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Endothelial Vasodilator Angiotensin Receptors are Changing in Mice with Ageing

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Abstract

Background: The vascular function of Angiotensin II-type-2 receptors in adults is controversial. We sought their location and function in mouse aortic rings at young and old mice.

Materials and Methods: Male C57Bl mice (aged 4 and 14 months) were killed by CO2. The descending thoracic aorta was cleaned and dissected into rings. Aortic rings were mounted in Krebs' solution at 37 °C and then setup in a multi-myograph. Also segments of aorta were incubated with or without antagonists then TMR-Angiotensin II and/or QAPB were added.

Results: At 4 months, angiotensin II, at low concentrations, caused losartan-sensitive contraction; higher concentrations (100nmol/L) caused relaxation sensitive to endothelial denudation, L-NAME or PD123319. Angiotensin II-type-1 receptors blockade plus L-NAME revealed PD123319-sensitive contraction. At old mice, aortic relaxation to angiotensin II was lost. At young mice, Losartan and PD123319, together but not separately, abolished binding of fluorescent TMR-angiotensin II, to endothelium and smooth muscle, indicatin Angiotensin II-type-1 and Angiotensin II-type-2 receptors in both cell types. In contrast, at 14 months endothelial fluorescence was eliminated by losartan.

Conclusions: Aortic endothelium of young adult mice has Angiotensin II-type-2 receptors that release vasodilator nitric oxide. This is lost in old age, explaining age-related loss of vasodilatation by Angiotensin II. Aortic smooth muscle has pro-contractile Angiotensin II-type-1 and Angiotensin II-type-2 receptors in young and old mice. Reciprocal actions of angiotensin II are, due to Angiotensin II-type-1 and Angiotensin II-type-2 receptors situated on different cell types but only at young ages, Angiotensin II-type-1 receptors of unknown function are present on endothelium.

Keywords: Angiotensin II; Angiotensin receptors; L- NAME; Nitric oxide; Mouse aorta; Aging

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Introduction

The potential significance of type-2 angiotensin receptors (AT2-R) emerged from the realization that, in contrast to angiotensin converting enzyme inhibition, which should prevent all actions of angiotensin II, type-1 angiotensin receptor (AT1-R) blockade, could allow or enhance actions via other AT-receptors. Consequently, attenuating the angiotensin system in these two different ways should produce different cardiovascular consequences. That stimulated the search for physiological properties of AT2-R1; 2 and the interesting finding for hypertension pathology that vasodilator AT2-R are dysfunctional in the spontaneously hypertensive rat (SHR) mode l3. Thus a deficit of AT2-R could contribute to hypertension, highlighting the need to understand the biology of these receptors.

However, hypotheses on the vascular roles and interactions of AT1-R and AT2-R have become complex. Two issues of particular relevance are, first, the lack of information on localization and colocalization of the two receptor types in the various vascular cell types and, secondly, the belief that AT2-R are more significant during development than in adult life and so might regulate growth and development of blood vessels rather than have acute actions on vascular tone. (1, 2-6) There is little information on the relative localization of AT1-R and AT2-R in the blood vessel wall, either between cell types or within cells.

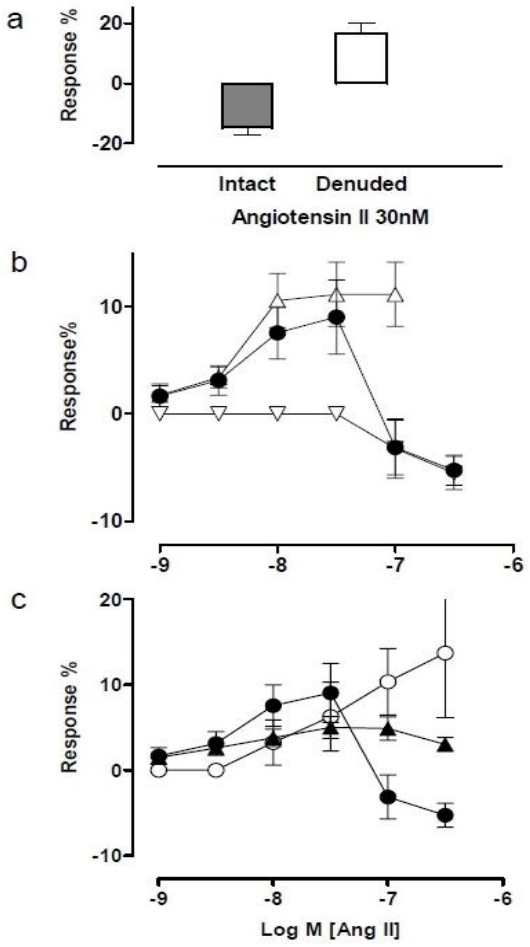

Figure 1. Responses to angiotensin II in 4 month old mouse aorta in the presence of 5-HT-induced tone.

a. A single concentration of 30nmol/L produced relaxation that was changed to contraction by removing the endothelium. (t test, $p< 0.05$) n=18

b. Cumulative addition of angiotensin II produced contraction at low concentrations and relaxations at high concentrations (● filled circles). The contractions were abolished by Losartan 1µmol/L (Δ upward pointing open triangles). Relaxation was eliminated by the AT2-R antagonist PD123319 1µmol/L (∇ downward pointing open triangles). n=11

c. Blockade of nitric oxide synthesis by L-NAME 0.1mmol/L (▲upward pointing filled triangle) abolished relaxation (t test, $p<0.05$ at Angiotensin 0.1 and 0.3 µmol/L) but did not significantly affect contraction produced by lower concentrations of Angiotensin II. In the presence of both L-NAME and losartan, contraction remained and became variable at high concentration (○open circles, n=18). Control response as in b (● filled circles). Data shown are means and s.e.

Consequently, the site of AT1-R / AT2-R interaction is not clear: some studies concentrate on the concept that the subtypes reside in the same cells so that their interactions, synergistic or antagonistic, occur intra-cellularly at the molecule-molecule or transcription regulation level; others concentrate on inter-cellular physiological antagonism between opposing responses originating from different cell types, such as endothelium (AT2-R) versus smooth muscle (AT1-R).

The literature is surprisingly unclear regarding the straightforward demonstration of AT2-R-mediated endothelial responses *in vitro*. In the rat, AT2-R mediates nitric oxide-mediated vasodilatatory responses *in vivo* (7-8) and *in vitro* in perfused mesenteric arcade 9. However, these complex preparations cannot prove an endothelial source for nitric oxide, and, no vasodilator effects of angiotensin II were found in the relatively simpler strips of mouse blood vessels, (10-11) so the mechanisms underlying the vasodilator effects of angiotensin II remain to be clarified.

Interpretation was further complicated by concepts arising from work with transgenic manipulation of the receptors. AT2-R knockout mice showed hypersensitivity to angiotensin II's pressure response *in vivo* and aortic contraction *in vitro* (12-13). This would be consistent with loss of an AT2-R-mediated endothelial response that is physiologically antagonistic to pro-contractile AT1-R. However, alternative, more complex explanations for the interaction have been proposed. For example, that AT2-R counteracts the AT1-R-mediated vascular contractile action of angiotensin II through downregulation of AT1-R (14-15). This potentially shifts the localization of "vasodilator" AT2-R to vascular smooth muscle cells and makes it important to know whether, in native cells, the two receptor types produce their opposing actions in the same or in separate cells.

Resolving these issues should clarify the potential differences between AT1-R-blockade and ACEI (Angiotensin Converting Enzyme I) in the treatment of cardiovascular disease. Comparison of the two treatments in clinical studies suggests that there is no advantage between each alone or the two combined, provided that dosage of each is sufficient (16-18, 35). This contrasts with animal models, where the combination of ACEI with AT1-R blockade produces different outcomes from either treatment alone (19-

20). A factor here could be age, since studies of animal models are invariably carried out on young adults, while human cardiovascular disease study populations are relatively old. Together with the concept that AT2-R is more relevant in development, this led us to seek AT2-R in older animals, where receptors of importance for development might be lost, as well as in the usual young adult population.

We sought to establish the vascular location of binding sites for angiotensin II in arterial cells, to demonstrate whether each cell type has AT1-R and/or AT2-R, to establish functional evidence for which cells and receptors are involved in the multiple interacting actions of angiotensin II (to shed light on inter- versus intra-cellular interactions of the two receptors) and to determine whether AT2-R are still present and functional at older age. Our aim was to establish the localization and function of AT1-R and AT2-R using the mouse aorta as a model. We used standard pharmacologically selective antagonists for the AT1-R and AT2-R receptors to identify the contractile and relaxant responses to angiotensin II, using rings of aorta. We then developed a new approach to localization of receptors using a fluorescent derivative of angiotensin II and identified the receptor types using the same antagonists. By validating each other, these two approaches made the analysis more robust.

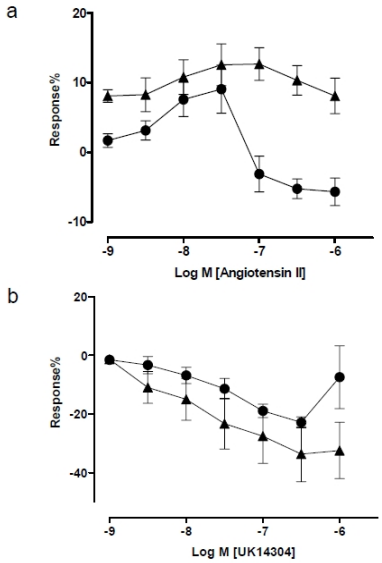

Figure 2. Responses to angiotensin II in 14 month old mouse aorta in the presence of 5-HT-induced tone.

a. At 14 months old (n=5), cumulative addition of angiotensin II produced only contraction at all concentrations (▲upward pointing filled triangles) compared with the biphasic response in younger mice (● filled circles). Responses were significantly different at all concentrations of ≥0.1µmol/L ($p<0.05$)

b. In contrast the α_2-adrenoceptor agonist UK14304 (n=5) produced relaxation over a similar concentration range in young (● filled circles) and old (▲upward pointing filled triangles). Data shown are means and s.e.m.

Our initial objective was to detect opposing AT1-Receptor-mediated (direct smooth muscle) constrictor and AT2-Receptor-mediated (indirect via endothelium) dilator responses but we were also open to the possibility of each receptor being in other cell types. We also employed endothelial denudation and inhibition of nitric oxide synthase (NOS) to identify endothelial nitric oxide-mediated responses.

Materials and Methods

Male C57Bl mice (aged 4 and 14 months) were killed by CO2. The descending thoracic aorta was cleaned of connective tissue, removed and dissected into rings (2-3 mm in length).

Contractile Responses

Aortic rings were mounted in Krebs' solution (NaCl 118.4mM, KCl 4.7mmol/L, CaCl2 2.5mmol/L, KH2PO4 1.2 mmol/L, MgSO4 1.2 mmol/L, NaHCO3 25mmol/L and glucose 11.1mmol/L bubbled with 95% O2 5% CO2 to pH 7.4) at 37 °C in a multi-myograph (myo-interface, model 600M, DMT, Aarhus) using 40. M stainless steel wires (21, 22) (Mulvany and Halpern. 1976, 1977), placed under a resting tension of 1g and left to equilibrate for 30-45 minutes. Endothelium was removed, where appropriate, by gently rubbing the intimal surface.

Reproducible contractile responses were first obtained to 5HT (0.1mol/L). At the plateau of contraction to 5HT acetylcholine (1 mol/L) was added to assess endothelial integrity. Criteria for functional endothelium was >50% and for denuded endothelium was <5% relaxation. In "endothelium-denuded" aortic rings acetylcholine produced no relaxation and, indeed, produced contraction (data not shown). Acetylcholine-induced relaxation was abolished by L-NAME (0.1mmol/L) in intact aortic rings (data not shown).

Tissues were tested with increasing cumulative concentrations of angiotensin II in 0.5 log unit increments from 1nmol/L-100nmol/L or 1 mol/L, added at 4 minute intervals either without or with additional 5HT-induced submaximal tone (50-75% of maximum).

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.

Visualization of endothelial AT receptors

The method was an adaptation of that used to visualise vascular–adrenoceptors (23-24) previously applied to endothelium by Shafaroudi et al. (25- 27). The endothelium lies on the autofluorescent

corrugated 3-dimensional surface of the internal elastic lamina. This provides a reference surface for identification of endothelial cells. The adrenoceptor ligand BODIPY-FL labeled prazosin (QAPB) binds to aortic endothelial cells (25-26) and so provides a counterstain to indicate the location of endothelial cells when competitors are used to antagonize AT receptor binding.

Tissue preparation

2-3mm segments of aorta were incubated for 30 min with or without antagonists then TMR-Angiotensin II (fluorescent angiotensin II) (0.1 mol/L) and/or QAPB (BODIPY-FL labeled prazosin) (0.1 mol/L) were added for 60 min. Following incubation, without washing, aortic segments were cut open and placed endothelial side up in the sample well of a glass slide 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. Excitation/emission was 567nm/610nm for TMR-Angiotensin II and 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 a pinhole setting of 1.5 Image size of 512 x 512 pixels equates to a field size of 289 m x 289 m. Each procedure was carried out in triplicate on at least three different mice. 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 to the provisions of the UK Animals (Scientific procedures) Act 1986.

Drugs

All drugs were of analytical grade and were dissolved in distilled water (H2O), ethanol or DMSO (Dimethyl sulfoxide**)** (as indicated below).

Norepinephrine dilution included 23.mol/L EDTA to prevent oxidation. Norepinephrine (H2O), Angiotensin II (H2O), Acetylcholine chloride (H2O), PD123319 (H2O), 5-Hydroxytryptamine (H2O), U46619 (ethanol), L-NAME (N-Nitro-L-Arginine Methyl Ester) (H2O), Rauwolscine **(**17α-hydroxy-20α-yohimban-16β-carboxylic acid methyl ester) (H2O) [Sigma-Aldrich Co; Poole, UK]. UK14304 (DMSO) [Pfizer Central Research, Sandwich, UK], Losartan (DMSO) [MERCK USA]. BODIPY-FL labeled prazosin (H2O) (abbreviation QAPB); Excitation /Emission, 488nm/515nm [Molecular Probes, Eugene, OR, USA], Trimethyl Rhodamine-Angiotensin II-Human (H2O) (abbreviation TMR-Angiotensin II); Excitation/Emission, 567nm/610nm [Phoenix Europe, Karlsruhe, Germany].

Statistical analysis

Values are means ± Standard error mean from n experiments. Statistical and graphical analysis was carried out using Excel 97 and GraphPad Prism 3.00. Data used to plot the concentration response curves are the mean contractions induced at each concentration of the agonist.

Results

Contractile Responses

Dual effects of angiotensin II in young adult mice

In 4-month old aorta, in the presence of induced tone a single concentration of angiotensin II of 30nmol/L produced relaxation which was reversed to contraction by removing the endothelium (Figure 1a). With the tone raised, the angiotensin II concentration-response curve was biphasic.

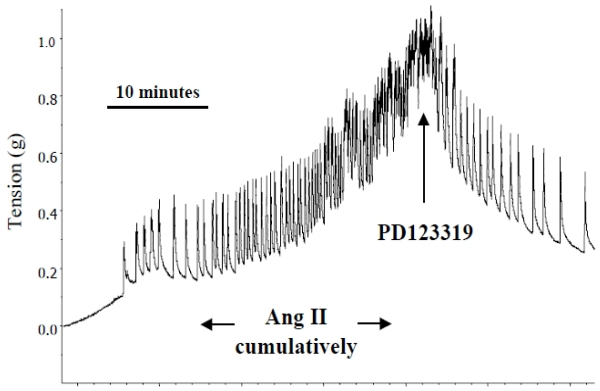

Figure 3. Trace of AT2-R-mediated contraction to Angiotensin II in aorta of 14 month old mouse. Losartan was added for 30 min then tone was raised with U46619 (at start of trace). After 20 minutes equilibration, rhythmic contraction was established and the cumulative addition of Angiotensin II produced concentration-related contraction and increased frequency of rhythmic activity. At the equilibrium of contraction to the highest concentration, PD123319 (1μmol/L) produced a large reduction in tone and slowed rhythmic activity.

Low concentrations of angiotensin II (1-10nmol/L) induced contraction whilst higher concentrations (0.1-1mol/L) induced relaxation (Figure 1b). When the cumulative addition of angiotensin II reached 30 nmol/L it produced a short-lived contraction that returned to the original elevated baseline (the contraction is plotted on the graph in Figure 1b). When the next concentration of 100nmol/L was added it produced straightforward relaxation. All contractions were abolished by the AT1-R antagonist Losartan (1mol/L, Figure 1b). All relaxation was eliminated by the AT2-R antagonist PD123319 (ditrifluoroacetate**)** (1mol/L, Figure 1b) or by L-

NAME (L-N^G-Nitroarginine methyl ester) (Figure1c). In the presence of L-NAME angiotensin produced only contraction. After L-NAME and losartan (2-butyl- 4- chloro-1- [*p*- (*o*- 1*H*-tetrazol- 5- ylphenyl) benzyl] imidazole-5-methanol monopotassium salt). Together, angiotensin II still produced contractions which were of variable size but were not significantly different from either losartan alone or L-NAME alone, suggesting an AT2-R-mediated contractile response (Figure 1c).

Response to angiotensin II in aorta of older mice

In 14-month old mouse aorta, only contraction, with no relaxation, was observed to either single (not shown) or cumulative concentrations of angiotensin II (n=18) (Figure 2a). Endothelium-mediated relaxant responses to acetylcholine (not shown) or to α2-adrenoceptor-mediated activation by UK14304 (Figure 2b) were present and of similar size at 4 months and 14 months.

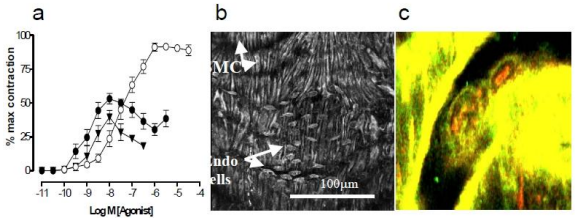

Figure 4. The pharmacological action and cellular binding of TMR-angiotensin II in 4 month old mouse aorta.
a. TMR-angiotensin II (▼downward pointing filled triangles), angiotensin II (● filled circles) and norepinephrine (○ open circles) produced concentration-related contractions (n=5). The maximum for noradrenaline was significantly greater than for the other two agents. ($p<0.05$) Data shown are means and s.e.m.
b. 3D reconstruction from confocal images taken from the intimal side of the opened aorta pre-incubated in TMR-Angiotensin II. The endothelial cells binding TMR-Angiotensin II can be seen lying on the top of the underlying smooth muscle cells. Both cell types were rendered visible by the bound ligand, indicating the widespread distribution of the receptors.
c. Confocal 2D image of endothelial cells adjacent to internal elastic lamina pre-incubated with TMR-angiotensin II (green) and the α-adrenoceptor ligand QAPB (red). Endothelial cells express a high concentration of intracellular AII receptors whilst a-adrenoceptors are located mainly on the cell surface. The large area of strong yellow colour represents autofluorescence of the elastic lamina. Yellow inside the cells represents co-localisation of AT and adrenoceptors.

In young mice, in order to uncover pro-contractile AT2-R, it was necessary to block both AT1-R (smooth muscle contraction) and NOS (endothelial AT2-R-mediated relaxation) (Figure 1c). In older mice, however, it was possible to demonstrate pro-contractile AT2-R in the presence of losartan alone. In that case, PD123319 reversed angiotensin II-mediated contraction without the need for L-NAME (Figure 3). It was subsequently shown this was due to the absence of endothelial AT2-R (hence no vasodilator effect) but the continued presence of pro-contractile smooth muscle AT2-R, as shown by fluorescent TMR-angiotensin II (Trimethyl Rhodamine-Angiotensin II-Human- fluorescent angiotensin II).

Visualization of Receptors

The pharmacological similarity of TMR-angiotensin II to Angiotensin II was demonstrated in 4-month old mouse aorta. Angiotensin II and TMR-Angiotensin II both produced concentration-related contractions, which declined at high concentrations. In comparison, norepinephrine produced a larger maximum with no decline at high concentrations (Figure 4a).

At 4 months, TMR- Angiotensin II produced fluorescence on both endothelial cells and smooth muscle cells. Figure 4b shows a 3D reconstruction from confocal images taken from the intimal side of the opened aorta.

The endothelial cells binding TMR-Angiotensin II can be seen lying on the top of the underlying smooth muscle cells. Both cell types were rendered visible by the bound ligand, indicating the widespread distribution of the receptors. Receptor-ligand binding was present over the whole surface of the smooth muscle cells and was also located intracellular. In endothelial cells the intracellular binding was largely perinuclear and was more apparent than surface binding. In figure 4b the internal elastic lamina that separates the endothelial and smooth muscle layers has been removed by image processing to reveal the two cell layers since this image is intended to illustrate the relationship of the cell types. In order to analyse the presence or absence of binding it was necessary to show unprocessed images which also provide the reference surface of the lamina.

In figure 4c this is shown as a 2D image through an endothelial cell. The cell was counter stained by a ligand for α2-adrenoceptors (QAPB) that bound to surface receptors as well as intracellular receptors, allowing more detailed visualization of the intracellular binding of TMR-Angiotensin II.

The intense natural fluorescence of elastin impedes visualization of cellular detail of endothelial cells except in such a 2D view that highlights the cells against the black background of the empty vascular lumen. To gauge the cell population that binds TMR-angiotensin II we reverted to 3D views that make a virtue of elastin fluorescence since it shows the laminar surface against which cells that bind TMR-Angiotensin II are either visible or not (Figure 5).

Using this approach, in aorta from 4-month old mice losartan alone did not abolish fluorescence and left a clear image of endothelial cells lying on the laminar surface (Figure 5b). However, the combination of

both PD123319 and losartan abolished TMR-Angiotensin II fluorescence leaving a clear lamina (Figure 5c). Thus the removal of losartan-resistant binding by PD123319 indicates the presence of endothelial AT2-R.

In smooth muscle cells, as in the endothelial cells, abolition of TMR-Angiotensin II fluorescence required both losartan and PD123319 (not shown). In aorta from 14-month old mice TMR-Angiotensin II bound to endothelial cells (Figure 5d). In endothelium, the result was different from that in younger mice: losartan, on its own, abolished fluorescence, indicating that all endothelial binding sites were AT1-R and that AT2-R were absent (Figure 5e). The effects of successive application of antagonists produced a similar effect on smooth muscle to that in younger mice indicating the presence of both AT1-R and AT2-R. In figure 5f, smooth muscle binding in the presence of losartan (i.e. AT2-R) is shown in a 2D slice of a 3D image.

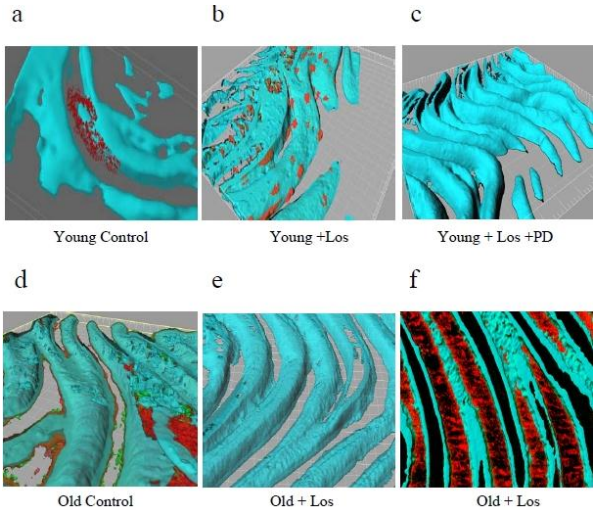

Figure 5. Image analysis of fluorescent TMR-angiotensin II binding to young (a-c) and old (d-f) mouse aorta. Red indicates the binding of TMR-Angiotensin II. Cyan blue was chosen to colour the iso-surfaces representing the autofluorescent folded internal elastic lamina (b-f).
a) Many endothelial cells bind TMR-angiotensin II
b) In the presence of losartan, TMR-Angiotensin II still binds to endothelial cells on the surface of the internal elastic lamina.
c) The presence of both losartan and PD prevents TMR-Angiotensin II binding to ECs.
d) In old animals both TMR-Angiotensin II and QAPB bind to endothelial cells.
e) Losartan alone is sufficient to prevent binding of TMR-Angiotensin II to endothelial cells.
f) When the image is optically sliced to allow visualisation of smooth muscle cells located just below the surface of the IEL, losartan-resistant binding on smooth muscle cells is shown

Discussion

The mouse aorta model allows observation and analysis of the main facets of AT2-R *in vitro* vascular biology that have been found in other species. It allows comparison of these properties between cell types and shows that they change across the life-span. Fluorescent ligand binding showed both AT1-R and AT2-R in cells of endothelium and medial smooth muscle. We identified functional responses for AT1-R in smooth muscle (contraction), AT2-R in smooth muscle (contraction) and AT2-R in endothelium (release of vasodilator nitric oxide). Functional responses of the AT1-R that were found in endothelium remain undetected.

The use of fluorescent TMR-angiotensin II with selective antagonists to identify receptor subtypes worked well. All vascular cell types bound fluorescent TMR-angiotensin II and it was necessary to incubate in both AT1-R and AT2-R antagonists to eliminate this binding, indicating that both receptor types exist in each cell type. This accounts for the principal known effects and locations of the arterial effects of angiotensin II, i.e. pro-contractile smooth muscle AT1-R and pro-dilator AT2-R.

Fluorescent ligand binding to the smooth muscle cells in the presence of losartan showed the AT2-R that are responsible for the contractile response to angiotensin II which is seen after blocking both AT1-R and the nitric oxide production that is activated by endothelial AT2-R. An AT2-R-blocker-sensitive vasoconstrictor response has been demonstrated in young SHR rat resistance arteries, either without AT1-R blockade3 (6 weeks old) or with AT1-R blockade 26 (11-12 weeks old). At corresponding ages this response was not detected in normotensive WKY rats (3, 28).

However, these earlier studies did not report whether the combination of AT1-R blockade and suppression of endothelial factors release (by any means) uncovered an AT2-R-mediated contraction in WKY. It may, then, be that endothelial and smooth muscle AT2-R-mediated responses cancelled each other out in the normal rat, but in the young SHR the lack of endothelial AT2, as shown by immunohistochemistry for AT2-R (26), uncovered the contractile AT2-R. Contractile AT2-R may, therefore, be a more common physiological response exposing it may require the suppression not only of AT1-R but also of the counteracting endothelial AT2-R actions. Note that in terms of absolute and relative age the rats in these earlier studies were all even younger than the younger group of mice used in the current study.

We found losartan-sensitive TMR-Angiotensin II binding on endothelial cells, which presumably indicates endothelial AT1-R. None of our experiments demonstrated a function for these. In old mice these binding sites were present but no relaxant response to angiotensin II was found. Their function, thus, remains to be determined but, on the

basis that it could involve modulation of the release of endothelial vasomodulators or could modify cell growth.

Our data shows that physiological antagonism occurs between angiotensin II's actions on endothelial AT2-R and smooth muscle AT1-R. This answers the question of whether the opposing actions of AT1-R and AT2-R arise from different cell types, i.e. they do. However, both the functional and the binding data indicate that the smooth muscle cells contain AT1-R and AT2-R, each of which causes contraction. This opens the possibility that receptor heterodimers could form, influencing the excitation-contraction coupling process, as has been suggested for recombinant receptors (29, 31).

The separation of the sites of the opposing actions of the two receptor subtypes to different cells remains consistent with a possible upregulation of AT1-R in the AT2-R knockout mouse, (12-13) but suggests that it might be due to the loss of the physiologically antagonistic signal from nitric oxide rather than an intracellular interaction between the AT receptor subtypes. The proposal that AT2-R-mediated vasodilatation is due to AT2-R situated on smooth muscle cells is negated by our evidence in two ways. First there is AT2-R on endothelial cells. The lack of evidence for their presence had been part of the argument that they must therefore be on other cell types. Secondly, in old mice, where there is AT2-R on smooth muscle cells but not on endothelium, there is no vasodilatation to angiotensin II.

Vasodilator AT2-R in mice has been relatively neglected compared with AT2-R in other species. Our data shows that aortic smooth muscle was relaxed indirectly via nitric oxide released by endothelial, PD123319-sensitive AT2-R. This contrasts with the lack of a relaxant response reported when spiral strips of aorta were employed (11; 14). We used wire myograph-mounted aortic rings to ensure minimal damage to the vascular endothelium. It was the relative fragility of drug-induced relaxant responses in spiral strips that led Furchgott & Zawadski (30) to discover endothelium derived relaxant factors. The same factor seems to apply here and may have reduced the effort applied to mouse models.

To the question of whether AT2-R are of relevance in development but not in adult life, our data shows that their contractile function was present at 4 and 14 months old, while their vasodilator effect was present at 4 but not 14 months. In mouse terms, 14 months old could be considered "middle age" since, in controlled conditions, mice typically survive to between 2 and 3 years of age. Thus, the age-dependent decline in AT2-R, perceived over fetal development, continues during adulthood. This is not due to a general decline of endothelial responses since the response via α2-adrenoceptors was maintained. In rats, the pro-vasodilator AT2-R is present at a relatively young age and its absence in the SHR model and can be restored by antihypertensive treatment (28). Thus the endothelial AT2-R response seems to be a normal regulatory factor in young adults that can be modulated by disease or drugs. The loss of AT2-R with age thus adds another aspect to age-related cardiovascular disease, since normal ontogenetic loss of endothelial AT2-R-mediated vasodilatation would contribute to increased peripheral resistance and decreased compliance. Thus, AT1-R-blockade may have an advantage over ACEI at younger ages since it would spare the vasodilator, beneficial effects of AT2-R but this effect would be lost with increasing age. This can explain why animal models, which are invariably carried out at a very young age, show different outcomes to ACEI and AT1-R blockade (19-20, 32). In contrast, comparisons of AT1-R blockade and ACEI in the clinic, invariably using patients in middle or old age, show no advantage to each alone or to the combination; provided that dosage of each is complete (16, 18, 33-34). This can now be explained by the age factor, if human follows the same trend as mice, i.e. a decline of endothelial AT2-R with age. Thus, a model based on older mice would be more realistic as a basis for human rennin-angiotensin system pathology. Conversely, there might be a divergence in the effects of AT1-R blockade and ACEI in human children and young adults.

Perspectives

Mouse aorta presents a model of the location and function of AT1-R and AT2-R. Both receptors are present on both vascular smooth muscle and on endothelium, where they mediate contraction and vasodilatation, respectively, except that the endothelial function of AT1-R remains unknown. This allows investigation of how these receptors interact to produce functional consequences and how this relationship changes over the life-span. It allows modeling of the functional consequences of drugs that modify Angiotensin II production or differently affect the two receptor types, and raises issues of interaction between drugs affecting the Renin Angiotensin System (RAS) and other vasoactive drugs, such as those affecting endothelial factors. The loss of endothelial AT2-R with age has consequences for all of these issues. An endothelial vasodilator action via AT2-R should be beneficial, so its loss with age is deleterious and should be a target for replacement, e.g. through gene therapy. Its loss potentially changes the effects of drugs that have a differential action on the two receptor types, e.g. AT1-R might have a more beneficial effect on the

young, or individuals with good survival of endothelial AT2-R, through sparing AT2-R-mediated vasodilatation. This depends on how the mouse model translates to the human. It should be determined whether age-dependent loss of endothelial AT2-R occurs in humans and is associated with cardiovascular disease. Loss may be a normal ontogenetic phenomenon that is exaggerated in some individuals with deleterious consequences. These issues should also be sought in other vessels to delineate consequences for resistance, compliance and capacitance.

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References

1. Johren O, Dendorfer A, Dominiak P. Cardiovascular and renal function of angiotensin II type-2 receptors. Cardiovasc Res. 2004; 62(3):460-7. PMID: 15158138

2. Carey RM. Update on the role of the AT (2) receptor. Curr Opin Nephrol Hypertens. 2005; 14(1):67-71. PMID: 15586018

3. Touyz RM, Endemann D, He G, Li JS, Schiffrin EL. Role of AT(2) receptors in angiotensin II-stimulated contraction of small mesenteric arteries in young SHR. Hypertension. 1999; 33(1 Pt 2):366-72. PMID: 9931131

4. Touyz RM, Schiffrin EL. Signal transduction mechanisms mediating the physiological and pathophysiological actions of angiotensin II in vascular smooth muscle cells. Pharmacol Rev.2000; 52(4): 639-72. PMID: 11121512

5. Vanhoutte PM. Endothelial AT (2)-receptors: chicken or egg? Br J Pharmacol. 2002; 136(4):481-3. PMID: 12055125

6. Widdop RE, Jones ES, Hannan RE, Gaspari TA. Angiotensin AT (2) receptors: cardiovascular hope or hype? Br J Pharmacol. 2003; 140(5): 809-24. PMID: 14530223

7. Scheuer DA, Perrone MH. Angiotensin Type-2 Receptors Mediate Depressor Phase of Biphasic Pressure Response to Angiotensin. Am J Physiol. 1993; 264(5 Pt 2):R917-23. PMID: 8498601

8. Munzenmaier DH, Greene AS. Opposing actions of angiotensin II on microvascular growth and arterial blood pressure. Hypertension. 1996; 27(3 Pt 2):760-5.PMID: 8613237

9. Katada J, Majima M. AT (2) receptor-dependent vasodilation is mediated by activation of vascular kinin generation under flow conditions. Br J Pharmacol. 2002; 136(4):484-91. PMID: 12055126

10. Tsutsumi Y, Matsubara H, Masaki H, Kurihara H, Murasawa S, Takai S, et al. Angiotensin II type 2 receptor overexpression activates the vascular kinin system and causes vasodilation. J Clin Invest. 1999; 104(7):925-35.

11. Russell A, Watts S. Vascular reactivity of isolated thoracic aorta of the C57BL/6J mouse. J Pharmacol Exp Ther. 2000; 294(2):598-604. PMID: 10900237

12. Ichiki T, Labosky PA, Shiota C, Okuyama S, Imagawa Y, Fogo A, et al. Effects on Blood-Pressure and Exploratory-Behavior of Mice Lacking Angiotensin-Ii Type-2 Receptor. Nature. 1995; 377(6551): 748-50. PMID: 7477267

13. Hein L, Barsh GS, Pratt RE, Dzau VJ, Kobilka BK. Behavioral and Cardiovascular Effects of Disrupting the Angiotensin-Ii Type-2 Receptor Gene in Mice. Nature. 1995; 377(6551):744-7. PMID: 7477266

14. Tanaka M, Tsuchida S, Imai T, Fujii N, Miyazaki H, Ichiki T, Naruse M, Inagami T. Vascular response to angiotensin II is exaggerated through an upregulation of AT1 receptor in AT2 knockout mice. Biochem Biophys Res Commun. 1999; 258(1):194-8. PMID: 10222259

15. Akishita M, Yamada H, Dzau VJ, Horiuchi M. Increased vasoconstrictor response of the mouse lacking angiotensin II type 2 receptor. Biochem Biophys Res Commun. 1999; 261(2):345-9. PMID: 10425188

16. Solomon SD, Skali H, Anavekar NS, Bourgoun M, Barvik S, Ghali JK, et al. Changes in ventricular size and function in patients treated with valsartan, captopril, or both after myocardial infarction. Circulation. 2005; 111(25): 3411-9. PMID: 15967846

17. Van de Wal RMA, Van Veldhuisen DJ, van Gilst WH, Voors AA. Addition of an angiotensin receptor blocker to full-dose ACE-inhibition: controversial or common sense? Eur Heart J. 2005; 26(22):2361-7. PMID: 16105846

18. McMurray JJV. The role of angiotensin II receptor blockers in the management of heart failure. Eur Heart J Suppl. 2005; 7: J10-J14.

19. Zornoff LA, Paiva SA, Matsubara BB, Matsubara LS, Spadaro J. Combination therapy with angiotensin converting enzyme inhibition and AT1 receptor inhibitor on ventricular remodeling after myocardial infarction in rats. J Cardiovasc Pharmacol Ther. 2000; 5(3): 203-209. PMID: 11150409

20. Nakamura Y, Yoshiyama M, Omura T, Yoshida K, Izumi Y, Takeuchi K, et al. Beneficial effects of combination of ACE inhibitor and angiotensin II type 1 receptor blocker on cardiac remodeling in rat myocardial infarction. Cardiovasc Res. 2003; 57(1): 48-54. PMID: 12504813

21. Mulvany MJ, Halpern W. Mechanical-Properties of Vascular Smooth-Muscle Cells Insitu. Nature. 1976; 260(5552): 617-9. PMID: 1264225

22. Mulvany MJ, Halpern W. Contractile Properties of Small Arterial Resistance Vessels in Spontaneously Hypertensive and Normotensive Rats. Circ Res. 1977; 41(1): 19-26. PMID: 862138

23. Briones AM, Daly CJ, Jimenez-Altayo F, Martinez-Revelles S, Gonzalez JM, McGrath JC, et al. Direct demonstration of beta(1)- and evidence against beta(2)- and beta(3)- adrenoceptors, in smooth muscle cells of rat small mesenteric arteries. Br J Pharmacol. 2005; 146(5):679-91.

24. Pediani JD, Colston JF, Caldwell D, Milligan G, Daly CJ, McGrath JC. Beta- arrestin- dependent spontaneous alpha (1a)-adrenoceptor endocytosis causes intracellular transportation of alpha-blockers via recycling compartments. Mol Pharmacol. 2005; 67(4): 992-1004. PMID: 15626751

25. Malekzadeh Shafaroudi M, McBride M, Deighan C, Wokoma A, MacMillan J, Daly CJ, et al. Two "knockout" mouse Models demonstrate that aortic vasodilatation is mediated via alpha (2A)-adrenoceptors located on the endothelium. J Pharmacol Exp Ther. 2005; 314(2):804-10. PMID: 15878998

26. McGrath JC, Deighan C, Briones AM, Shafaroudi MM, McBride M, Adler J, et al. New aspects of vascular remodelling: the involvement of all vascular cell types. Exp Physiol. 2005; 90 (4): 469-75. PMID: 15894533

27. Malekzadeh Shafaroudi M, Daly Cj, McGrath JC. Multiple action sites of α_2-AR agonist (UK14304) on different adrenoceptors in mice aorta. Journal Damghan Univ Basic Sci. 2008; 1(3):111-22.

28. You D, Loufrani L, Baron C, Levy BI, Widdop RE, Henrion D. High blood pressure reduction reverses angiotensin II type 2 receptor-mediated vasoconstriction into vasodilation in spontaneously hypertensive rats. Circulation. 2005; 111(8): 1006-11. PMID: 15710752

29. AbdAlla S, Lother H, Abdel-tawab AM, Quitterer U. The angiotensin II AT2 receptor is an AT1 receptor antagonist. J Biol Chem 2001; 276(43): 39721-6. PMID: 11507095

30. Furchgott RF, Zawadzki JV. The Obligatory Role of Endothelial-Cells in the Relaxation of Arterial Smooth-Muscle by Acetylcholine. Nature. 1980; 288(5789): 373-6. PMID: 6253831

31. Heeneman S, Sluimer JC, Daemen MJAP. Angiotensin-Converting Enzyme and Vascular Remodeling. Circ Res. 2007; 101(5): 441-54.

32. Fujisaka T, Hoshiga M, Hotchi J, Takeda Y, Jin D, Takai S, Angiotensin II promotes aortic valve thickening independent of elevated blood pressure in apolipoprotein-E deficient mice. Atherosclerosis. 2013; 226(1): 82-7. PMID: 23177972

33. Yano H, Hibi K, Nozawa N, Ozaki H, Kusama I, Ebina T, et al. Effects of valsartan, an angiotensin II receptor blocker, on coronary atherosclerosis in patients with acute myocardial infarction who receive an angiotensin-converting enzyme inhibitor. Circ J. 2012; 76 (6): 1442-51. PMID: 22473458

34. Savarese G, Costanzo P, George J, Cleland F, Vassallo E, Ruggiero D, Rosano G, Perrone-Filardi P. A Meta-Analysis Reporting Effects of Angiotensin-Converting Enzyme Inhibitors and Angiotensin Receptor Blockers in Patients Without Heart Failure. J Am Coll Cardiol. 2013; 61(2):131-42. PMID: 23219304

35. Wong J, Molyneaux L, Constantino M, Twigg SM, Yue DK. Angiotensin-converting enzyme inhibition and angiotensin II receptor blockade in combination, a lesser evil in some? Diabetes Obes Metab 2010; 12 (12): 1072-8. PMID: 20977578