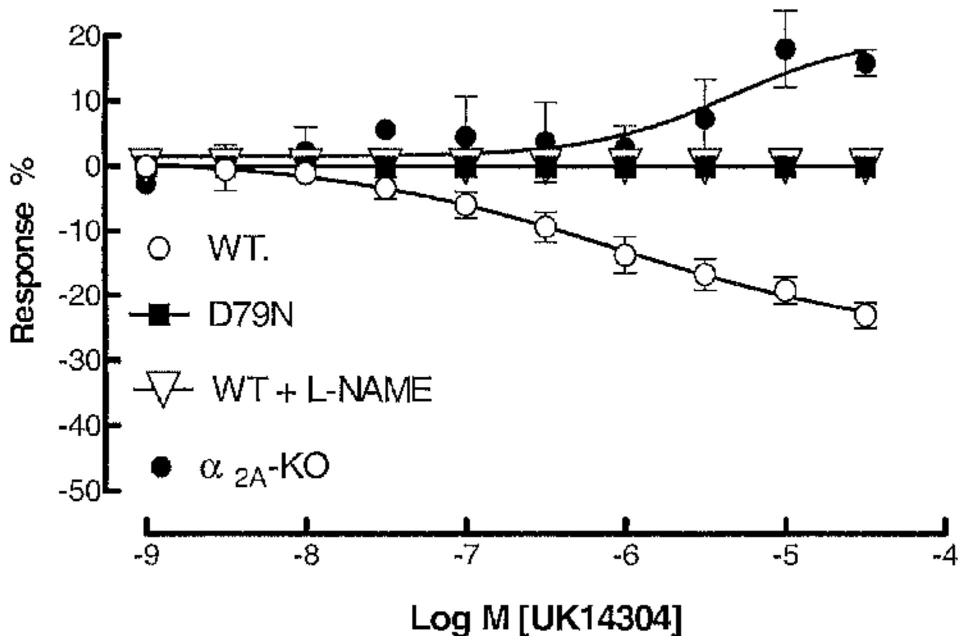


Fig 3-9: Effect of UK14304 (1μM) in presence and absence of L-NAME (0.1mM) in wild type (n=7) and Nashville D79N (n=9) four months mouse aorta. Two way ANOVA statistical analysis followed by Bonferroni's multiple comparison post test revealed significant difference between strains (P value=<0.001). However, there was not significant difference between D79N and D79N + L-NAME (P value>0.05).



P value α2A-KO & SWT. = 0.0094 <0.05 * P value D79N & SWT. = 0.0069 <0.05 *

Fig. 3-10: Relaxation response to UK14304 cumulatively in six months wild type in presence (∇) and absence (O) of L- NAME (0.1mM), Nashville D79N mouse (■) (n=6) and α_{2A}-KO (●) (n=4) carotid artery.

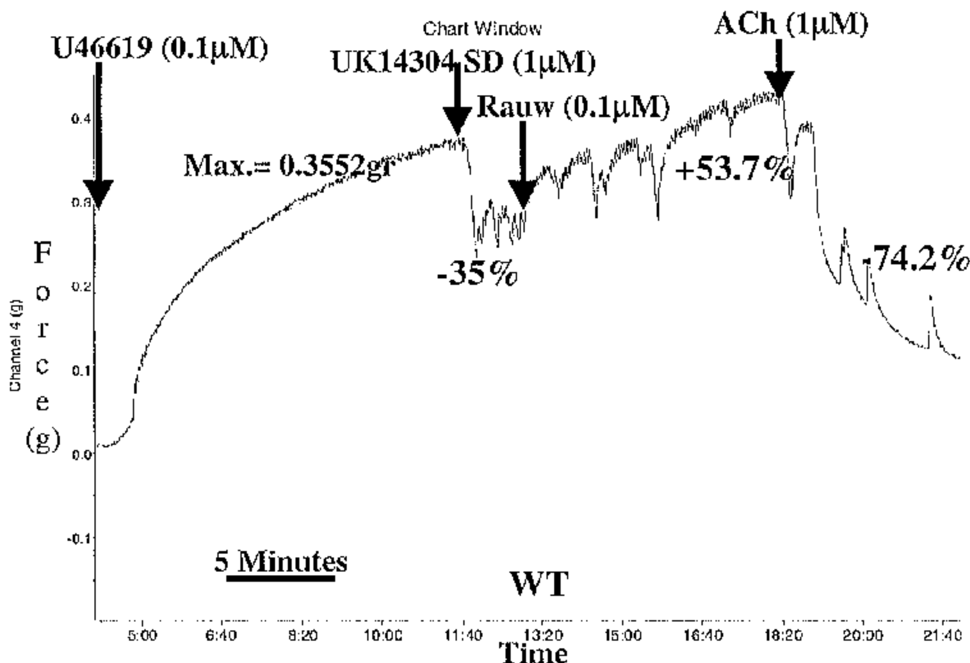


Fig. 3-11: Trace; Powerlab (4.2)- Four months wild type mouse carotid artery, effect of single concentration of UK14304 (1μM) on top of U46619 (0.1μM) and then adding rauwolscine (0.1μM). Rauwolscine could reverse -35% relaxation response to UK14304 to +53.7% contraction after five minutes. Channel had -74.2% relaxation to acetylcholine (1μM).

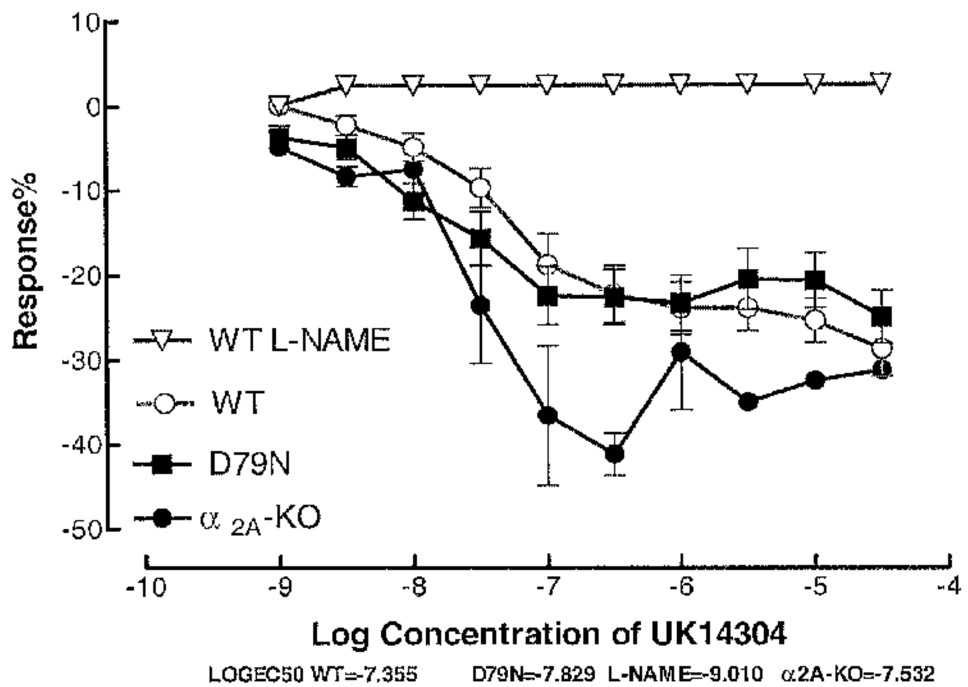


Fig. 3-12: Cumulative concentration of UK14304 on top of U46619 (0.1μM) in four months (n=7) wild type in presence (∇) and absence (O) of L-NAME (100μM), Nashville D79N (■) (n=5) and α_{2A}-KO (•) (n=4) mouse superior mesenteric artery.

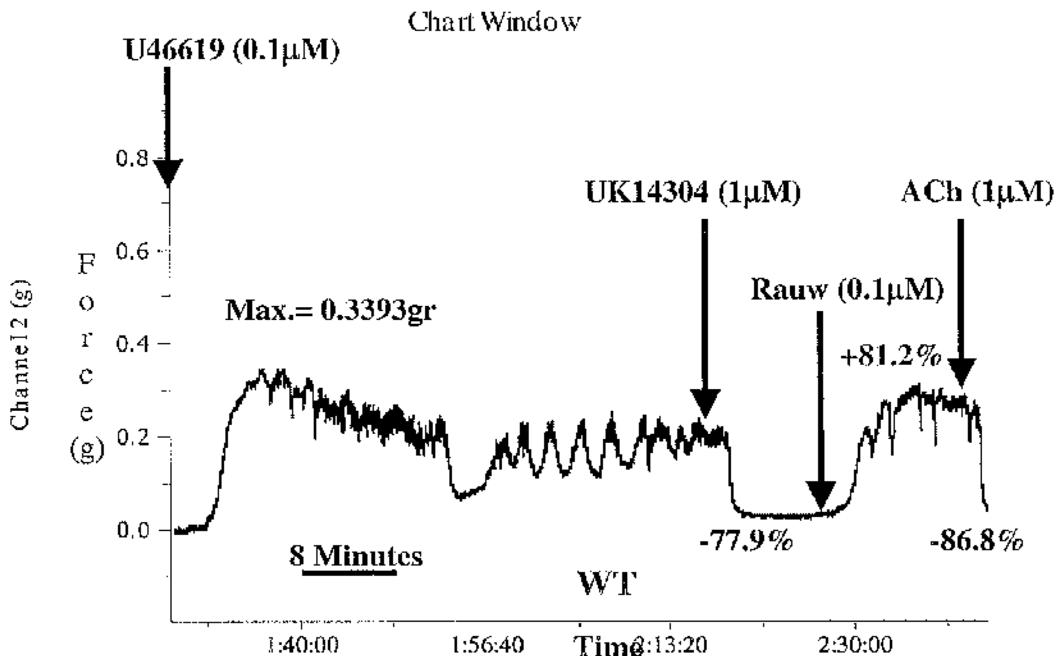


Fig. 3-13: *Trace*; Powerlab (4.2)- Effect of single concentration of UK14304 (1μM) and rauwolscine (0.1μM) on mouse superior mesenteric artery. Rauwolscine could reverse relaxation to UK14304 (-77.9%) to contraction (+81.2%).

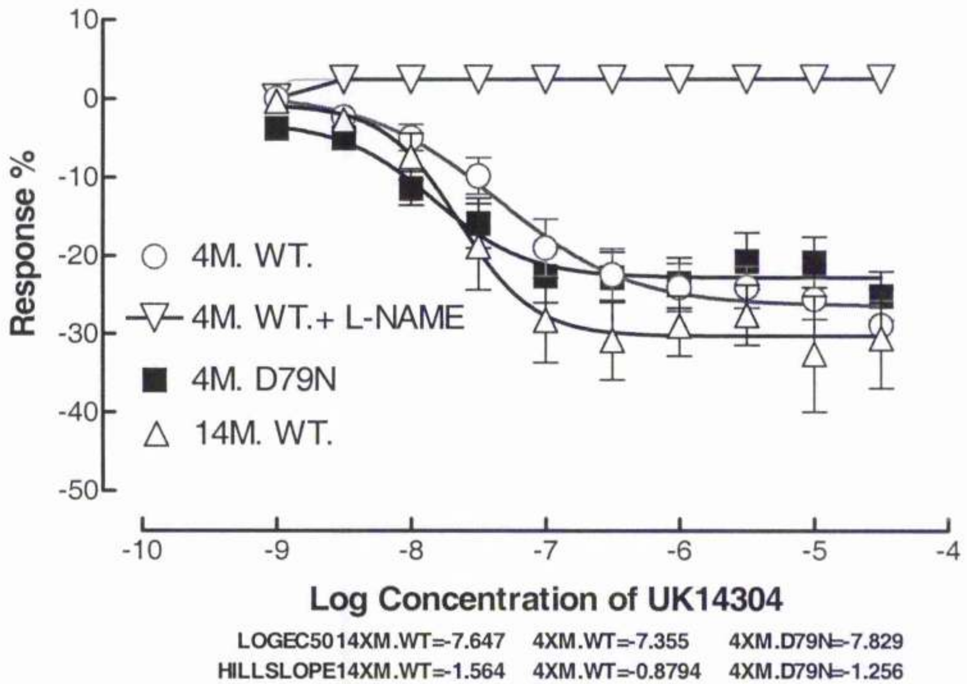


Fig. 3-14: Cumulative concentration of UK14304 on top of U46619 (0.1μM) in four (n=7) and fourteen (Δ) (n=5) months wild type in presence (∇) and absence (O) of L-NAME (100μM) and four months Nashville D79N (■) (n=5) mouse superior mesenteric artery.

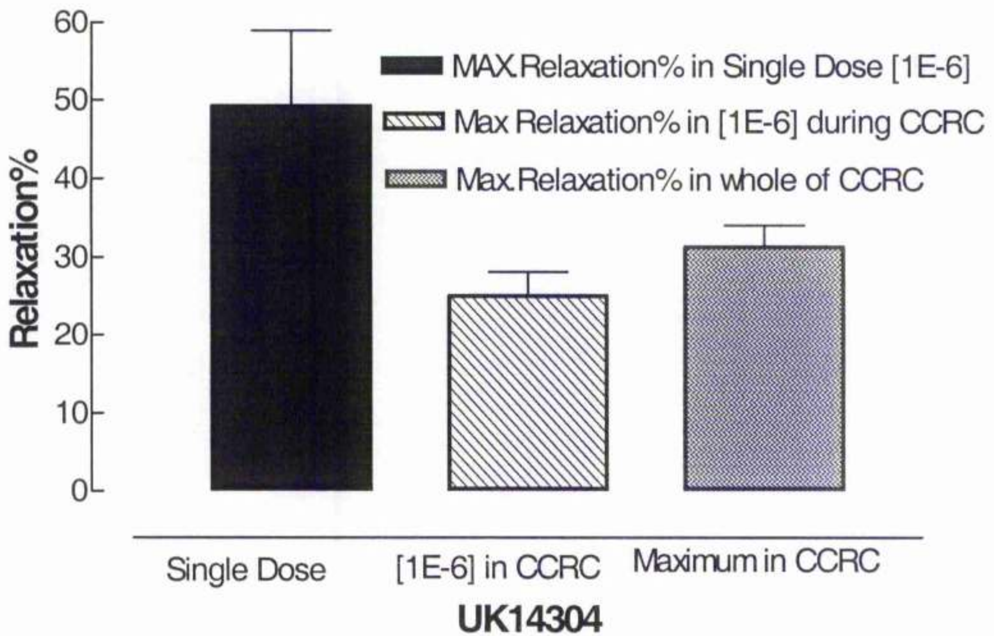


Fig. 3-15: Comparison between relaxation response to UK14304 in different situation in four months wild type mouse superior mesenteric artery (n=6).

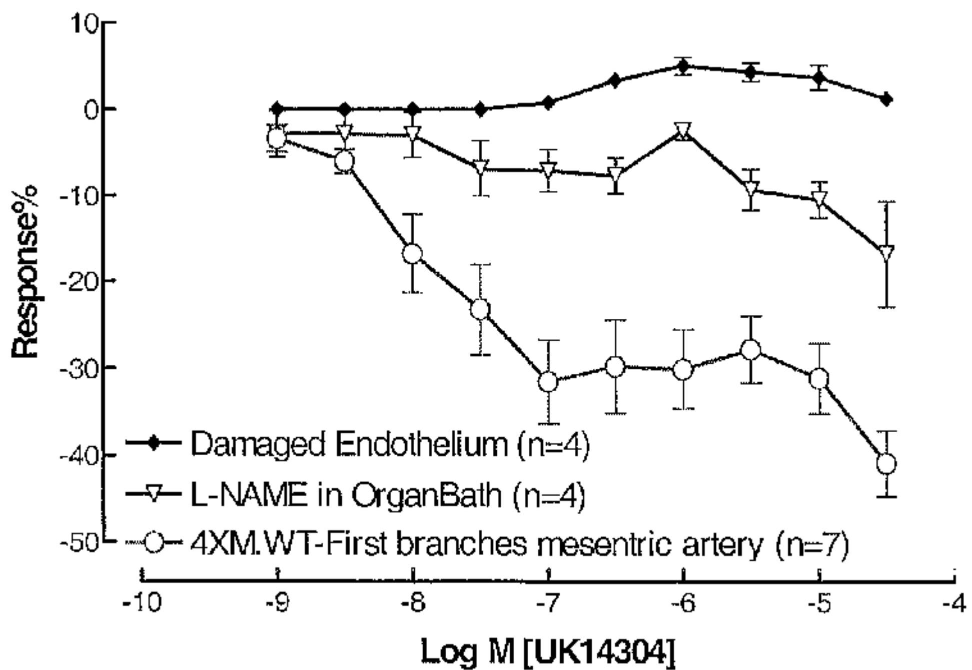


Fig. 3-16: CCRC to UK14304 in four months wild type mouse first branches of mesenteric artery (Ó) (n=7), In presence of L-NAME (∇) (0.1mM) and damaged endothelium (♦) (n=4).

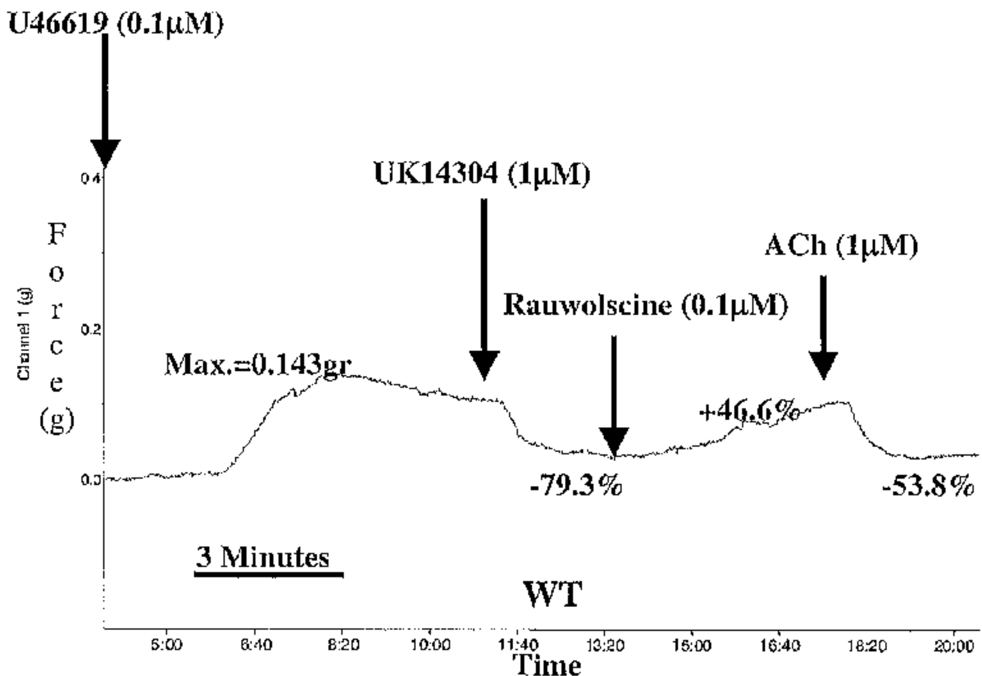


Fig. 3-17: *Trace*; From Powerlab (4.2)- Four months wild type mouse first branch mesenteric artery. UK14304 (1μM) could provide -79.3% relaxation response on top of U46619 (0.1μM) preconstriction. Rauwolscine reversed it to +46.6% contraction. Vessel had more than 50% relaxation response to Acetylcholine.

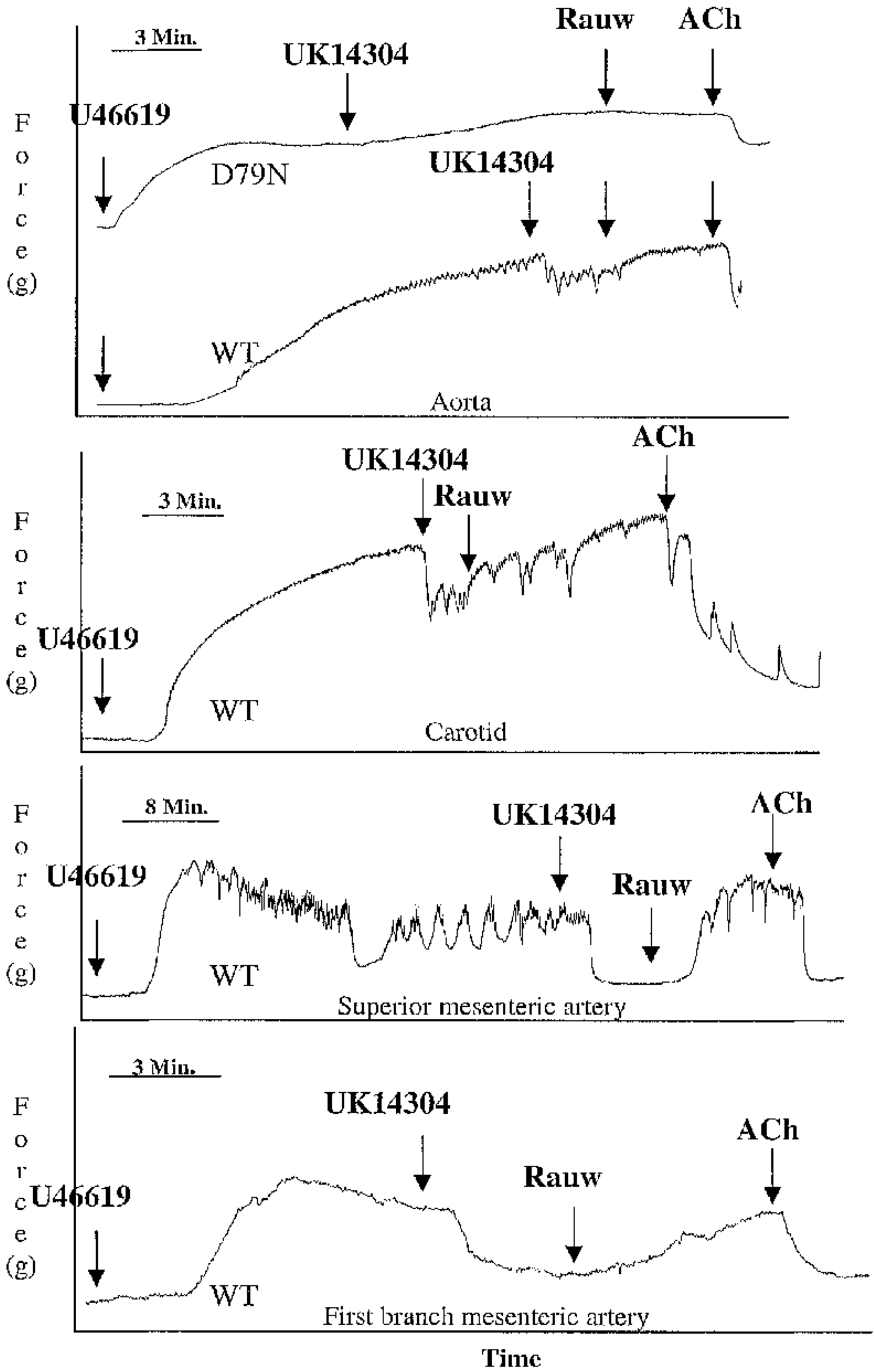


Fig. 3-18: Traces together; Effects of UK14304 (1μM) and rauwolscine (0.1μM) on four months wild type aorta, carotid, superior and first branch mesenteric arteries.

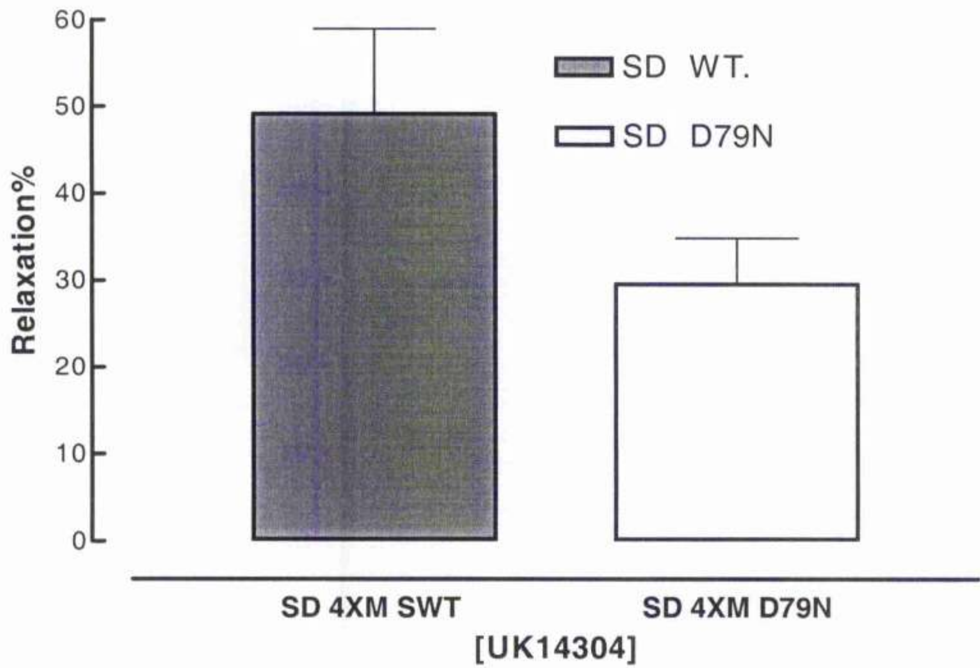


Fig. 3-19: Comparison between relaxation responses to single concentration of UK14304 (1μM) in four months WT (n=7) and Nashville D79N (n=5) mouse superior mesenteric artery.

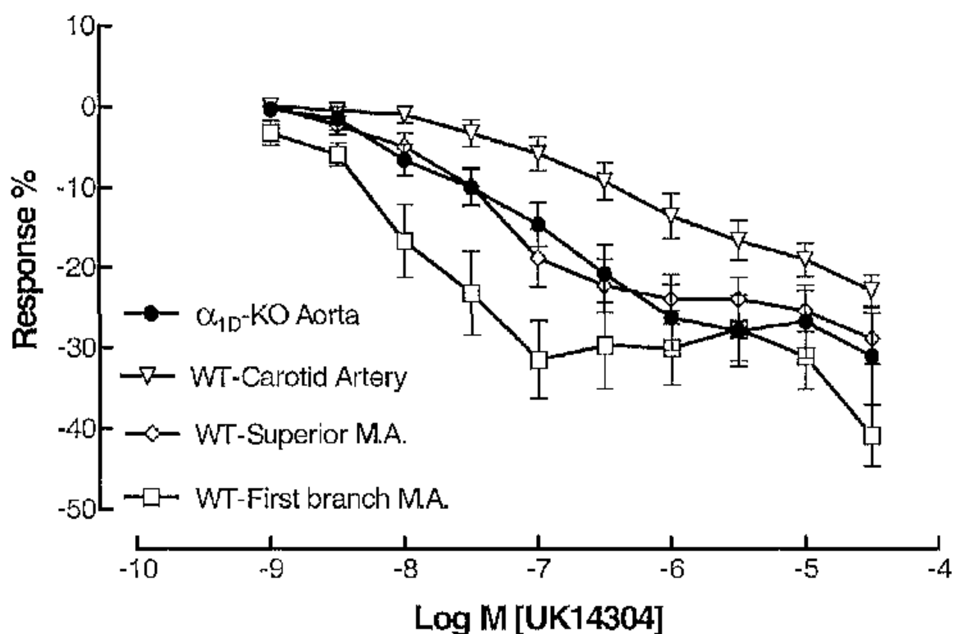


Fig. 3-20: Preconstricted, CCRC to UK14304 in four months α_{1D}-KO aorta (◆), wild type carotid artery (∇), superior (◊) and first branches (❑) mesenteric Artery (n=7).

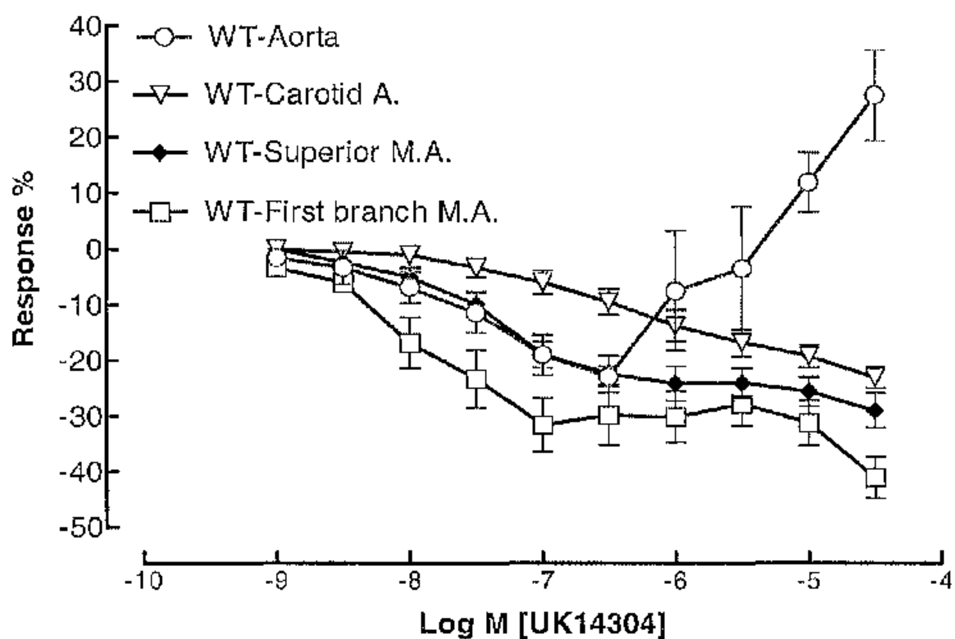


Fig. 3-21: Preconstricted, CCRC to UK14304 in four months wild type aorta (Ó), carotid artery (∇), superior (◆) and first branches (❑) mesenteric artery (n=7).

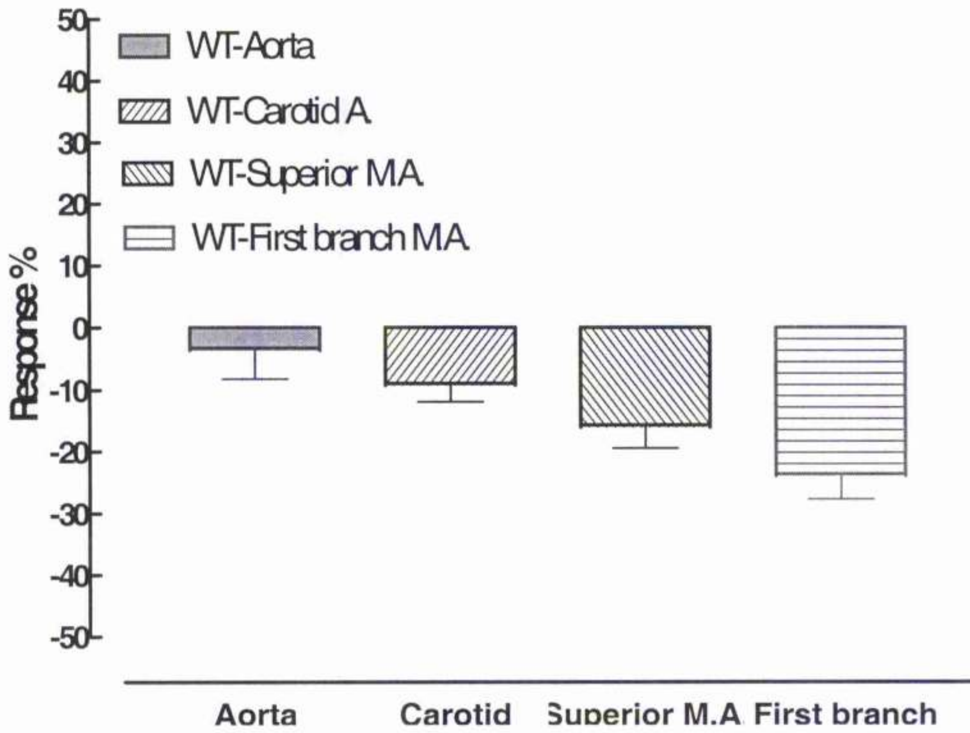


Fig. 3-22: Preconstricted- Comparison in size of response and potency in CCRC to UK14304 in four months wild type aorta, carotid artery, superior and first branches of mesenteric artery (n=7).

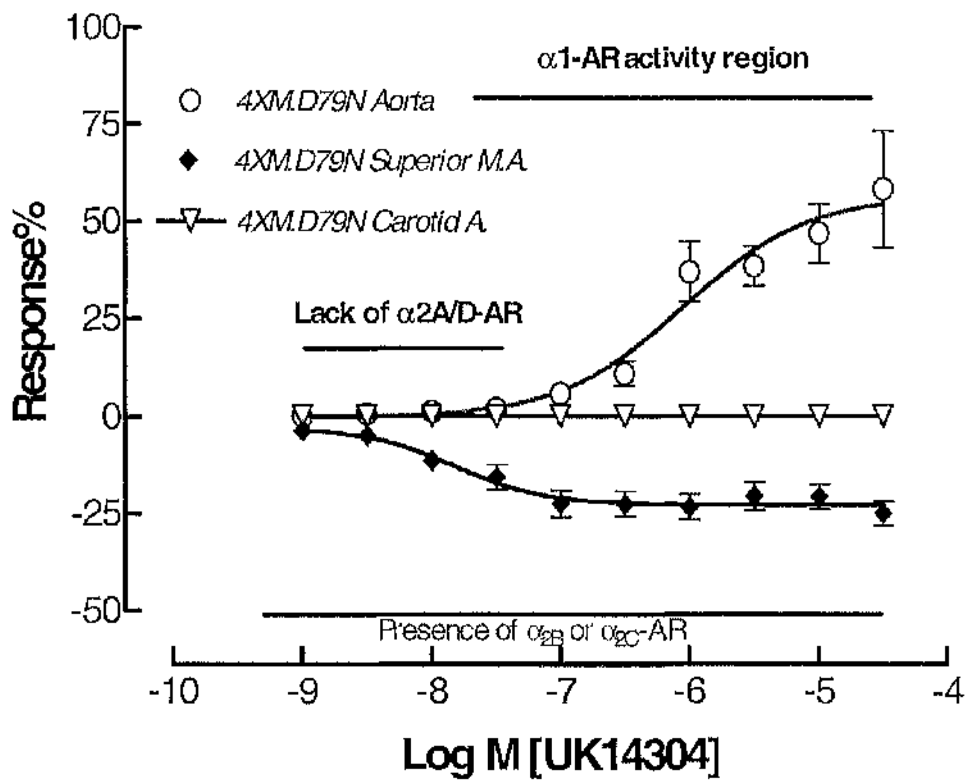


Fig. 3-23: Cumulative response curve to UK14304 in Nashville D79N mouse aorta (O), carotid artery (∇) and superior mesenteric (◆) artery (n=5). .

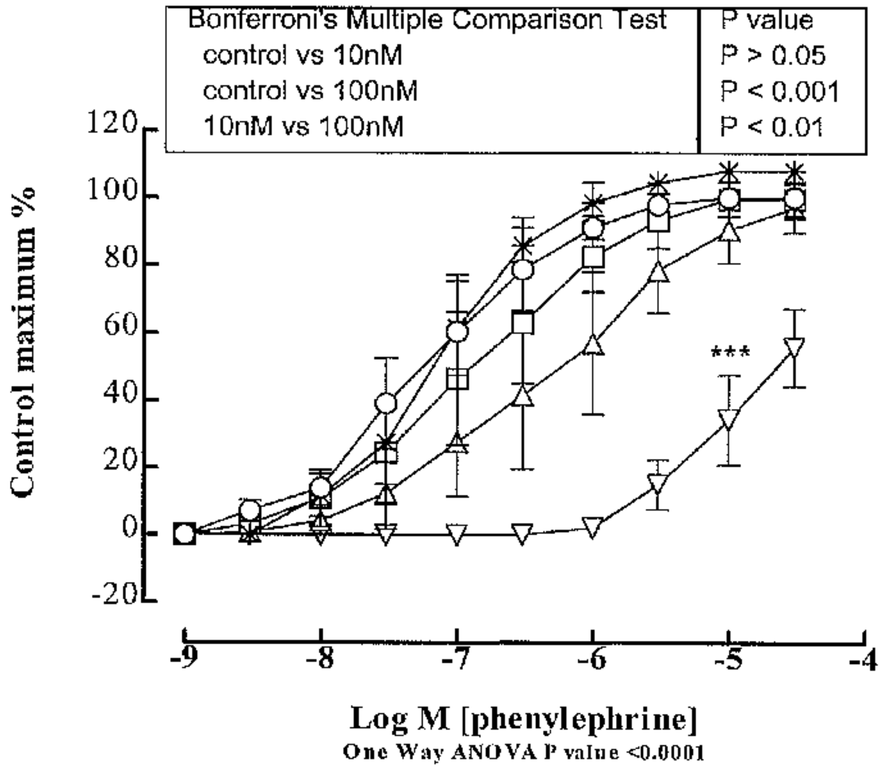


Fig. 3-24: QAPB antagonism on cumulative concentration response to Phenylephrine in young rat aorta. control (O), time control (*), 1nM (Φ), 10nM (≅) and 100nM (∇) of QAPB (Graph from Dr. C. Daly & S. p. MacGrory) (n=6).

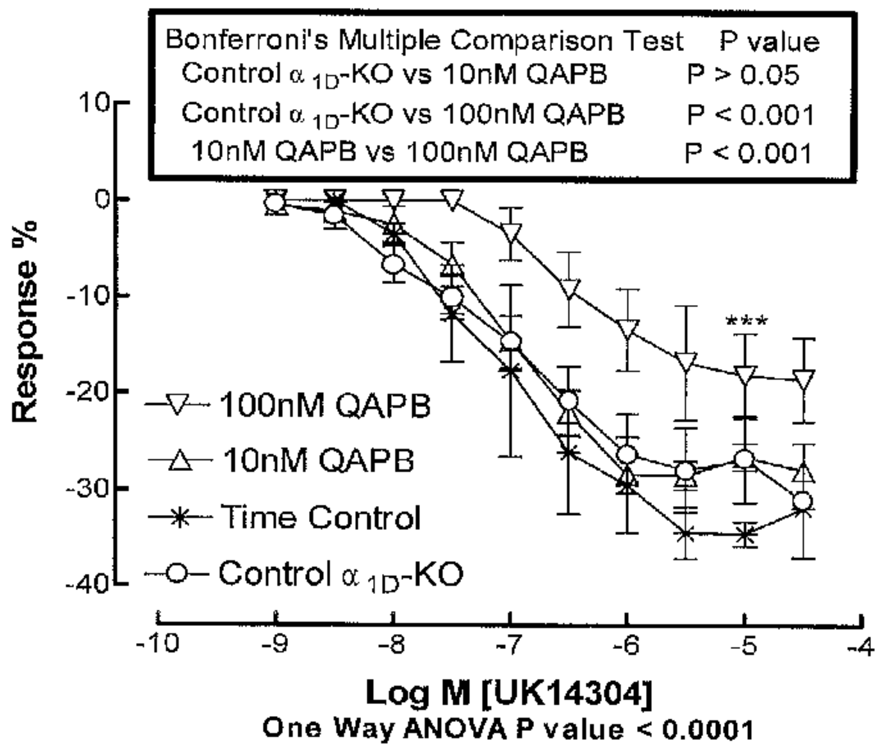


Fig 3-25: QAPB affinity on α_2-AR in α_{1D}-KO mouse aorta (n=5).

Image 3-1: Four months wild type mouse aorta endothelial cells treated by Losartan (AT_1–antagonist) (10μM) and then stained with QAPB (0.1μM) and Rho-Ang II-Human (50nM). Green colour has localised α-AR and Red AT_2-Receptor (n=6).

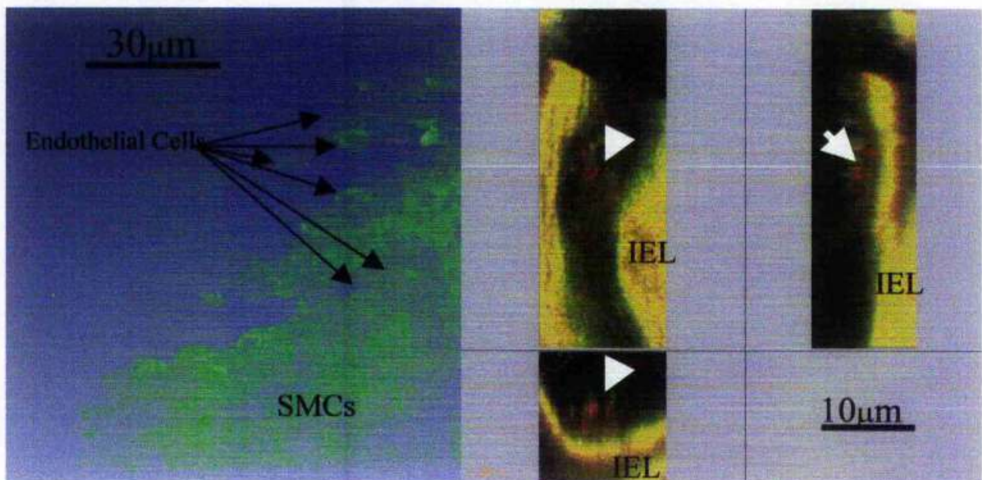


Image 3-2: Four months wild type mouse aorta endothelial cells stained with QAPB (0.1μM). α-ARs are localised by green colour in both Endothelial Cells (ECs) Smooth Muscle Cells (SMCs) (n=6).

Image 3-3: Four months wild type mouse aorta endothelial cells treated by both of Losartan (10μM) and BMY7378 (0.1μM) then stained with QAPB (0.1μM) and Rho-Ang II-Human (50nM). The Green colour granules reveal α_1-ARs (α_{1A} & α_{1B}) subtypes or α_2-ARs and Red granules illustrate AT_2-Receptors inside the cell, around the nucleus (arrow). Yellow colour is due to overlapping of Red and Green on Internal Elastic Membrane (IEM).

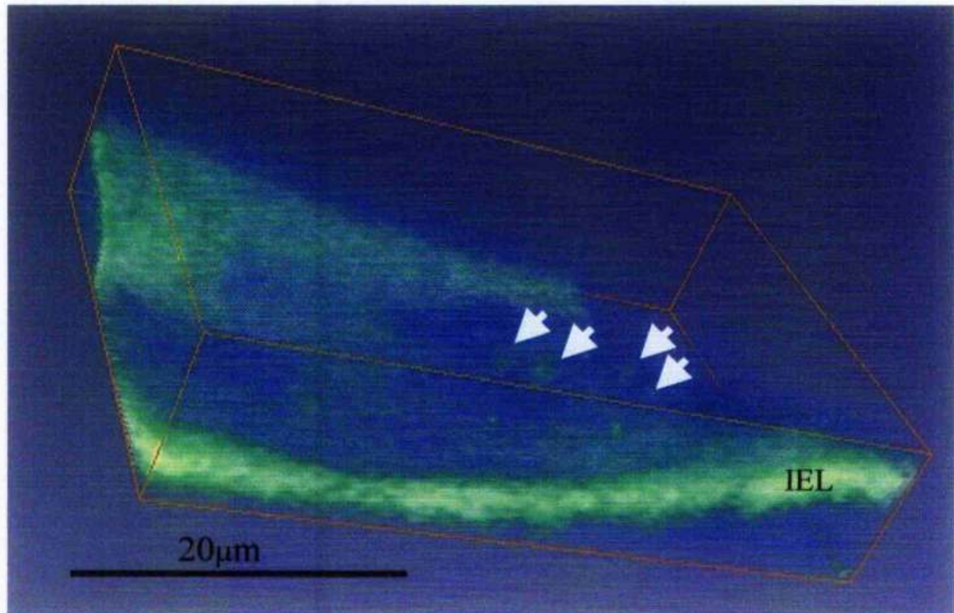


Image 3-4: Amira 3D model image of an endothelial cell stained with QAPB (0.1µM) after treatment by BMY7378 (0.1µM). α_1-AR subtypes (α_{1A}-AR α_{1B}-AR) (White arrows) are localised on a base of Internal Elastic Lamina (IEL) around the nucleus. Four months wild type mouse aorta

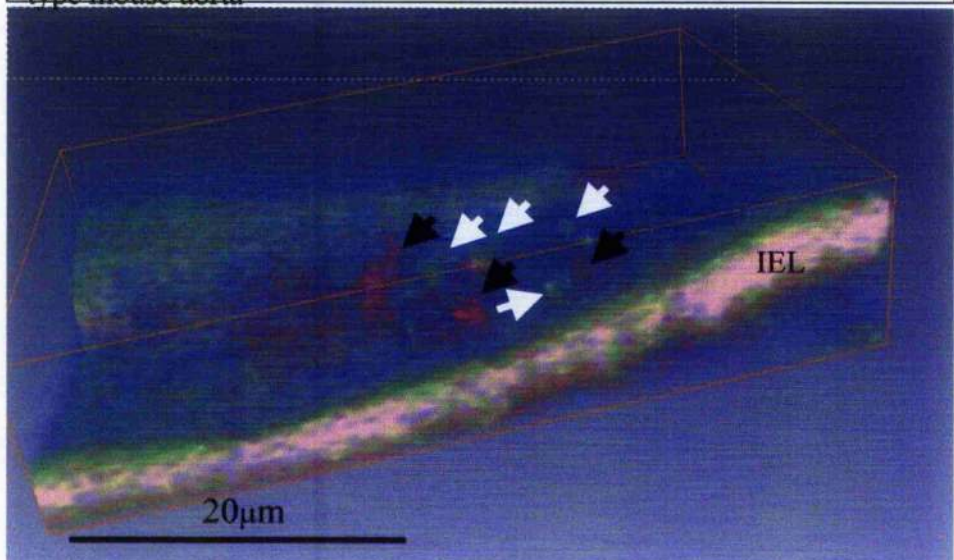


Image 3-5: Amira 3D model image of an endothelial cell stained with QAPB (0.1µM) and Rho-Ang II-Human (50nM) after treatment by Losartan (10µM) and BMY7378 (0.1µM). α_1-AR subtypes (α_{1A}-AR α_{1B}-AR), α_2-ARs (White arrows) and AT_2-Receptor (Black arrows) are localised on a base of Internal Elastic Lamina (IEL) around the nucleus. Four months wild type mouse aorta

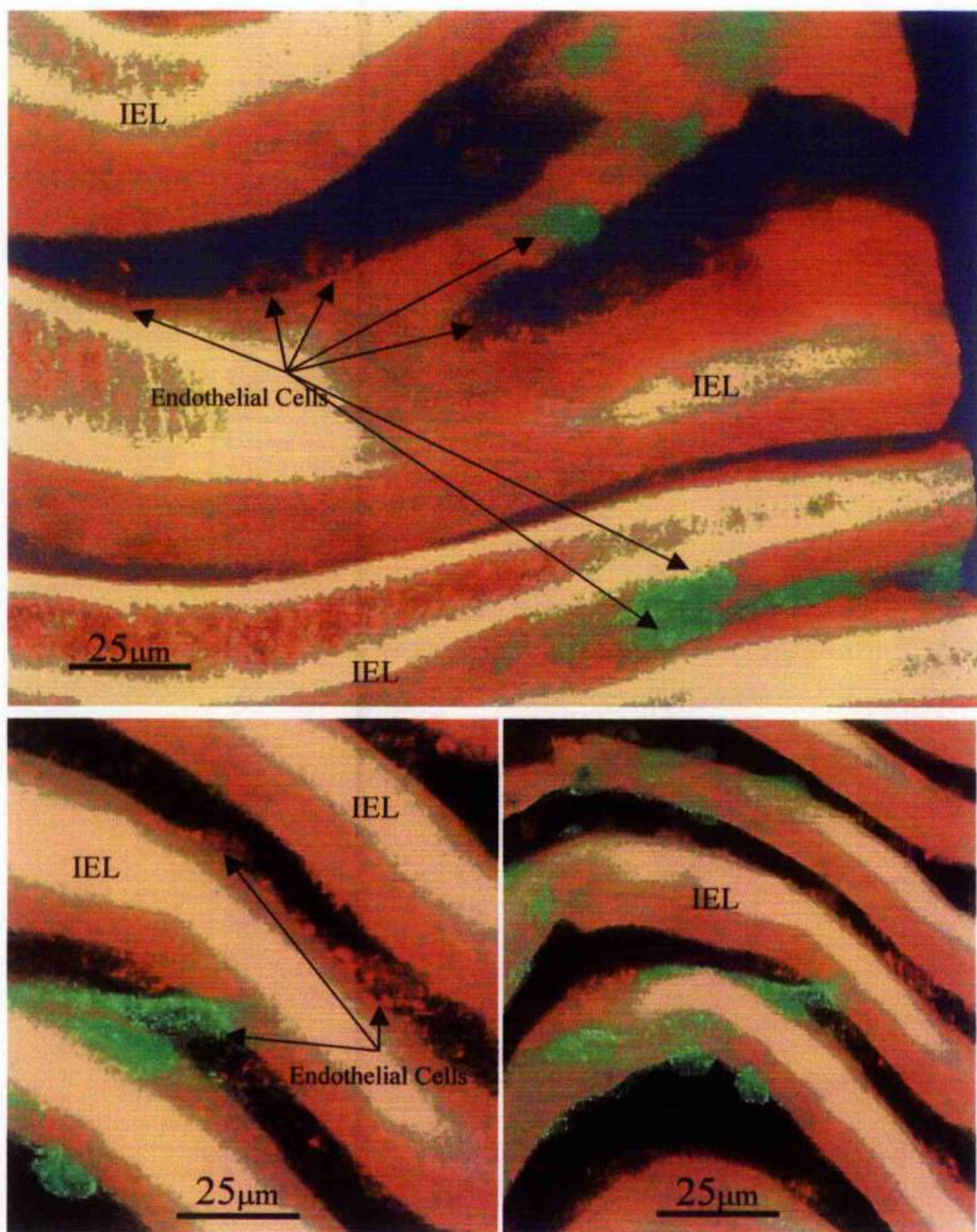


Image 3-6: Four months wild type mouse aorta treated with Losartan (10μM), Rauwolscine (1μM) + BMY7378 (1μM) then stained with QAPB (0.1μM) and Rhodamin-Ang II (50nM). After treating with high concentrations of BMY7378 + Rauwolscine QAPB shows presence of α_{1A} or α_{1B}-AR in some endothelial cells (Green colour). However, a majority of endothelial cells are stained only with Rhodamine-Ang II (Red colour) which shows AT_2-Receptors in endothelial cells. This reveals a mosaicism related to α_1-ARs in endothelial cells of young mouse aorta (n=6).

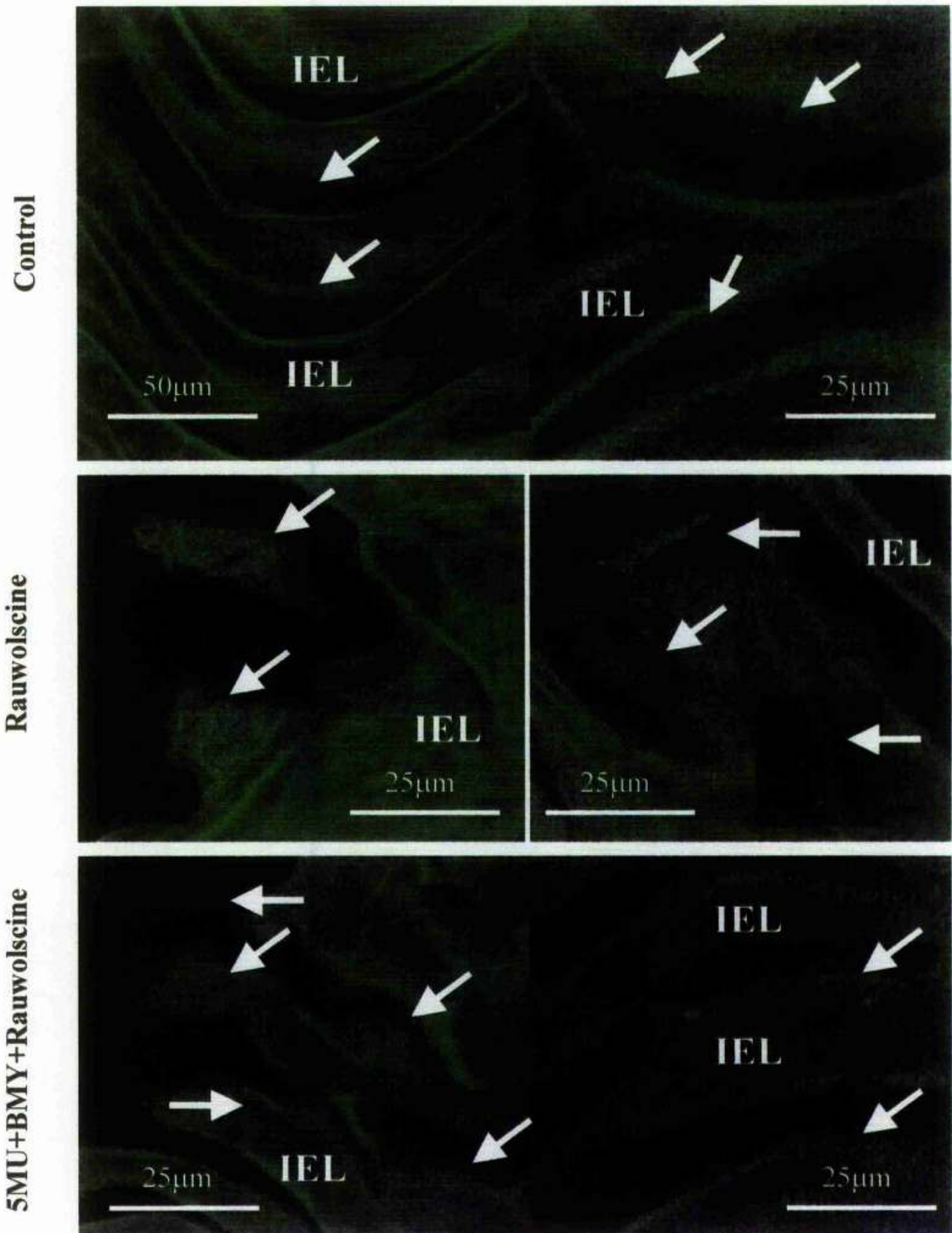


Image 3-7: Four months wild type mouse aorta, stained with QAPB (0.1μM).
First row: **control**
Second row: **Treated with Rauwolscine (0.1μM).**
Third row: **Treated with 5MU (0.1μM), BMY7378 (0.1μM) and Rauwolscine (0.1μM).**
Antagonists affected on intact tissues one hour before staining. Endothelial cells appeared in all three raw (White arrows). Second raw shows presence of α-ARs on endothelial cells. Third raw suggests remaining QAPB intensity is due to α_{1B}-AR on mouse endothelial cells (n=6).

LSCM on four months α_{1B}-Knockout Mouse Aorta

Open lumen, live aorta tissues stained with QAPB (0.1μM) after treatment by BMY7378 (0.1μM), 5MU (0.1μM), Rauwlscine (0.1μM) alone or in combination and compared with unstained tissue's autofluorescence.

LSCM method details: Excitation wavelength = 488nm, Barrier = 515nm, Pixel * Lines = 512 * 512 (1μm = 1.77 Pixel) Objective = X40 OIL, Speed: 166-500 IPS, Gain =15, Iris = 1.5, Laser Power = 50%, Step = 0.35μm

All the z-series images developed in Metamorph (Version 4.2) and made as 3D images in Amira (Version 3.2) software.

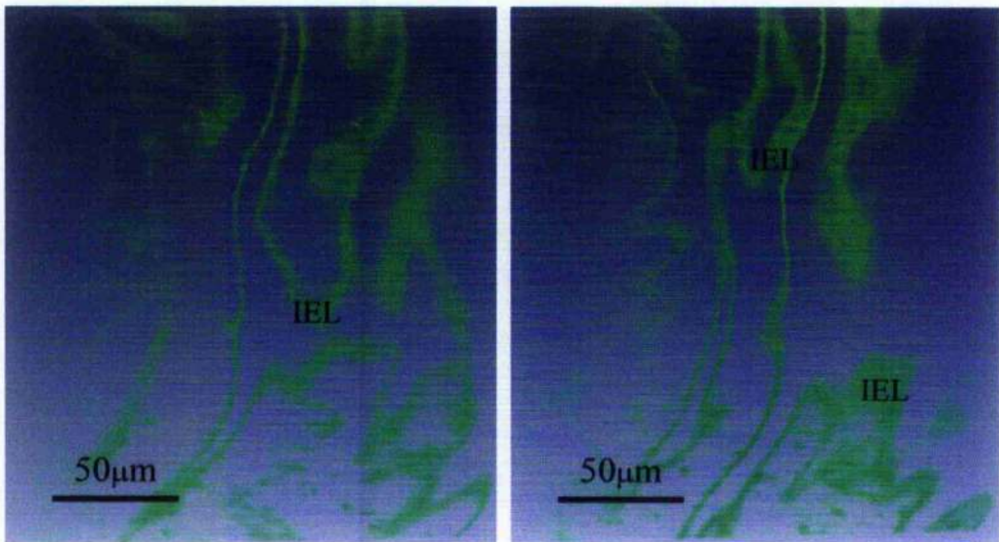


Image 3-8: Unstained α_{1B}-Knockout mouse aorta (Only Autofluorescence) (n=3).

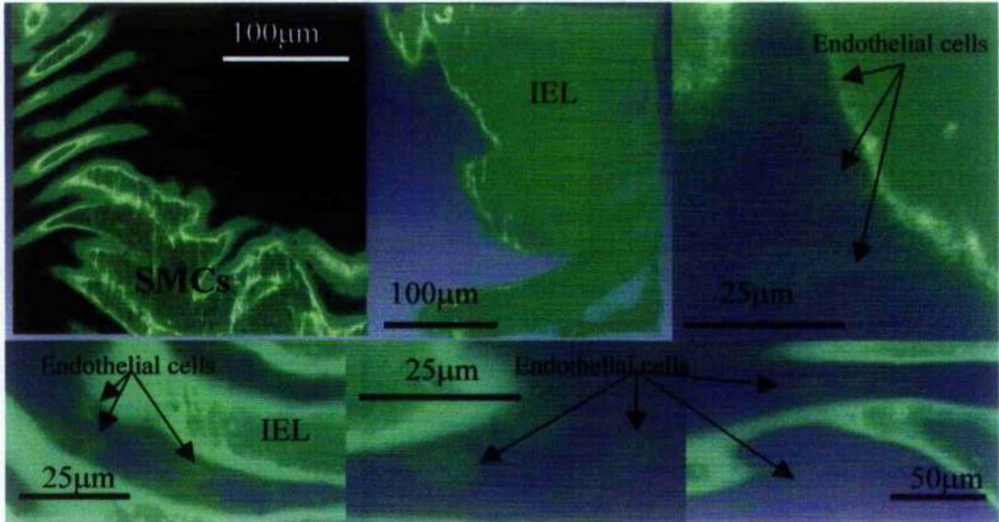


Image 3-9: Control-Four months α_{1B}-Knockout mouse aorta, stained with QAPB (0.1µM). Both the Endothelial and Smooth Muscle Cells (SMCs) stained perfectly (n=3).

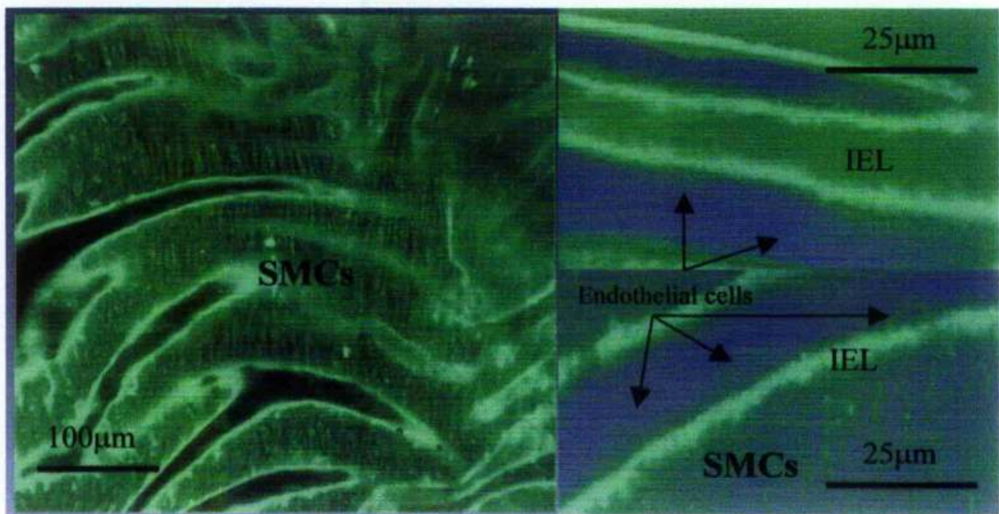


Image 3-10: Only BMY7378 (0.1µM) {α_{1D}-AR antagonist} pretreatment, on four months α_{1B}-Knockout mouse aorta, stained with QAPB (0.1µM). Both the Endothelial and Smooth Muscle Cells stained perfectly. Remaining QAPB staining should be α_{1A}-AR or α_2-ARs. Also we already have shown using D79N mice that one of the α_2-ARs present in mouse aorta endothelium is $\alpha_{2\,A/D}$ -AR (n=3).

LSCM method details: Excitation wavelength= 488nm, Barrier= 515nm, Pixel * Lines= 512 * 512, Objective= X40 OIL, Speed: 166-500 IPS, Gain=15 (<30), Iris= 1.5, Laser Power= 50%, Step= 0.35µm

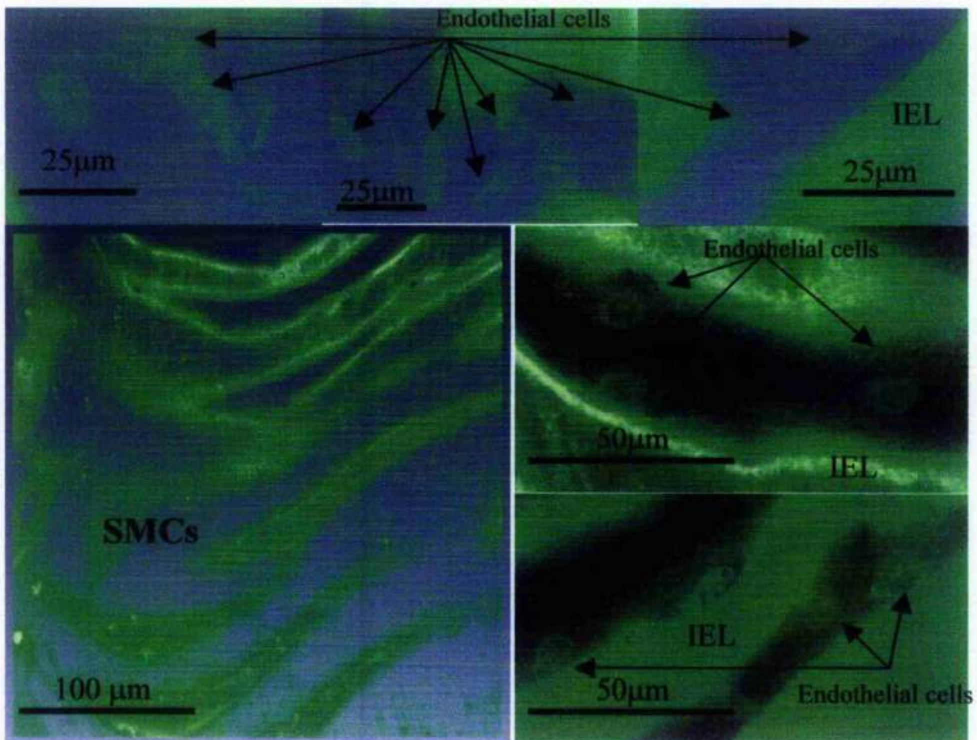


Image 3-11: BMY7378 (0.1μM) {α_{1D}-antagonist} and 5MU (0.1μM) {α_{1A}-antagonist}, on four months α_{1B}-Knockout mouse aorta, stained with QAPB (0.1μM). Both the Endothelial and Smooth Muscle Cells are stained less than in the control mice . Since α_{1B}-AR expression is absent in α_{1B}-KO mouse, all α_1-AR is eliminated and the remaining QAPB staining indicates α_2-AR (n=3).

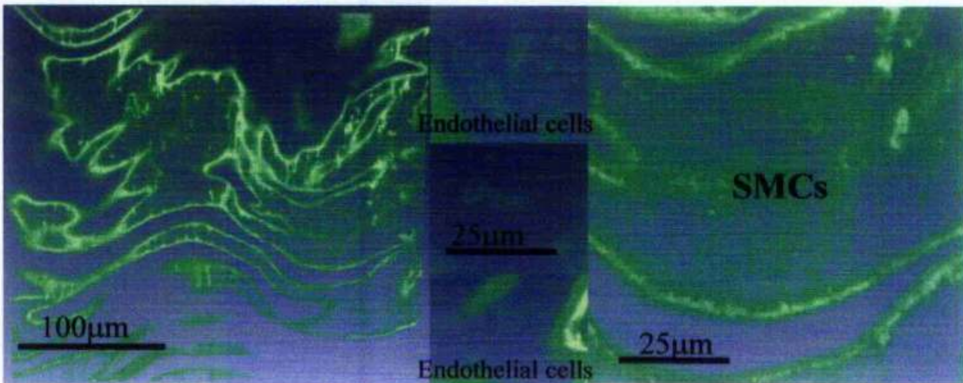


Image 3-12: Only Rauwolscine (0.1μM) {α2-AR antagonist}, on four months α_{1B}-Knockout mouse aorta, stained by QAPB (0.1μM). Green in both Endothelial and Smooth Muscle Cells shows α_1-AR expression in both cell types. However, there is a reduction in QAPB-Binding overall compared with control and BMY7378 treated cases (n=3).

LSCM method details: Excitation wavelength= 488nm, Barrier= 515nm, Pixel * Lines= 512 * 512, Objective= X40 OIL, Speed: 166-500 IPS, Gain=15 (<30), Iris= 1.5, Laser Power= 50%, Step= 0.35μm

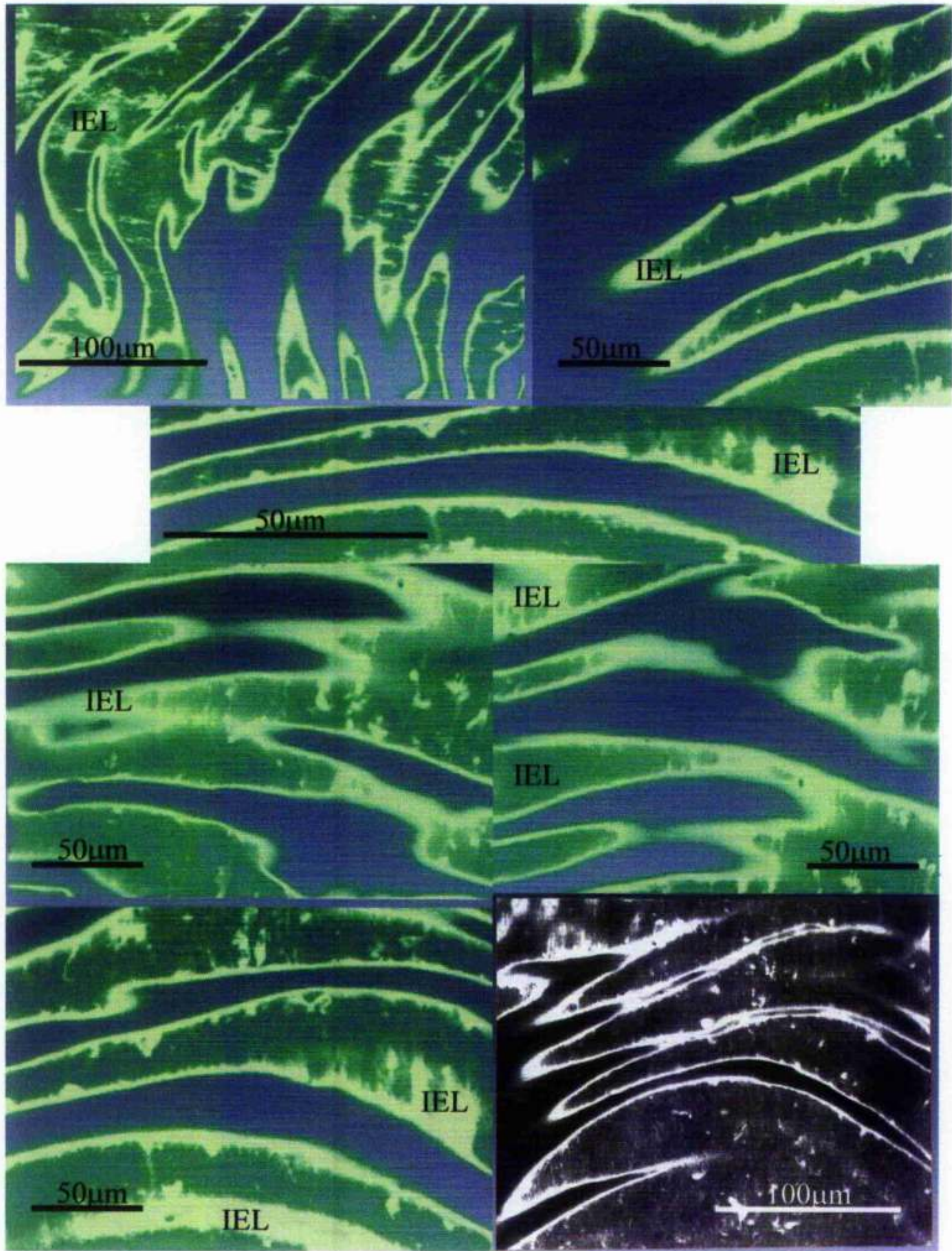


Image 3-13: BMY7378 (0.1μM) {α_{1D}-antagonist} and 5MU (0.1μM) {α_{1A}-antagonist} and Rauwolscine (0.1μM) {α_2-AR antagonist} together on four months α_{1B}-Knockout mouse aorta, stained with QAPB (0.1μM). All the endothelial cells have disappeared through the treatment with antagonist drugs. The characteristic lines of punctuate staining in smooth muscle cells is also lost indicating that this combination of antagonist ligands is validated as removing all α-AR when α_{1B}-AR are absent (n=3).

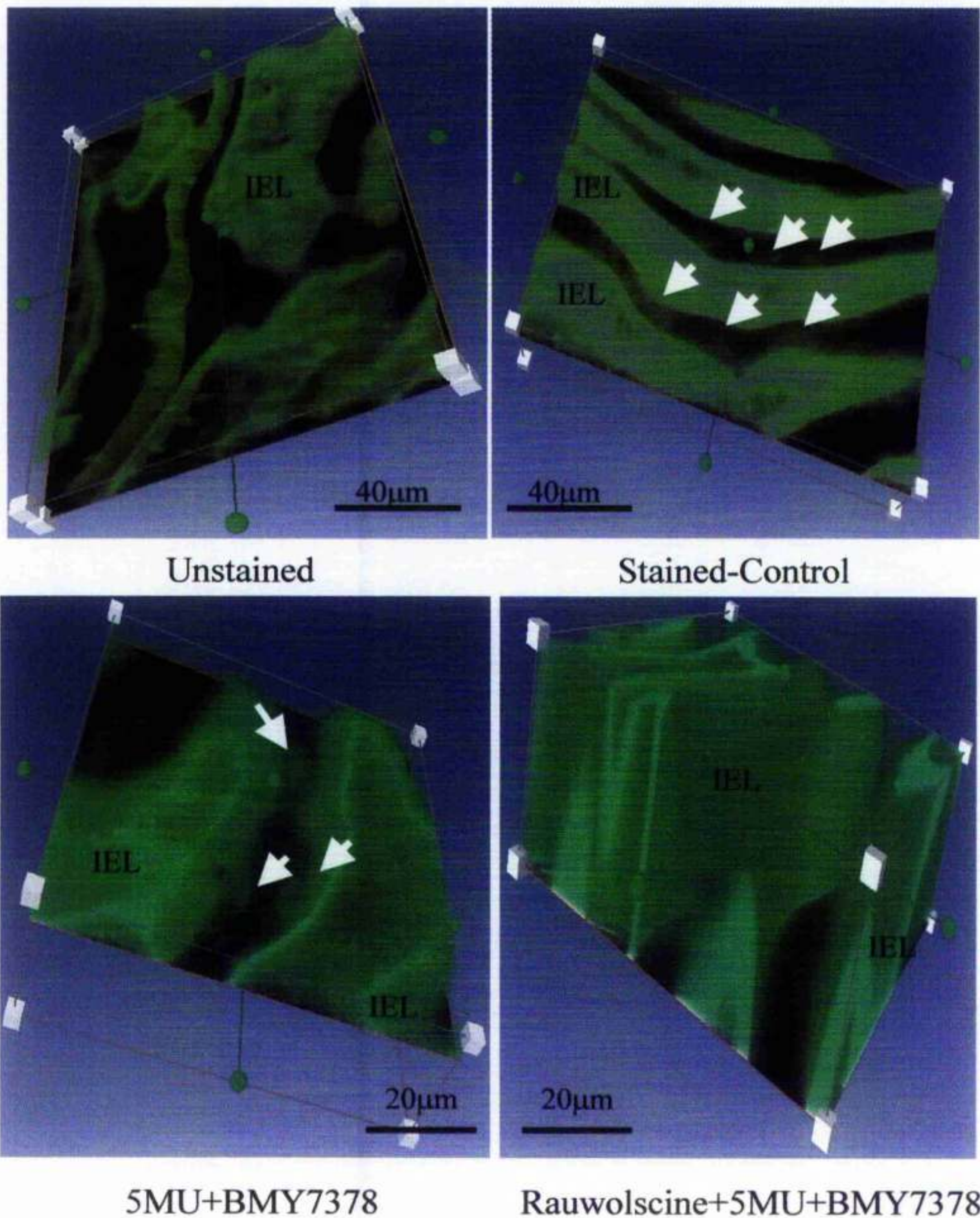


Image 3-14: Summary of 3D Orthoslice and Voltex together in four months α_{1B}-Knockout mouse aorta. Unstained control (top left) shows elastin autofluorescence. White arrows indicate QAPB binding to endothelial cells in control (top right). All staining disappears after treatment with Rauwolscine (0.1μM), 5MU (0.1μM) and BMY7378 (0.1μM) (bottom right). When only α_1-AR antagonists are present (bottom left) endothelial binding remains indicating the presence of α_2-AR (n=3).

LSCM method details: Excitation wavelength= 488nm, Barrier= 515nm, Pixel * Lines= 512 * 512, Objective= X40 OIL, Speed: 166-500 IPS, Gain=15 (<30), Iris= 1.5, Laser Power= 50%, Step= 0.35μm

LSCM on α_{2A}-Knockout Mouse Aorta

Opened lumen, live aorta tissues stained with QAPB (0.1μM) after treatment by BMY7378 (0.1μM), 5MU (0.1μM), Rauwolscine (0.1μM) alone and together compared with unstained (autofluorescence).

LSCM method details: Excitation wavelength = 488nm, Barrier = 515nm, Pixel * Lines = 512 * 512 (1μm = 1.77 Pixel) Objective = X40 OIL, Speed: 166-500 IPS, Gain =15, Iris = 1.5, Laser Power = 50%, Step = 0.35μm

All the z-series images developed in Metamorph (Version 4.2) and made as 3D images in Amira (Version 3.2) software.

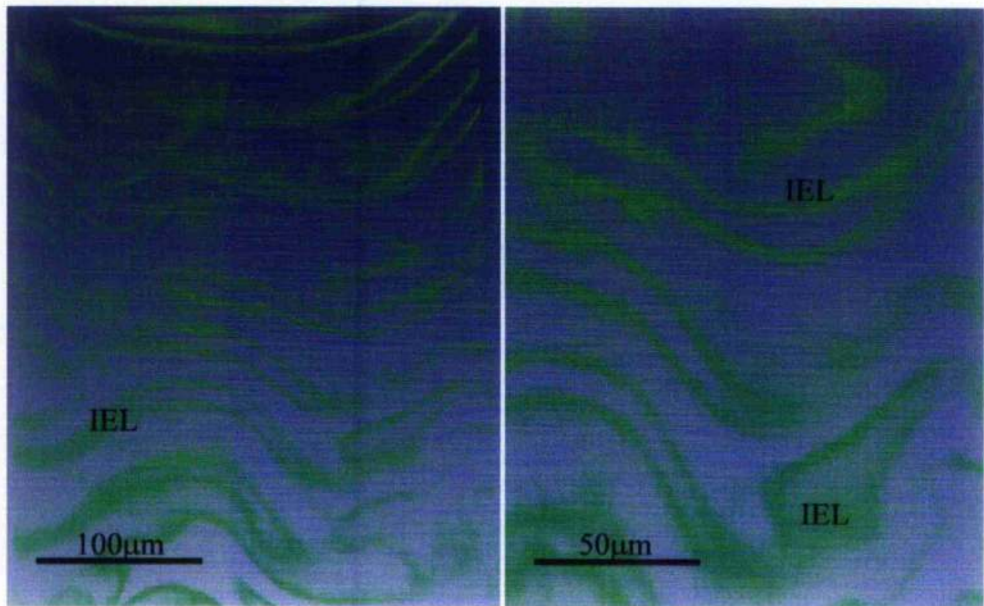


Image 3-15: Unstained α_{2A}-Knockout mouse aorta (Only Autofluorescent) (n=3).

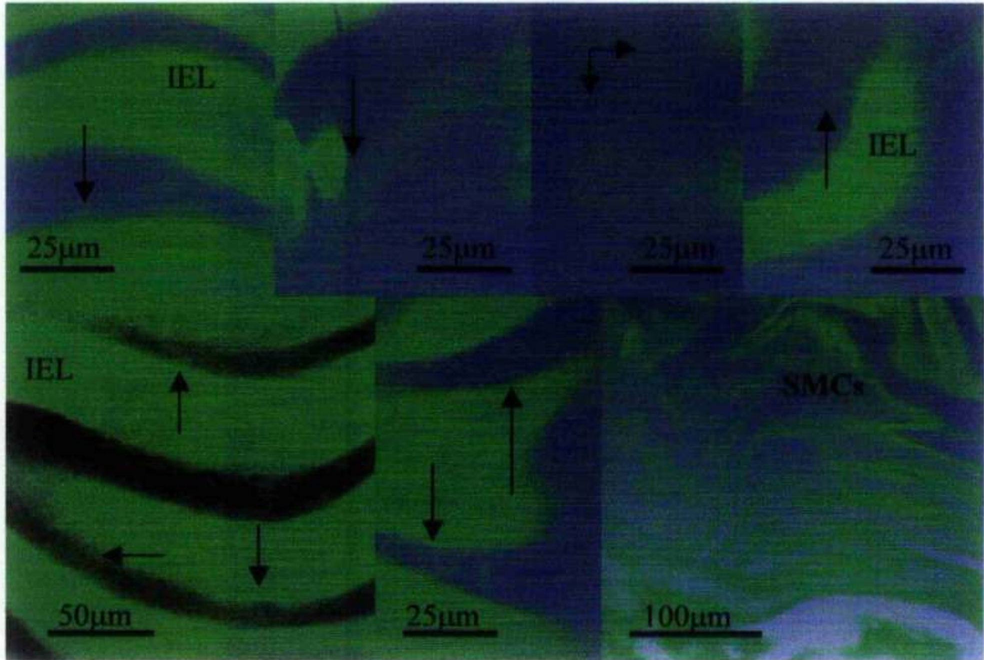


Image 3-16: Control-Four months α_{2A}-Knockout mouse aorta, stained with QAPB (0.1μM). Both the Endothelial (arrows) and smooth muscle cells are poorly stained (n=3).

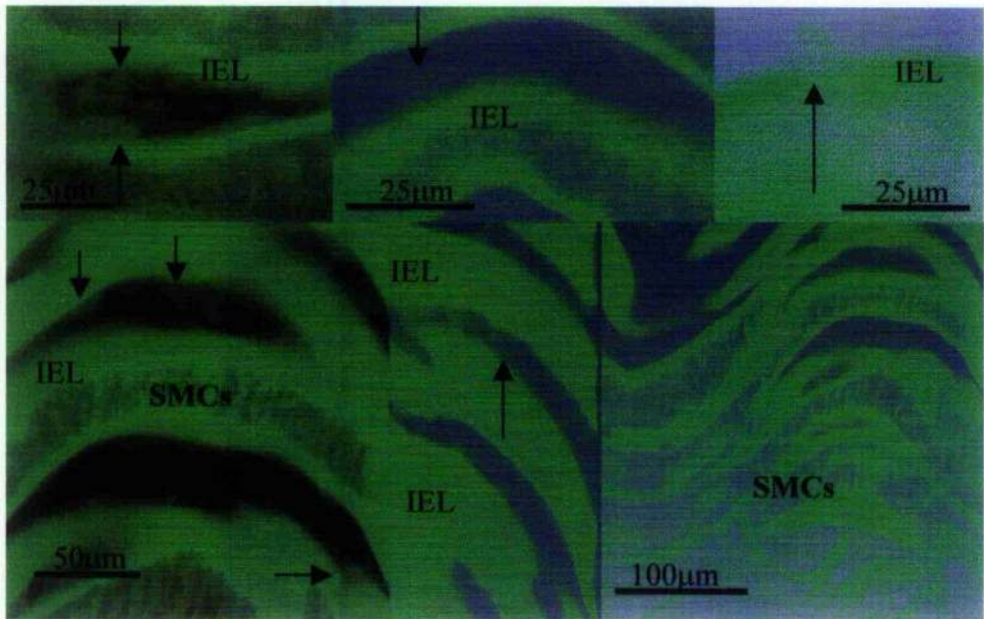


Image 3-17: Only Rauwolscine (0.1μM) {α_2-AR antagonist}, on α_{2A}-Knockout mouse aorta, stained by QAPB (0.1μM). Green light in Both Endothelial (arrows) and Smooth Muscle Cells shows α_1-AR expression in both cell types (n=3).

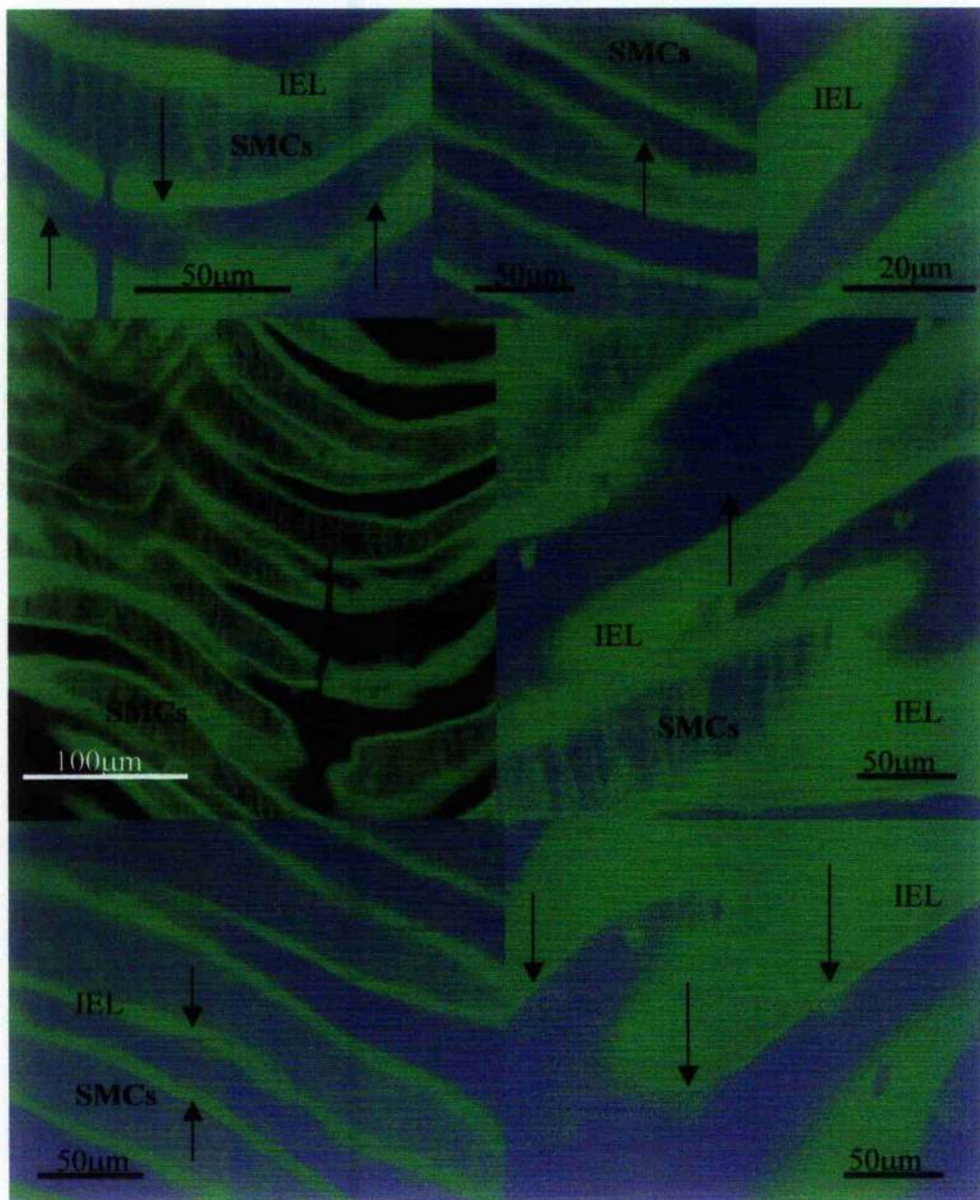


Image 3-18: BMY7378 (0.1µM) {α_{1D}-antagonist} and 5MU (0.1µM) {α_{1A}-antagonist}, together on α_{2A}-Knockout mouse aorta, stained with QAPB (0.1µM). Some of the Endothelial cells (arrows) stained suggesting that the remaining QAPB-binding may be α_2-AR or α_{1B}-AR. In smooth muscle cells QAPB intensity reveals the presence of α_{1B}-AR or α_2-AR (n=3).

LSCM method details: Excitation wavelength= 488nm, Barrier= 515nm, Pixel * Lines= 512 * 512, Objective= X40 OIL, Speed: 166-500 IPS, Gain=15 (<30), Iris= 1.5, Laser Power= 50%, Step= 0.35µm

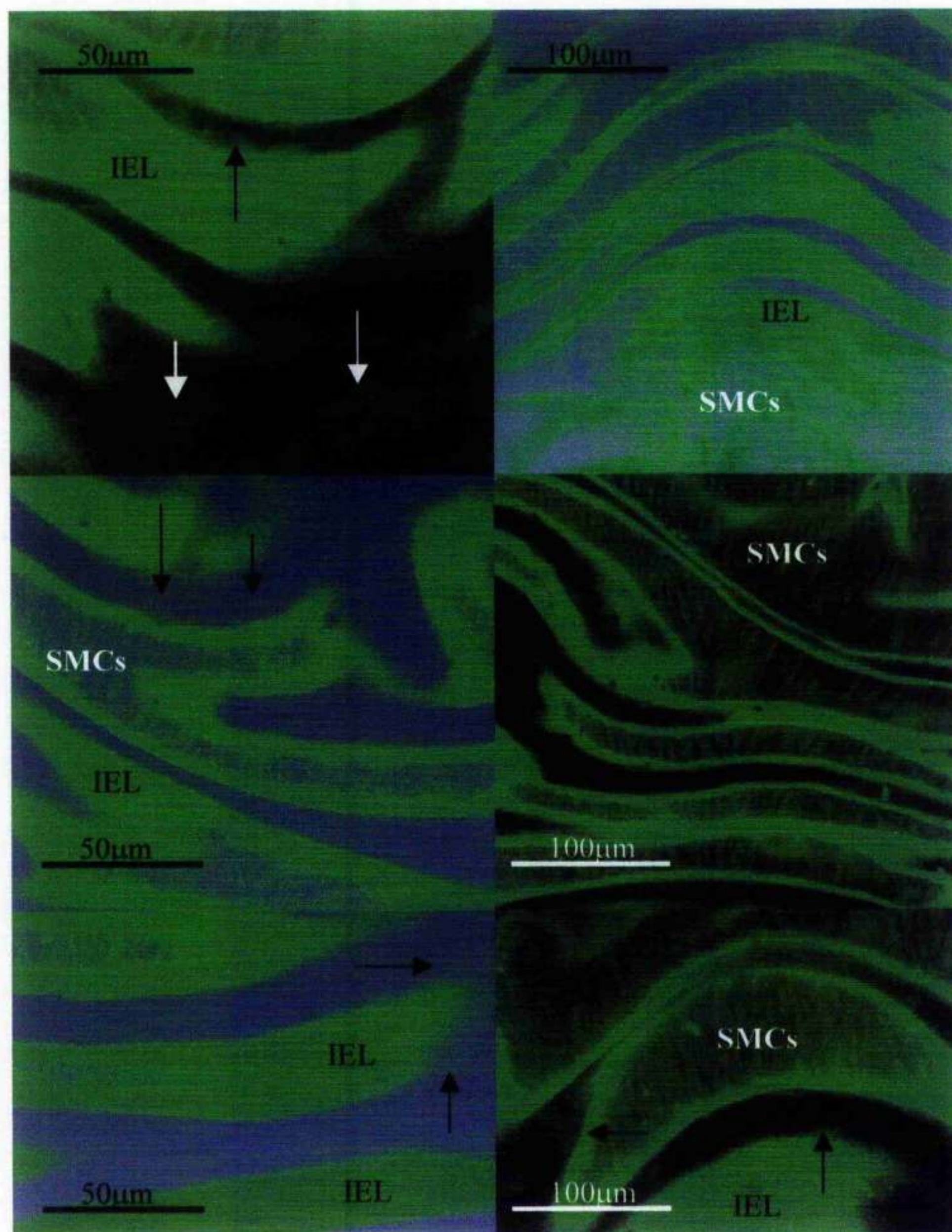


Image 3-19: BMY7378 (0.1µM) {α_{1D}-antagonist} and 5MU (0.1µM) {α_{1A}-antagonist} and Rauwolscine (0.1µM) {α_2-AR antagonist} on α_{2A}-Knockout mouse aorta, stained with QAPB (0.1µM). A majority of the Endothelial cells have disappeared through the treatment with antagonist drugs. However, some Endothelial cells still show QAPB-binding (arrows). This is likely to indicate α_{1B}-AR in this knockout mouse: compare with Image 3-13 in which the same combination of antagonists eliminates binding when the α_{1B}-AR has been knocked out. Smooth Muscle Cells are stained with QAPB, again probably due to α_{1B}-AR in smooth muscle cells (n=3).

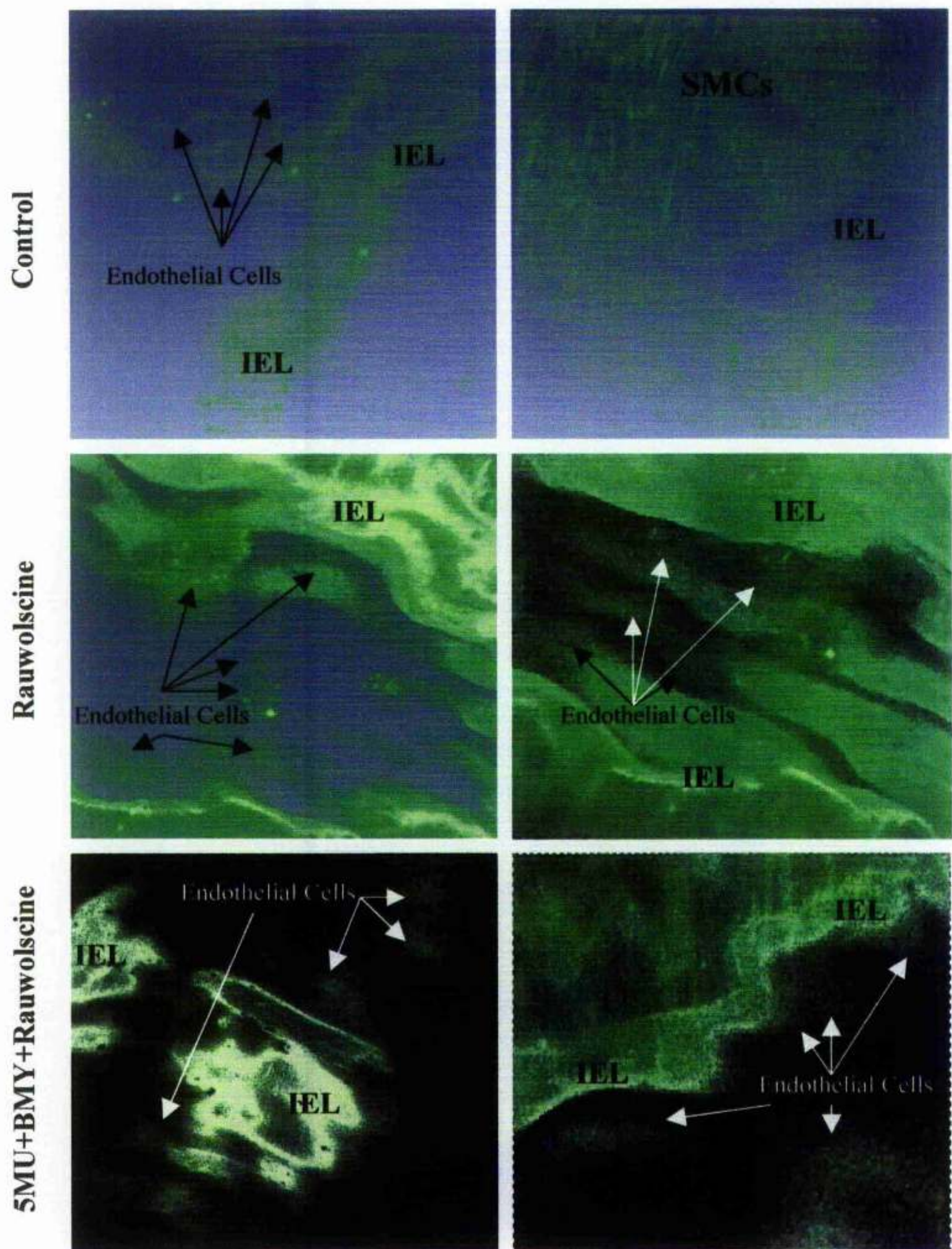


Image 3-20: Four months wild type mouse superior (main) mesenteric artery, stained with QAPB (0.1μM).

First row: **control**

Second row: **Treated with Rauwolscine (0.1μM).**

Third row: **Treated with 5MU (0.1μM), BMY7378 (0.1μM) and Rauwolscine (0.1μM).**

Antagonists affected on intact tissues one hour before staining. Endothelial cells appeared in all three raw (arrows). Second raw shows presence of α-ARs on endothelial cells. Third raw suggests remaining QAPB intensity is due to α_{1B}-AR on mouse endothelial cells (n=5).

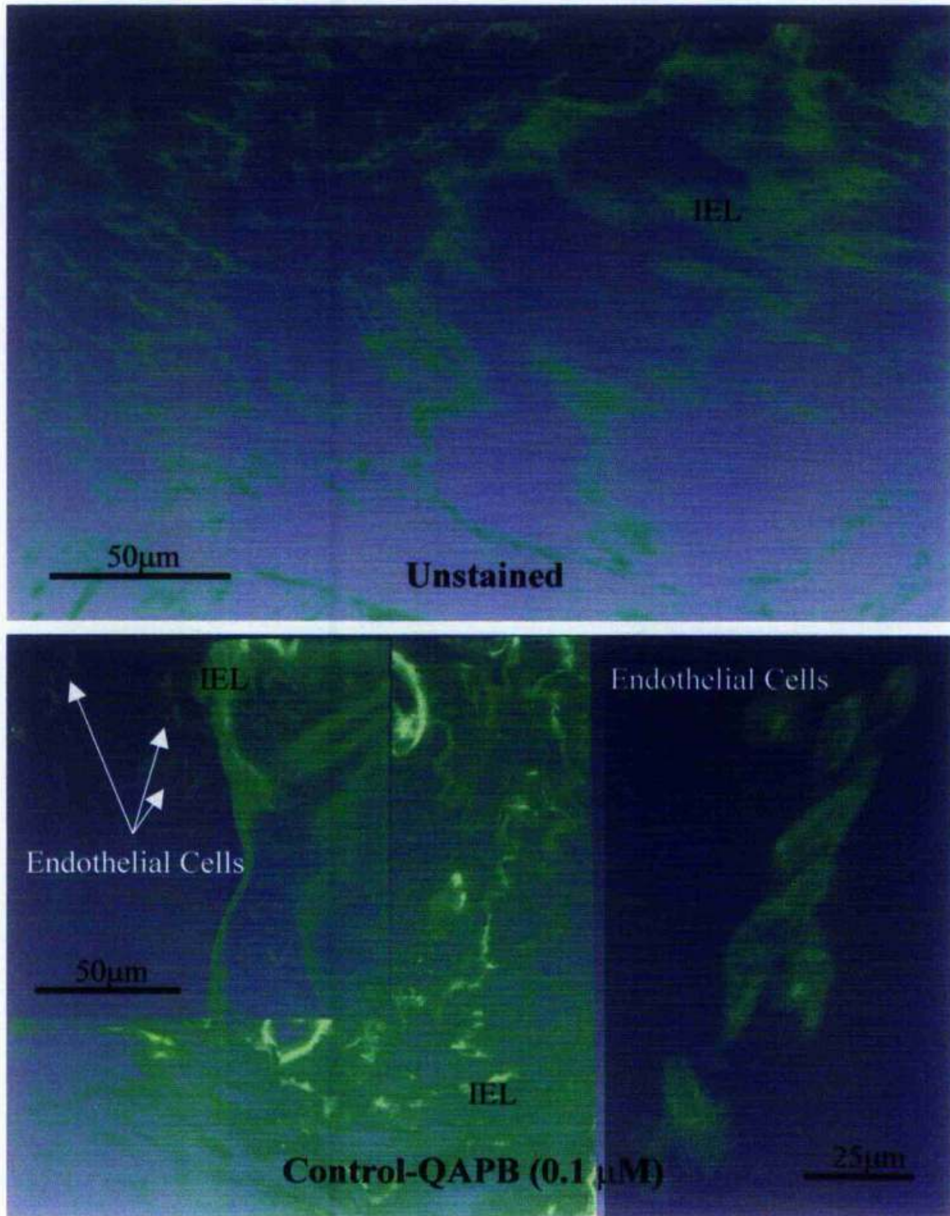


Image 3-21: Control-Four months α_{1B}-KO mouse superior mesenteric artery QAPB-binding present in Endothelial Cells (n=3).

LSCM method details: Excitation wavelength= 488nm, Barrier= 515nm, Pixel *Lines= 512 * 512, Objective= X40 OIL, Speed: 166-500 IPS, Gain=15 (<30), Iris= 1.5, Laser Power= 50%, Step= 0.35μm

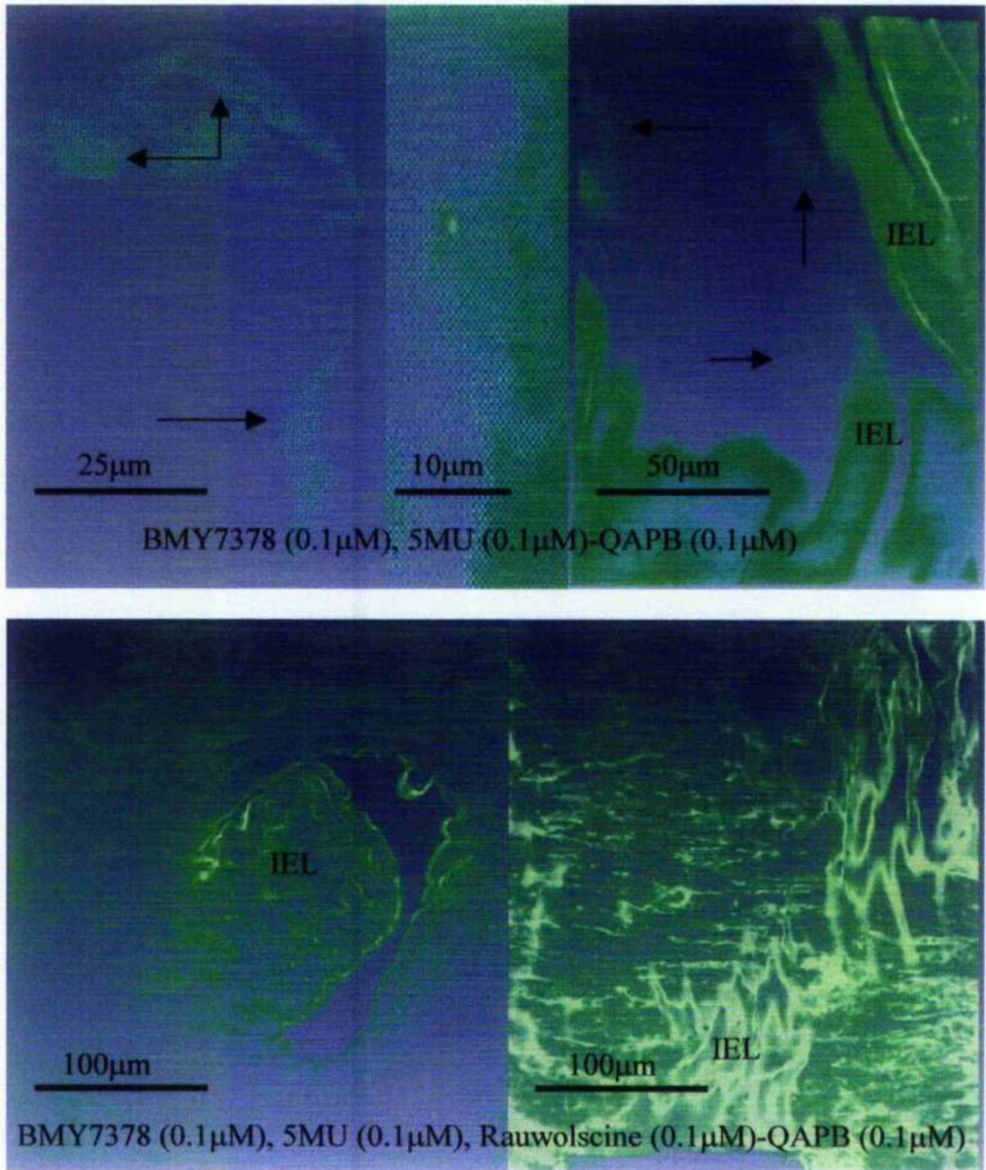


Image 3-22: Four months α_{1B}-KO mouse superior mesenteric artery. The endothelial cells (arrows) show QAPB-binding in the presence of α_1-AR antagonists (upper panels) but in the additional presence of rauwolscine, endothelial binding disappears (lower panels).

QAPB (0.1μM) after treatment by BMY7378 (0.1μM), 5MU (0.1μM) and/or Rauwolscine (0.1μM) (n=3).

LSCM on α_{2A}-Knockout Mouse Superior Mesenteric Artery

Opened lumen, live superior mesenteric artery tissues stained with QAPB (0.1μM) after treatment by BMY7378 (0.1μM), 5MU (0.1μM), Rauwlscine (0.1μM) alone and together compared with unstained (autofluorescence).

LSCM method details: Excitation wavelength = 488nm, Barrier = 515nm, Pixel * Lines = 512 * 512 (1μm = 1.77 Pixel) Objective = X40 OIL, Speed: 166-500 IPS, Gain =15, Iris = 1.5, Laser Power = 50%, Step = 0.35μm

All the z-series images developed in Metamorph (Version 4.2) and made as 3D images in Amira (Version 3.2) software.

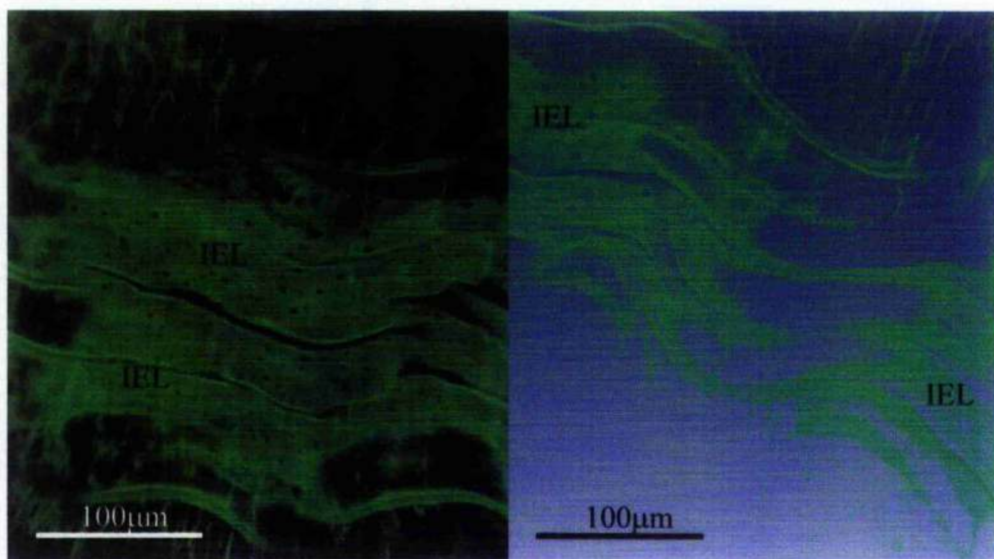


Image 3-23: Unstained α_{2A}-Knockout mouse superior mesenteric artery. Internal Elastic Lamina (IEL) autofluorescence (n=3).

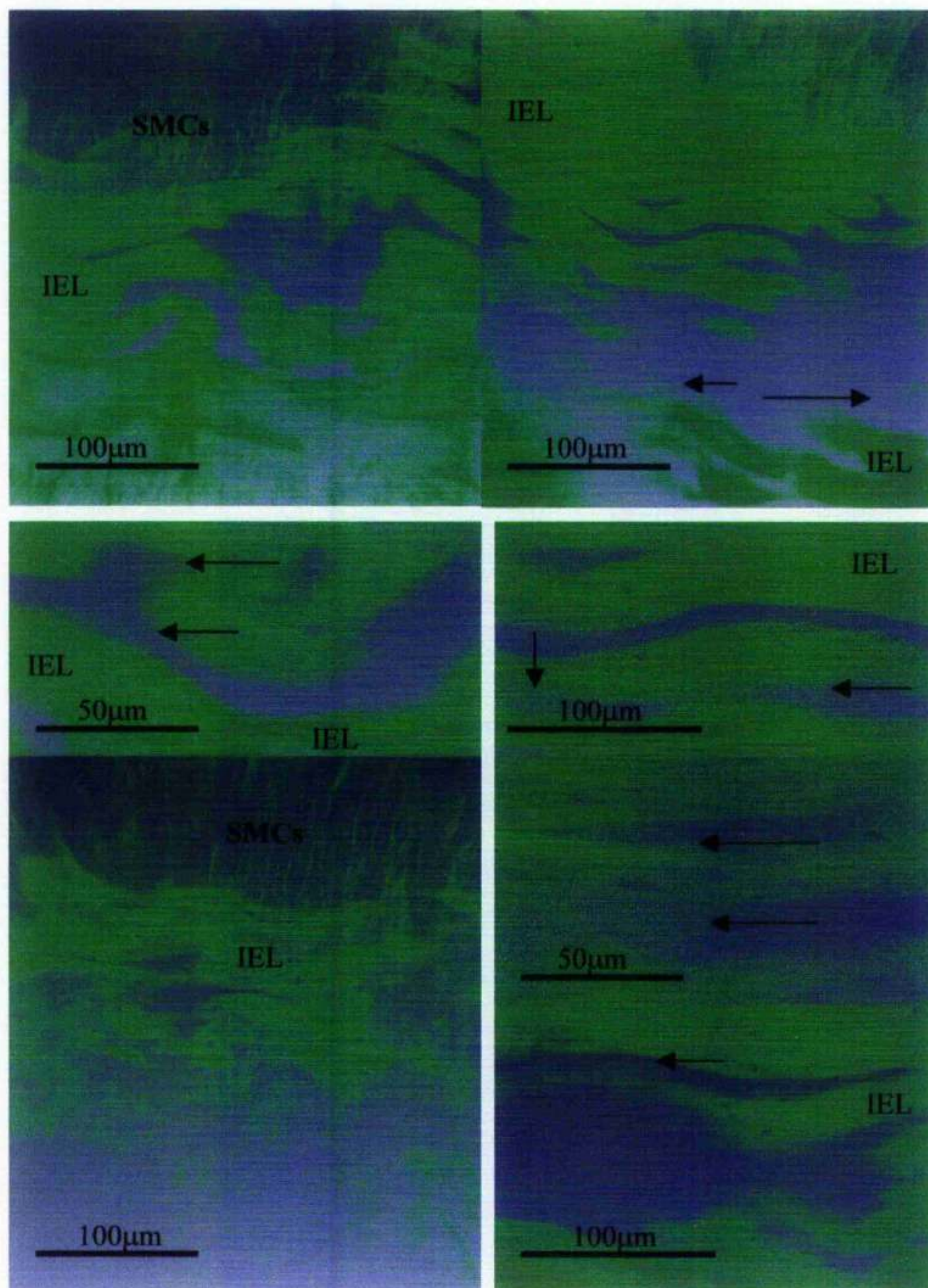


Image 3-24: Control-Four months α_{2A}-Knockout mouse superior mesenteric artery QAPB-binding present in the Endothelial Cells (arrows) (n=3).

LSCM method details: Excitation wavelength= 488nm, Barrier= 515nm, Pixel * Lines= 512 * 512, Objective= X40 OIL, Speed: 166-500 IPS, Gain=15 (<30), Iris= 1.5, Laser Power= 50%, Step= 0.35µm

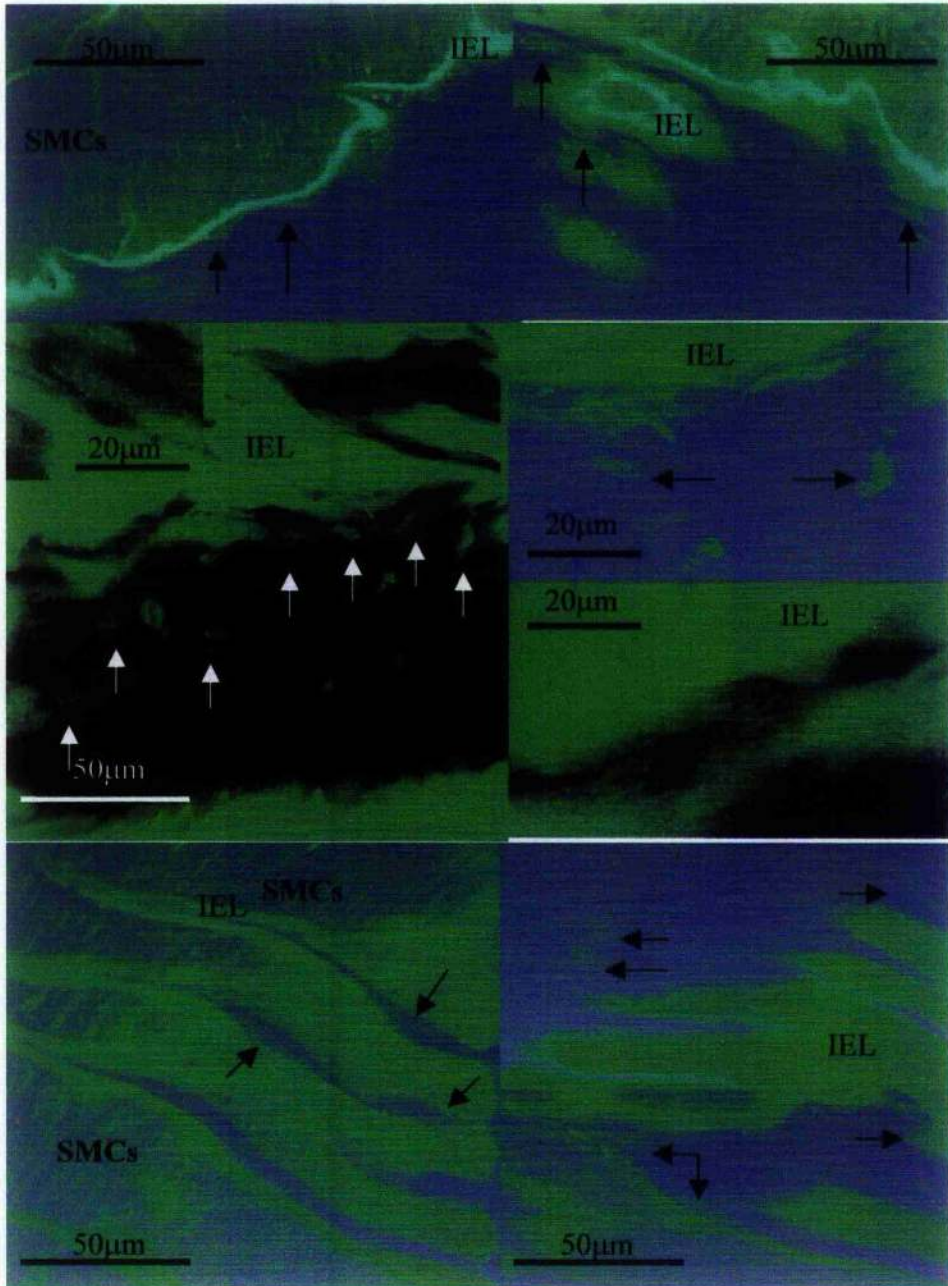


Image 3-25: BMY7378 (0.1μM) {α_{1D}-antagonist} and 5MU (0.1μM) {α_{1A}-antagonist}, on α_{2A}-Knockout superior mesenteric artery, stained with QAPB (0.1μM), still plenty of endothelial cells stained. This suggests that remaining QAPB-binding is α_2-AR or α_{1B}-AR in endothelium. In Smooth Muscle Cells QAPB intensity reveals also presence of α_{1B}-AR or α_2-AR (n=3).

LSCM method details: Excitation wavelength= 488nm, Barrier= 515nm, Pixel * Lines= 512 * 512, Objective= X40 OIL, Speed: 166-500 IPS, Gain=15 (<30), Iris= 1.5, Laser Power= 50%, Step= 0.35μm

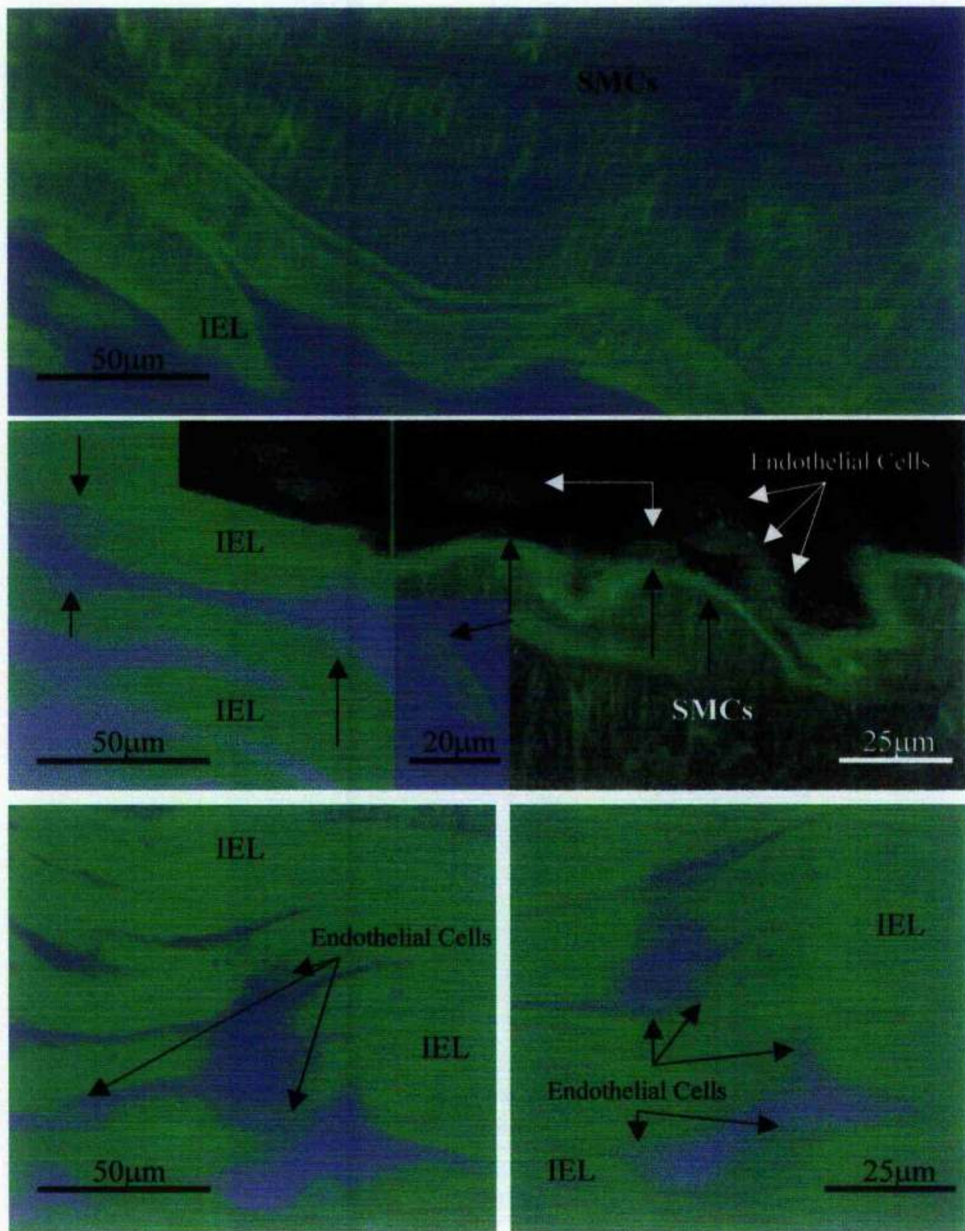


Image 3-26: Only Rauwolscine (0.1μM) {α_2-AR antagonist}, on α_{2A}-Knockout superior mesenteric artery, stained by QAPB (0.1μM). Green light in both Endothelial (arrows) and Smooth Muscle Cells shows α_1-AR expression in these cells (n=3).

LSCM method details: Excitation wavelength= 488nm, Barrier= 515nm, Pixel * Lines= 512 * 512, Objective= X40 OIL, Speed: 166-500 IPS, Gain=15 (<30), Iris= 1.5, Laser Power= 50%, Step= 0.35μm

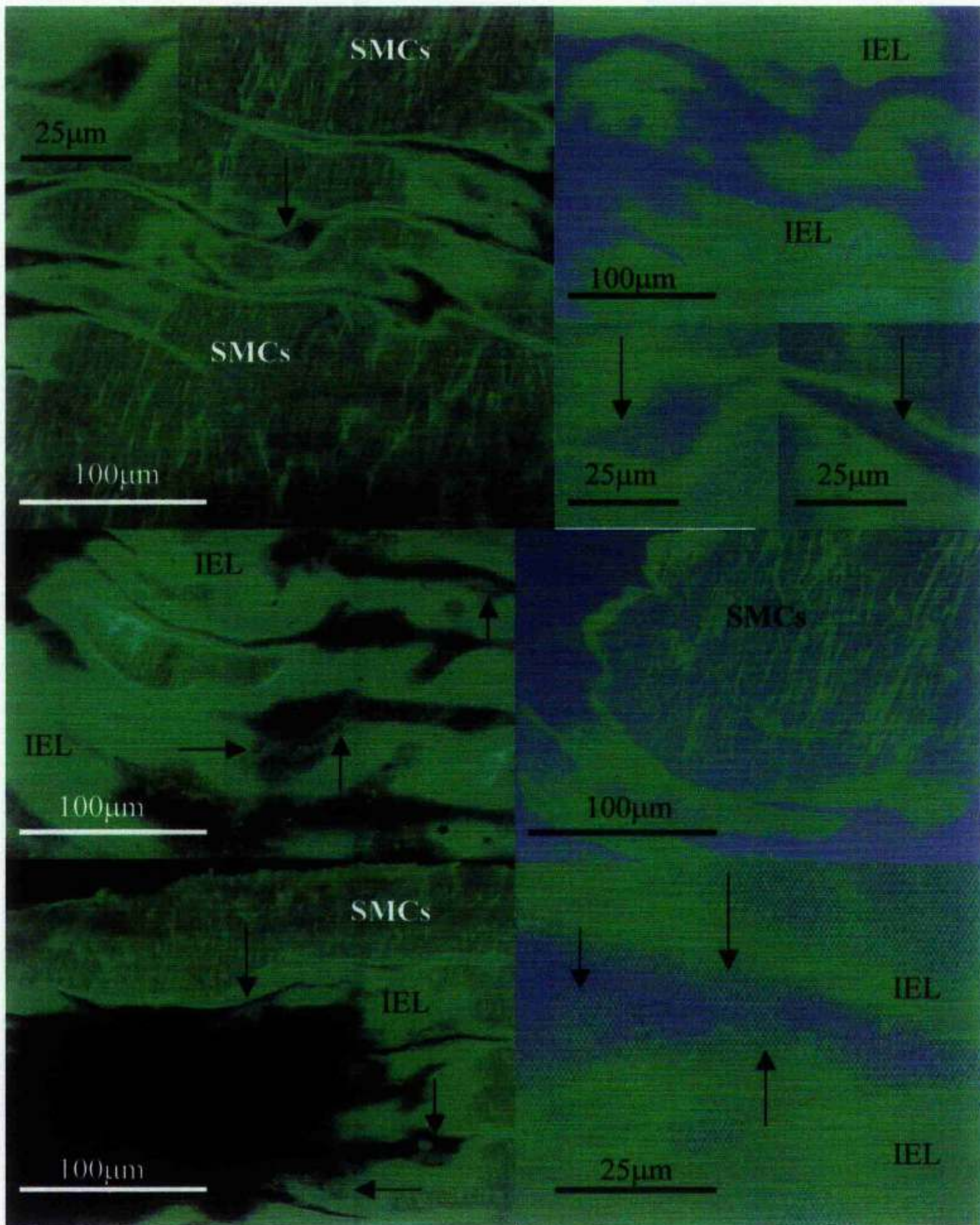


Image 3-27: BMY7378 (0.1μM) {α_{1D}-antagonist} and 5MU (0.1μM) {α_{1A}-antagonist} and Rauwolscine (0.1μM) {α_2-AR antagonist} on α_{2A}-Knockout superior mesenteric artery, stained with QAPB (0.1μM). In contrast to the α_{1B}-KO mouse (image 3-20), the endothelial cells still appear after treatment with antagonist drugs indicating the presence of α_{1B}-AR in the endothelium of this vessel. Smooth muscle cells stained with QAPB again probably due to α_{1B}-AR (n=3).

3-5. Discussion:

α_2-adrenoceptor-activated vasodilator responses:

We have demonstrated an α_2-adrenoceptor-activated, endothelium/NO mediated vasodilator response in the aorta, carotid artery and mesenteric arteries in the main mammalian model organism, the mouse. This is an important observation because knowledge of this phenomenon has been scanty and regarded as "species-related". The ability to focus on this phenomenon in the mouse and other model species should accelerate appreciation of its role in man.

The demonstration and analysis of vasodilator responses via α_2-adrenoceptors proved straightforward in wire myograph-mounted, pre-constricted arteries from young adult mice. Aorta, carotid and mesenteric arteries showed rauwolscine-sensitive relaxant responses to UK14304 as their most sensitive response. Subsequent analysis in aorta and carotid showed that this represented the vasodilator phenotype of the $\alpha_{2A/D}$-adrenoceptor involving endothelium-mediated release of nitric oxide. In mesenteric arteries, however, resistance to the knockout or the D79N mutation of the $\alpha_{2A/D}$-AR points to another subtype of α_2-adrenoceptor.

The carotid artery proved the most straightforward since it showed only relaxant responses to UK14304 and these were susceptible to rauwolscine, to knockout or to mutation and to blockade of NOS, indicating an $\alpha_{2A/D}$-adrenoceptor-mediated release of nitric oxide and no other obvious action. All other arteries showed some variation from this that could be accounted for by activation of other adrenoceptors but which, taken in isolation, would confuse the picture.

In the aorta there was a contractile response to UK14304 at a higher concentration range. This response was sensitive to knockout of the α_{1D}-adrenoceptor indicating it to be mediated by the α_{1D}-adrenoceptor, the dominant contractile adrenoceptor in this vessel (Yamamoto & Koike, 2001; Tanoue *et al.*, 2002; Daly *et al.*, 2002). Agonists that are generally considered to be "α_2-adrenoceptor -selective" are often partial agonists at α_1-adrenoceptors; this has been reported in large arteries in the rat (Martin *et al.*, 1986). This reinforces the conclusion of Vandeputte et al (2003) that in mouse aorta constrictor α_{1D}- and dilator $\alpha_{2A/D}$- adrenoceptors act in opposition. Like aorta the carotid has dominant contractile α_{1D}-adrenoceptors in rat (Hussain & Marshall, 1997) and mouse (Daly *et al.*, 2002) but the receptor reserve is lower than in aorta, as shown by its lower sensitivity to α_1-adrenoceptor agonists. Therefore, as an α_1-adrenoceptor partial agonist, UK14304 will occupy and activate α_{1D}-adrenoceptors in these vessels but it will not generate a sufficient signal to cause contraction; in the $\alpha_{2A/D}$-KO, however, even the carotid showed a small contraction to UK14304 once the endothelial inhibitory influence was removed.

The experimental objectives of this study were met equally well by the $\alpha_{2A/D}$-KO and D79N mice. The former is a straightforward "knockout" in which the receptors will not be expressed while the latter produces a mutated version of the $\alpha_{2A/D}$-adrenoceptor that has been shown to produce phenotypes that carry, in effect, a functional $\alpha_{2A/D}$-adrenoceptor knockout due both to poor functionality of the mutated receptor and its low expression levels (MacMillan *et al.*, 1998). Both eliminated the α_2-adrenoceptor-mediated vasodilatation in aorta and carotid but not in the two mesenteric artery preparations. This produces definitive evidence that the endothelial α_2-adrenoceptor response is mediated via the $\alpha_{2A/D}$-adrenoceptor and validates the tentative pharmacological analysis in rat and pig (Bockman *et al.*, 1993; 1996) that the α_2-

adrenoceptor-mediated endothelial response, first demonstrated by Cocks & Angus (1983), is mediated by the $\alpha_{2A/D}$-adrenoceptor subtype. However, the $\alpha_{2A/D}$-adrenoceptor was not the only subtype mediating such responses when further vessels were investigated.

The main mesenteric artery and its first branch showed clear rauwolscine-sensitive relaxant responses to UK14304 that were present in the $\alpha_{2A/D}$-KO and D79N mice leaving the subtype unclassified, but either α_{2B}- or α_{2C}- adrenoceptors. This is novel. In the published work on the blood pressure of conscious mice with knockouts of the three subtypes, only elimination of the $\alpha_{2A/D}$-adrenoceptors caused the loss of the vasodepressor action of clonidine (Link *et al.*, 1996; MacMillan *et al.*, 1996). However an endothelial vasodilatation to α_{2B}-adrenoceptors or α_{2C}-adrenoceptors might be submerged within the overall blood pressure response to countervailing pressor and depressor influences of α_2-adrenoceptor agonists. Mesenteric vessels in rat are the most commonly employed models of vascular resistance and this is likely to be repeated in mouse models so the observation of a possible non-$\alpha_{2A/D}$-adrenoceptor-mediated vasodilatation is significant.

There were subtle differences in the responses found in the two strains which may repay further investigation but lie outside our current objectives, viz. in aorta, the $\alpha_{2A/D}$-KO and D79N mice both lost the relaxant effects of UK14304, but the contractile response to UK14304 that survived had a different concentration-response relationship in the two strains; this was reflected also in the carotid which showed a small contraction only in the $\alpha_{2A/D}$-KO; in the first branch mesenteric artery, compared with aorta, carotid and main mesenteric arteries vasodilator responses to UK14304 were slightly bigger.

In contrast to the vasodilator effect, we found no evidence for a contractile α_2-adrenoceptor in mouse aorta, carotid, main mesenteric or first branch mesenteric

arteries. The literature in other species shows that α_2-adrenoceptor-mediated vasopressor responses are easily demonstrated *in vivo* (Docherty & McGrath, 1980) but are difficult to show *in vitro*, particularly in large conducting arteries (McGrath *et al.*, 1989). This is now reinforced and extended in the mouse. In the rat the clearest example of a vascular α_2-adrenoceptor-mediated contraction *in vitro* is in the tail artery (Medgett, 1995; Templeton *et al.*, 1989; Xiao & Rand, 1989) and this applies also to the mouse (Chotani *et al.*, 2000, McBride *et al.*, 2002). The few examples in other species include vessels such as saphenous artery and vein and ear vein (Demey & Vanhoutte, 1981; Daly *et al.*, 1988) for which we have, so far, not identified technically feasible counterparts in the mouse.

Localisation of α_2-adrenoceptors:

The visualisation of a fluorescent ligand binding to endothelial cells and its competitive elimination by the α_2-adrenoceptor-antagonist rauwolscine provides compelling direct evidence for the endothelial location of the α_2-adrenoceptors that mediate vasodilatation. The existence of myoendothelial connections places doubt on whether a smooth muscle response that depends upon endothelium is actually initiated there since depolarisation of smooth muscle cells might influence the endothelium (Dora *et al.*, 2000) (Oishi *et al.*, 2001). Until now the vascular localisation of α_2-adrenoceptors has relied on low resolution autoradiography that indicated the presence of receptors in smooth muscle but not endothelium, a finding repeated even for acetylcholine (Stephenson *et al.*, 1988). Our present study shows direct proof of α_2-adrenoceptor binding sites on endothelial cells. Thus location and function are in accord.

Relevance of vascular endothelial α_2-adrenoceptors:

These results show that the blood pressure lowering effect of α_2-adrenoceptor agonists should include a peripheral endothelium/nitric oxide-mediated direct vasodilatation in addition to any effects on sympathetic nerve traffic or post-ganglionic transmission. Until now the blood pressure lowering effect of α_2-adrenoceptor agonists, such as clonidine, has been ascribed entirely to action at neuronal α_2-adrenoceptors, either those in the CNS regulating sympathetic nervous system output or pre-junctional receptors on peripheral sympathetic post-ganglionic terminals. Deletion or mutation of the $\alpha_{2A/D}$-adrenoceptors results in the loss of the falls in both heart rate and blood pressure caused by intravenous administration of α_2-adrenoceptor agonists such as UK14304 and clonidine in the conscious mouse (MacMillan *et al.*, 1996; Altman *et al.*, 1999). It has been assumed that both arise entirely from withdrawal of sympathetic tone. The present data shows that these agonists produce vasodilatation via endothelial α_2-adrenoceptors. In major conducting arteries, such as aorta and carotid this would successively increase vascular compliance, reduce systolic pressure, reduce afterload, reduce cardiac output and, hence, lower mean arterial blood pressure. In resistance arteries, as represented by the mesenteric first branch artery, a straightforward hypotensive action through reduced peripheral resistance could be expected. It should, therefore, be expected that the vasodepressor action of α_2-adrenoceptor activation would be a combination of endothelial activation and sympathoinhibition.

This is important since activation of endothelial relaxant and hypotrophic factors is another distinction between the mechanisms of action of α_2-adrenoceptor agonists and beta blockers at the various level. This might confer advantages in the treatment of particular cardiovascular diseases since activation of endothelial α_2-AR would cause

vasodilatation that is independent of sympathetic tone. In contrast β-blockers will attenuate of β-adrenoceptor mediated vasodilatation leading to vasoconstriction.

Vascular endothelial or smooth muscle α_2-adrenoceptors may, therefore, be as relevant as are pre-junctional receptors to the hypothesis that α_2-adrenoceptors confer protection from heart failure (Brede *et al.,* 2002). Their correct function may be essential to regulation of blood flow in critical vascular beds. For example, this may be the explanation for the proposed physiological role of nitric oxide released by α_2-adrenoceptors in protecting the rat kidney from excessive adrenergic vasoconstriction (Zou & Cowley, 2000). A range of such countervailing actions may be of great importance for the protection of the specialised functions of many vascular beds in the face of a generalised sympathetic activation in "fight or flight".

The demonstration of vascular α_2-adrenoceptors completes the initial analysis in the mouse of the distribution of functional vascular adrenoceptors from all three families (α_1-, α_2- and β-adrenoceptors). The two best characterised subtypes of β-adrenoceptors (β_1- and β_2-) are distributed heterogeneously throughout the vascular tree, both causing vasodilatation, a finding that contradicts the earlier generalisation that α_1-adrenoceptors dominate in blood vessels (Chruscinski *et al.,* 2001). Vasoconstrictor α_1-adrenoceptors have a dominance of α_{1D}-adrenoceptors in large arteries but of α_{1A}-adrenoceptors in small arteries, while α_{1B}-adrenoceptors play a minor role (remodelling and initiation of contraction) in all vessels (Daly *et al.*, 2002). We now show a sharp phenotypic divide between arteries whose endothelial vasodilator response is mediated by $\alpha_{2A/D}$-adrenoceptors and by other subtypes of α_{2A}-adrenoceptors. A third group, typified by the tail artery displays a dominance of vasoconstrictor α_2-adrenoceptors comprising at least $\alpha_{2A/D}$-adrenoceptors and α_{2C}-adrenoceptors (Chotani *et al.,* 2000; McBride, 2002).

This consolidates the statement of Phillipp et al (2002) that for α_2-adrenoceptor subtypes "one receptor is not enough".

Chapter Four

The multiple action sites of UK14304 at different adrenoceptors.

4-1. Abstract:

1. α_1 and α_2-adrenoceptor (α-AR) responses were studied in mouse aorta. In other species various subtypes from these receptor families mediate effects directly on smooth muscle and indirectly via endothelium. The pharmacological interactions are very complicated so in order to simplify and clarify pharmacological analysis in this study, we used genetically modified Nashville D79N ($\alpha_{2A/D}$-AR mutant-C57BL$_6$), α_{1D}-AR Knockout (129/sv/C57BL$_6$) mice compared with controls (129/sv/C57BL$_6$) {WT}. This enabled us to analyse the rather complicated action of UK14304.

2. Mouse aorta were cut into 2mm rings, mounted on a wire myograph (Mulvany *et al* 1976) and used to acquire single or cumulative concentration response curves to UK14304 in presence and absence of L-NAME.

3. Cumulative concentration response curves to UK14304 without pre-constriction showed a small relaxation, which was followed at higher concentration by a contraction. Damage to the endothelium or L-NAME allowed lower concentrations of UK14304 to cause contraction. L-NAME treatment revealed contraction on which the UK14304 contraction could be superimposed. However the tone was sensitive to BMY7378, suggesting that it is due to an action involving α_{1D}-AR and indicating the presence of constitutively active α_{1D}-AR on smooth muscle cells whose action is revealed by withdrawal of the influence of Nitric Oxide, which in turn would appear to be constitutive. However, in α_{1D}-AR Knockout L-NAME contractile response was smaller. UK14304 could not produce any contractile effect on top of the L-NAME contractile response, and this was sensitive to 5MU but not BMY7378. This revealed also the possible presence of some constitutively active α_{1A}-AR on smooth muscle cells.

UK14304 acted as a partial agonist of α_{1D}-AR, causing weak contraction in high concentration that was absent in the α_{1D}-KO and antagonising α_{1D}-mediated contraction to phenylephrine in both intact and denuded vessels. In the presence of tone UK14304 produced relaxation that was absent in D79N or removal of the endothelium. Thus UK14304 contracts smooth muscle directly via α_{1D}-AR and relaxes smooth muscle via an endothelial effect indirectly.

4. This indicates overall that the mouse aorta has a multiple population of α-adrenoceptors capable of initiating contraction or relaxation and thus it can be used to analyse mechanisms involved with such receptors.

4-2. Introduction:

Adrenoceptors are targeted by the catecholamines adrenaline and noradrenaline. They mediate biological effects in different parts of the body including CNS and cardiovascular system. To date, nine distinct adrenergic receptor subtypes have been cloned from several species: α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, α_{2C}, β_1, β_2 and β_3 (Bylund 1994). For several adrenergic receptors, their precise physiological functions and their therapeutic potential have not been clear. Despite the fact that $\alpha2$-adrenoceptors serve a number of physiological roles in vivo and have great therapeutic potential, no sufficiently subtype-selective ligands are available experimentally and clinically yet. Recently, transgenic and knockout mice have added considerable information about specific function of the three α_2-AR subtypes in mouse models carrying targeted mutation or deletions in the genes encoding for α_2-adrenoceptors (Docherty 1998; Hein 2001; MacDonald 1997; Macmillan 1998; Rohrer and Kobilka 1998).

Distribution of α_1-AR subtypes varies in different parts of the cardiovascular system. According to the literature, there are at least four types of α_1-AR in the cardiovascular system as defined by functional data (α_{1A}, α_{1B}, α_{1L} and α_{1D}). If we consider embryonic development, we can postualte that a majority of conductive arteries, which developed directly from embryonic branchial arches, contain the primordial version of α_1-AR called α_{1D}-AR (for example: Aorta and Carotid artery). However, the arteries which are more distant from heart are often created from local mesenchyma, and contain further versions of α_1-AR called α_{1A}-AR, α_{1B}-AR e.g. resistance or distributing arteries like subcutaneus arteries, coronary arteries, renal, tail and mesenteric arteries (Sandra 1999).

According to Faber et al (1998), in Rat aorta distribution of α_1-AR in adventitia and media is completely different. The dominant α_1-AR in adventitia is α_{1A}-AR (α_{1A}-AR 44%, α_{1B}-AR 37% and α_{1D}-AR 19%), whereas, in media the α_{1D}-AR is the dominant adrenoceptor (α_{1D}-AR 55%, α_{1B}-AR 26% and α_{1A}-AR 19%) (Faber 1998). In mouse aorta also α_{1D}-AR is dominant in media and has a main contractile role (Yamamoto & Koike 2001; Tanoue 2002; Daly 2002). Also it has been suggested that α_{1B}-AR often have growth or remodelling roles in the cardiovascular system, whereas, α_{1A} and α_{1D}-AR often play contractile roles in vasculature tissues. However, in many resistance or distributing arteries the presence of α_{1B}-AR may be a necessity for starting the contractile response (Daly 2002).

The involvement of the $\alpha_{2A/D}$-adrenoceptor was analysed using a mouse harbouring the Nashville D79N mutation of that receptor, which serves as a functional knockout, in part due to expression of the mutant receptors (that replace the native receptors) at lower expression levels (MacMillan 1996).

The reason for the delay between availability of α_2-adrenoceptor KO mice and the first analysis of the direct vasodilator action of α_2-adrenoceptors in blood vessels lies in the multiplicity of actions via the family of nine adrenoceptors {Three families (α_1, β, α_2)} and the interactions and synergism between these and other factors. The direct vascular actions of α_2-adrenoceptors are subject to modulation in both directions by other vasoactive factors. In particular, because the agonists that are partly selective for α_2-adrenoceptors tend to be partial agonists, responses tend to have a relatively small receptor reserve so that agonists may not be able to achieve a maximal, or even a threshold, response.

We have employed the major conducting arteries in which α_2-adrenoceptor-mediated vasodilatation has been found in other species (reviewed by Guimareas & Moura 2001)

and use rings of arteries mounted on a wire myograph to ensure minimal damage to the vascular endothelium.

Hypothesis:

1. Is UK14304 (selective α_2-ARs agonist) a partial agonist at contractile α_1-ARs?

If yes, incubation with UK14304 (1μM) should shift CCRC to Phenylephrine to the right in tissue rings.

2. If UK14304 can shift CCRC to Phenylephrine to the right, which subtype or subtypes of α_1-ARs are actually involve in this phenomenon?
3. Can UK14304 produce a contraction response via subtypes of α_1-ARs (α_{1A}, α_{1B} and α_{1D}-ARs)?

The first step of this study examined effects of incubation with UK14304 on CCRC to Phenylephrine in young intact and denuded mice aorta compared with other α_1-AR antagonists (e.g. Prazosin and BMY7378). The removal of endothelium potentially simplifies the situation.

The next step tried to find the source of contraction response to both high concentrations of UK14304 and to L-NAME. We hypothesised that constitutive release of NO might reveal constitutive actions of α_1-AR.

We then tested effects of BMY7378 (more selective α_{1D}-AR antagonist) and 5MU (more selective α_{1A}-AR antagonist) on contractile effect to UK14304 in wild type and α_{1D}-AR knockout. The α_{1D}-AR knockout has the potential to make a great deal of difference since it may eliminate both the direct action of UK14304 and the constitutive activity of α_{1D}-AR, thus altering the action of L-NAME. This may lead us to finding which subtype of α_1-ARs involves in contractile effect of UK14304.

4-3. Material and methods:

We chose Young mice (four months) aorta. Both intact and denuded aorta rings are used in this project.

We used genetically modified Nashville D79N ($\alpha_{2A/D}$-AR mutant-C57BL$_6$), α_{1D}-AR Knockout (129/sv/C57BL$_6$) mice compared with controls (129/sv/C57BL$_6$) {WT}, which were killed by CO2, and the thoracic aorta (descending part) removed. Connective tissue including adipose tissue was then removed and the samples dissected into rings (2 mm in length). According to the protocol, in some cases endothelium was removed by scrubbing the inside of aorta lumen by a needle or human hair (denuded aorta) and others remained intact. Tissue rings then were mounted in Krebs solution (NaCl 118.4mM, KCl 4.7mM, $CaCl_2$ 2.5mM, KH_2PO_4 1.2mM, $MgSO_4$ 1.2mM, $NaHCO_3$ 25mM & glucose 11.1mM bubbled with 95% O_2 5% CO_2 to pH 7.4) at 37°C in a multi-myograph (myo-interface, model 600M and 610 Version 2.2, Aarhus) using 40 micron stainless steel wires at 37°C. An isometric force transducer (Linseis L6512B, Belmont Instruments and Powerlab software "Version 4.2.2 for windows 98" which was installed on a pentium III computer) that was calibrated at one gram for sixty small boxes (mm) (0.016gr for each unit based on one volt sensitivity) and was used to measure force development (Mulvany & Halpern, 1976 1977). The aortic rings were placed under 1gr initial tension and left to equilibrate for 30-45 minutes. Reproducible responses were obtained to noradrenaline (0.1μM) or phenylephrine (0.1μM) or U46619 (10nM) according to protocol before commencing further experiments. Tissues were contracted with different agonists cumulatively according to protocols in 0.5 log unit increments beginning with (1nM) up to (30μM). At the plateau of contraction, acetylcholine (1μM) was added to test the endothelium. 50% relaxation response was the criterion for functional endothelium. Also at the end of each experiment

endothelium integrity was checked by the same concentration of acetylcholine (bigger than 50% relaxation responses still required). The tissues were washed every 5 minutes at the end of each step of the experiment and left for 45-75 minutes as a resting period. After this intervening resting period, the next step of the experiment was started. All the antagonists were added at least 30 minutes before agonists.

Drugs:

All drugs were of analytical grade and were dissolved in either distilled water (H_20), ethanol or DMSO as indicated below. Noradrenaline dilution included 23μM EDTA to prevent oxidation.

noradrenaline (H_2O), phenylephrine (H_2O), acetylcholine chloride (H_2O), 5HT (H_2O), U46619 (ethanol), L-NAME (N-Nitro-L-Arginine Methyl Ester) (H_2O), rauwolscine (H_2O) [Sigma-Aldrich Co; Poole, UK]. UK14304 (DMSO). BMY7378 [Sigma-Aldrich Co; Poole, UK] (H_2O),

Statistics:

Values are means ± Standard error mean from n experiments. Difference between maximal contraction response to CCRC to agonist in presence and absence of antagonists were compared with one-way and two way ANOVA followed by Bonferroni's post test and two-tailed unpaired and paired t-test. Statistical and graphical analysis was carried out using **Excel 97 and GraphPad Prism 3.00** for PC. Data used to plot the dose response curves are the mean contraction induced at each concentration of the drugs, and hence, the maximum response shown graphically differs from the maximum calculated from individual tissue maximum.

4-4. Results:

Hypothesis:

From chapter three results we found that high concentration UK14304 could produce contractile effect in mice aorta, which were antagonised by BMY7378 (0.1μM) (Fig. 4-1 & 4-2).

Cumulative concentrations of UK14304 from (1nM) to (30μM) in presence and absence of BMY7378 (0.1uM) (more selective α_{1D}-AR antagonist) showed in wild type mouse aorta that BMY7378 not only could delay contraction to high concentration of UK14304, but also increased the relaxation range (Fig. 4-2). In order to clarify and simplify α_{1D}-AR involvement in contraction to high concentration of UK14304 we carried out the same protocol on young Japanese α_{1D}-Knockout transgenic mouse. In α_{1D}-KO transgenic mouse there was no contractile response to high concentration of UK14304 at all (n=7) (Fig. 4-3 & 4-4).

Contractile effect of UK14304 was greater in Nashville D79N mouse aorta (Fig.4-5) and this was antagonised by prazosin (non-selective α-ARs antagonist) (See chapter three).

If UK14304 acts as a partial agonist of α_1-ARs then incubation with UK14304 (1μM) should shift CCRC to phenylephrine to the right, and if UK14304 can shift it, it is important to distinguish which subtype or subtypes of α_1-ARs is actually involved.

In young aorta Phenylephrine was more potent in denuded (EC50 = 0.09μM) than in intact (EC50 = 0.64μM) (Fig. 4-6). A single concentration of UK14304 (1μM) produced contraction (mean 0.39g) on denuded aorta, but not intact, indicating that an inhibitory influence of endothelium on smooth muscle cells can negate the contractile effect (Fig. 4-6).

UK14304 (1μM) shifted the control curve to phenylephrine to the right in both intact (EC50 0.64μM → 10μM, shift 15.6 folder) and denuded (EC50 0.09μM → 0.1μM, shift 1.1 folder), young aorta (Fig 4-6). These observations indicate that UK14304 can act as an antagonist at α_{1D}-AR. Since it also has a contractile effect it is classified as a partial agonist.

Treating with L-NAME (0.1mM) also caused contraction. Contraction to UK14304 came down to 65% after addition of BMY7378 (0.1μM) (Fig. 4-7, Trace). This suggests that constitutively active α_{1D}-AR can activate contraction but this is normally suppressed by endothelial nitric oxide that, in turn, must be constitutively released. This is confirmed by the further observation that in α_{1D}-AR Knockout, the L-NAME-induced contractile response was very small (Fig. 4-8, Trace). In this case UK14304 could not produce any contractile effect on top of the (very small) L-NAME-induced contractile response indicating that its contractile effect requires the presence of α_{1D}-AR and is not revealed even when nitric oxide is suppressed (Fig. 4-9, Trace).

The small L-NAME-induced contraction was sensitive to 5MU but not BMY7378. This revealed also the possibility of the presence of some constitutively active α_{1A}-AR on smooth muscle cells (Fig. 4-8 & 4-9, Trace).

This also shows that UK14304 acts selectively at the α_{1D}-AR being the main adrenergic receptor in mouse aorta, which is responsible for the pressor response to noradrenaline and phenylephrine (Fig. 4-1).

Chapter Four Graphs and images

Multiple action sites of UK14304 on different adrenoceptors in mice aorta.

Graphs created in Prism (Version 3) and Traces in Powerlab (Version 4.2)

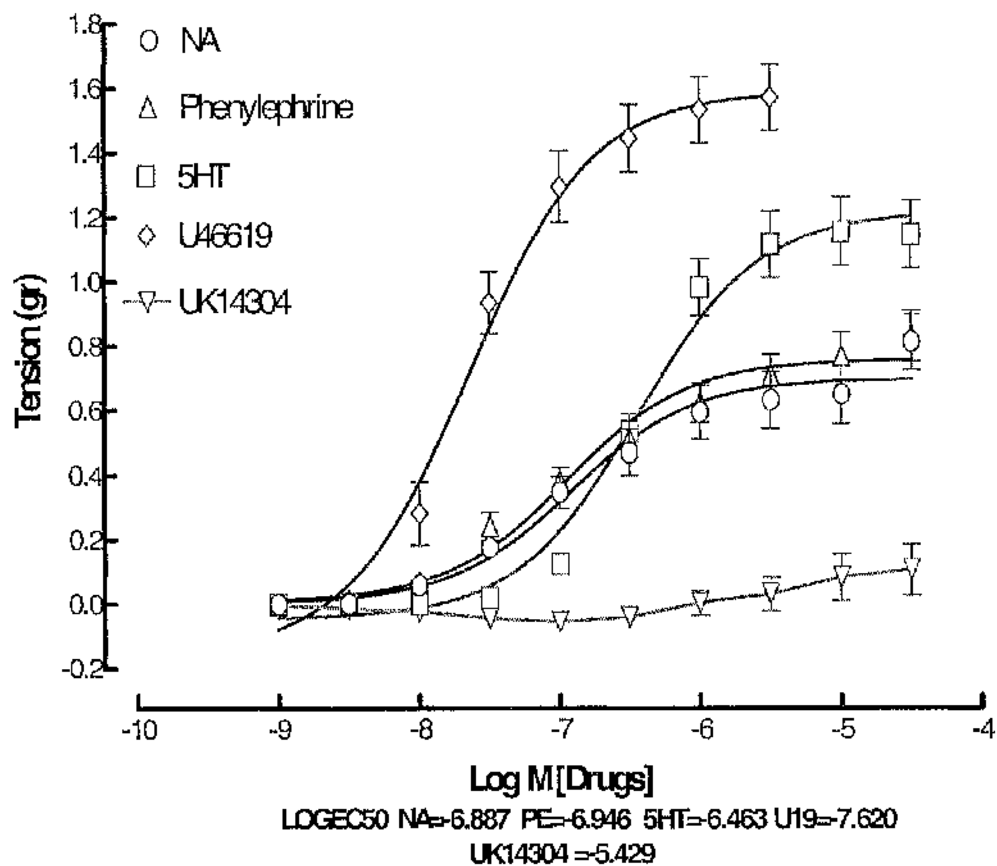


Fig. 4-1: Comparison between CCRC to noradrenaline (O), phenylephrine (Δ), 5HT (□), U46619 (◇) and UK14304 (∇) in four months wild type mouse aorta (n=7).

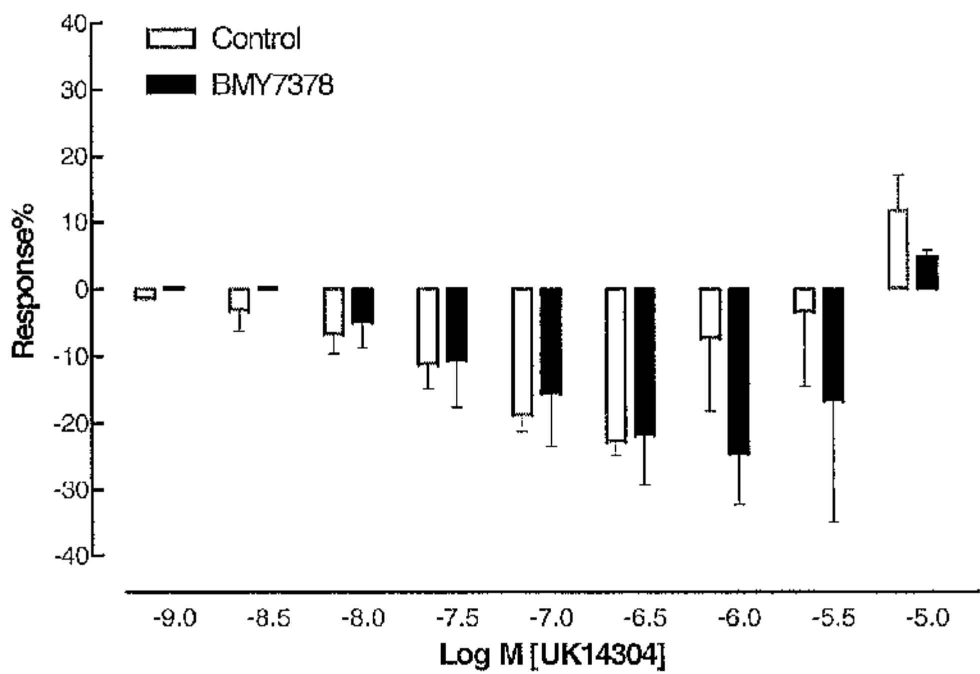


Fig. 4-2: comparison between cumulative concentration response to UK14304 in four months wild type in presence and absence of BMY7378 (0.1μM) in mouse aorta (n=5).

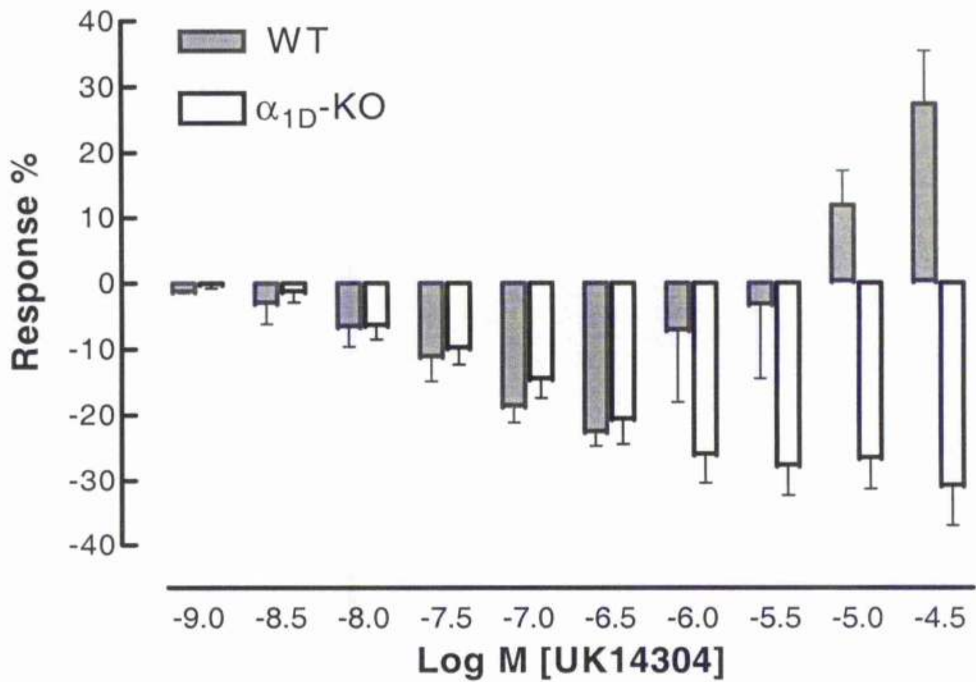


Fig. 4-3: Comparison between four months wild type (control) and α_{1D}-Knockout mouse aorta in cumulative response to UK14304 (n=7) on top of U46619 pre-constriction.

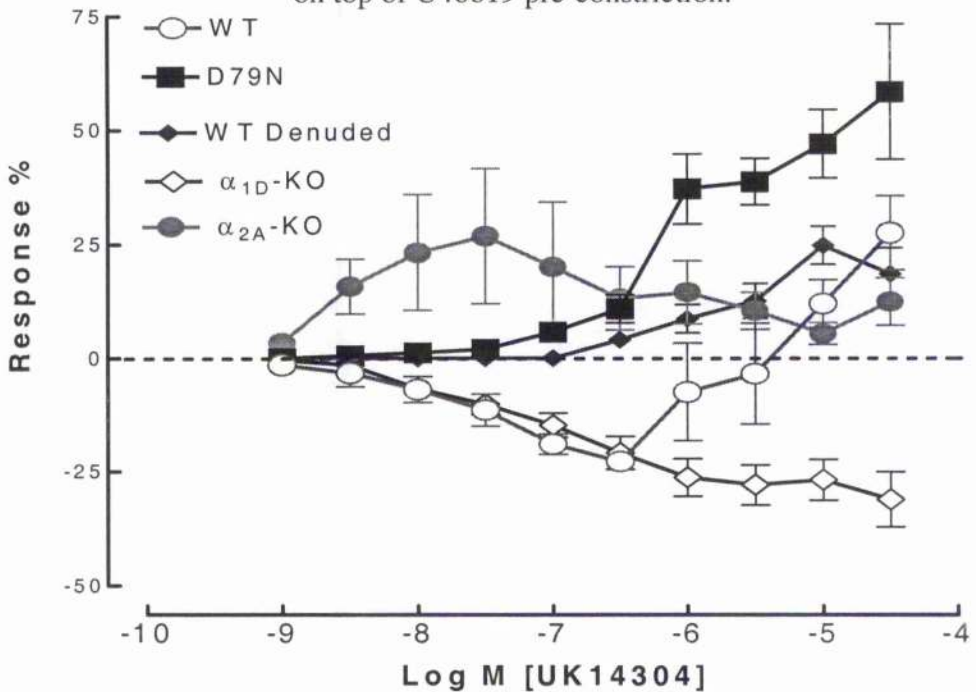


Fig 4-4: Preconstricted, CCRC to UK14304 in four months wild type {intact (O) and denuded (◆)} (n=5), Nashville D79N (■) (n=4), α_{1D}-KO (◈) (n=7) and α_{2A}-AR Knockout (n=4) (●) mouse aorta. Each point represents mean ± standard error.

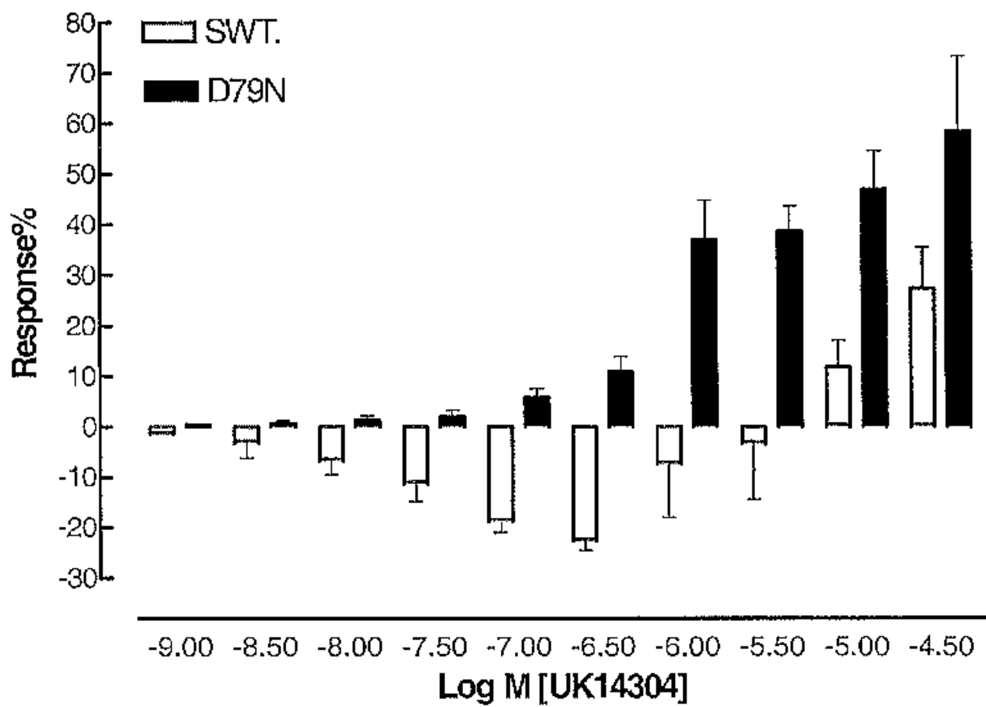


Fig. 4-5: Effect of cumulative concentration of UK14304 on four months wild type (n=5) and Nashville D79N (n=4) mouse aorta.

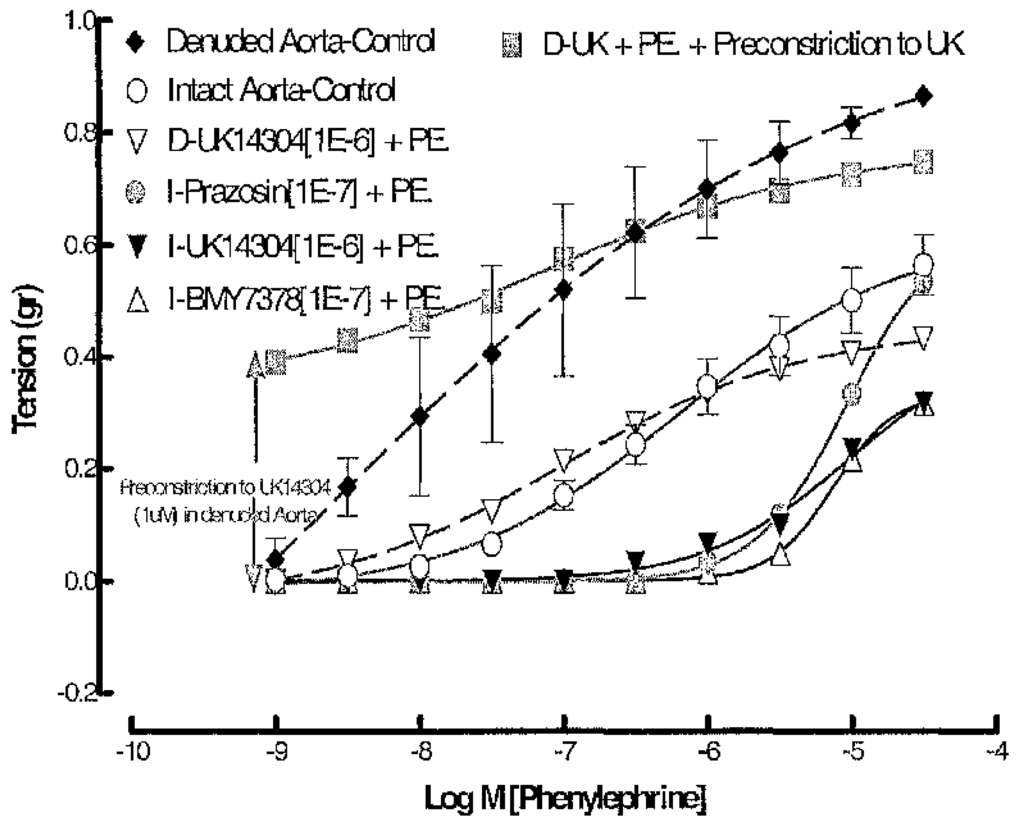


Fig 4-6 : Effect of UK14304 (▼) (1μM), prazosin (●) (0.1μM) and BMY7378 (Δ) (0.1μM) to shift CCRC to phenylephrine in four months wild type {intact (O) and denuded (◆)} mouse aorta. In denuded aorta UK14304 could produce pre-constriction (■) response due to lack of the endothelium (n=3).

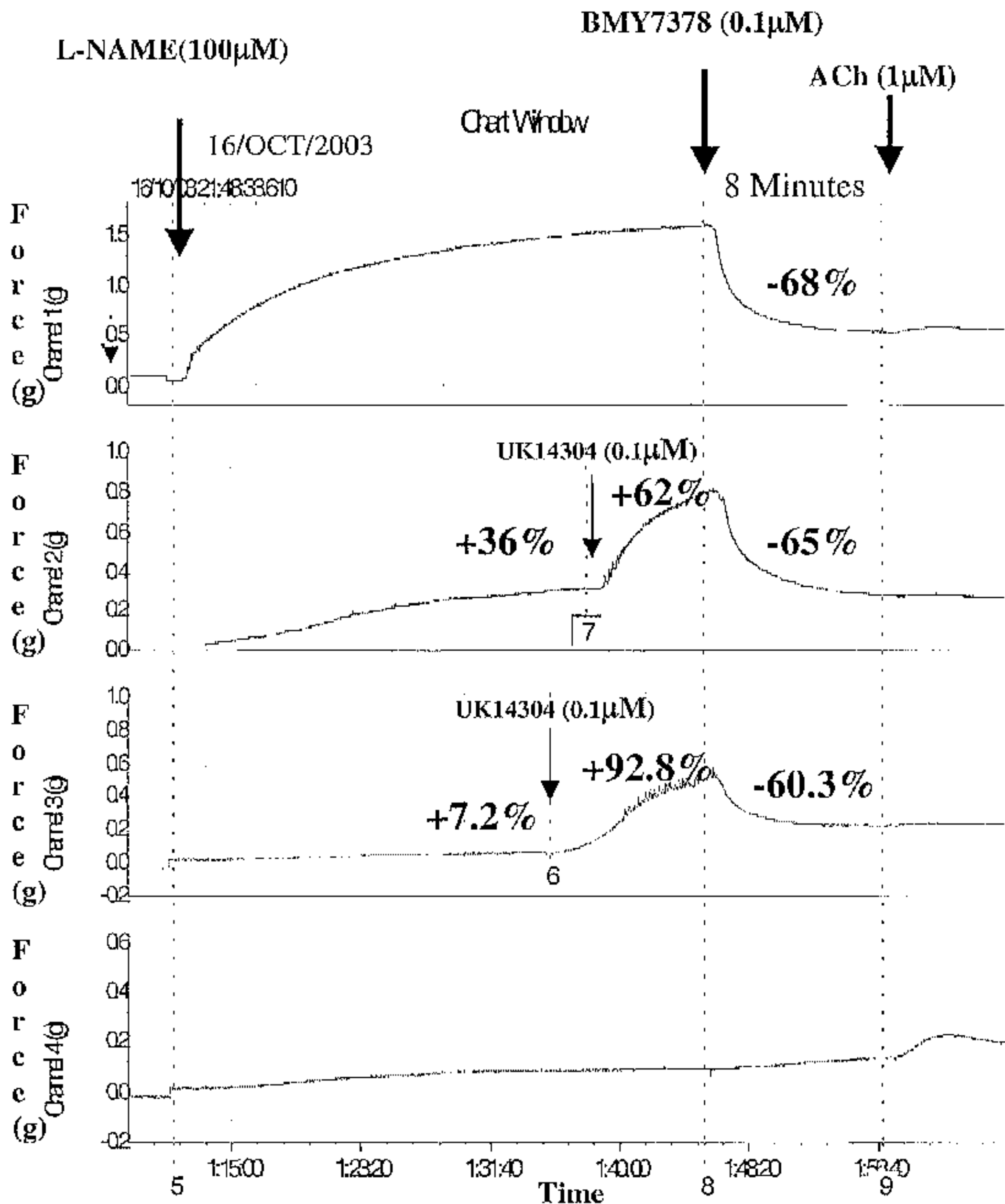


Fig. 4-7: ***Trace***; Using L-NAME (0.1mM) revealed the presence of constitutively active α_{1D}-AR in four months wild type aorta. More than 60% of contractile response to L-NAME is removed by BMY7378 (0.1μM). Incubating with BMY7378 may block this constitutively active receptors which situated on smooth muscle cells of media as well as endothelium and reduced maximum response to phenylephrine or noradrenaline as well as delay in response to α_{1D}-AR agonists in young wild type mouse aorta. As chart has shown, UK14304 (1μM) could produce more than 76% contraction on top of preconstriction response to L-NAME in mean which again removed by BMY7378 (selective α_{1D}-AR antagonist). This phenomenon also revealed that UK14304 acts as a partial agonist of α_{1D}-AR in young mouse aorta.

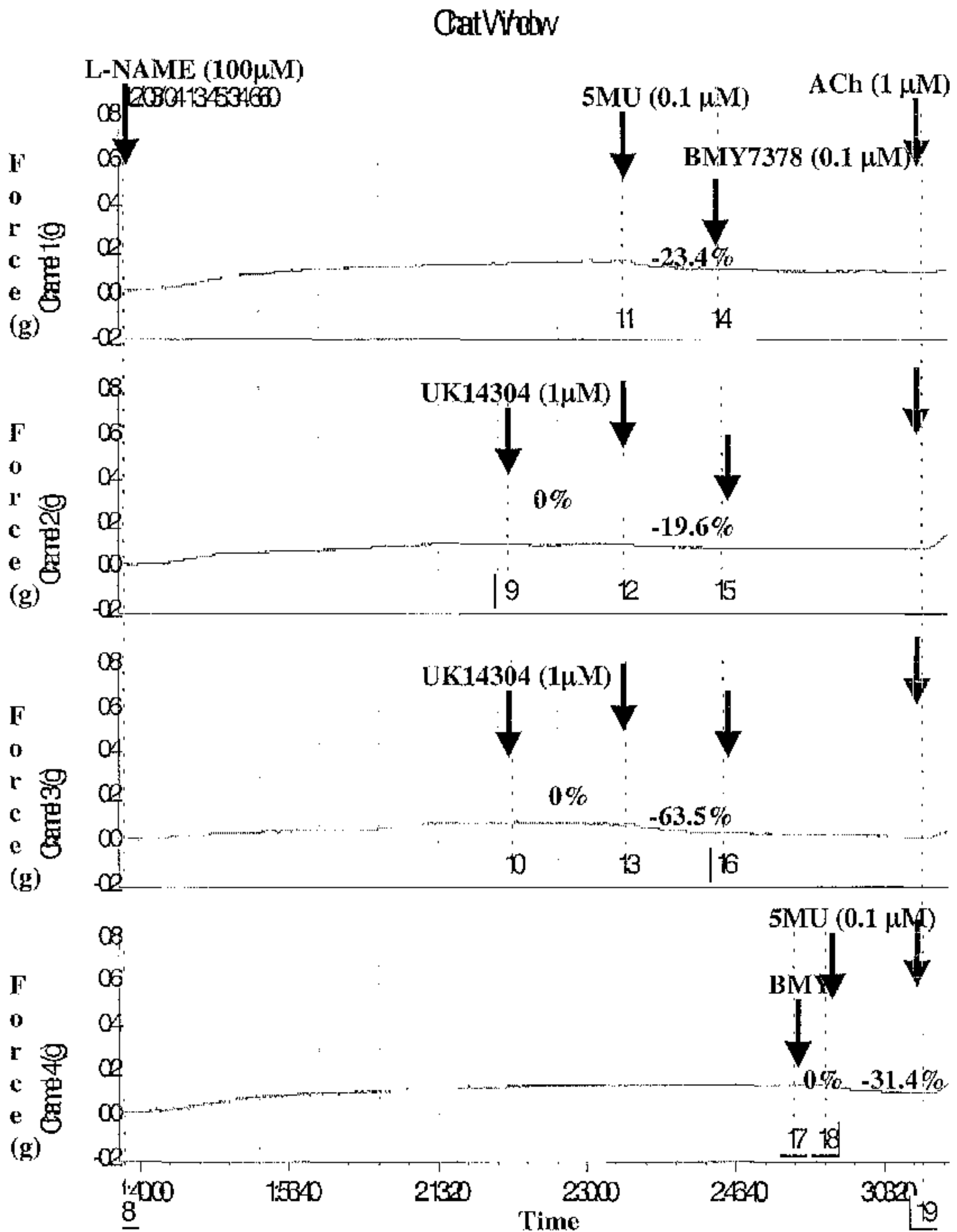


Fig. 4-8: ***Trace;*** Four months α_{1D}-Knockout mouse aorta. Using L-NAME (0.1μM) revealed the presence of constitutively active α_{1A}-AR. There was neither contractile response to UK14304 (0.1μM) nor relaxation to BMY7378 (0.1μM). However, 5MU (0.1μM) could stimulate 34.4% in mean relaxant effect. All the channels had contractile response to ACh (1μM). This phenomenon also has shown more selectivity of 5MU on α_{1A}-AR as well as that UK14304 is only partial agonist of α_{1D}-AR and not α_{1A}-AR.

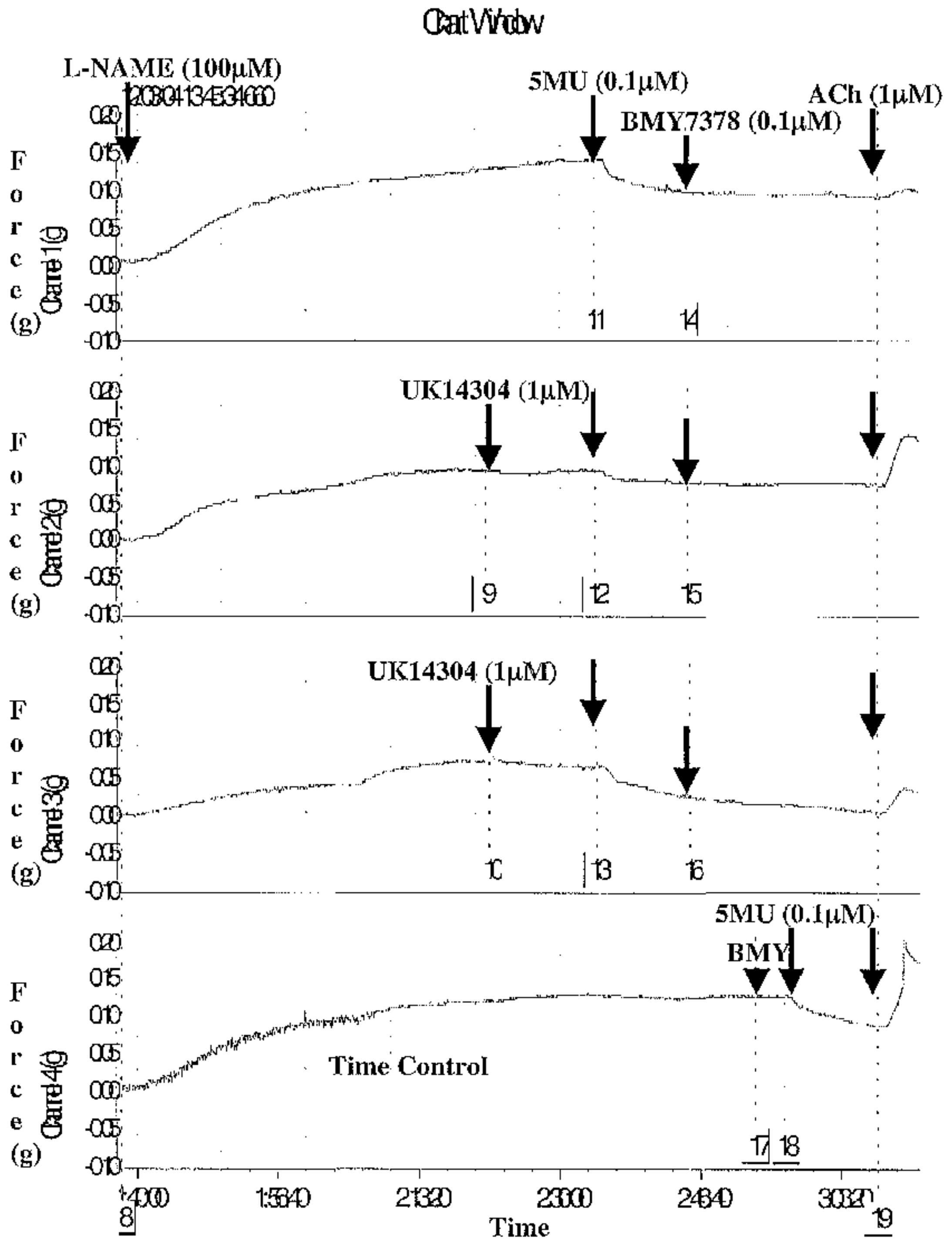


Fig. 4-9: ***Trace;*** **High Magnification (sixteen times) of figure 4-8.**
Four months α_{1D}-Knockout mouse aorta. Using L-NAME (100μM) revealed the presence of constitutively active α_{1A}-AR. There was neither contractile response to UK14304 (1μM) nor relaxation to BMY7378 (0.1μM). However, 5MU (0.1μM) could stimulate 34.4% in mean relaxant effect. All the channels had contractile response to ACh (1μM). This phenomenon also has shown that UK14304 is only partial agonist of α_{1D}-AR and not α_{1A}-AR.

4-5. Discussion:

Patients with high blood pressures have an increased risk for cardiovascular events. Drugs that selectively decrease blood pressure may be of interest for these patients.

It may be possible to develop drugs with a higher selectivity for large arteries. Such drugs may be good candidates to decrease high blood pressure without substantially decreasing mean and diastolic blood pressures.

Continuing understanding of molecular mechanisms of such drugs not only helps to identify better drugs for these targets but should also provide an insight into developing further drugs with better selectivity and less toxicity.

Drugs particularly in high concentration may be non-selective between receptor. However, distinguishing which type of receptor can respond to high concentration of a drug is important. G-protein-coupled receptor (GPCR) subtypes are differentially distributed in smooth muscle cells. However, it remains unclear how this affects the subtype selectivity of particular drugs. Sugawara et al (2002) carried out flow cytometry analysis with the fluorescent ligand, BODIPY FL-prazosin, to study the relationship between the subcellular distribution of subtype receptors and the subtype-selective character of ligands using α_{1A}- and α_{1B}-adrenoceptors (ARs). α_{1A}-ARs predominantly localise inside the cell, while α_{1B}-ARs on the cell surface. This study has illustrated that location of receptor in addition to the affinity of the drug for the receptor should be taken into account in assessing the subtype selectivity of a drug.

Despite the importance of adrenoceptors to regulation of vasculature tonicity and blood pressure, among adrenergic receptors only β-adrenoceptors have sufficient subtype-selective ligands. Selective agonists for the β_2-adrenoceptors play an important role in asthma therapy (e.g. Salbutamol), and β_1-receptor antagonists (e.g. proparanolol) are in

the first–line medication for hypertension, coronary heart disease or chronic heart failure (Brophy 2001; Frishman and Lazar 1990).

α_2-adrenoceptors were initially characterised as presynaptic receptors that could provide a negative feedback loop to regulate noradrenaline release (Starke 1975). Soon scientists found postsynaptic function for α_2-adrenoceptors. Using pharmacological antagonists, revealed that α_{2A}-adrenoceptor has a major inhibitory presynaptic role to release of noradrenaline from sympathetic neurons as part of a feedback loop (Trendelenburg 1997). Chapter three of this thesis also explained direct α_2-adrenoceptors vasodilator effects via nitric oxide release through the endothelium in various arteries of mouse.

Agonists that are generally considered to be "α_2-adrenoceptor-selective" are often partial agonists at α_1-adrenoceptors; this has been reported in large arteries in the rat (Naghadeh 1996).

UK14304 is a non-selective α_2-AR agonist. However, experiments on wild type and α_{1D}-Knockout mice using BMY7378 {selective α_{1D}-AR antagonist} and 5MU {selective α_{1A}-AR antagonist} have revealed that in high concentration (higher than 0.3μM) UK14304 acts as a partial agonist for α_{1D}-AR which, disappeared in α_{1D}-AR Knockout mice.

Hence, finding selective agonists or antagonists for α_2-adrenoceptor subtypes may play an important role to use them clinically in order to find hopeful view to control blood pressure related to cardiovascular disease.

Chapter Five

Interaction, Ageing and Receptors balance

5-1. Abstract:

1. Contractile and relaxant effects were studied on wire myograph-mounted mouse aorta, carotid, and main mesenteric arteries with a view to determining sites, mechanisms of action and involvement of adrenoceptor subtypes at different ages and strains.

2. In aorta and superior mesenteric arteries, noradrenaline (NA) and phenylephrine (PE) produced responses related to age and strain.

3. Comparisons of young D79N with WT showed reduced contractile responses to PE, suggesting reduced functionality of α_1-ARs in D79N. Laser scanning confocal microscopy showed that QAPB-binding intensity was reduced in the presence of in both control and BMY7378. This suggests a regulation of α_1-AR dependent on functional α_2-AR.

4. The AT_2 mediated relaxation response to angiotensin II in young mice disappeared with age. Conversely, α_2-AR mediated relaxation was greater in older mice. This shows a remarkable age switch in the vasodilator influence of the renin-angiotensin II and adrenergic systems, in mouse major conductive arteries, in favour of adrenergic.

5-2. Introduction:

Studies carried out on Drosophila melanogaster (Vinegar or fruit Fly) have shown that changes in genetic environment (mutant or knockout gene) can affect other part of the gene pool and may appear as changes in expression of one or more gene alleles lead to changing in phenotypes. For example: mutations in the XPD gene (Xeroderma Pigmentosum group D), which require for excision repair of UV-damaged DNA and the mechanism with which the cell reads the genetic information and converts it into proteins, lead to the highly cancer-pron skin disease XP, trichothiodystrophy (TTD) and Cockayne syndrome in human (Weeda 1997; Brabont 2001). In Drosophila melanogaster mutation in XPD gene which control the number of hairs on the body of the fly could lead to a longer reproductive period from normal (12 days) to more than 45 days and these changes will delete in nature in a short time (Sandoval and Zurita 2001).

At the end of the twentieth century geneticists completed human, Drosophila melanogaster and yeast gene maps. However, we don't know too much about the internal genetic environment, particularly in mammals.

From 20 years ago providing and using transgenic or knockout animals has been considered as a useful model to clarify and simplify responses to drugs acting at receptors (Gordon and Ruddle 1982). Particularly, during the past decade, scientists started to produce double knockout animals (animals which have not expression or lower expressions of two subtypes of a family receptors). However, these transgenic animals often show poor survival. Experiences with knockout mice have shown that not only is there variability in expression of other receptors, which may not even belong to the same family but also the expression of knockout gene may not be the same in

different parts of the body. If ageing change receptor expression levels. We may face a lot of changes in phenotypes compared with the normal case.

The balance between α-adrenergic (α-AR) receptors plays a key role in regulation of smooth muscle cells (SMCs) contraction response. Some receptors have opposing effects.

For example; α_1-AR contraction responses acts on SMCs via three different subtypes (α_{1A}. α_{1B} and α_{1D}), whereas α_2-AR oppose this contraction via another three subtypes (α_{2A}, α_{2B} and α_{2C}) which are either situated on presynaptic sympathetic nerve terminals inhibits NA release (Ruffolo 1991) (Philipp 2002) or on endothelial cells acting via endothelium nitric oxide release (See chapter three). Furthermore α_2-AR on SMCs can facilitate α_1-AR-mediated contraction (Dunn 1991).

The presynaptic effect of α_2-AR agonists (Dexmedetomidine or UK14304) was completely absents in α_{2A}-AR knockout mice vas deferens preparation. Deletion of α_{2A}-AR subtype in Nashville D79N (Macmillan 1996) and α_{2A}-AR knockout caused an increase in sympathetic activity with resting tachycardia. The results suggest a major role for α_{2A}-AR in regulating Norepinephrine release in synaptic clefts.

These confirm that α_{2B} or α_{2C}-AR may function as presynaptic autoreceptors to inhibit transmitter release (Joun 1999). Thus, using gene-targeting strategies indicates independent functions for each of the three subtypes of α_2-AR (Link 1996; MacMillan 1996). However, deletion of one of them may lead to increased expression of another subtype revealing this subtypes involvement in the specific function (Philipp 2002).

The first step in this study was comparing three different responses at young (four months) and old (fourteen to sixteen months) mice ages in aorta and superior mesenteric arteries.

1) Contractile adrenoceptor responses (α_1-ARs).

2) Relaxant adrenoceptor receptors (α_2-ARs).

3) Angiotensin II contraction and relaxation effects (AT_1 & AT_2-R).

Next step tried to analysis the same responses in knockout mice in order to see whether the loss of one receptor results in a change in the functional responses to the other receptors.

1) First at the young age.

2) Second at the old age.

This design helps us to see how the differences between strains at the young age further changes as the animal ages.

5-3. Material and Methods:

a) Wire myography:

Male mice (aged 4, 14 and 16 months) were killed by CO_2 and the descending thoracic aorta, carotid, main (Superior) mesenteric artery, the were removed, cleaned of connective tissue then dissected into rings (2 mm in length). The strains of mice employed were the Swiss wild type and $\alpha_{2A/D}$-knockout mutant, D79N ($C57BL_6$-Homozygous) (MacMillan 1996, 1998), α_{2A}-knockout ($C57BL_6$/129/sv/Adra $2a^{Tm1Lcl}$) (Docherty 2003, Dublin) which had been back-crossed on to $C57BL_6$. Endothelium was removed, where appropriate, by rubbing the intimal surface with a human hair or small needle. Tissues were then mounted in Kreb's solution (NaCl 118.4mM, KCl 4.7mM, $CaCl_2$ 2.5mM, KH_2PO_4 1.2mM, $MgSO_4$ 1.2mM, $NaHCO_3$ 25mM & glucose 11.1mM bubbled with 95% O_2 5% CO_2 to pH 7.4) at 37°C in a multi-myograph (myo-interface, model 600M or 610M DMT, Aarhus) using 40 micron tungsten or stainless steel wires (Mulvany & Halpern. 1976, 1977). Aortic rings were placed under a resting tension of 1g; Carotid artery 0.333 g, superior (Main) mesenteric artery 0.25 g; and left to equilibrate for 30-45 minute. Reproducible responses were obtained to, NA (0.1μM), phenylephrine (0.1μM), or U46619 (10nM), according to protocol, before commencing experiments. Tissues were tested with increasing concentrations of agonists' in 0.5 log unit increments from 1nM-30μM. Agonist addition was cumulative also in the other arteries.

At the plateau of contraction to noradrenaline (1μM), acetylcholine (1μM) was added to assess endothelial integrity. Criteria for functional endothelium was >50% and for denuded endothelium was < 5% relaxation. At the end of each experiment endothelium

was re-checked using the same criteria and samples included only if meeting criteria at both times. All four arteries relaxed to Acetylcholine (1μM). In all arteries L-NAME (100μM) could abolish the relaxation response to acetylcholine.

Tissues were washed at 5 minutes intervals following each experimental protocol and given a 60 minutes recovery period. Following the rest period, second cumulative concentration response curve (CCRC) obtained.

b) Laser Scanning Confocal Microscopy (LSCM):

Tissue preparation:

2-3mm segments of aorta from WT and D79N mice were incubated for 60 minutes in both QAPB (1μM) and rhodamine-angiotensin II-Human (50nM) in the presence and absence of BMY7378 (0.1μM), introduced 30 minutes prior to incubation with the fluo-ligand. Following incubation, without washing, aortic segments were cut open and placed endothelial side up in the sample well of a glass slide. The well containing the tissue and prior incubation media was sealed with a glass coverslip (No. 1.5 for confocal use).

Image capture:

Serial optical sections were collected on a Biorad 1024 & Radiance 2100 confocal laser scanning microscope. The Excitation/Emission parameters used were 488/515nm for QAPB and 567/610nm For Rhodamine Angiotensin II

(Rho-Ang II-H). In all experiments the laser power; gain and offset (contrast and brightness) were kept constant. The distance between optical sections was maintained a 0.5μm for each image stack. Tissues were visualised using a x40 oil immersion objective on which the numerical aperture is 1.00 and therefore optimal pinhole setting is 1.5. Image size was set to 512 x 512 pixels, which equates to a field size of 289μm x 289μm.

Image analysis:

3D volumes (image stacks) were transferred to either MetaMorph (Universal Imaging) or Amira (TGS) software packages for subsequent analysis and volume visualisation respectively. 3D volumes containing two channels of data are psuedocoloured green and red for QAPB & Rho-Ang II-H respectively. Where two channels co-localise and their intensities are roughly equivalent, the co-localised area is displayed in yellow. Spatial localisation of fluorescent signals was achieved using orthogonal viewing of the XY, XZ & YZ planes. 3D views were rendered using the Amira 'Voltex' module.

Drugs:

All drugs were of analytical grade and were dissolved in either distilled water (H_20), ethanol or DMSO as indicated below. Noradrenaline dilution included 23μM EDTA to prevent oxidation.

noradrenaline (H_2O), phenylephrine (H_2O), acetylcholine chloride (H_2O), 5HT (H_2O), U46619 (ethanol), L-NAME (N-Nitro-L-Arginine Methyl Ester) (H_2O), rauwolscine (H_2O) [Sigma-Aldrich Co; Poole, UK], UK14304 (DMSO) [Pfizer, Sandwich, UK]. BMY 7378 (H_2O) [Sigma-Aldrich Co; Poole, UK], 5MU (H_2O [Sigma-Aldrich Co; Poole, UK].

Fluorescent compounds:

Fluorescent prazosin (QAPB) (DMSO) [Molecular Probes INC; EUGENE-USA], Rhodamine-Angiotensin II-Human (Rho-Ang II-H) (H2O) [Phoenix Pharmaceuticals INC; Germany].

Statistics:

Values are means ± Standard error mean from n experiments. Differences between maximal contraction response to CCRC to agonist in presence and absence of drugs were compared with one-way and two-way ANOVA followed by Bonferroni's post test and two-tailed unpaired and paired t-test. Statistical and graphical analysis was carried out using Excel 97 and GraphPad Prism 3.00 for PC. Data used to plot the concentration response curves are the mean contractions induced at each concentration of the agonist.

5-4. Results:

Contractile α_1-adrenoceptor responses:

Comparison between contractile responses to cumulative concentrations of phenylephrine (PE) in four and fourteen months wild type mouse aorta revealed no significant reduction due to ageing (Fig. 5-1). The same result was obtained in wild type superior mesenteric artery (CCRC to PE), in which a two tailed unpaired t-test confirmed no significant reduction between four and fourteen months (Fig. 5-5).

The experiment was repeated using the Non-selective α-AR agonist, noradrenaline (NA), cumulatively. Here also, there was no significant increase in responses for aorta and superior mesenteric artery due to ageing, confirmed by two tailed unpaired t-test analysis (Fig. 5-2 and 5-6).

Comparing CCRC curves to phenylephrine and noradrenaline together related to age in aorta (Fig. 5-3 and 5-4) and superior mesenteric artery (Fig. 5-7 and 5-8) illustrates that the potency of noradrenaline and phenylephrine reversed with ageing. However, this change was not significant. Closer inspection also reveals that NA curves are biphasic in both arteries, though more in aorta.

Table 5-1: Aorta: responses to phenylephrine and noradrenaline with age.

Response/Ages Aorta	PE EC50	PE Hillslope	NA EC50	NA Hillslope
Four months	0.11μM	0.5493	0.12μM	0.5728

Fourteen months	0.24μM	0.4246	0.10μM	0.2326

Table5-2: Superior mesenteric artery: responses to phenylephrine and noradrenaline with age.

Response/Age Mesenteric artery	**PE** EC50	**PE** Hillslope	**NA** EC50	**NA** Hillslope
Four months	**1.7μM**	**0.8481**	**1.76μM**	**1.045**
Fourteen months	**3.18μM**	**1.031**	**2.73μM**	**0.6399**

Relaxant α_2-adrenoceptors responses:

Responses to UK14304 (selective α_2-AR agonist) were also different in young wild type mouse aorta compared with old ones (P value: 0.0044<0.05). At the young age there was a relaxation response to low concentration of UK14304 reversing to contraction at concentrations higher than 0.3μM, the latter blocked by BMY7378 (0.1μM) (See chapter three). In fourteen months wild type relaxation responses were larger, although they waned at high concentration. They never contracted (Two tailed unpaired t-test P value: 0.0044<0.05, Significant) (Fig. 5-9).

Responses to cumulative concentrations of UK14304 on wild type superior mesenteric artery, where responses are solely relaxant at both ages, increased (two tailed unpaired t-test, P value: 0.3815>0.05, Not significant) between four and fourteen months.

However, this change was not significant (Fig. 5-10). Thus, we uncovered more sensitivity and greater relaxant response to an α_2-AR agonist only in aorta.

Angiotensin II responses (contraction and relaxation):

Cumulative concentrations of angiotensin II (Ang II) had a dual effect on preconstricted young mouse aorta. Low concentrations caused around 10% increase in mean contraction. At higher than 30nM a relaxant effect started to appear which continued to around 6% under the base preconstriction to 5HT (0.1μM). This relaxation was blocked by L-NAME (100μM) or denudation of endothelium or PD123319 (See Chapter two). However, this dual effect did not appear in fourteen months wild type mouse aorta, and was replaced by only a greater contractile response to Ang II (Two tailed unpaired t-test P value: $0.0025<0.05$, Significant), revealing a change in the balance between AT_1 and AT_2 receptor expression related to ageing in favour of AT_1 (Fig. 5-11).

In young wild type superior mesenteric artery cumulative concentration of Ang II provided only contraction. In fourteen months this contraction was not significantly (Two tailed unpaired t-test P value: $0.1944<0.05$) greater than at four months (Fig. 5-12).

Therefore, we faced greater expression of AT_1 receptors only in old aorta compared with four months.

We already showed in chapter three that there were no interactions between AT_2 angiotensin II receptor and α_2-adrenoceptors in relaxation (See chapter three).

Contractile α_1-adrenoceptor response in Knockout mice:

Cumulative concentration response curves to the selective α_1-agonist, Phenylephrine, revealed significant differences in maximum contractile responses to α_1-AR stimulation in young aorta confirmed with ONE WAY ANOVA statistical analysis followed by

Bonferroni's post test (P value: 0.0001<0.05) between three different strains of mice (Wild Type>Nashville D79N > α_{2A}-AR Knockout) (Fig.5-13).

Statistical analysis also confirmed a difference between CCRC to Phenylephrine between young wild type and α_{2A}-AR Knockout carotid artery (two tailed unpaired t-test P value: 0.0185<0.05). However, there was no significant difference between strains in superior mesenteric artery (two tailed unpaired t-test P value: 0.493<0.05) (Fig.5-14 and 5-15).

Therefore, young α_{2A}-Knockout strains revealed lower contraction via α_1-AR compared with wild type in aorta and carotid.

Melissa McBride, also has reported that the maximum contractile response to noradrenaline and Phenylephrine in D79N {Functionally $\alpha_{2A/D}$-AR knockout} tail artery were significantly smaller than in wild type (Melissa McBride 2003).

Relaxant α_2-adrenoceptors response in Knockout mice:

Comparing young wild type and α_{1D}-AR knockout (KO) mouse aorta showed a significant difference in response to cumulative UK14304 in high concentrations (Two tailed unpaired t-test P value: 0.0395<0.05) (Fig. 5-16 also see chapter three) revealing dominance of relaxation in the KO. However, the sixteen months α_{1D}-AR Knockout mouse had a significantly smaller relaxation response to UK14304 compared with four months Knockout mice (Two tailed unpaired t-test P value: 0.0157<0.05) (Fig. 5-17). Thus, the "enhanced" relaxation response in old wild type mice is lost when the dominant contractile α_{1D}-AR is knocked out (Two tailed unpaired t-test P value: P value: 0.0004<0.05) (Fig. 5-18).

Visualisation results on α_1-AR binding site with QAPB (FL-Prazosin) related to strains:

Laser Scanning Confocal Microscopy on young wild type and D79N mouse aorta in presence and absence of the most selective α_{1D}-AR antagonist, BMY7378 (0.1μM), revealed significant difference between QAPB intensity in strains. The objective was to highlight the α_2-AR.

In Nashville D79N mouse aorta visual inspection shows a marked reduction in QAPB binding sites in both control and treated with BMY7378 (Images 5-1 and 5-2) intact vessels. In order to show cells that lack QAPB binding, Rhodamine–Angiotensin II-Human with different Excitation/Emission wavelength 567/610 was used along with QAPB (Excitation/Emission is 488/515). In this way cells with no-QAPB binding appeared as red coloured cells. This shows that the difference is not due to a loss of cells but to a diminished population of receptors that bind QAPB and perhaps, to a reduction in number of cells expressing them.

Interaction, ageing and receptors balance

Interaction between α-AR and angiotensin II receptors

Aorta and Mesenteric arteries

Graphs created in GraphPad prism (Version 3)

Images created in Amira (Version 3.2)

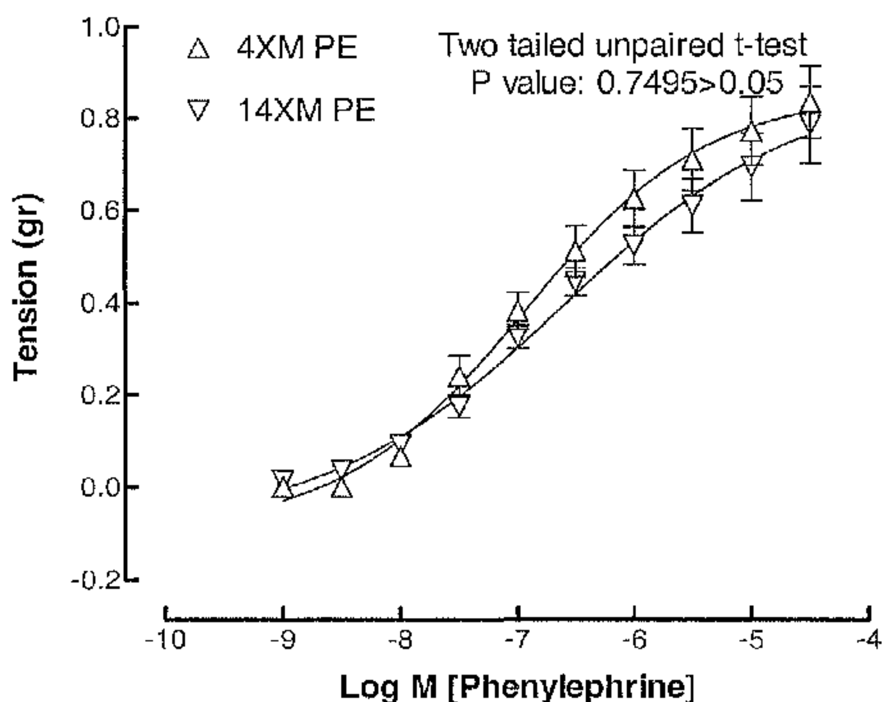


Fig. 5-1: Comparison between CCRC response in four (△) and fourteen (▽) months wild type mouse aorta to phenylephrine (n=7).

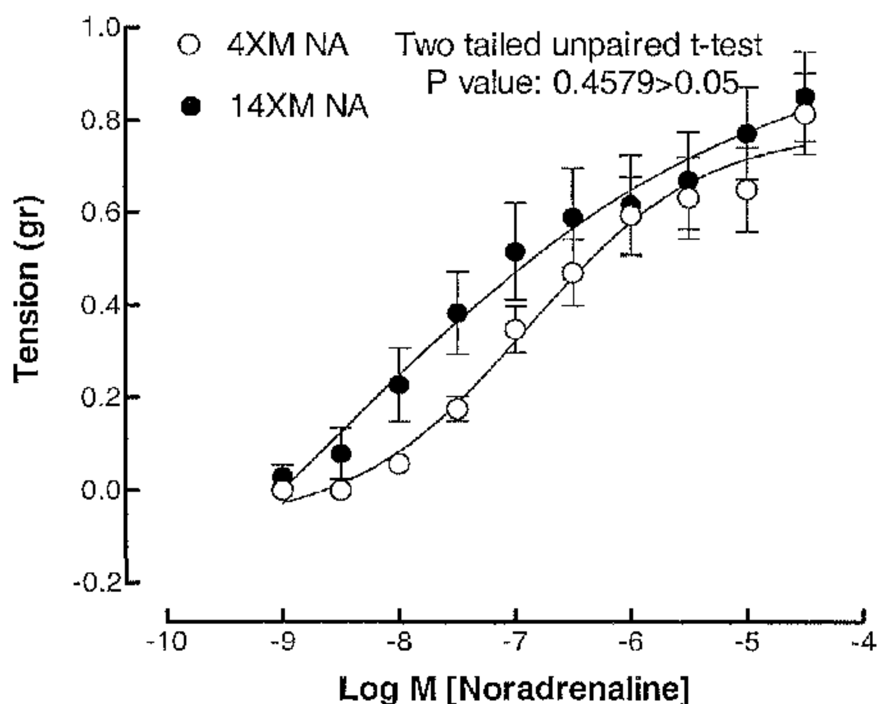


Fig. 5-2: Comparison between CCRC response in four (O) and fourteen (•) months wild type mouse aorta to noradrenaline (n=7).

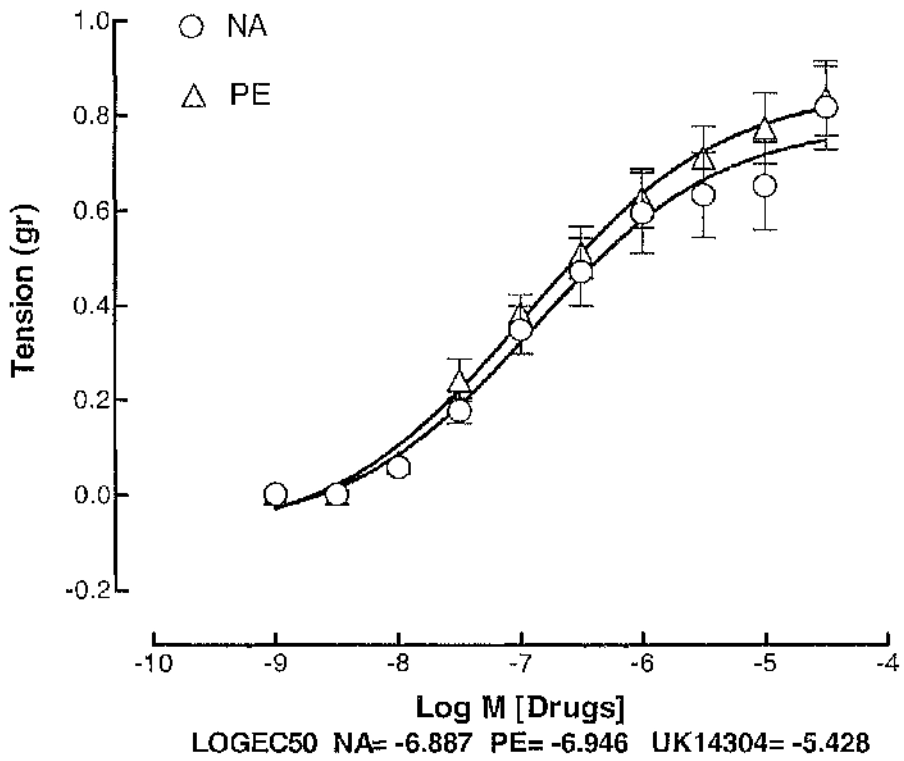


Fig. 5-3: CCRC to noradrenaline (O) and phenylephrine (Δ) in four months wild type mouse aorta (n=7).

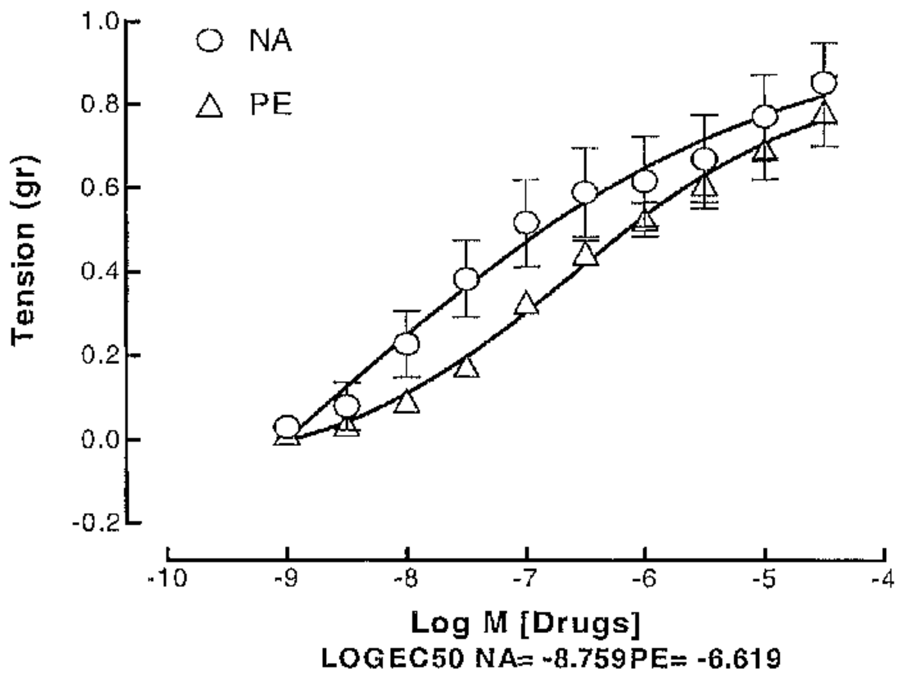


Fig. 5-4: CCRC to noradrenaline (O) and phenylephrine (Δ) in fourteen months wild type mouse aorta (n=6).

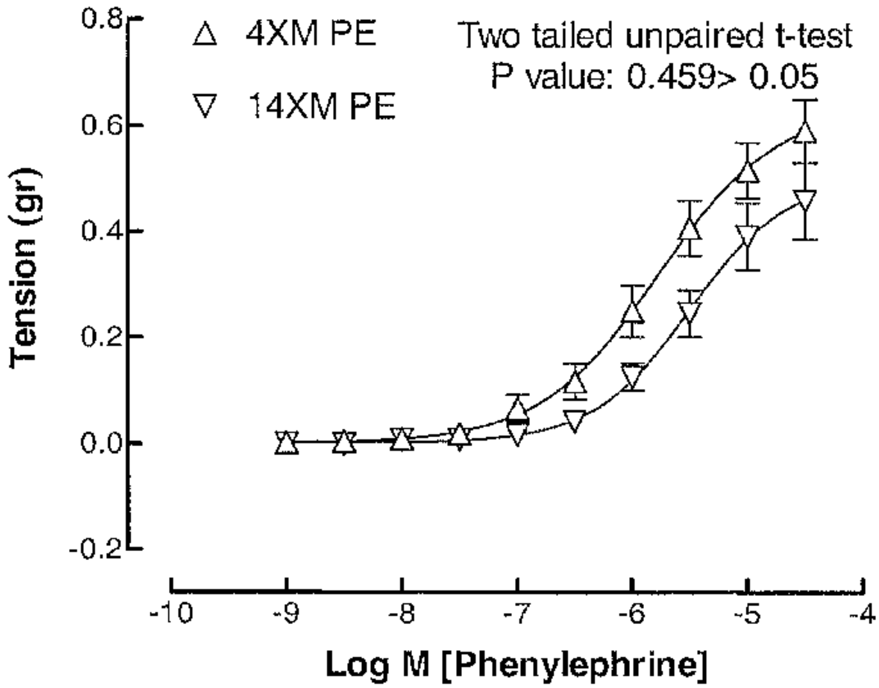


Fig. 5-5: Comparison between CCRC response in four (△) and fourteen (▽) months wild type superior mesenteric artery to phenylephrine (n=6).

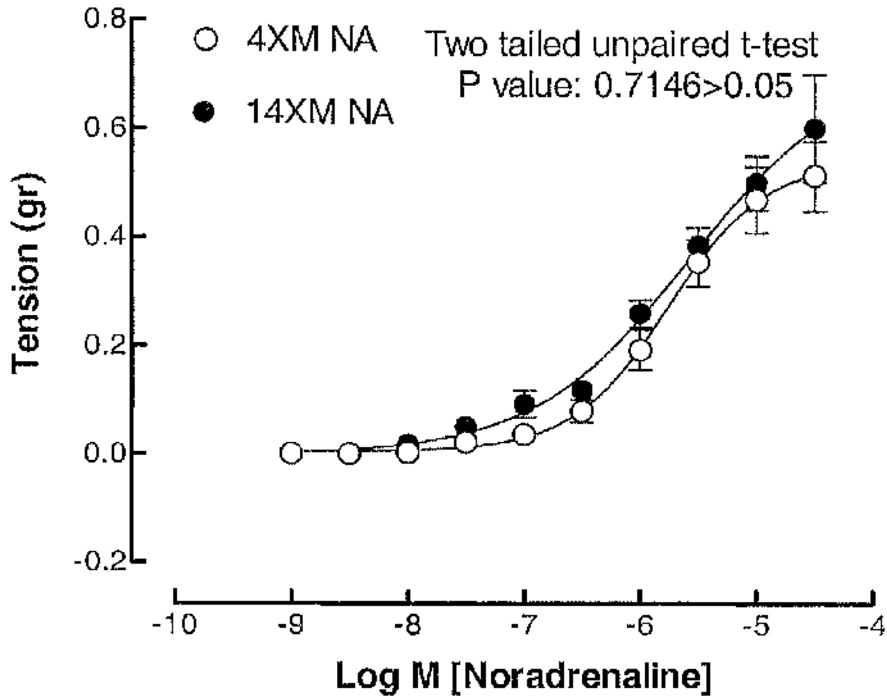


Fig. 5-6: Comparison between CCRC response in four (O) and fourteen (•) months wild type superior mesenteric artery to noradrenaline (n=6).

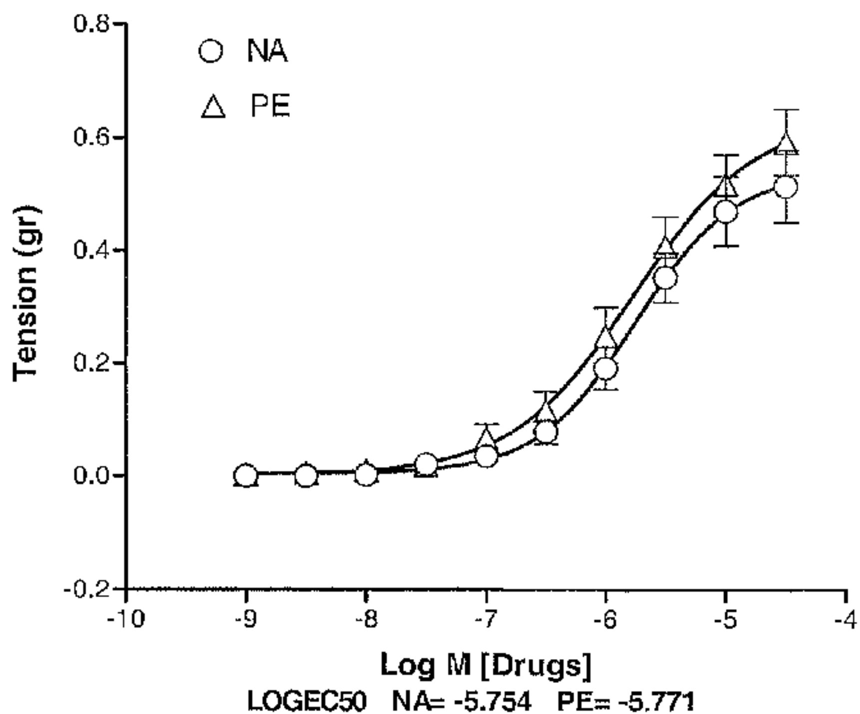


Fig. 5-7: CCRC to noradrenaline (O) and phenylephrine (△) in four months wild type mouse superior mesenteric artery (n=6).

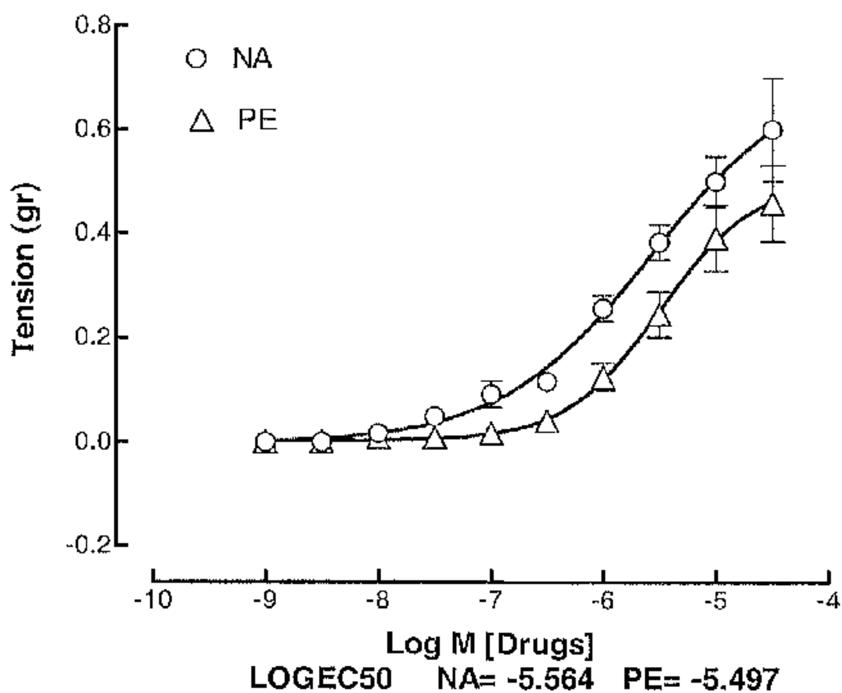


Fig. 5-8: CCRC to noradrenaline (O) and phenylephrine (△) in fourteen months wild type mouse superior mesenteric artery (n=4).

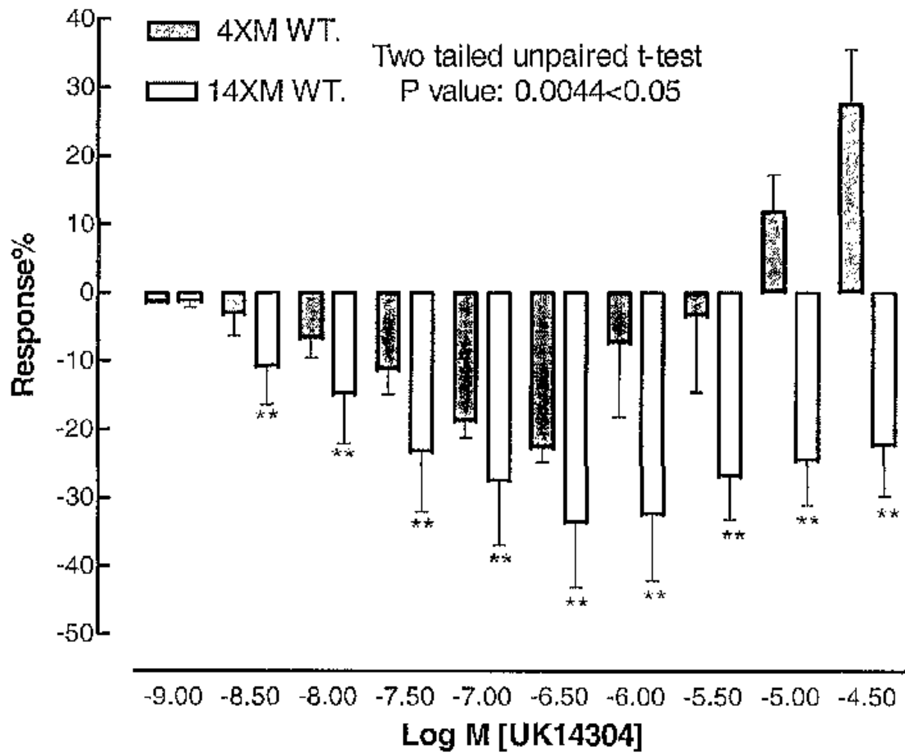


Fig. 5-9: Comparison between four and fourteen months wild type mouse aorta in response to CCRC to UK14304 (n=5).

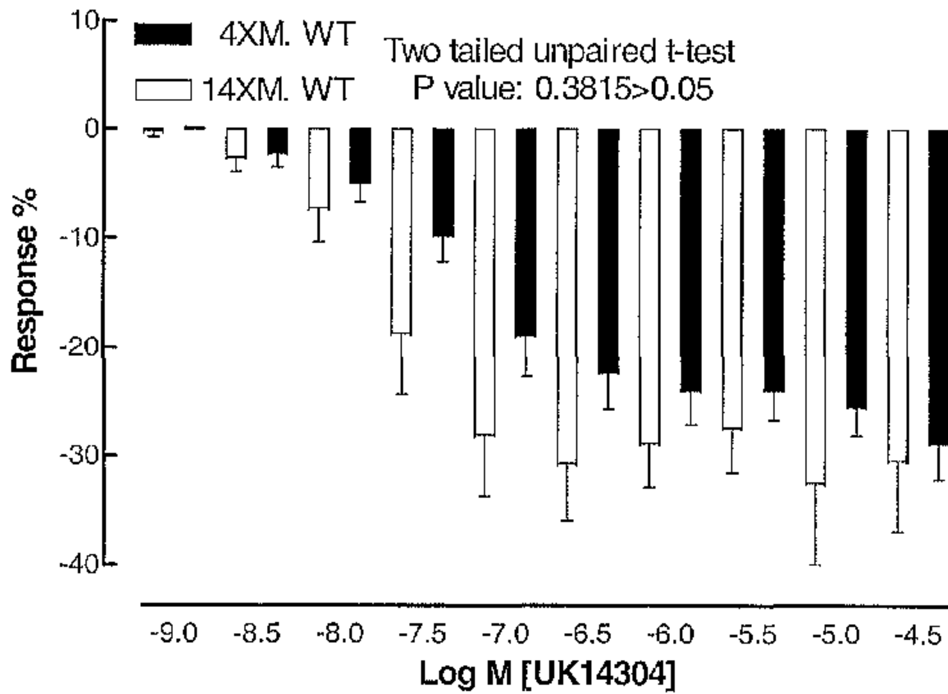


Fig. 5-10: Comparison between four and fourteen months wild type mouse superior mesenteric artery in response to UK14304 cumulatively (n=6).

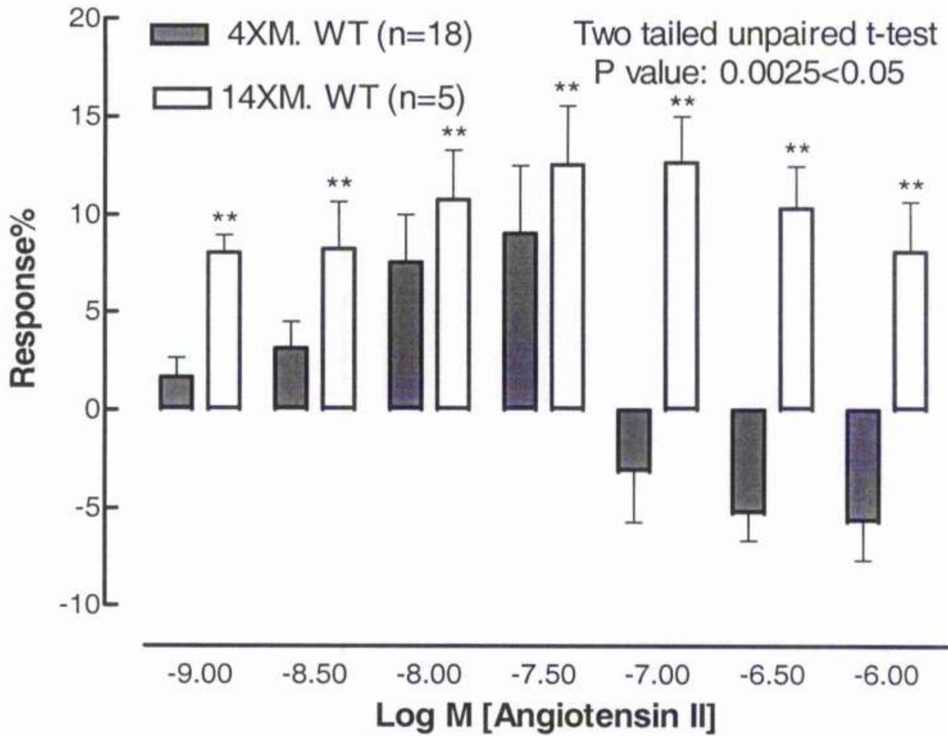


Fig. 5-11: Comparison between four and fourteen months wild type mouse aorta in response to angiotensin II cumulatively (n=18).

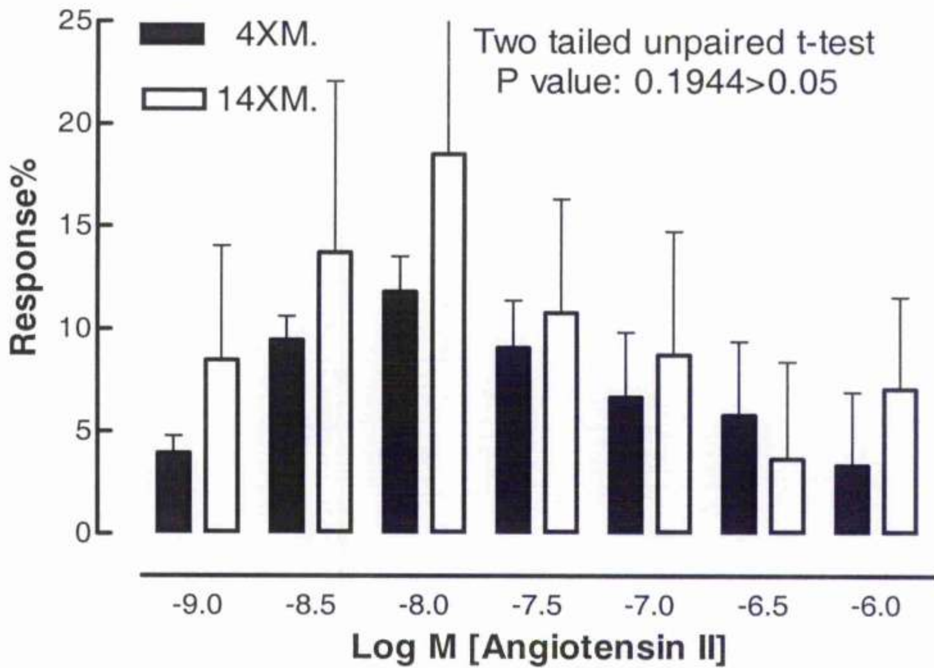
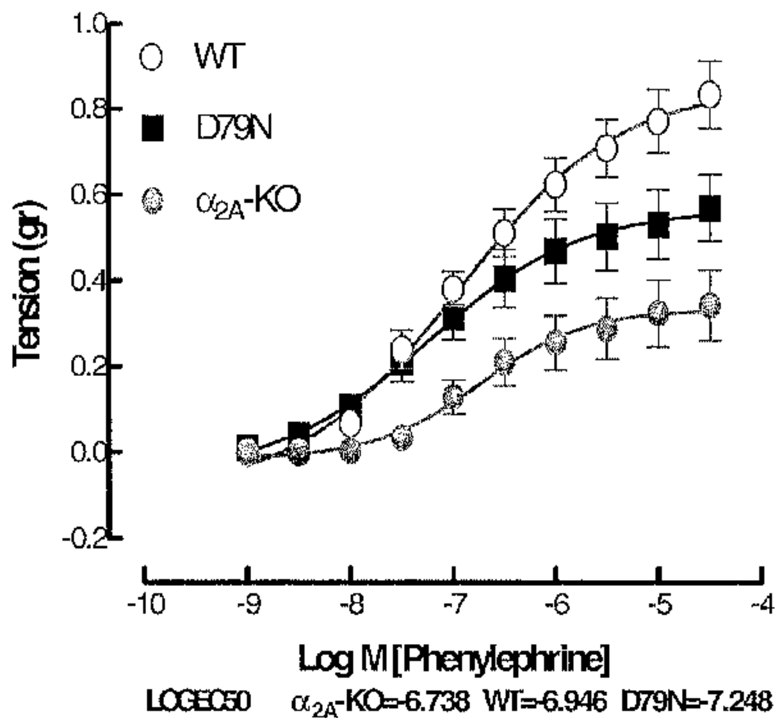


Fig. 5-12: Comparison between four and fourteen months wild type mouse superior mesenteric artery in response to angiotensin II cumulatively (n=6).



One Way ANOVA

Parameter	Value
P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	18.67
R squared	0.6747

Bonferoni's Multiple Comparison Test	P value
α_{2A}-KO aorta vs WT aorta	< 0.001
α_{2A}-KO aorta vs D79N aorta	< 0.01
WT aorta vs D79N aorta	> 0.05

Fig. 5-13: Comparison between response to CCRC to phenylephrine in wild type (O) , D79N, (■) and α_{2A}-KO (●) mouse aorta (n=7).

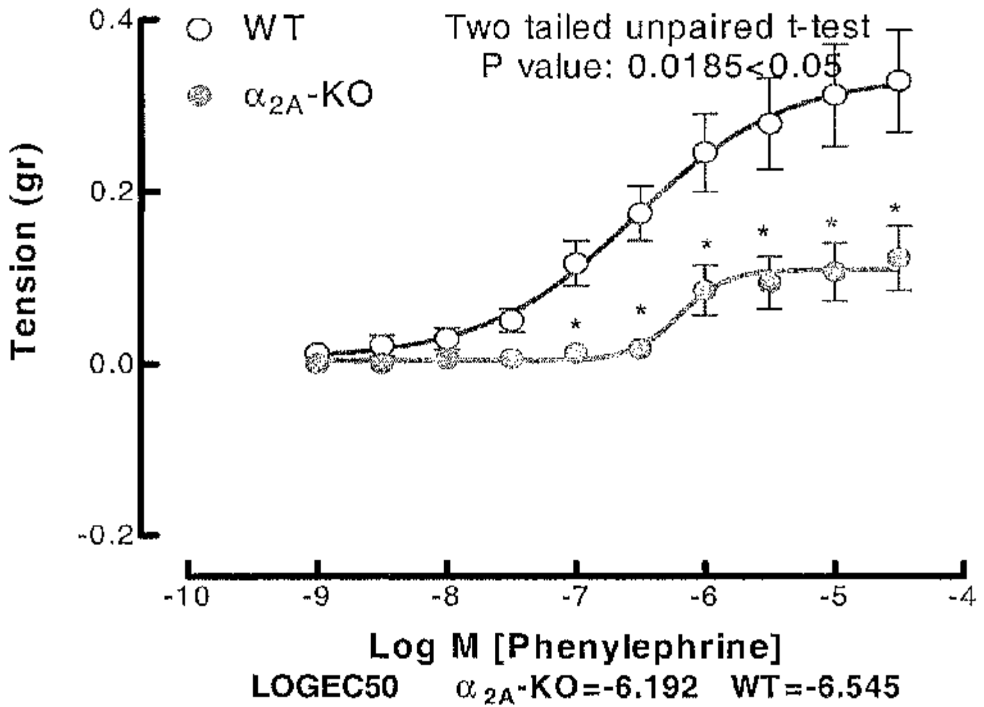


Fig. 5-14: Comparison between response to CCRC to phenylephrine in wild type (O) and α_{2A}-KO (●) carotid artery (n=4).

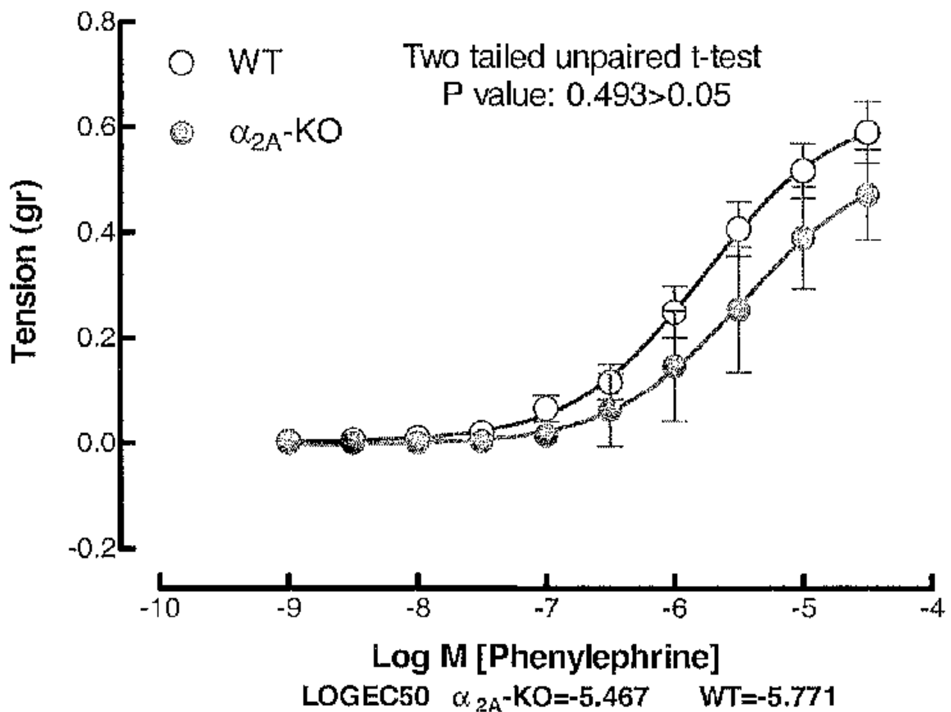


Fig. 5-15: Comparison between CCRC to phenylephrine in wild type (O) and a_{2A}-KO (●) superior mesenteric artery (n=6).

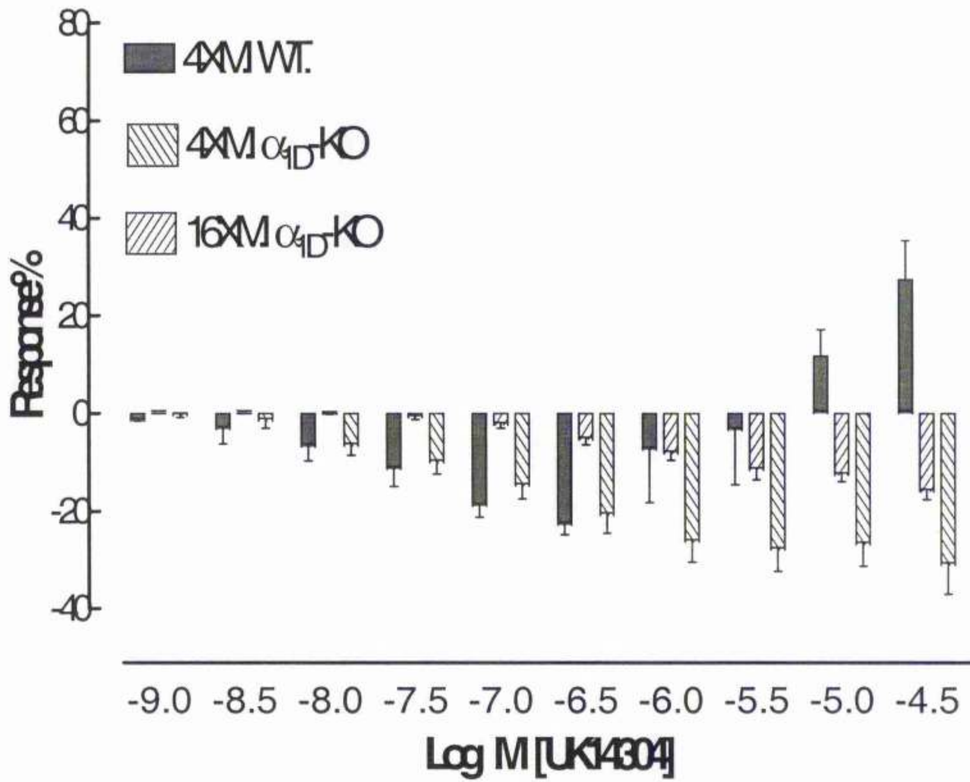


Fig. 5-16: Comparison between young & old WT and α_{1D}-KO mouse aorta in response to UK14304 cumulatively on top of U46619 pre-constriction (n=7).

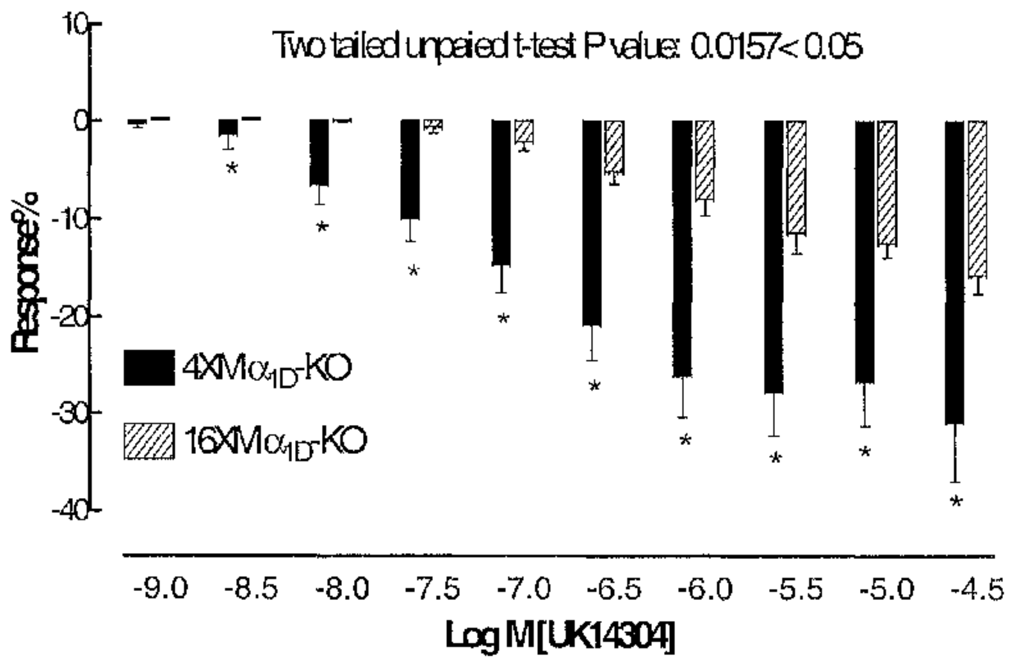


Fig. 5-17: Comparison between young & old α_{1D}-KO mouse aorta in response to UK14304 cumulatively on top of U46619 preconstriction (n=7). There is significant difference (P value<0.0097) by two tailed unpaired student t-test between them.

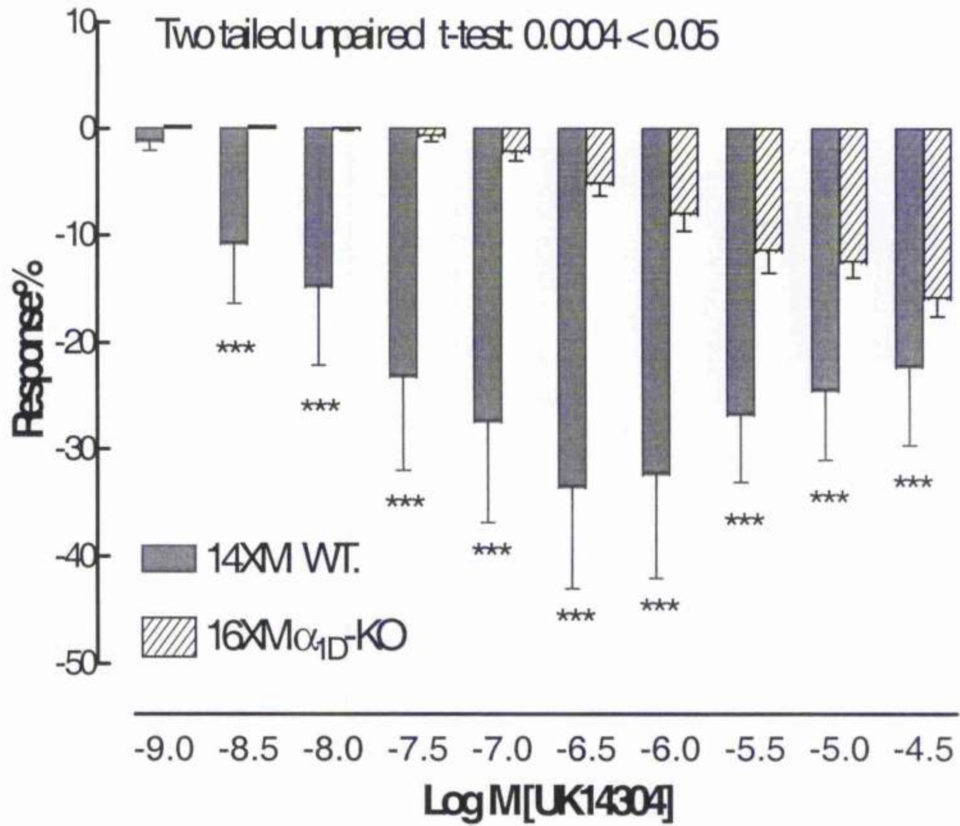


Fig. 5-18: comparison between old wild type and α_{1D}-KO mouse aorta in response to UK14304 cumulatively on top of U46619 preconstriction (n=7). There is significant difference (two tailed Unpaired t-test P value: 0.0004< 0.05) between them.

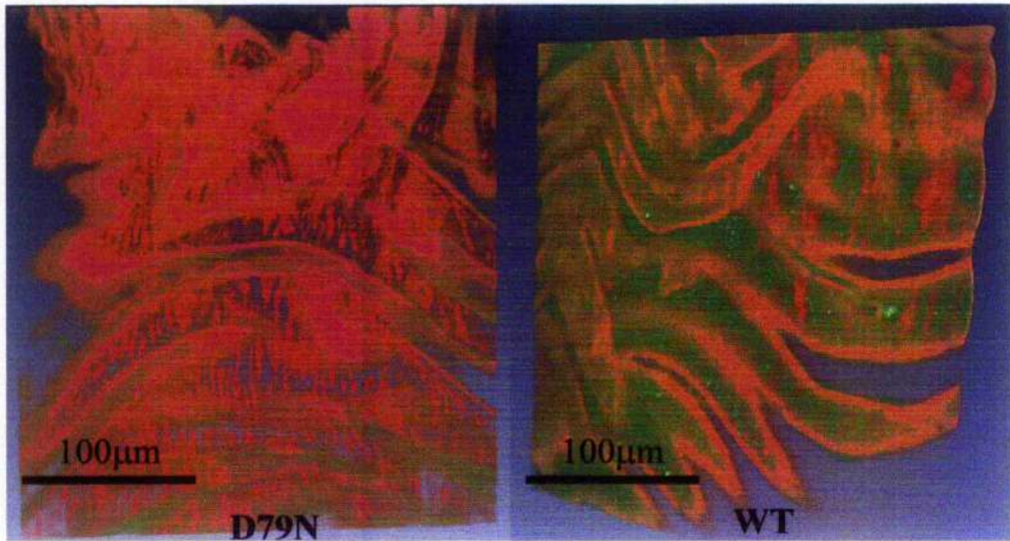


Image 5-1: Four months wild type (WT) and D79N mouse aorta Smooth Muscle Cells which treated by Both of Losartan (10μM) and BMY7378 (0.1μM), then stained with Rhodamine-Ang II-Human (50nM) and QAPB (0.1μM). As the images show Rho-Ang II-H effected more stronger on D79N smooth muscle cells compared with WT . Both of these images created by Amira (Version 3.2), Both Voltex, in the same condition (n=4).

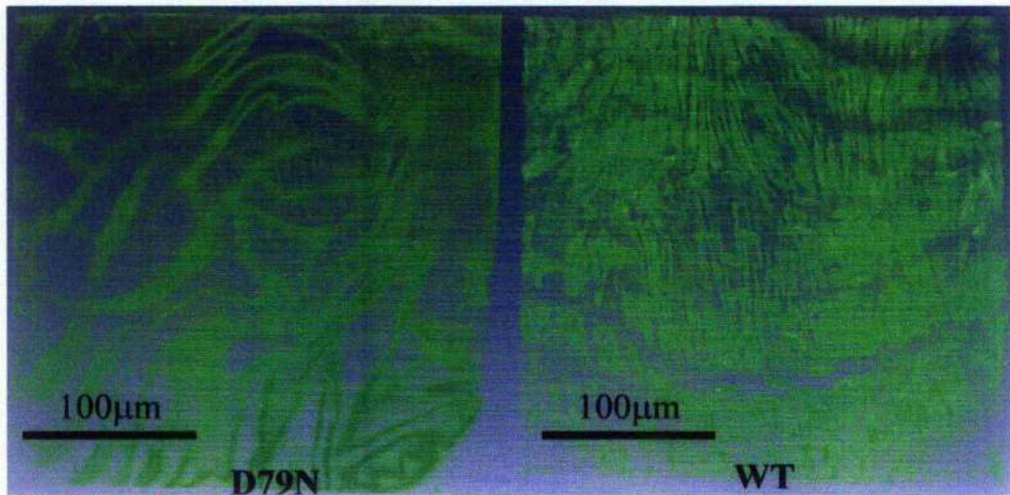


Image 5-2: Four months WT and D79N mouse aorta smooth muscle cells which only stained with QAPB (0.1μM). As the images show, QAPB-binding intensity is much stronger in wild type compared with D79N. Both of the images created in by Amira (Version 3.2) Voltex QAPB, in the same condition (n=4).

5-5. Discussion:

In the course of determining the basic properties of these receptors, we have found some strain- and age-related interactions between responsiveness to α_2-adrenoceptor- and AT_2-activated, endothelium/NO- mediated vasodilator responses and α_1-adrenoceptor-mediated contraction in the aorta and main (superior) mesenteric arteries.

For α_1-AR, ageing did not significantly change responses to PE (α_{1D}-AR in aorta and α_{1A}-AR in mesenteric arteries), so the receptors and their signalling system do not appear to be greatly modified by ageing. Nevertheless, contraction to UK14304 decreased, suggesting either a reduction in receptor number or that its response was overwhelmed by an increased endothelial action (see below).

α_2-AR-mediated relaxant responses increased with age, so either their signalling system becomes enhanced with age or their expression level on endothelial cells increases. The nitric oxide release system showed no overt signs of changing with age according to responses to ACh so perhaps a change in expression level of α_2-AR should be considered and examined in future work. The only other equivalent system that we tested in old age was relaxation via AT_2 receptors and this declined markedly with age, a sign perhaps that this system suffers a loss of receptors as age progresses.

There were signs of a reciprocal balance between α_1 and α_2-AR. Circumstances that delete or lower expression of one of them seems to affect the other's expression. Essentially, genetic elimination of the $\alpha_{2A/D}$-AR reduced responsiveness to α_1 while genetic elimination of the α_{1D}-AR led to attenuation of the age-related increase in endothelial $\alpha_{2A/D}$-AR-mediated relaxation

Endothelial α_2-ARs and AT_2-Receptors appear to work independently, according to our attempts to detect synergism. However, there seems to be an age-related balance between expression of angiotensin II receptors and α_2-adrenoceptors at least in mouse aorta, adrenoceptors becoming dominant with age. It is not known whether these events are connected, but a consequence will be that the AT_1 receptor will dominate the effects of Ang II as age progresses.

Changes related to ageing in mouse arteries show an apparent *balance* between subtypes of adrenoceptors as well as between adrenoceptors and angiotensin II receptors. We hypothesise that, in these cases, deleting expression of one receptor may reflect a change in the balance of receptors that occurs in ageing. In turn this may reflect a natural balance between vasoconstrictor and vasodilator elements in mouse arteries.

Essentially, we found unexpected effects on the other receptor of knocking out either the α_{1D}-AR or the $\alpha_{2A/D}$-AR.

In a first example, we found an unexpected difference between young and old α_{1D}-AR knockout mice, namely a fall in relaxant response to activation of the $\alpha_{2A/D}$-AR by UK14304, suggesting that the presence of α_1-ARs (Contractile receptors) is necessary to maintain an active population of α_2-ARs (relaxant receptors).

Perhaps related to this, we found that in the D79N mouse aorta, the maximum contractile response to cumulative concentrations of Phenylephrine in was around 30% less than in normal mice, an effect that was also found, by Dr. Melissa McBride, in the tail artery. This is more difficult to explain since we do not know the loss of which aspect of the $\alpha_{2A/D}$-AR is responsible for the loss of the α_{1D}-AR response, but given the age-related effect noted above, the *balance* between the two receptors in their vasoconstrictor and vasodilator actions may be a potential mechanism.

The reduced expression of α_1-ARs in D79N aorta, as suggested by the functional experiments, is supported by the imaging experiments. QAPB binding intensity was lower in aortic smooth muscle cells of D79N. Furthermore, while the binding to endothelial cells was reduced in D79N, as would be expected from lower expression of $\alpha_{2A/D}$-AR, there was evidence of a loss of other α_1-AR subtypes: treatment with BMY7378 could completely remove QAPB-intensity in a majority of D79N aorta smooth muscle cells but not in wild type. This suggests a lower expression of the other subtypes of α_1-ARs (α_{1A}-AR and α_{1B}-AR) in D79N mouse aorta compared with wild type. In turn this may suggest a general downregulation of endothelial adrenoceptors in response to elimination of the major type, i.e. $\alpha_{2A/D}$-AR.

The second example of interest was the age-related loss of the relaxant AT_2 response in aorta, which suggests a greater vasodilatory role for the Renin-Angiotensin-System (RAS) in young mice arteries compared with old ones. Thus, this places more functional responsibility on other dilator receptors including adrenoceptors. It is also known that vasodilator β-ARs decline with age (Arribas 1997). Thus, in old mice, more responsibility is placed on α_2-adrenoceptor. This may provide a more secure way to protect against acute high blood pressure. AT_2 receptors have a slow action so need at least 5-15 minutes to dilate the vasculature. In that situation, adrenoceptors may have a more effective and faster role for smooth muscle cell relaxation, in the challenging circumstances for ageing blood vessels where atherosclerosis may have reduced lumen cross-sectional area.

In old wild type mice, the contractile effect of the AT_1-Receptor is stronger even at low concentrations where the AT_2 response is not detected at four months (Fig.5-11). Since AT_2-Receptor expression is believed to down-regulate AT_1-R effects (Matsubara 1998)

(Horiuchi *et al.*, 1999) (De. Gasparo *et al.*, 2000), it is possible that the loss of AT_2 and gain of AT_1 are linked. This could have consequences for the effectiveness, in old age, of drugs that influence the angiotensin system such as ACE inhibitors or AT-receptor blockers. In this respect it is also interesting that dilator α_2-AR takes a greater role in old mice. Therefore, increasing α_2-AR expression could be a compensation for less expression of AT_2 in normal condition and might place contractile AT_1 and dilator catecholamines in sharper opposition as age proceeds.

Theoretical Context

It is possible to simplify the balance between adrenoceptors by the following formulas:

IF: S = SMCs and E = Endothelium

$$\alpha\text{-ARs response} = (\alpha_{1S} - \alpha_{1E}) - (\alpha_{2E} - \alpha_{2S})$$

For angiotensin II receptors we can summarise reaction of vascular bed in following formula:

$$\text{Angiotensin II receptors response} = AT_{1S} - (AT_{2E} - AT_{2S})$$

So for balance between these two separate systems, two above formula can summarise in following formula:

$$\alpha\text{-ARs response} \pm \text{angiotensin II receptors response} = \text{Constant}$$

Minus (–) for young due to greater expression of AT_2 in endothelium and Plus (+) for old mice due to absence of AT_2 relaxant effect.

Thesis General Discussion and Conclusion

Angiotensin II receptors responses:

The AT-Receptor (AT-R) responses in the mouse arteries were investigated using classical pharmacology. There was contraction of vascular smooth muscle via losartan-sensitive AT_1 receptors and indirect relaxation via NO released by endothelial, PD123319-sensitive AT_2 receptors. This confirms the general interpretation of Tanaka et al. (1999) using an AT_2 knockout mouse that loss of relaxation in the KO shows this subtype to be responsible for relaxation. Our data also shows that the standard AT subtype-selective antagonists distinguish the receptors well in this simple mouse preparation.

In conducting arteries and large veins from other species the contractile responses via α-AR and AT receptors show considerable synergism (Dunn *et al*, 1991). Establishing this in mouse vessels to enable further investigation of this phenomenon was one of our initial objectives. Both receptors, on their own, invoked weak contractile responses, as in vessels showing synergism. However, it soon emerged that the characteristic interaction was not synergism but negative interaction between excitatory and inhibitory effects. This showed up clearly in the interaction between noradrenaline and angiotensin II. Despite the potential for involvement of up to eleven receptors, the dominant interaction was between contractile response of noradrenaline via α_{1D}-AR and the relaxant effect of angiotensin via endothelial AT_2.

The contractile effect of UK14304 that was uncovered by blocking the endothelial α_2-AR-mediated response was found to be mediated by α_{1D}-AR. The response was absent in the α_{1D}-AR KO mouse and was antagonised by BMY7378 (Shafaroudi *et al*, 2002). In rat first order mesenteric arteries it was reported that AT_2 could invoke relaxation in the presence of losartan and that this was sensitive to bradykinin 2-Receptor blockade.

Thus, a proportion of the relaxant effect of Ang II was indirectly due to bradykinin-2 receptors in rat first branch mesenteric artery (Berthiaume *et al*, 1997) which is opposed by α_{1A}–AR contractile effect (Rokosh and Simpson 2002) (Yamamoto & Koike 2001). However, angiotensin II could stimulate up to 13% relaxation even in the absence of losartan in first order branches of mouse mesenteric arteries. Clearly the interaction between angiotensin and catecholamines will depend on the dominant receptor populations in the particular blood vessel. However, the dominant influence of the AT_2 endothelium-mediated response in mouse aorta and first branch mesenteric artery is new and unexpected.

These results revealed greater dominance of the AT_2 receptor in small resistance arteries compared with large conductive arteries like aorta, carotid and superior mesenteric arteries. Thus, this may make smaller arteries more susceptible to relaxation by angiotensin II and noradrenaline due to direct effect on endothelium AT_2 and α_2-AR stimulation respectively (see chapter three).

In conclusion, mouse aortic and first branch of mesenteric arteries have both smooth muscle AT_1 receptors that initiate contraction and endothelial AT_2 that promote the release of nitric oxide, detectable as an invoked smooth muscle relaxation. This provides a useful system for the analysis of these receptors. The dominant catecholamine-angiotensin interaction is between contractile α_{1D}-AR/or α_{1A}-AR and relaxant AT_2.

Localisation of Angiotensin II-Receptors

Laser Scanning Confocal microscopy on live dissociated cells and intact vessels could localised both AT_1 and AT_2 receptors using selective antagonists (losartan and PD123319 respectively) and fluorescent rhodamine label (Rhodamine Ang II-H). Our

data shows the AT_2 receptor expressed more inside both smooth muscle and endothelial cells compared with AT_1, which localised more on the cell membrane. This can explain the slow onset of AT_2-R relaxation, which is reflected in our pharmacological data.

α_2-adrenoceptor-mediated vasodilator responses

We have also demonstrated an α_2-adrenoceptor-activated, endothelium/NO mediated vasodilator response in the aorta, carotid artery and mesenteric arteries in the important mammalian model organism, the mouse. Clarification of the receptor interaction in the mouse and other model species should accelerate appreciation of its role in man.

Analysis of vasodilator responses via α_2-adrenoceptors proved straightforward in wire myograph-mounted, pre-constricted arteries. Aorta, carotid and mesenteric arteries showed rauwolscine-sensitive relaxant responses to UK14304 that were susceptible to endothelial damage or inhibition of NOS. This represents the vasodilator phenotype of the $\alpha_{2A/D}$-adrenoceptor in aorta and carotid arteries since it was absent in the knockout and the D79N mutation of this receptor.

The aorta contracts to high concentrations of UK14304. This is sensitive to knockout of the α_{1D}-adrenoceptor, the dominant contractile adrenoceptor in this vessel (Yamamoto, 2001; Tanoue, 2002; Daly, 2002).

α_2-adrenoceptor -selective agonists are often partial agonists at α_1-adrenoceptors; e.g. rat aorta (Hussain and Marshal, 1997). This reinforces the conclusion of Vandeputte et al (Vandeputte and Docherty, 2003) that in mouse aorta constrictor α_{1D}- and dilator $\alpha_{2A/D}$- adrenoceptors act in opposition. Like aorta the carotid has contractile α_{1D}-adrenoceptors (Surprenant, 1992; Daly, 2002). It did not exhibit contraction to

UK14304 in the WT but did in the $\alpha_{2A/D}$-KO, reflecting the greater sensitivity in this strain, found also in aorta.

The experimental objectives of this study were met equally well by the $\alpha_{2A/D}$-KO and D79N mice. The $\alpha_{2A/D}$-KO is a straightforward "knockout" in which the receptors are not expressed. The D79N is a mutated version of the $\alpha_{2A/D}$-adrenoceptor that is unable to activate K+ currents but retains inhibition of voltage-gated Ca^{2+} channels and cAMP production (Surprenant, 1992). However, because it is expressed at a reduced density relative to wild-type $\alpha_{2A/D}$-adrenoceptors it is considered a "functional $\alpha_{2A/D}$-adrenoceptor knockout" rather than a selective uncoupler of a particular activation pathway (MacMillan, 1996). Both the $\alpha_{2A/D}$-KO and D79N mutant did not exhibit the α_2-adrenoceptor-mediated vasodilatation shown in aorta and carotid artery from WT mice. This produces definitive evidence that an endothelial α_2-adrenoceptor response is mediated via the $\alpha_{2A/D}$-adrenoceptor and validates the tentative pharmacological analysis in large arteries of the rat and pig (Bockman, 1996; Gumimaraes, 2001) of an endothelial $\alpha_{2A/D}$-adrenoceptor subtype.

The phenotype of this vasodilator α_2-adrenoceptor in these large arteries contrasts with the pre-junctional α_2-adrenoceptor in that only one subtype, the $\alpha_{2A/D}$-adrenoceptor, was responsible whereas both $\alpha_{2A/D}$- and α_{2C}-adrenoceptors were implicated in the pre-junctional modulation of noradrenaline release and both had to be knocked out to eliminate that response (Hein, 2001) There was no evidence of compensatory up-regulation of another α_2-adrenoceptor subtype.

Subtle differences in the two "knockout" strains may repay further investigation but lie outside our current objectives. Both strains lost the aortic relaxant effects of UK14304, but the surviving contractile responses had different concentration-response relationships. This was reflected in carotid, which showed a small contraction only in

the $\alpha_{2A/D}$-KO. These minor differences are likely to be due to different adaptations of the strains to the genetic manipulations. Nothing was found that compromised our current conclusions.

We found no evidence for a contractile α_2-adrenoceptor in either artery. In other species α_2-adrenoceptor-mediated vasopressor responses are easily demonstrated *in vivo* (Docherty & McGrath, 1980) but difficult to show *in vitro* (McGrath, 1989), This is now reinforced and extended in the mouse. The clearest example of α_2-adrenoceptor-mediated vasoconstriction *in vitro* is in tail artery (McBride, 2002).

Localisation of α_2-adrenoceptors

We visualised the fluorescent ligand, QAPB, binding to aortic endothelial cells and eliminated this binding with the α_2-adrenoceptor antagonist, rauwolscine. We validated this by showing that this fluorescent ligand is a functional antagonist of aortic relaxation to UK14304. This provides compelling direct evidence for the endothelial location of the α_2-adrenoceptors that mediate vasodilatation. There is controversy surrounding whether the initial step in the release of endothelial relaxant factors is activation of receptors on the endothelial cells or receptors on smooth muscle cells that signal to the endothelium. The existence of myoendothelial connections could transmit depolarisation from smooth muscle cells to the endothelium (Oishi, 2001; Dora, 2001). Previous vascular localisation of receptors relied on autoradiography, indicating α_2-adrenoceptors in the medial layer but not on endothelium (Stephenson, 1987). Our present study shows direct proof of α_2-adrenoceptor binding sites on endothelial cells as well as the "mosaic" nature of the endothelium which may related to myoendothelial connections (Images FC-1and FC-2). Thus location and function are in accord and a direct endothelial effect should be reconsidered.

A peripheral endothelium/nitric oxide-mediated direct vasodilatation to α_2-adrenoceptor agonists must now be considered in addition to any centrally-mediated sympatho-inhibitory effects or pre-junctional inhibition of post-ganglionic sympathetic transmission.

We now show vasodilatation may be induced via endothelial α_2-adrenoceptors. In major conducting arteries, such as aorta and carotid this would reduce blood pressure via a reduced after-load. Thus, the vasodepressor action of α_2-adrenoceptor activation would be a combination of endothelial activation and sympathomimetic inhibition.

This is important because activation of endothelial relaxant and hypotrophic factors are aspects of α_2-adrenoceptor agonists that distinguish them from beta blockers. This might confer specific advantages in the treatment of different cardiovascular diseases since α_2-adrenoceptor agonists could cause endothelium-mediated vasodilatation that is additional to their effect on sympathetic tone. In contrast the vasodilatory or hypotensive effect of β-adrenoceptor blockade is dependent on central inhibition of sympathetic tone and in blood vessels themselves will actually antagonise local vasodilatation mediated by β-adrenoceptors, resulting in peripheral vasoconstriction. α_2-adrenoceptor agonists would, therefore, be advantageous where vasodilatation is beneficial but where there is already little sympathetic tone or where circulating catecholamines are blocked or not stimulating a beneficial β-adrenoceptor-mediated vasodilatation.

Age related receptor population changing in normal and knockout mouse

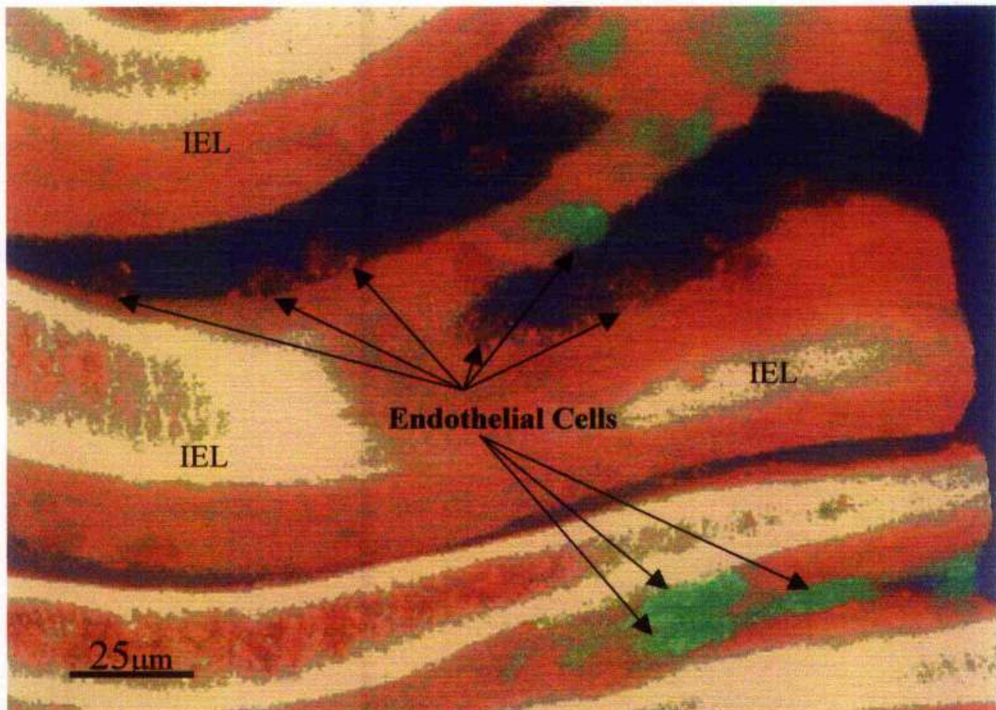
We found some strain- and age-related interactions between responsiveness to α_2-adrenoceptor- and AT_2-activated, endothelium/NO- mediated vasodilator responses and $\alpha 1$-adrenoceptor-mediated contraction in the mouse aorta.

Despite the lack of significant difference in α_1-ARs with ageing, α_2-AR mediated relaxation increased with age. Endothelial α_2-ARs and AT_2-receptors appear to work independently, according to our attempts to detect synergism. However, there seems to be an age-related balance between expression of angiotensin II receptors and α_2-adrenoceptors at least in mouse aorta, adrenoceptors becoming dominant with age. Loss of the relaxant AT_2 response in aorta suggests a greater vasodilatory role for the Renin-Angiotensin-System (RAS) in young mice arteries compared with old ones.

Therefore, increasing α_2-AR expression could be a compensation for lower expression of AT_2 in WT mice and might place contractile AT_1 and dilator catecholamines in sharper opposition as age proceeds.

Essentially, we found unexpected effects on the other receptor of knocking out either the α_{1D}-AR or the $\alpha_{2A/D}$-AR.

We found a difference between young and old α_{1D}-AR knockout mice, namely a fall in relaxant response to activation of the $\alpha_{2A/D}$-AR by UK14304, suggesting that the presence of α_1-ARs (contractile receptors) is necessary to maintain an active population of α_2-ARs (relaxant receptors) and vice versa. The reduced expression of α_1-ARs in D79N aorta, as suggested by the functional experiments, is supported by the imaging experiments. QAPB binding intensity was lower in aortic smooth muscle cells of D79N.



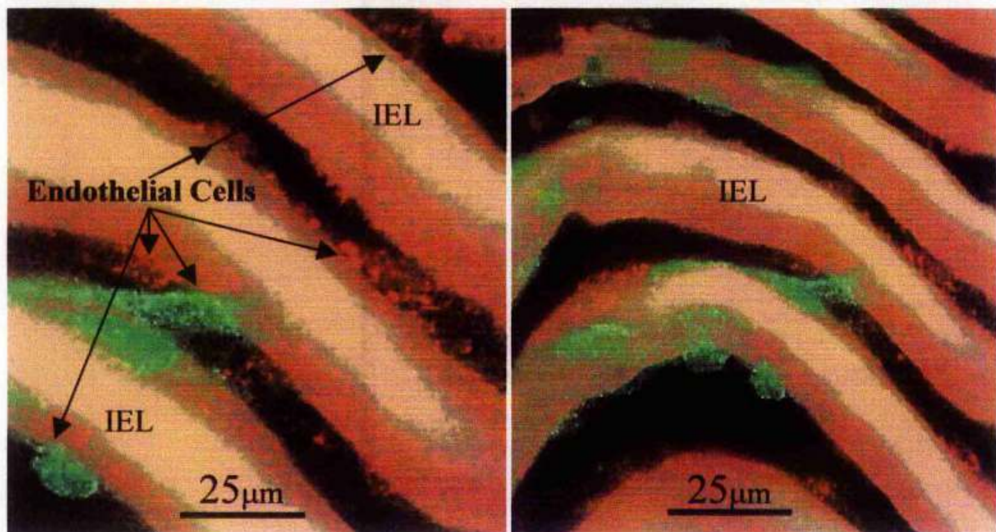


Image FC-1: Four months wild type mouse aorta treated with Losartan (10μM), Rauwolscine (1μM) + BMY7378 (1μM) then stained with QAPB (0.1μM) and Rhodamin-Ang II (50nM). Some endothelial cells only stained Red and some others still showing QAPB-binding (α_{1A}- or α_{1B}-ARs) (n=6) {Mosaicism}. This reveals a mosaicism related to α1-ARs in endothelial cells of young mouse aorta.

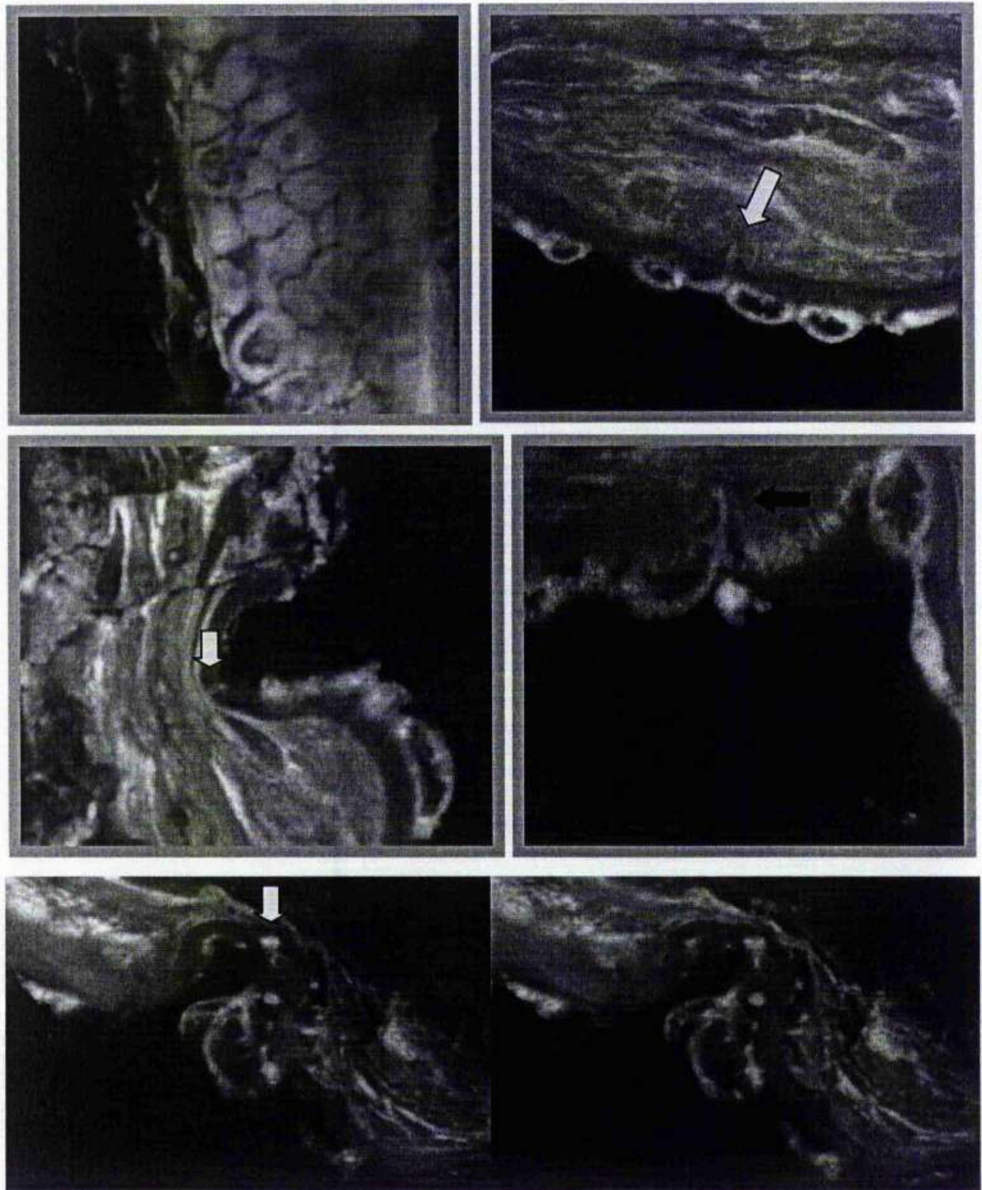


Image FC-2: Young rat mesenteric artery endothelial cells which connected to smooth muscle cells (myoenothelial connection) through the Internal Elastic Lamina (IEL) fenestrations. (images belongs to Jose Maria Gonzales, University of Valencia-Spain, which stained with special Lipophilic Tracers $DiOC_{18}$ (3), (1μM) {Excitation/Emission 484nm/501nm} for two hours at 37ºC and taken at X40 zoom, 1280*1024, 0.02-0.04 μ/Pixel).

Appendix

Characteristic of drugs used in the project:

R	Drugs-Solubility	MW	Characters	Effects
1	Noradrenaline (EDTA 23mM & water)	319.3	α_1-agonist α_2-agonist	Contraction
2	Phenylephrine (water)	203.7	Selective α_1 agonist	Contraction
3	U19 (TXA2) (water)	350.4	Thromboxane R. agonist	Contraction
4	Prazosin (water)	419.9	Non-Selective α-antagonist	α- blocker
5	Phenoxybenzamine		α_1 & α_2 antagonist	α_1 & α_2 blocker
6	UK14304 (DMSO)	292.14	Selective α_2 agonist (minor α1 agonist)	Contraction if the receptors are situated on smooth muscle cells & relaxation on endothelial cells.
7	Rauwolscine	390.9	Selective α_2 antagonist	α_2 adrenoceptor blocker
8	Yohimbine	390.9	Selective α_2 antagonist (minor α1 antagonist)	α_2 adrenoceptor blocker
9	Nifedipine	346.3	L-type calcium channel blocker	Relaxation of tone. Blocker of response to some agonists.

10	Acetylcholine (water)	181.7	Na+ channel agonist Muscarinic	Contraction on smooth muscle cells. But, in Endothelium presence cause relaxation in vascular smooth muscle cells via Nitric Oxide production..
11	L-name (N-Nitro-L-Arginine methyl Ester) (water)	269.7	Inhibit Nitric Oxide synthase	If Endothelium present in vessels can provide contraction in response to Acetylcholine. Blocks any vasoresponses involving NO production.
12	Angiotensin (water)	1046.2	AT_1 and AT_2 agonist	Contraction on smooth muscle (AT_1) Relaxation on smooth muscle (AT_2)
13	Losartan (DMSO)	461	AT1 -antagonist	No contraction response to Ang II
14	PD 123319 (water)	736.7	Selective AT_2 antagonist	No relaxation response to Ang II
15	5MU (water)	401.5	Selective α_1-antagonist	More selective for α_{1A}
16	BMY 7378 (Water)	458.43	Selective α_1-antagonist	***More selective for*** α_{1D}

Preparing Krebs solution (PSS):

Stock solution (Two Litres):

PSS Solution A:

278.20g NaCl

14.00g KCl

5.77g $MgSo_4$

PSS Solution B:

84.00g $NaHCo_3$

6.40g KH_2Po_4

Procedure:

Krebs solution (PSS) can be prepared and kept for a maximum of 2 days (including the day of preparation). Do not reuse Krebs solution, which has been previously heated.

To make Krebs (PSS) solution (2Lit):

1- Add 100ml of stock A and 100ml stock B to a volumetric flask.

2- Add distilled water to approximately ¾ the volume to the flask.

3- Weight out 4g of Glucose ($C_6H_{12}O_6$) and add it to the flask.

4- Bubble the solution with 95% O_2 and 5% CO_2 for five minutes.

5- Add 5ml of Calcium Chloride (1Molar) to the bubbled solution and bubble it for further five minutes (This will equilibrate the pH and stop the $CaCl_2$ precipitation out of the solution.

6- Add 2ml EDTA (23mM) to the solution (this will provide slower degradation of noradrenaline).

Preparing Krebs solution with ultra-potassium (Potassium Krebs or KPSS):

KPSS Solution A:

92.23g KCl

1.443g Mg SO_4

KPSS Solution B:

84.00g $NaHCO_3$

6.40g KH_2PO_4

To make KPSS solution (One Litre- 125mM):

1- Add 50ml of KPSS stock solution A and 50ml stock B to a one litre volumetric flask.

2- Add distilled water to approximately ¾ the volume to the flask.

3- Weight out 2g of Glucose ($C_6H_{12}O_6$) and add it to the flask.

4- Bubble the solution with 95% O_2 and 5% Co_2 for five minutes.

5- Add 2.5ml of Calcium Chloride (1Molar) to the bubbled solution and bubble it for further five minutes (this will equilibrate the pH and stop the $CaCl_2$ precipitation).

Note: Do not add EDTA to KPSS solution.

We can keep KPSS solution for at least two weeks in a fridge (Refrigerator).

Cell Dissociation Method:

Preparation of Buffers One and Two and combination of enzymes, which dissolve in Buffer Two:

Buffer One:

Final Concentration in solution	Name of Component	mg/1Lit	mg/5Lit
137mM	NaCl	8006.28	40031.4
5mM	KCl	372.75	1863.75
1mM	$MgCl_2$	1ml of 1Molar	5ml
1.8mM	$CaCl_2$	1.8mls of 1Molar	9mls
10mM	HEPES	2383mg	11915mg
0.1%	Bovine Serum Albumin (BSA)	1mg	5mg
pH to 7.4	1N NaOH only for pH balance	add Drop by Drop	Drops

Buffer Two:

Final Concentration in solution	Name of Component	mg/1Lit	mg/5Lit
80mM	Sodium Glutamate	169	845
54mM	NaCl	3155.76	15778.8
5mM	KCl	372.75	1863.75
1mM	$MgCl_2$	1ml of 1Molar	5mls
0.1mM	$CaCl_2$	100μL of 1Molar	500μL
10mM	HEPES	2383mg	11915mg

10mM	Glucose ($C_6H_{12}O_6$)	1801.6mg	9008mg
0.2mM	EDTA	74.44mg	372.2mg
0.1%	Bovine serum albumin (BSA)	1mg	5mg
pH to 7.4	1N NaOH only for pH balance	add Drop by Drop	Drops

Preparing Enzymes in 500µL of Buffer Two:

Buffer 2A:

Papain; 1.7mg + 500µL of Buffer Two

Dithioerythritol; 0.7mg + 500µL of Buffer Two

Note: Add two Ependorffs together. So we have 1ml of Buffer 2A now.

Buffer 2B:

Collagenase II; 1mg + 500µL of Buffer Two

Hyaluronidase; 1mg + 500µL of Buffer Two

Note: add two Ependorffs together. So we have 1ml of Buffer 2B now

Results from Craig J. Daly et al., (2002) "A knockout approach indicates a minor vasoconstrictor role for vascular α1B-adrenoceptors in mouse." *Physiology Genomices.* **9**: 85-91.

Table 1. Comparison between potency of 3 antagonists vs. the α1-adrenoceptor agonist phenylephrine in mouse aorta, carotid, mesenteric and caudal arteries.

Artery	Prazosin	5MU	BMY7378	Conclusion on α1-AR subtype
Aorta (WT)	9.8 (1.1)	8.3 (0.9)	8.8 (1.0)	1D, 1B
Aorta (α_{1B}-KO)	10.6 (0.8)	8.1 (1.0)	9.3 (1.0)	1D
Carotid (WT)	9.6 (0.9)	7.5 (1.1)	9.7 (0.4)	1D, 1B
Carotid (α_{1B}-KO)	10.3 (0.9)	7.6 (1.1)	9.6 (0.9)	1D
Mesenteric (WT)	9.0 (1.0)	8.9 (0.9)	7.0 (0.37)	1A, 1B
Mesenteric (α_{1B}-KO)	8.9 (1.0)	9.4 (0.7)	ND	1A
Caudal (WT)	8.8 (1.1)	8.3 (1.4)	ND	1A, 1B
Caudal (α_{1B}-KO)	9.2 (1.2)	8.5 (1.1)	ND	1A
α1A-AR (recomb)	9.0	9.2	7.1	
α1B-AR (recomb)	9.0	7.2	6.8	
α1D-AR (recomb)	9.0	7.9	9.3	
Human SMRA	9.2	8.5	6.5	1A, ?

Values shown are pA_2 calculated by the method of Arunlakshana and Schild, with the slope of the regression line shown in parenthesis. Recombinant (recomb) receptor binding data taken from Mackenzie et al and Jarajapu et al, WT, wild type; KO, α_{1B}-AR knockout; recomb, recombinant; 5MU, 5-methylurapidil; AR, adrenoceptor; SMRA, skeletal muscle resistance artery; ND, not determined.

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