JPET Fast Forward. Published on May 5, 2005 as DOI: 10.1124/jpet.105.085944

JPET#85944

Two “knockout” mouse models demonstrate that aortic vasodilatation is mediated via α_{2A}-adrenoceptors located on the endothelium.

Majid Malekzadeh Shafaroudi, Melissa McBride, Clare Deighan, Alexis Wokoma, Joyce Macmillan, Craig J. Daly & John C. McGrath

Autonomic Physiology Unit, Institute of Biomedical and Life Sciences, West Medical Building, University of Glasgow, Glasgow G12 8QQ, Scotland (MM, CD, AW, JM, CJD, JCM), Sari Medical Faculty, Khazar Boulevard, Mazandaran, IRAN (MMS)

JPET#85944

Running title: Endothelial vasodilator α_{2A}-adrenoceptors

Corresponding author:

Professor J.C. McGrath
Autonomic Physiology Unit
Institute of Biomedical and Life Sciences
West Medical Building
University of Glasgow
Glasgow
G12 8QQ
UK

Tel: +44 141 330 4483

Fax: +44 141 330 2923

Email: i.mcgrath@bio.gla.ac.uk

Text Pages: 26
Tables: 0
Figures: 4
References: 30
Abstract: 234 words
Introduction: 701 words
Discussion:1175 words

Non-standard Abbreviations:
QAPB - Quinazoline Piperazine Bodipy;
CCRC - Cumulative concentration response curve

Section assignment: Cardiovascular

JPET Fast Forward. Published on May 5, 2005 as DOI: 10.1124/jpet.105.085944
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

UK14304-mediated vasodilator responses were studied on wire myograph mounted mouse aorta to determine the cells involved, mechanisms of action and subtypes of α_2-adrenoceptors. In the presence of induced tone, UK14304 produced 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 α_{2A}-adrenoceptor knockout mouse and the D79N mouse, a functional knockout of the α_{2A}-adrenoceptor, these relaxant effects of UK14304 were lost, indicating the involvement of the α_{2A}-adrenoceptor. 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 for a contractile α_2-adrenoceptor-mediated response. A fluorescent ligand, Quinazoline Piperazine Bodipy (QAPB), antagonised the relaxant action of UK14304. This compound could be visualised on aortic endothelial cells and its binding could be prevented by rauwolscine, providing direct evidence for the presence of α_2-adrenoceptors on the endothelium. Norepinephrine reduced tone in the α_{1D}-adrenoceptor knockout and controls, an effect blocked by rauwolscine and L-NAME but not by prazosin. This suggests that norepinephrine activates endothelial α_2-adrenoceptors. In conclusion, the endothelium of mouse aorta has an α_{2A}-adrenoceptor that responds to norepinephrine, promotes the release of nitric oxide, causing smooth muscle relaxation, and which can be directly visualised. Knockout or genetic malfunction of this receptor should increase arterial stiffness, exacerbated by raised catecholamines, and contribute to heart failure.

Introduction

All three α_2-adrenoceptors have distinct, yet poorly defined, roles in the control of the vascular system. The limited selectivity of agonists and antagonists has therefore prompted the use of transgenic mouse models. The subtypes are α_{2A}, α_{2B}, α_{2C}: the mouse orthologue of the human α_{2A}-adrenoceptor is sometimes called the α_{2D}- or $\alpha_{2A/D}$-adrenoceptor; we will use the generic term α_{2A}-adrenoceptor (Alexander et al., 2004). They have two direct pharmacological effects on blood vessels that can modify vascular tone: a direct vasopressor action (reviewed in Wilson et al., 1991; Guimaraes & Moura, 2001) and vasodilatation via endothelium-derived relaxant factors (Cocks and Angus, 1983; Vanhoutte, 2001). They also reduce sympathetic traffic centrally and inhibit transmitter release from sympathetic post-ganglionic nerves (Starke, 2001), although this is not well established as a physiological phenomenon in blood vessels. Available pharmacological data and knockout studies, though not definitive, present evidence for, at least, α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors for vasoconstriction, α_{2A}- and α_{2C}-adrenoceptors for sympatho-inhibition and α_{2A}-adrenoceptors for endothelial vasodilatation, though none of the relevant studies has considered all three actions and each chooses a different example preparation (Bockman et al., 1996; Link et al., 1996; Hein et al., 1999; McCafferty et al., 1999; Chotani et al, 2000; Guimaraes and Moura, 2001; Vanhoutte, 2001).

Thus, there is no consensus for the α_2-adrenoceptor subtypes responsible for direct vascular actions, constrictor or dilator. Yet both phenomena are potentially significant for the therapeutic use of α_2-adrenoceptor agonists and antagonists and for the physiological and pathophysiological roles of α_2-adrenoceptors in the cardiovascular system.

JPET Fast Forward. Published on May 5, 2005 as DOI: 10.1124/jpet.105.085944
This article has not been copyedited and formatted. The final version may differ from this version.

A pathophysiological role for α_2-adrenoceptors has recently emerged from the demonstration that polymorphisms of α_{2A}- and α_{2C}-adrenoceptors are linked with cardiovascular disease (Brede at al., 2002; Small et al., 2002). Hypotheses for the aetiology have focussed almost exclusively on the concept that malfunction of α_2-adrenoceptors regulating the release of neurotransmitters from sympathetic nerves could be deleterious to the cardiovascular system. However, this could just as rationally be attributable to endothelial α_2-adrenoceptors. The first specific clinical implication is that synergistic polymorphisms of β_1- and α_{2C}-adrenoceptors, a combination over-represented in the American population of African descent, can increase the risk of congestive heart failure (Small et al., 2002, Small et al., 2004). Since interpretation of this aetiology involves the use of genetically modified mice (Brede at al., 2002; Liggett, 2004) it is timely to elucidate the mechanisms underlying vascular α_2-adrenoceptors using this species to establish the physiological and, hence, potential pathophysiological roles of the different α_2-adrenoceptor subtypes and to determine whether endothelial α_2-adrenoceptors need to be considered.

There is also controversy surrounding whether the initial step in the release of endothelial relaxant factors is direct activation of receptors on the endothelial cells or indirectly through activation of receptors on smooth muscle cells that then signal to the endothelium via myo-endothelial connections (Dora, 2001). This is compelling since it is consistent with earlier vascular localisation of receptors by autoradiography, which indicated α_2-adrenoceptors in the medial layer but not on endothelium (Stephenson and Summers 1987).

Analysis of α_2-adrenoceptors in the aorta is complicated by the presence of a powerful α_1-adrenoceptor-mediated contraction, even when using relatively selective

agonists. Vandeputte et al. (2003) showed that, in mouse aorta, the complex response to norepinephrine contains constrictor α_{1D}- and dilator α_{2A}- adrenoceptor components acting in opposition. This factor was accounted for by isolating the α_2-adrenoceptor-mediated response in a strain of mice in which the dominant contractile adrenoceptor in this vessel, the α_{1D}-adrenoceptor, (Daly et al., 2002) was knocked out (Tanoue at al., 2002). "α_2-adrenoceptor-selective" agonists are often partial agonists at α_1-adrenoceptors (Docherty & McGrath, 1980; Wilson et al, 1991).

The objectives of the present study were to: establish the vasodilator phenotype for α_2-adrenoceptors in mouse large arteries; identify by two independent transgenic models whether the α_{2A}-adrenoceptor subtype is involved; establish the functional involvement of the endothelium and nitric oxide; determine whether the receptors are located on the endothelium; demonstrate that the phenomenon is activated by a physiological agonist, norepinephrine.

We demonstrate that α_{2A}-adrenoceptors can account for the entire α_2-adrenoceptor-mediated vasodilator response in mouse aorta. A combination of pharmacology, transgenic models and fluorescent ligand binding shows that the site is on the endothelial cells and that the mechanism involves the α_{2A}-adrenoceptor subtype, which activates the release of endothelial nitric oxide.

Materials and Method

Experimental Rationale

Rings of aorta mounted on a wire myograph ensured minimal disruption to endothelium. A previous study of mouse aorta found no evidence for α_2-adrenoceptor-mediated vasodilatation (Russell and Watts, 2000) but employed strips, which are susceptible to endothelial damage (Furchgott and Zawadski, 1980).

Receptor "knockout" strains were used to simply pharmacological interpretation where non-selectivity of drugs limits analysis. These were: the α_{2A}-adrenoceptor knockout mouse (Altman et al., 1999); mice harbouring the D79N point mutation of the α_{2A}-adrenoceptor, which serves as a "functional knockout" in some systems due to low expression of the mutant receptor and dysfunction due to disengagement of G protein-coupling to potassium but not calcium currents (MacMillan et al., 1996; MacMillan et al., 1998; Surprenant et al., 1992; Ceresa and Limbird, 1994).

Aortic contraction, by agonists, is a confounding factor. We eliminated this using the α_{1D}-adrenoceptor knockout (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 (Cavalli et al., 1997) and selective antagonists of the α_{1A}-adrenoceptor and α_{1D}-adrenoceptor subtypes. Concentrations of these drugs were selected, from pharmacological analysis of α_{1A}- and α_{1D}-adrenoceptor-mediated responses in these same vessels, to be clearly (approx. 10 fold) above the affinity constant for the desired receptor but below that for the

others (Daly et al., 2002). We showed that QAPB (0.1μmol/L) antagonised the α_2-adrenoceptor-mediated relaxation to UK14304 before employing this concentration to visualise α_2-adrenoceptors on the vascular endothelium.

Myography

Male mice (4 months) were killed by CO_2 inhalation. Descending thoracic aortae were isolated and set up in Krebs on wire myographs with 5ml baths to which drugs were added directly, as previously described (Daly et al., 2002). Strains were: α_{2A}-adrenoceptor mutant D79N mouse (MacMillan et al., 1996; MacMillan et al., 1998), back-crossed on to C57/BL6 (gift: Professor Lee Limbird); α_{2A}-adrenoceptor knockout, C57/BL6; ($\alpha_{2A/D}$-knockout, Jackson Laboratories, Bar Harbor, Maine, U.S.A.) (gift: Professor J.R. Docherty); α_{1D}-adrenoceptor knockout (Tanoue et al., 2002), background of 129sv/C57/BL6 (gift: Professor Gozoh Tsujimoto); α_{1B}-adrenoceptor knockout (Cavalli et al., 1997; Cotecchia et al., 1997), background of 129sv/C57/BL6 (gift: Professor Susanna Cotecchia). We compared several aspects of adrenergic pharmacology between 129sv/C57/BL6 controls and the C57/BL6 without finding significant differences. Thus, in this study we used the 129sv/C57/BL6 as control. The investigation conforms with the *Guide for the care and use of laboratory animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and with the provisions of the UK Animals (Scientific procedures) Act 1986.

Endothelium was removed, where appropriate, by rubbing the intimal surface with a roughened metal probe. Reproducible responses were obtained to norepinephrine (0.1μmol/L), phenylephrine (0.1μmol/L), or U46619 (10nmol/L), according to protocol, before commencing experiments.

At the plateau of contraction to phenylephrine (0.1μmol/L) or norepinephrine (1μmol/L), acetylcholine (1μmol/L) was added to assess endothelial integrity. Criteria for functional and dysfunctional endothelium were respectively >50% and < 5% relaxation at start and finish of experiment. L-NAME (0.1mmol/L) abolished relaxation to acetylcholine.

Tissues were tested with increasing cumulative concentrations of UK14304 in 0.5 log unit increments from 1nmol/L-30μmol/L. After a 60 min rest period, test drugs were added for at least 30 minutes before construction of a second cumulative concentration response curve (CCRC).

Visualisation of endothelial α_2-adrenoceptors

We employed the fluorescent ligand, QAPB, an analogue of prazosin with high affinity for α_1-adrenoceptors (pK_i: 8.1-8.9) (McGrath et al., 1996; Daly et al., 1998; Mackenzie et al., 2000) but which also has moderate affinity for α_2-adrenoceptors (pK_i 7.3-7.8; Dr. C.M. Milligan, personal communication, (method of Brown et al., 1993)). Binding to the three α_1-adrenoceptors was eliminated by employing vessels from the α_{1B}-adrenoceptor-knockout mouse and blocking the other two subtypes with selective antagonists: for α_{1A}-adrenoceptors, 5-methylurapidil, (5MeU); for α_{1D}-adrenoceptors, BMY7378. This allowed us to visualise putative α_2-adrenoceptors. We then confirmed that they were α_2-adrenoceptors by preventing this binding with rauwolscine.

Laser Scanning Confocal Microscopy (LSCM)

Tissue preparation: 2-3mm segments of aorta from α_{1B}-knockout mice were incubated for 30 min in BMY7378 (0.1μmol/L) and 5MeU (0.1μmol/L), with or

JPET Fast Forward. Published on May 5, 2005 as DOI: 10.1124/jpet.105.085944
This article has not been copyedited and formatted. The final version may differ from this version.

without rauwolscine (0.1μmol/L), then QAPB (0.1μmol/L) was added for 60 min. Following incubation, without washing, aortic segments were cut open and placed endothelial side up in the sample well of a slide 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. Excitation/Emission 488/515nm for QAPB. Laser power, gain and offset (contrast and brightness) were kept constant. Tissues were visualised using a x40 oil immersion objective numerical aperture 1.00 and therefore optimal pinhole setting 1.5. Image size 512 x 512 pixels equates to a field size of 289μm x 289 μm. Each procedure was carried out in triplicate on at least three different mice.

Drugs

All drugs were of analytical grade and were dissolved either in distilled water, ethanol or DMSO. Phenylephrine (H_2O), norepinephrine hydrochloride (23μM EDTA), acetylcholine chloride (H_2O), BMY7378 (H_2O), propranolol (H_2O), 5-methyl-urapidil (H_2O), U46619 (ethanol), L-NAME (N-Nitro-L-Arginine methyl ester) (H_2O), rauwolscine (H_2O) [Sigma-Aldrich Co; Poole, UK], Quinazoline Piperazine Bodipy (DMSO), [Molecular Probes, Eugene, OR, USA], UK14304 (DMSO) [Pfizer, Sandwich, UK].

Statistics

Values are means ± Standard error of the mean from n experiments. Differences in maximal contraction response to agonist in presence and absence of drugs were compared using one-way ANOVA or by Student's t-test. Statistical and graphical analysis was carried out using Excel 97 and GraphPad Prism 3.00.

Results

α_2-adrenoceptor-mediated relaxation

Rings were preconstricted with a concentration of U46619 required to produce ~75% of the maximum contraction. At the pre-constriction plateau, UK14304 (1µmol/L) was added, causing a marked rapid fall in tone (vasodilatation; fig 1a). UK14304-induced relaxations were blocked in the presence of rauwolscine (fig 1b) but not prazosin (fig 1c).

CCRCs for the α_2-adrenoceptor agonist UK14304 versus U46619 pre-constriction had two phases (fig 2a). UK14304 produced vasodilator responses at low concentrations (1nmol/L-0.3µmol/L) and vasoconstriction at higher (>0.3µmol/L) concentrations. In aorta taken from either α_{2A}-knockout or D79N mice the vasodilator response was not present (fig 2a).

Removal of the endothelium also abolished the vasodilator response to low concentrations of UK14304 (fig 2b), confirming the likely involvement of α_2-adrenoceptors on the endothelium. In aorta, from α_{1D}-knockout mice, the vasoconstrictor effect of high concentrations of UK14304 was absent (fig 2b).

In a separate series of experiments, a single concentration of UK14304 (1µmol/L) was tested against U46619 (1-10nmol/L) in the presence and absence of the nitric oxide synthase inhibitor L-NAME (0.1mmol/L). In control tissues UK14304 produced a reduction in tone (7.4% ± 4.7; n = 7). In the presence of L-NAME, UK14304 produced an increase in tone (44.2% ± 0.7; n = 7).

Localisation of α_2-adrenoceptors in aortic endothelial cells

JPET Fast Forward. Published on May 5, 2005 as DOI: 10.1124/jpet.105.085944
This article has not been copyedited and formatted. The final version may differ from this version.

QAPB (0.1μmol/L), a fluorescent α-adrenoceptor ligand, inhibited UK14304-induced relaxation of mouse aorta (figure 3a, $p<0.001$). To visualise QAPB binding to α_2-adrenoceptor sites, the three α_1-adrenoceptor subtypes were first eliminated by employing the α_{1B}-adrenoceptor-knockout mouse and incubating vessels in the α_{1D}-adrenoceptor antagonist BMY7378 (0.1μmol/L) and the α_{1A}-adrenoceptor antagonist 5MeU (0.1μmol/L). Confocal fluorescence microscopy revealed QAPB (0.1μmol/L) binding to endothelial cells, which line the grooves of the (unpressurised) internal elastic lamina (figure 3b & 3c). In the presence of rauwolscine (0.1μmol/L), no QAPB binding could be detected (figure 3d).

Norepinephrine-induced vasodilatation

Experiments were performed in the presence of either β-blockade (0.1μmol/L propranolol) alone or β-blockade and α_1-blockade (0.1μmol/L prazosin) to determine the physiological significance of the α_2-adrenoceptor-mediated vasodilatation. In the presence of pre-constrictor U46619, β-blockade and in the absence of the α_{1D}-adrenoceptors, norepinephrine (1μmol/L) produced a marked vasodilatation (figure 4a). Norepinephrine's vasodilator effect was blocked in the presence of L-NAME (0.1mmol/L; figure 4b). In control mice, following β-blockade and α_1-blockade, norepinephrine (1μmol/L) caused a relaxation of U46619-induced tone (fig 4c). In aorta taken from D79N mice, the vasodilator action of norepinephrine was also absent (fig 4d). In α_{1D}-knockout aorta, following β-blockade, rauwolscine (0.1μmol/L) significantly inhibited the norepinephrine-induced relaxation (fig 4e, $p<0.0001$, un-paired t-test).

JPET Fast Forward. Published on May 5, 2005 as DOI: 10.1124/jpet.105.085944
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

This study has established a vasodilator phenotype for α_2-adrenoceptors in the mouse that involves nitric oxide release of endothelial origin. The α_{2A}-adrenoceptor subtype alone is responsible with no evidence for a residual response by another subtype after its elimination. Having established the properties of the phenomenon using a selective synthetic agonist UK14304, we demonstrated that the physiological ligand for the receptors norepinephrine was highly effective once its other actions had been eliminated. Since conducting arteries are not (or are sparsely) innervated, but the endothelial cells are in intimate contact with the plasma, we propose that circulating catecholamines inhibit tone (stiffness) of these vessels via endothelial α_{2A}-adrenoceptors and that dysfunction of this action would exacerbate vasoconstrictor factors causing an increased load on the heart.

The aortic phenotype of the vasodilator α_2-adrenoceptor contrasts with the pre-junctional α_2-adrenoceptor in that only one subtype, the α_{2A}-adrenoceptor, was responsible whereas both α_{2A}- and α_{2C}-adrenoceptors were implicated in the pre-junctional modulation of norepinephrine release and both had to be knocked out to eliminate that response (Hein et al., 1999). There was no evidence of compensatory up-regulation of another α_2-adrenoceptor subtype.

The ability to focus on the α_{2A}-adrenoceptor-activated, endothelium/NO-mediated vasodilator response in the mouse and other model species should accelerate appreciation of its role in man. Endothelial α_2-adrenoceptors have not been reported in man but we can find no evidence of this having been pursued.

The endothelial α_2-adrenoceptors should be considered as possible physiological players since they are activated by norepinephrine. The correct function

of α_{2A}-adrenoceptors may be essential to regulation of blood flow in critical vascular beds, in the face of a generalised sympathetic activation in "fight or flight". e.g. nitric oxide released by α_2-adrenoceptors protects the kidney from excessive adrenergic vasoconstriction (Zou and Cowley, 2000). Norepinephrine and epinephrine both circulate freely in the plasma and have intimate contact with endothelial cells. In our demonstration that natural ligands can activate endothelial α_2-adrenoceptors we used norepinephrine rather than epinephrine to avoid activating vasodilatory β-adrenergic receptors. All evidence points to similar potency of these two catecholamines at α_2-adrenoceptors.

Pharmacological analysis of vasodilator responses via α_2-adrenoceptors was simplified by the use of selective agonists and receptor knockouts. Aorta had relaxation as its most sensitive response to UK14304 and this was susceptible to endothelial removal or inhibition of NOS. This represents the conducting artery vasodilator phenotype of the α_{2A}-adrenoceptor since it was absent in the knockout and the D79N mutation of this receptor. The loss of the response in both of these strains strengthens the case. It produces definitive evidence that an endothelial α_2-adrenoceptor response is mediated via the α_{2A}-adrenoceptor and validates the pharmacological analysis in large arteries of the pig (Bockman et al., 1996) of an endothelial α_{2A}-adrenoceptor subtype.

The effect of UK14304 in the α_{1D}-adrenoceptor-knockout mouse was interesting because, by eliminating the contractile response, the entire concentration response curve for vasodilatation was isolated.

We visualised the fluorescent ligand, QAPB, binding to aortic endothelial cells and eliminated this binding with the archetypal α_2-adrenoceptor antagonist

rauwolscine. We validated this by showing that this fluorescent ligand is a functional antagonist of aortic relaxation to UK14304 at the concentration employed for visualisation. This provides compelling direct evidence for the endothelial location of the α_2-adrenoceptors that mediate vasodilatation. This direct proof of α_2-adrenoceptor binding sites on endothelial cells suggests that earlier autoradiography, that indicated no endothelial binding of tritiated rauwolscine (Stephenson and Summers 1987) provided a false negative result due to the small volume of endothelial tissue to which radioligand can bind, coupled with the low receptor expression level on endothelium relative to a high level in the arterial media. We now show that α_2-adrenoceptors are located on the endothelium

Demonstrating that a natural ligand, norepinephrine, could activate the endothelial α_2-adrenoceptors was straightforward once the confounding factors had been clarified using the selective agonist, antagonists and knockout strains. Norepinephrine produces, *in vitro*, a powerful contractile response that overwhelms its vasodilator actions. Its relaxant endothelial α_2-adrenoceptor-mediated effect could be seen clearly in the α_{1D}-adrenoceptor-knockout and shown to be rauwolscine sensitive. Demonstrating that the receptor involved is the α_{2A}-adrenoceptor is more difficult since the α_{1D}-adrenoceptor is present in the α_{2A}-adrenoceptor "knockout" strains. However, comparing the normal mouse with the D79N in the presence of prazosin and propranolol, it was possible to show the complete absence of a dilator response in D79N in contrast to a relaxant or multiphasic response in normal mice. Together with the other evidence this strongly supports that norepinephrine activates vasodilator α_{2A}-adrenoceptors.

JPET Fast Forward. Published on May 5, 2005 as DOI: 10.1124/jpet.105.085944
This article has not been copyedited and formatted. The final version may differ from this version.

A peripheral endothelium/nitric oxide-mediated direct vasodilatation to α_2-adrenoceptor agonists must now be considered as a potential depressor mechanism to intravenous α_2-adrenoceptor agonists in addition to any centrally mediated sympatho-inhibitory effects or pre-junctional inhibition of post-ganglionic sympathetic transmission. We suggest that emphasis on the latter action should be reconsidered. Deletion or mutation of the α_{2A}-adrenoceptors eliminates the reduction in heart rate and blood pressure caused by intravenous α_2-adrenoceptor agonists such as UK14304 and clonidine in the conscious mouse (MacMillan et al., 1996; Altman et al., 1999). This has been assumed to arise entirely from withdrawal of sympathetic tone, though no evidence for a change in sympathetic tone is available. There is also no evidence for pre-junctional α_2-adrenoceptors on mouse vascular sympathetic nerves. In the pithed rat, the vascular response to sympathetic nerve stimulation was the least sensitive of several organ systems to inhibition by clonidine (Docherty and McGrath 1980). Thus, if the mouse is like the rat, inhibition of norepinephrine release from nerve endings in blood vessels may not be a major action of α_2-adrenoceptor agonists. In conducting arteries vasodilatation via endothelial α_2-adrenoceptors would reduce blood pressure via a reduced after-load. Thus, there may be an endothelial component to the vasodepressor action of α_2-adrenoceptor activation and this may combine with any sympatho-inhibition that exists.

Large artery endothelial α_2-adrenoceptors should be relevant to the hypothesis that α_{2A}-adrenoceptors confer protection from heart failure (Brede at al., 2002). Excess mortality in the α_{2A}-adrenoceptor-knockout strain was attributed to heart failure due to a combination of enhanced left ventricular hypertrophy, fibrosis and elevated circulating catecholamines (Brede at al., 2002). The present work predicts

that in the α_{2A}-adrenoceptor-knockout the loss of large artery vasodilatation via α_{2A}-adrenoceptors combined with unopposed vasoconstriction to elevated catecholamines would create a highly deleterious situation of reduced arterial compliance and consequent increased cardiac work. Lack of innervation makes it likely that sympatho-inhibition has a role and that the endothelial α_{2A}-adrenoceptor effect will be responsible.

The α_{2C}-adrenoceptor-knockout mouse is susceptible to a similar pathology (Brede at al., 2002). Combined with the link between the polymorphisms of this receptor and susceptibility to heart failure (Small et al., 2002), this has focussed attention on this receptor. The present work indicates that α_{2C}-adrenoceptors are not involved in mouse large artery endothelial vasodilatation and the lack of innervation excludes sympatho-inhibition, so there is no evidence for a vasodilator action by this subtype in large arteries. However, α_{2C}-adrenoceptors could be involved in innervated small arteries that regulate blood pressure through the peripheral resistance, either through endothelial or nerve mechanisms. Thus, heart failure may be exacerbated by different mechanisms according to which α_2-adrenoceptor is “abnormal”.

References

Alexander SPH, Mathie A and Peters JA (2004) Guide to Receptors and Channels; Adrenoceptors, α_2. *Br J Pharmaco.* **141**:Suppl. 1: S9.

Altman JD, Trendelenburg AU, MacMillan L, Bernstein D, Limbird L, Starke K, Kobilka BK and Hein L (1999) Abnormal regulation of the sympathetic nervous system in α_{2A}-adrenergic receptor knockout mice. *Mol Pharmacol* **56**:154-161.

Bockman CS, Gonzalez-Cabrera I and Abel PW (1996) Alpha-2 adrenoceptor subtype causing nitric oxide-mediated vascular relaxation in rats. *J Pharmacol Exp Ther* **278**:1235-1243.

Brede M, Wiesmann F, Jahns R, Hadamek K, Arnolt C, Neubauer S, Lohse MJ and Hein L (2002) Feedback inhibition of catecholamine release by two different [alpha]2-adrenoceptor subtypes prevents progression of heart failure. C*irculation* **106**:2491–2496.

Brown CM, MacKinnon AC, Redfern WS, Hicks PE, Kilpatrick AT, Small C, Ramcharan M, Clague RU, Clark RD, MacFarlane CB and Spedding, M (1993) The pharmacology of RS-15385-197, a potent and selective alpha 2-adrenoceptor antagonist. *Br J Pharmacol* **108**:516-525

Cavalli A, Lattion A-L, Hummler E, Nenniger M, Pedrazzini T, Aubert J-F, Michel MC, Yang M, Lembo G, Vecchione C, Mostardini M, Schmidt A, Beermann F and

Cotecchia S (1997) Decreased blood pressure response in mice deficient of the alpha (1b)-adrenergic receptor. *Proc Nat. Acad Sci. USA* **94**:11589-11594.

Ceresa BP and Limbird LE (1994) Mutation of an aspartate residue highly conserved among G-protein-coupled receptors results in nonreciprocal disruption of alpha 2-adrenergic receptor-G-protein interactions. *J Biol Chem* **269**:29557–29564.

Cocks TM and Angus JA (1983) Endothelium-dependent relaxation of coronary arteries by noradrenaline and serotonin. *Nature* **305**: 627-630.

Daly CJ, Deighan C, McGee A, Mennie D, Ali Z, McBride M and McGrath JC (2002) A knockout approach indicates a minor vasoconstrictor role for vascular α_{1B}-adrenoceptors in mouse. *Physiol Genomics.* **9**:85-91.

Daly CJ, Milligan CM, Milligan G, Mackenzie JF and McGrath JC (1998) Cellular localisation and pharmacological characterisation of functioning α_1-adrenoceptors by fluorescent ligand binding and image analysis reveals identical binding properties of clustered and diffuse populations of receptors. *J Pharmacol Exp Ther* **286**:984-990.

Docherty JR and McGrath JC (1980) The factors influencing the time course of drug action at α-adrenoceptors: an investigation of the effects of clonidine in the pithed rat. *Br J Pharmacol* **68**:225-234.

Dora KA (2001) Cell-cell communication in the vessel wall. *Vasc Med* **6**:43-50.

Furchgott RF and Zawadski JV (2001) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle. *Nature* **1980**:288:373-376.

Guimaraes S and Moura D (2001) Vascular Adrenoceptors: an update. *Pharmacol Rev* **53**:319-356.

Hein L, Altman JD and Kobilka BK (1999) Two functionally distinct α_2-adrenergic receptors regulate sympathetic neurotransmission. *Nature* **402**:181-184.

Liggett SB (2004) Genetically modified mouse models for pharmacogenomic research. *Nat Rev Genet* **5**(9):657-663.

Link RE, Desai KL, Hein L, Stevens ME, Chruscinski A, Bernstein D, Barsh GS and Kobilka BK (1996) Cardiovascular regulation in mice lacking alpha 2-adrenergic receptor subtypes b and c. *Science* **273**:803-805.

McCafferty GP, Naselsky DP, Hieble JP (1999) Characterization of postjunctional alpha-adrenoceptors in the pithed mouse. *Gen Pharmacol* **33**(1):99-105.

Mackenzie JF, Daly CJ, Pediani JD and McGrath JC (2000) Quantitative imaging in live human cells reveals intracellular alpha(1)-adrenoceptor ligand-binding sites. *J Pharmacol Exp Ther* **294**(2):434-443.

MacMillan LB, Hein L, Smith MS, Piascik MT and Limbird LE (1996) Central hypotensive effects of the α_{2A}-adrenergic receptor subtype. *Science* **273**:801-803.

MacMillan LB, Lakhlani P, Lovinger D and Limbird LE (1998) α_2-adrenergic receptor subtypes: subtle mutation of the α_{2A}-adrenergic receptor in vivo by gene targeting strategies reveals the role of this subtype in multiple physiological settings. *Recent Prog Horm Res* **53**:25-34.

Russell A and Watts S (2000) Vascular reactivity of isolated thoracic aorta of the C57BL/6J mouse. *J Pharmacol Exp Ther* **294** (2):598-604.

Small KM, Mialet-Perez J, Seman CA, Theiss CT, Brown KM and Liggett SB (2004) Polymorphisms of cardiac presynaptic alpha$_{2C}$-adrenergic receptors: Diverse intragenic variability with haplotype-specific functional effects. *Proc Natl Acad Sci U S A* **101**(35):13020-13025.

Small KM, Wagoner LE, Levin AM, Kardia SLR and Liggett SB (2002) Synergistic polymorphisms of β_1- and α_{2C}-adrenergic receptors and the risk of congestive heart failure. *N Engl J Med* **347**:1135-1142.

Starke K (2001) Presynaptic autoreceptors in the third decade: focus on α_2-adrenoceptors. *J Neurochem* **78**:685-693.

Stephenson JA and Summers RJ (1987) Autoradiographic analysis of receptors on vascular endothelium. *Eur J Pharmacol* **134**(1):35-43.

Surprenant A, Horstman DA, Akbarali H and Limbird LE (1992) A point mutation of the alpha 2-adrenoceptor that blocks coupling to potassium but not calcium currents. *Science* **257**(5072):977-980.

Tanoue A, Nasa Y, Koshimizu T, Shinoura H, Oshikawa S, Kawai T, Sunada S, Takeo S and Tsujimoto G (2002) The α_{1D}-adrenergic receptor directly regulates arterial blood pressure via vasoconstriction. *Journal of Clinical Investigation* **109**:765-775.

Vandeputte C, McCormick PA and Docherty JR (2003) Responsiveness to noradrenaline in aorta from wild-type, nitric oxide synthase-2, nitric oxide synthase-3 and $\alpha_{2A/D}$-adrenoceptor knockout mice. *Eur J Pharmacol* **466**:129-136.

Vanhoutte PM. Endothelial adrenoceptors (2001) *J Cardiovasc Pharmacol* **38**(5):796-808.

Wilson VG, Brown CM and McGrath JC (1991) Are there more than two types of α-adrenoceptors involved in physiological responses? *Exp Physiol* **76**:317-346.

Zou A-P and Cowley AW Jr (2000) α_2-Adrenergic receptor-mediated increase in NO production buffers renal medullary vasoconstriction. *Am J Physiol* **279**:R769-R777.

Footnotes

This work was supported by the EC project VASCAN-2000 (QLG-CT-1999-00084); Iranian Government Ministry of Education (MMS); British Heart Foundation, Junior Fellowship (CD) and Postgraduate Scholarship (MM), The Anne B. McNaught Bequest and The Muirhead Trust of Glasgow University.

Professor J.C. McGrath

Autonomic Physiology Unit

Institute of Biomedical and Life Sciences

West Medical Building

University of Glasgow

Glasgow

G12 8QQ

UK

[1]M.M.S and M.M contributed equally to this work.

JPET Fast Forward. Published on May 5, 2005 as DOI: 10.1124/jpet.105.085944
This article has not been copyedited and formatted. The final version may differ from this version.

Figure Legends

Figure 1. Representative tracings of vasodilator responses in mouse isolated aorta. Left hand panels show the full response. Right hand panels show a magnified view of the boxed area on the left. The addition of U46619 is shown by the open arrows. a) The effect of UK14304 (1μmol/L; closed arrows), to cause vasodilatation, in U46619 (1-10nmol/L) pre-constricted segments of aorta. b) The action of UK14304 in the presence of rauwolscine (0.1μmol/L). c) The action of UK14304 in the presence of prazosin (0.1μmol/L).

Figure 2. Concentration response curves for UK14304 in aorta taken from various mouse strains. a) A comparison of the effect of UK14304 in control (wild type) (○; n = 6); α_{2A}-adrenoceptor-knockout (●; n = 5) & D79N mice (△; n = 6). b) Comparison of the effect of UK14304 alone (○; n = 6); in the absence of endothelium (●; n = 5) or in aorta taken from α_{1D}-knockout mice (△; n = 7).

Figure 3. Localisation of α_2-adrenoceptors in aortic endothelial cells. a) Antagonism of the relaxant effect of UK14304 by the fluorescent ligand QAPB (0.1μmol/L; ***$p<0.0001$, one way ANOVA followed by Bonferroni post test). Two successive cumulative concentration response curves, 1st without and 2nd with antagonist, were constructed for each vessel segment. Control (1st curve, ❑); time control (2nd curve, ●); QAPB 10nM (▲), 0.1μmol/L (▼), n = 5. The fluorescent α-adrenoceptor ligand, QAPB (0.1μmol/L), binds to aortic endothelial cells on the surface of the internal elastic lamina (black arrow). b) & c) QAPB binding to endothelial cells is

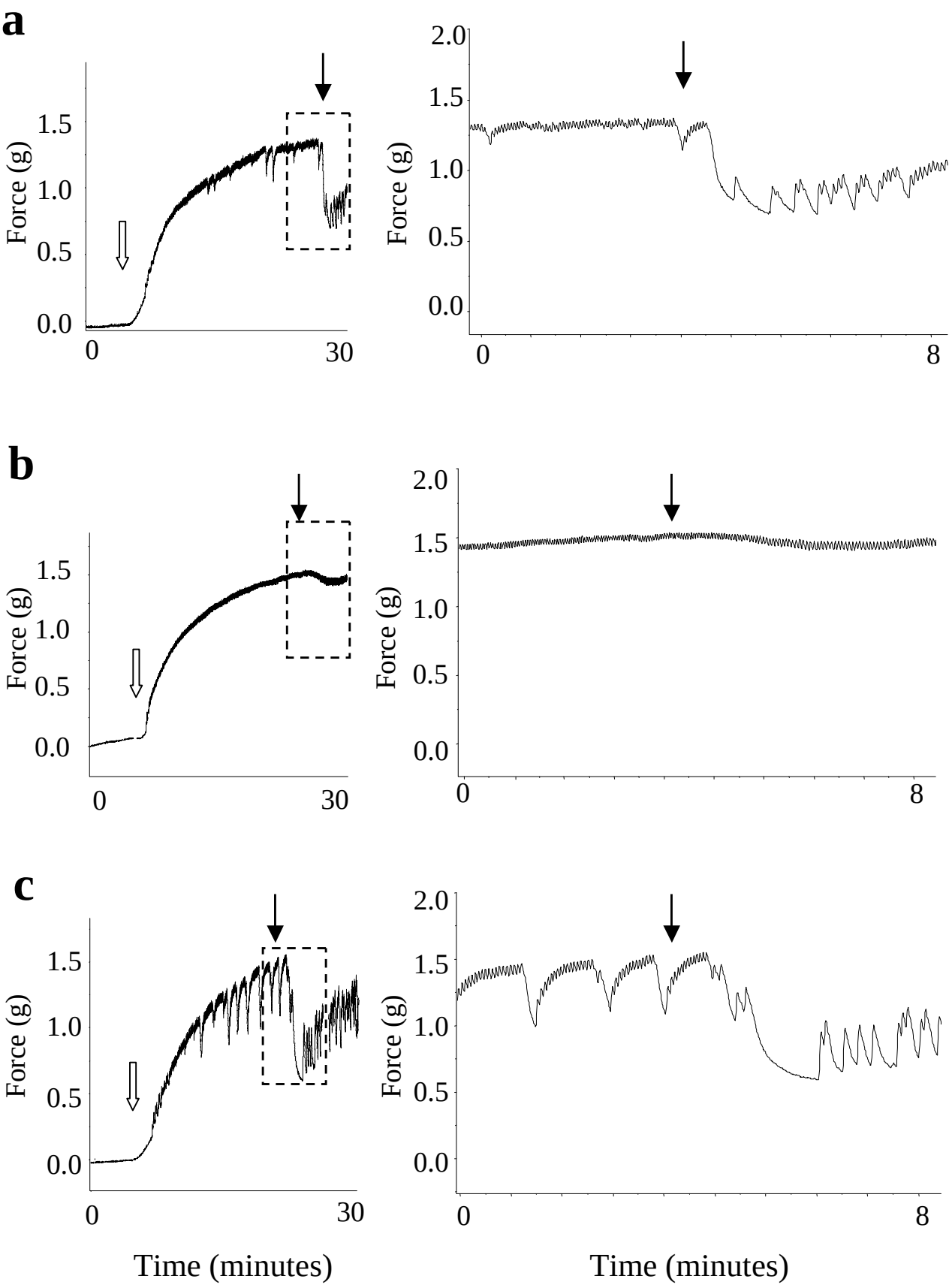
JPET Fast Forward. Published on May 5, 2005 as DOI: 10.1124/jpet.105.085944
This article has not been copyedited and formatted. The final version may differ from this version.

clearly visible (white arrows). d) Undetectable QAPB binding in the presence of rauwolscine (0.1µmol/L).

Figure 4. Norepinephrine (1µmol/L) relaxes aorta (arrows) following pre-constriction with U46619. a) α_{1D}-knockout in the presence of propranolol (0.1µmol/L). b) L-NAME (0.1mmol/L) in the α_{1D}-knockout and in the presence of propranolol (0.1µmol/L). c) Control mouse in the presence of prazosin & propranolol (0.1µmol/L); d) D79N in the presence of prazosin & propranolol (0.1µmol/L). e) Degree of relaxation produced in the α_{1D}-knockout by norepinephrine (1µmol/L) under control conditions and in the presence of rauwolscine (0.1 µmol/L) n = 4 mice, ***$p<0.0001$, unpaired t-test.

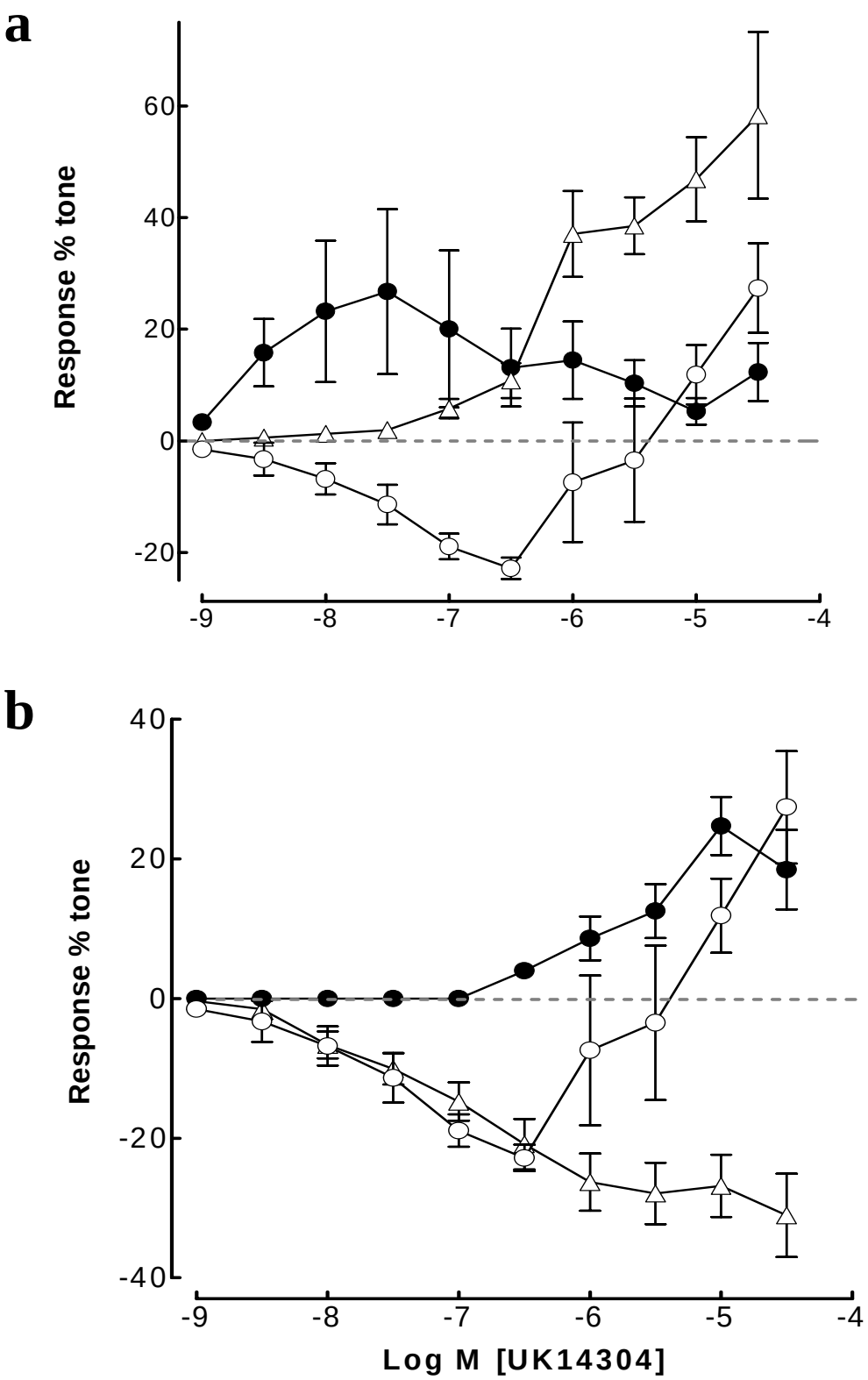
JPET Fast Forward. Published on May 5, 2005 as DOI: 10.1124/jpet.105.085944
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 1



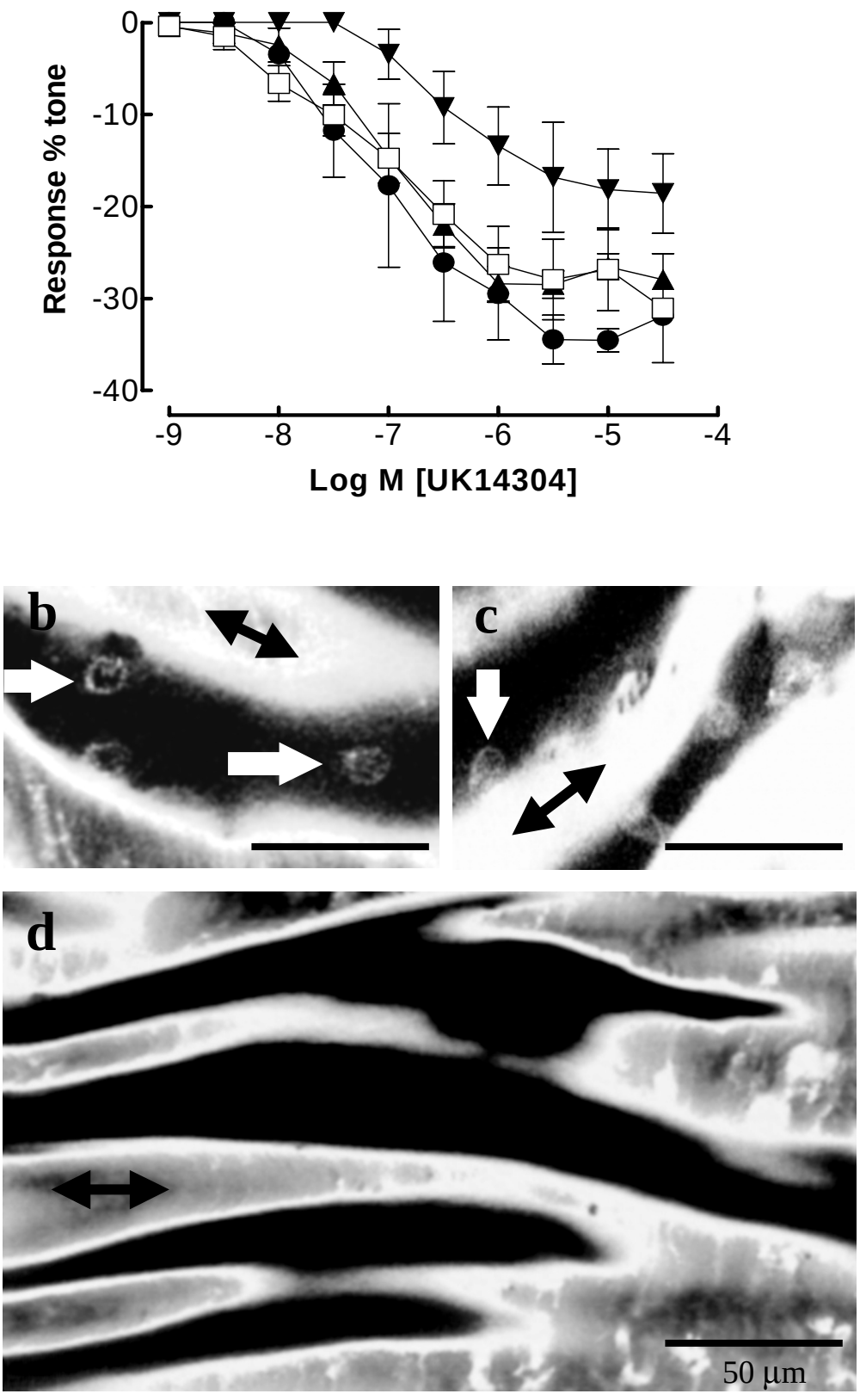
JPET Fast Forward. Published on May 5, 2005 as DOI: 10.1124/jpet.105.085944
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 2



JPET Fast Forward. Published on May 5, 2005 as DOI: 10.1124/jpet.105.085944
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 3



JPET Fast Forward. Published on May 5, 2005 as DOI: 10.1124/jpet.105.085944
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 4