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Pre- and postjunctional effect of rosiglitazone on the isolated rat aorta and heart

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Pre- and postjunctional effect of rosiglitazone on the isolated rat aorta and heart

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Abstract

Rosiglitazone (RSG) increases the risk of ischemic cardiovascular events in type 2 diabetes patients by unknown mechanisms. We determined the influence of RSG on the angiotensin II (AII)-mediated facilitation of noradrenaline release from sympathetic terminals (prejunctional effects) and on AII-mediated vasoconstriction (postjunctional effects). For comparison, of RSG influence on noradrenaline-mediated pre- and postjunctional effects was studied.

In rat left ventricle isolated slices preloaded with ^{3}H-noradrenaline and electrically stimulated, RSG (3 μM) caused a shift to the left of the concentration-response curve for the facilitatory effect of AII on tritium overflow. The $pEC_{30\%}$ values of AII in absence and presence of RSG (3 μM) were 8.6±0.1 and 9.3±0.3, respectively (n=5 each, $P<0.05$, t-test). Rauwolscine antagonized the autoreceptor-mediated negative feedback of noradrenaline release with a $pEC_{30\%}$=8.7±0.3 (n=4) in controls and a $pEC_{30\%}$ =8.9±0.3, (n=4) in the presence of RSG.

In rat aorta strips, RSG had no effect on the AII or noradrenaline-mediated vasoconstriction. For AII pEC_{50} was 7.2±0.4 and 7.6±0.5 and E_{max} was 132±39 and 129±35 mN/g of tissue, n=3-5 each, in the absence and presence of RSG (3 μM), respectively. For noradrenaline, pEC_{50} was 6.9±0.5 and 7.0±0.5 and E_{max} was 158±49 and 149±48, n=4 each, in the presence and absence of RSG, respectively.

Data show that RSG selectively potentiates the prejunctional facilitatory effects of AII on sympathetic terminals. It is suggested that this increase in noradrenaline release might contribute to the cardiovascular harm of RSG.

Key words: angiotensin II, rosiglitazone, noradrenaline release, cardiovascular, rat aorta

Resumo

A rosiglitazona(RSG) aumenta o risco de eventos cardiovasculares isquémicos nos pacientes com diabetes mellitus tipo 2 por mecanismos desconhecidos. Determinamos a influência de RSG na facilitação da libertação de noradrenalina mediada pela angiotensina II (AII) nos terminais simpáticos (efeito pré-juncional) assim como na vasoconstrição mediada pela AII (efeito pós-juncional). Para comparação, estudamos a influência da RSG nos efeitos pré e pós-juncionais mediados pela noradrenalina.

Em fatias isoladas de ventrículo esquerdo de rato carregado com ^{3}H-noradrenalina e electricamente estimulado, RSG (3 μM) causou um desvio para a esquerda na curva de concentração-resposta para o efeito facilitatório da AII no fluxo tritiado. Os valores de $pEC_{30\%}$ de AII na ausência e presença de RSG (3 μM) foram 8.6±0.1 e 9.3±0.3, respectivamente (n=5 cada, P <0.05, t-test). A rauvolscina antagonizou o feedback negativo mediado por autoreceptores da liberação de noradrenalina com um $pEC_{30\%}$ =8.7±0.3 (n=4) em controlos e um $pEC_{30\%}$ = 8.9±0.3(n=4) na presença de RSG.

Em tiras de aorta de rato, a RSG não teve efeito na vasoconstrição mediada tanto pela AII como pela noradrenalina. Para a AII o pEC_{50} foi de 7.2±0.4 e 7.6±0.5 e o E_{max} foi 132±39 e 129±35 mN/g de tecido, n=3-5 cada, na ausência e na presença de RSG (3 μM), respectivamente. Para a noradrenalina, pEC_{50} foi 6.9±0.5 e 7.0±0.5 e o E_{max} foi 158±49 e149±48, n=4 cada, na ausência e presença de RSG, respectivamente.

Os resultados mostram que a RSG potencia selectivamente os efeitos pré-juncionais da AII nos terminais simpáticos. Podemos sugerir que este aumento de liberação de noradrenalina pode contribuir para os efeitos adversos cardiovasculares da RSG.

Palavras -chave: angiotensina II, rosiglitazona, liberação de noradrenalina, cardiovascular, aorta de rato.

Introduction

RSG is an antidiabetic agent that targets the peroxisome-proliferator-activated receptor γ (PPARγ), a nuclear receptor that regulates the expression of many genes. RSG is an insulin sensitizer, improves glycemic control in type 2 diabetic patients and induces adipocyte differentiation. However, some studies point out several cardiovascular adverse effects produced by RSG, such as a significant increase in the risk of myocardial infarction and with an increase in the risk of death by other cardiovascular causes (Nissen and Wolski, 2010). Other studies now confirm the risk of developing heart failure with thiazolidinediones, namely in patients of all baseline cardiovascular risks, (diabetes mellitus, vascular disease or heart failure). (Chaggar et al., 2009).

The PPARγ agonist RSG was recently withdrawn from the European market. The EMEA based this decision in the analysis of the RECORD trial, which showed that the addition of RSG to glucose-lowering therapy in people with type 2 diabetes increases the risk of heart failure, mainly in women (Home et al., 2009). Thiazolidinediones have cardiovascular effects through as yet poorly defined mechanisms (Higashi et al., 2010).

The mechanism for the cardiovascular harm linked to RSG is uncertain. Recent experiments from our group (Bastos et al. 2008) showed that RSG and AT_1-receptor antagonists had opposite effects on the prejunctional noradrenaline release-enhancing effect of AII. While AT_1-receptor antagonists reduced, RSG increased the facilitatory action of AII on noradrenaline release by sympathetic nerve stimulation. This was unexpected because some of the AT_1-receptor antagonist tested mimetized the effect of RSG on adipocyte differentiation in vitro. It is known that telmisartan, a structurally unique AII receptor antagonist used for the treatment of hypertension, can function as a partial agonist of PPARγ; influence the expression of PPARγ target genes involved in carbohydrate and lipid metabolism; and reduce glucose, insulin, and triglyceride levels in rats fed a high-fat, high-carbohydrate diet (Benson et al., 2004).

Since AII is a vasoconstrictor peptide that has a direct action on vascular smooth muscle cells and an indirect action mediated by facilitation of noradrenaline postganglionic sympathetic neurons, potentiation of AII might contribute to the increased cardiovascular risk associated with RSG. Cardiovascular diseases, like hypertension, cardiac heart failure and myocardial infarction depend on the activation of the sympathetic nervous system for their evolution (Sarafidis and Nilsson, 2006).

Other prejunctional studies have concluded that RSG increased the facilitatory action of AII on noradrenaline release in a concentration-dependent manner, without changing the release-enhancing effect of α_2-autoreceptor antagonists in the rat heart. We also know that the potentiation of AII by RSG was antagonized by GW9662, a selective PPARγ antagonist. These results suggest that a positive allosteric modulation of AII receptors by PPARγ agonists may occur in sympathetic terminals (Ferreira et al, 2010).

Nowadays, there are few studies that characterize the postjunctional effect of RSG in the AII modulating effects in the rat artery. Some studies demonstrated that RSG and pioglitazone treatment significantly restored the elevated systolic, mean arterial, diastolic blood pressure and attenuated the enhanced contractile responses to AII in thoracic aortic rings, and that pioglitazone and RSG significantly decreased the specific binding to AT_1-receptors in high fat diet fed rats. It was concluded that the role of hyperglycemia in the elevation of AII induced vascular responses in thoracic aorta isolated from insulin resistant rats and PPARγ agonists can attenuate these responses (Gaikwad et al., 2007).

Other studies conclude that RSG attenuated AII-induced blood pressure elevation and intracellular signaling on aorta and mesenteric vessels and that there was differential inhibition of AT_1-receptor receptors/phosphatidylinositol 3-kinase/Akt and extracellular signal-regulated kinase 1/2 in both vessels. Effects of PPARγ activators on these pathways could contribute to regression of vascular remodeling in models of hypertension and diabetes and, accordingly, in hypertensive diabetic patients (Benkirane et al., 2006). Also, other postjunctional studies indicate that RSG, when acting on rat aorta, has beneficial effects in reducing blood pressure and the aortic tunica media hypertrophy with consequent balance of the wall stress in metabolically programmed offspring. In rats with a low protein diet, it restores the expressions of AT_1-receptor and endothelial nitric oxide synthase nearly to the levels presented in rats with a normal protein diet (Torres et al., 2008).

On the other hand, there are studies that indicate an endothelium-dependent mechanism of RSG and pioglitazone, which can alter vascular function differentially over the same pathways on the endothelium where they have a dual action, increasing both production of vasoconstrictor prostanoids and NO. The balance between both vasoactive substances determines the vascular response to TZD (Llorens et al., 2007).

The aim of this work is to characterize the pre and postjunctional effects of PPARγ agonist RSG on AII receptors of the rat heart and aorta, helping to clarify a possible explanation for the increase of mortality associated with cardiovascular causes inherent to RSG. For comparison, the influence of RSG on noradrenaline-mediated pre- and postjunctional effects was studied.

Methods

For the prejunctional studies the experiments were carried out in left ventricle slices. Male Wistar-Kyoto rats weighing 200-250 g were killed by decapitation, the heart was rapidly removed and the left ventricle isolated and cut in slices of about 7x7 mm. The slices were rapidly placed in warmed, aerated (with 95% O_2 and 5% CO_2) modified Krebs-Henseleit solution containing ^{3}H-noradrenaline (0.2 μM) and agitated for 1 hour (incubation period). The referred Krebs- Henseleit solution had the following composition (mM): NaCl 119, $CaCl_2$ 2.52, KH_2PO_4 1.18, $MgSO_4$ 1.23, $NaHCO_3$ 25.0, glucose 10.0 (Mota and Guimaraes, 2003).

After incubation, the slices were mounted in perifusion chambers and perifused with Krebs-Henseleit solution, warmed at 37° C, aerated with 95% O_2 and 5% CO_2, for 1 hour at a flow rate of 0.8 ml/min. Then the perifusing solution was changed to Krebs-Henseleit solution containing cocaine (12 μM), with the other conditions remaining the same, and the tissues were perifused for 20 more minutes.

From t=80 min (t=0 being the onset of the perifusion) the perifusion fluid was collected continuously in samples of 5 min, during 100 min (a total of 20 samples).

In the experiments using AII, three periods of transmural electric stimulation (1 Hz, 2ms, 50 mA) during 5 min were applied at min 70 (S0), 100 (S1) and 150 (S2). The first period was not considered for the calculations; the second was taken as control (Scontrol) and the third was used to verify the influence of angiotensin on noradrenaline release (Sdrug).

In the experiments using rauwolscine, two further periods of transmural electric stimulation were applied at min 200 (S3) and 250 (S4). Rauwolscine was added at increasing concentrations before S2, S3 and S4 (Sdrug). Drugs were added to the perifusion fluid at t=125 min and were maintained until the end of the experience.

At the end of experiment, the tissues were weighted and kept in perchloric acid (2 ml at 0.2 M). Radioactivity was measured by scintillation counting (liquid scintillation counter 1209 Rackbeta; LKB Wallac, Turku, Finland) in aliquots with the perifusate after addition of 8 ml of scintillation mixture (Wallac OptiPhase “HiSafe” 3; Fischer Chemicals, Loughborough, UK).

The outflow of tritium was calculated as a fraction of the amount of tritium in the tissue at the start of the respective collection period (fractional rate of loss^{-1}). The fractional release per shock was calculated by dividing evoked tritium overflow by tritium present in the tissue at the beginning of the stimulation period and by the number of shocks. Drug effects are expressed as the ratio FR of tritium evoked by $S_{control}$ over that evoked by S_{drug}. Each result was corrected for time-dependent changes as determined in parallel drug-free control experiments.

For the calculation of the overflow induced by electrical stimulation those 5 min samples were taken into account in which the overflow of tritium exceeded that in the last prestimulation control sample (usually this applied to the 3 or 4 samples collected during and

after stimulation). The spontaneous outflow measured in the last pre-stimulation sample was assumed to represent the spontaneous outflow in subsequent samples; it was subtracted from the overflow determined in stimulation and post-stimulation samples. The 'total overflow of transmitter' was the sum of all increases (induced by a period of stimulation) above the spontaneous level of outflow of tritium.

For the postjunctional studies, a segment of the thoracic and abdominal aorta between the aortic arch and the bifurcation of the abdominal aorta into the femoral arteries was dissected and placed in Krebs-Henseleit solution without EDTA. After removing the excess fat, connective tissue and endothelium, the aorta was cut into helical strips approximately 2–3 mm in width and 15– 20 mm in length.

The artery strips were mounted in a 10-ml bath containing aerated modified Krebs-Henseleit solution at 37° for 1 h under a resting tension of 1.0 g. The mechanical responses were recorded on a Harvard Universal Oscillograph.

Concentration-response curves were obtained on each strip by non-cumulative additions of AII with half-log increments. After the response to a given concentration had reached the maximum, the tissue was repeatedly washed out. Each postjunctional experiment consisted of: (a) a complete concentration-response curve of AII without RSG and (b) a complete concentration-response curve of AII in the presence of 3μM RSG.

Statistics

$EC_{30\%}$ values represent the molar concentration of the drug under study that increased the evoked overflow by 30% and $pEC_{30\%}$ the negative logarithm of $EC_{30\%}$. pEC_{50} values represent the negative logarithm of the molar concentration of the agonist that causes 50% of the maximal contraction. The results are expressed as arithmetic means ±SEM, unless it is stated otherwise. A probability level of 0.05 was considered statistically significant (independent t-test with Newman-Keuls correction for multiple comparisons was used for statistical analyses). Curve fitting was done with the software GraphPad Prism (GraphPad Prism Software Inc., La Jolla, CA, USA).

Chemicals

The chemicals used in this study were: angiotensin II (Sigma, St. Louis, MO., USA); cocaine hydrochloride (Uquipa, Lisbon, Portugal); levo-[ring-2,5,6-3H]-noradrenaline (49.5 Ci/mmol; PerkinElmer, Boston, MA., USA); L-(-)noradrenaline bitartrate (Sigma); rauwolscine

hydrochloride (Tocris, Ellisville, MO., USA); rosiglitazone maleate (maleate of 5-[[4-[2-(methyl- 2-pyridinylamino)-ethoxy]phenyl]methyl]-2,4-thiazolidinedione) (Kemprotec, Middlesbrough, UK). Stock solutions of rosiglitazone (10 mM) were prepared in ethanol. All other solutions were prepared in water.

Results

In order to verify whether RSG modifies the prejunctional effect of AII, we compared the effect of noradrenalin release induced by AII in the presence and absence of RSG using electrical stimulation of the rat heart, which had previously been incubated with ^{3}H-noradrenaline. AII (1 – 100 nM) had no effect on the spontaneous efflux of ^{3}H-noradrenaline and caused a concentration-dependent increase on the fractional release per shock (Fig. 1). The maximal increase on ^{3}H-noradrenaline release induced by electrical stimulation in the presence of AII was 110±8% (n=18) in relation to control. RSG (3 μM) had no effect on the release of ^{3}H-noradrenaline induced by electrical stimulation. However the concentration-response curve to AII was shifted to the left in the presence of RSG. The potency of AII for the prejunctional effects expressed as $pEC_{30\%}$ values was significantly increased by RSG at a concentration of 3 μM (Table 1).

To verify whether RSG changes the postjunctional effect of AII, we compared the concentration-response curve for the contractile response of the rat aorta to AII in the presence and absence of RSG (Fig. 2). There was no significant shift to the left of this curve. The maximal tension caused by AII was similar in the absence (132±39 mN/g, n=5) and in the presence (129±35mN/g, n=5) of RSG. The potency of AII for contraction was not significantly changed by RSG (Table 1).

To see if this effect was specific of AII, and not mediated by other receptors, we also compared the effect of noradrenalin release induced by the α_2-adrenoceptor antagonist rauwolscine in the presence and absence of RSG using electrical stimulation of the rat heart, to conclude about the prejunctional responses to noradrenaline. RSG caused no shift of the concentration-response curve of rauwolscine. The $pEC_{30\%}$ value of rauwolscine in the presence of RSG (3 μM) was not different from that obtained in its absence (Table 1).

To look for the influence of RSG on the postjunctional effect of noradrenaline we determined concentration-response curves of the rat aorta to noradrenaline in the presence and absence of RSG. The pEC_{50} values in the presence and absence of RSG were not different (Table 1). The maximal effect of noradrenaline in the absence of RSG was 158±49 mN/g (n=4), in controls and of 149±48 mN/g (n=4) in the presence of RSG.

Discussion

Our results confirm that RSG increased the facilitatory action of AII on noradrenaline release-enhancing effect in the terminal sympathetic nerves of the rat heart. This might be a possible explanation for the cardiovascular adverse effects of RSG, which could be mediated by the sympathetic system, leading to hypertension, acute myocardial infarction and cardiac heart failure. As shown in figure 1, the concentration-response curve to AII in the presence of increasing concentrations of RSG was shifted to the left with increase of the maximum. This is in good agreement with Bastos et al. (2008), who reported that RSG and AT_1-receptor antagonists had opposite effects on the prejunctional noradrenaline release-enhancing effect of AII. This means that while AT_1-receptor antagonists reduced, RSG increased the facilitatory action of AII on noradrenaline release by sympathetic nerve stimulation.

Our results concerning the postjunctional effect of AII show that RSG at the same concentration that potentiate the prejunctional effect of AII, does not shift the contractile concentration-response curve to AII (Fig. 2). The possibility that using a higher concentration of RSG may lead us to find a statistically significant postjunctional effect cannot be discarded.

The difference between the pre and postjunctional results concerning the AII action in the presence and absence of RSG could be explained by a slight difference of the pre and postjunctional receptors of AII. It is well established that AII AT_1 receptors are different pre- and postjunctionally and also that prejunctional and postjunctional AII receptors most probably belong to AT_{1B} and AT_{1A} subtypes, respectively. (Guimaraes and Pinheiro, 2005)

Various studies have demonstrated that the vasoconstrictor effects of AII result from a direct action on vascular smooth muscles (Helmer, 1964) and from an indirect action mediated by facilitation of noradrenaline release from postganglionic sympathetic neurons (Guimaraes and Moura, 2001). These post and prejunctional effects of AII in the cardiovascular system are mediated by two types of receptors, AT_1 and AT_2 (Guimaraes and Pinheiro, 2005). AII produces virtually all its actions, like cardiovascular, neuronal, renal, endocrine and hepatic effects, by stimulation of AT_1 receptors. These receptors mediate the vasoconstrictor effects, as well as vascular smooth muscle proliferation, aldosterone release and the regulation of the fluid-electrolyte balance (Wong et al., 1991). On the other hand, the biological function of the AT_2 receptor remains unknown (Ozono et al., 1997). The AT_1 receptor is sensitive to the reference compound losartan, whereas the AT_2 receptor is sensitive to PD123177 and the related agent PD123319. A further subdivision of AT_1 receptors into AT_{1A} and AT_{1B} subtypes is based on binding studies indicating a population of AT_1 receptors for which PD123319 showed a high

affinity and losartan a lower affinity (Nap et al., 2004). Prejunctional receptors for AII most probably belong to AT_{1B} subtypes. (Guimaraes and Pinheiro, 2005).

AII, when acting on sympathetic neurons, induces an increase of exocytotic release of noradrenaline (Boehm and Kubista, 2002). This mediator acts on presynaptic AT_1-receptors (Guimaraes et al., 2001). It was also established that AII requires ongoing α_2-autoinhibition for the full extent of his noradrenaline release-enhancing effect (Trendelenburg et al., 2003). The facilitation of noradrenaline release caused by activation of AT_{1B}-receptors requires an ongoing α_2-autoinhibition (some degree of tonic autoinhibition) since the blockade of α_2-autoreceptors attenuates the noradrenaline release enhancing effect of AII (Mota and Guimaraes, 2003). Therefore, in order to verify if the noradrenaline release-enhancing effect of RSG was specific for AII, the interactions between RSG and α_2-antagonists on the prejunctional modulation of noradrenaline release were studied. Our results show that the release enhancing effects of rauwolscine were not changed in the presence of RSG 3 μM, when comparing with the control values (Table 1). These results indicate that RSG does not alter this alternative mechanism which enhances the release of RSG. We should be aware that there might be other mechanisms of presynaptic action of AII.

Bearing the same objective in mind, we tested if the postjunctional effects of noradrenaline could be mediated by the presence and absence of RSG. As can be seen on table 1, there is no difference in the rat aorta contraction that could be mediated by this drug. Therefore, the results concerning noradrenaline are coincident, pre and postjunctionally.

Since we are studying acute actions of RSG in the rat heart and aorta, and thiazolidinediones are PPARγ agonists that act on the nucleus, we might consider that its actions were subacute or chronic. Peroxisome proliferator-activated receptors (PPAR) are nuclear receptors acting as transcription factors on numerous target genes after heterodimerization with the retinoid X receptor (Kahn and McGraw, 2010). PPARγ is mainly involved in fat cell differentiation and insulin sensitivity, and is expressed in smooth muscle cells and myocardium, although PPARγ is scarce in the latter. This could mean that RSG could not induce acute actions in our study. However, in transfected COS-7 cells, Tohgo et al (2003) found that angiotensin AT_{1a} forms a stable receptor-β-arrestin complex, and activates a β-arrestin-bound pool of ERK2 more efficiently than α1b and β2 adrenergic receptors, which form transient receptor-β-arrestin complexes. So, it is now established that, beyond the G-coupled pathways mediated by AII and noradrenaline, these two agonists also act through a second pathway, thus generating a powerful intracytoplasmatic signal. Studies also suggest that the existence of one or more mechanisms of ERK activation that do not require G protein activation, and that, like the β-arrestin-dependent

activation of ERK, leads to localized ERK activation outside of the cell nucleus. (Tohgo et al., 2003)

Also, in a cell-free assay the binding affinity of the AT_1 receptor antagonists telmisartan and valsartan to the PPARγ is close to that of the PPARγ selective agonist RSG. This description of a PPARγ out-side the nucleus provide us the possibility that RSG can exert its noradrenaline enhancing effect acting on his well-known target. Otherwise, it will be hard to explain how in few minutes RSG is able to modify the release enhancing effect of AII acting on PPARγ localized in nucleus when its mechanisms supposes modifications in genes transcriptions (Storka et al., 2008).

In conclusion, results show that RSG potentiates the noradrenaline-release enhancing effect of AII presinaptically, without changing the release-enhancing effect of α_2- receptor antagonists. This might suggest a positive allosteric modulation of AII receptors by PPARγ agonists in sympathetic terminals. Our results also show that this concentration dependent effect of AII in the presence of RSG is more perceptible pre than postjunctionally, suggesting that the allosteric modulation is more pronounced at the AT_{1B} receptor than at the AT_{1A} receptor. These results might contribute to explain the mechanisms inherent to the adverse cardiovascular effects of RSG.

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Legends

Fig.1 Effects of RSG 3 μM in the presence of AII on noradrenaline release induced by electrical stimulation of the rat left ventricle slices incubated with ^{3}H-noradrenaline. The effect of drugs was expressed as the ratio of the FR evoked by S_{drug} over that evoked by $S_{control}$. Shown are means (n = 3-5) and best-fitting curves representing a 2-parameter logistic equation (software GraphpadPrism)

Fig.2 Effects of RSG 3 μM in the presence of AII on the contractile force of the rat aorta. The effect of drugs was expressed in mN/g. Shown are means (n = 3-5) and best-fitting curves representing a 2-parameter logistic equation (software GraphpadPrism)

Table 1: Influence of RSG on the potency of AII, noradrenaline and rauwolscine.

	Prejunctional Effect		Postjunctional Effect	
	Angiotensin II ($pEC_{30\%}$)	Rauwolscine ($pEC_{30\%}$)	Angiotensin II (pEC_{50})	Noradrenaline (pEC_{50})
Control	8.6 ± 0.1	8.7 ± 0.3	7.2 ± 0.4	6.9 ± 0.5
Rosiglitazone 3µM	9.3 ± 0.3 *	8.9 ± 0.3	7.6 ± 0.5	7.0 ± 0.5

Results are mean ± SEM (n=3-6)

* $p<0.05$ vs control

Figure 1

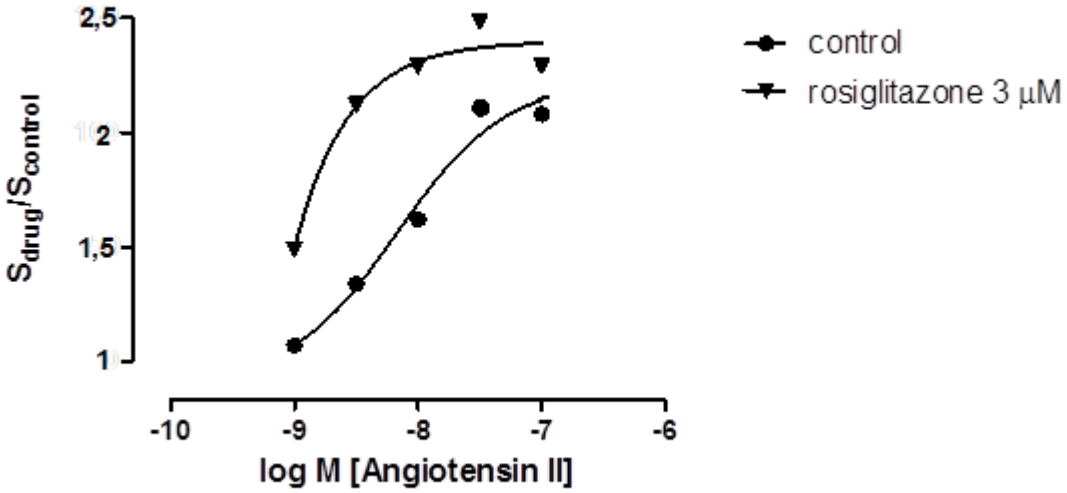
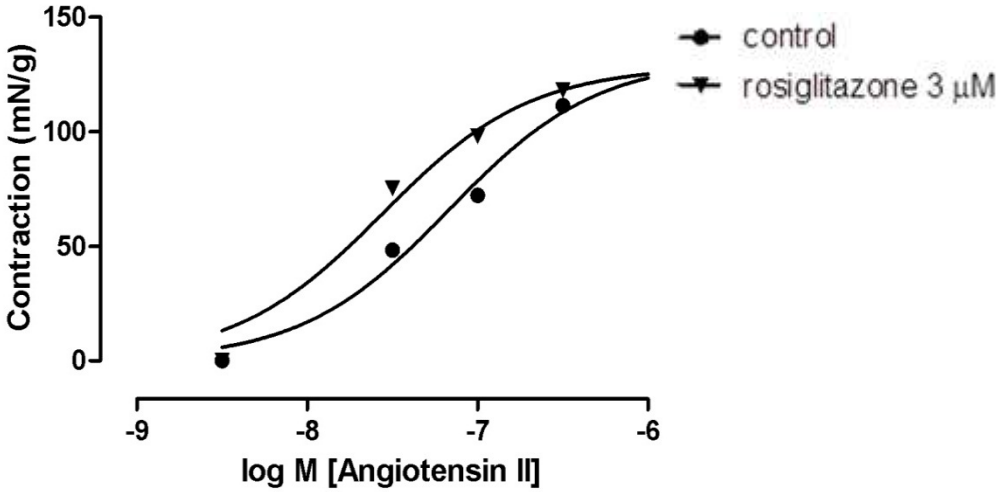

2,5
2
1,5
1
$S_{drug}/S_{control}$
-10
-9
-8
-7
-6
log M [Angiotensin II]
control
rosiglitazone 3 μM

Figure 2


150
100
50
0
Contraction (mN/g)
-9
-8
-7
-6
log M [Angiotensin II]
control
rosiglitazone 3 µM

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Book

South J, Blass B (2001) The future of modern genomics. Blackwell, London

Book chapter

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

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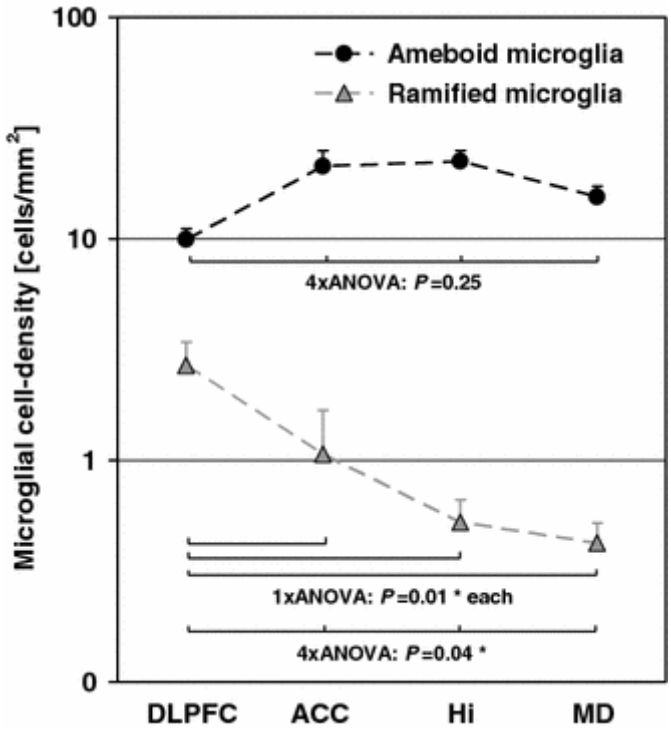

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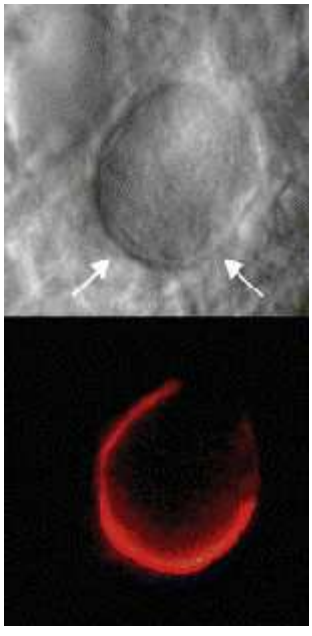

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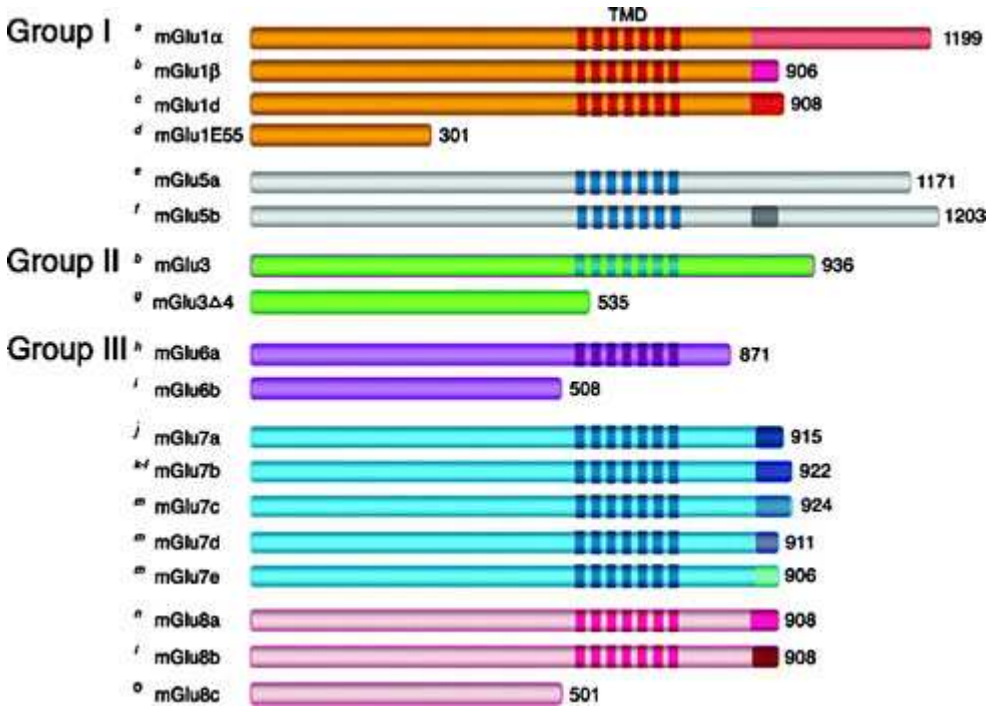

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