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Characterization of the sympathomimetic action of rosiglitazone

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Abstract

AT$_1$ receptor antagonists block the angiotensin II-enhancing effect on noradrenaline release from sympathetic neurons. In a cell-free assay the binding affinity of the AT$_1$ receptor antagonists telmisartan and valsartan to the gamma peroxisome proliferator-activated receptor (PPARy) is close to that of the PPARy selective agonist rosiglitazone. We tested whether rosiglitazone would also modify the prejunctional facilitatory effect of angiotensin II. Facilitation by $\alpha_2$-autoreceptor antagonists was also tested in the presence of rosiglitazone.

Left ventricular slices of rats were incubated with tritiated noradrenaline, perifused and electrically stimulated. The negative logarithm of the drug concentration that caused a 30% increase of control ($pEC_{50k}$) was calculated.

Angiotensin II caused a concentration-dependent increase of tritium overflow induced by electrical stimulation [$pEC_{50k}$=8.6±0.2 (mean±SEM, n=18); maximum increase=110±8%]. Rosiglitazone (0.3-3 μM) had no direct effect. The concentration-response to angiotensin II in the presence of fixed concentrations of rosiglitazone was shifted to the left with increase of the maximum ($pEC_{50k}$=8.8±0.2, 9.2±0.2 and 9.3±0.3; maximum increase=118±14%, 146±13% and 148±16%, in the presence of 0.3, 1 and 3 μM of rosiglitazone, respectively, n=4-6, each). The release-enhancing effects of neither rauwolscine nor phentolamine (1-300 nM) were changed by rosiglitazone.

Results show that rosiglitazone potentiates the noradrenaline-release enhancing effect of angiotensin II without changing the release-enhancing effect of $\alpha_2$-receptor antagonists suggesting that a positive allosteric modulation of angiotensin II receptors by PPARy agonists may occur in sympathetic terminals.

Keywords: rosiglitazone; angiotensin II; noradrenaline release; rauwolscine; phentolamine.
Resumo

Os antagonistas do receptor AT₁ bloqueiam o efeito facilitador da angiotensina II na libertação de noradrenalina dos neurônios simpáticos. Em preparações sem células intactas, a afinidade de ligação dos antagonistas dos receptores AT₁ telmisartan e valsartan para o receptor ativado por proliferadores dos peroxissomos gamma (PPARγ) é semelhante ao do seu agonista selectivo rosíglitazona. Verificámos neste trabalho se a rosíglitazona modifica o efeito facilitador pré-juncional da angiotensina II. A facilitação mediada pelos antagonistas α₂ também foi testada na presença de rosíglitazona.

As fatias de ventrículo esquerdo de rato foram incubadas com noradrenalina tritiada, perfundidas e electricamente estimuladas. Calculou-se o logaritmo negativo da concentração de fármaco que causou um aumento da libertação de noradrenalina tritiada de 30% do controlo (pEC₃₀).

A angiotensina II causou um aumento dependente da concentração do efluixo de tritio induzido por estimulação elétrica [pEC₃₀=8.6±0.2 (média±SEM, n=18); aumento máximo=110±8%]. A rosíglitazona (0.3-3 μM) não teve efeito directo. A curva de concentração-resposta da angiotensina II na presença de concentrações fixas de rosíglitazona foi desviada para a esquerda com aumento no valor máximo (pEC₃₀=8.8±0.2, 9.2±0.2 and 9.3±0.3; aumento máximo=118±14%, 146±13% e 148±16%, na presença de 0.3, 1 e 3 μM de rosíglitazona, respectivamente, n=4-6, cada). Os efeitos facilitadores da libertação quer da rauvolscina quer da fentolamina não foram alterados pela rosíglitazona.

Os resultados mostram que a rosíglitazona potencia o efeito facilitador que a angiotensina II tem sobre a libertação de noradrenalina, sem alterar o mesmo tipo de efeito facilitador que os antagonistas dos receptores α₂ têm sobre a libertação de noradrenalina, sugerindo que possa ocorrer nos terminais simpáticos uma modulação alostérica positiva dos receptores da angiotensina II pelos agonistas PPARγ.

Palavras-chave: rosíglitazona; angiotensina II; libertação de noradrenalina; rauvolscina; fentolamina.
Introduction

Peroxisome proliferator-activated receptors (PPARs) are nuclear ligand-activated transcription factors that increase or decrease the transcription of the target genes which, ultimately, lead to the up-regulation of the insulin-sensitizing factors and down-regulation of the insulin-resistant factors (Guo et al. 2006).

Rosiglitazone, a PPARγ agonist, is a widely used drug with tens of millions prescriptions for patients with type 2 diabetes (Psaty and Furberg 2007). However, rosiglitazone has been associated with serious adverse effects. A recent meta-analysis demonstrated that rosiglitazone is associated with a significant increase in the risk of myocardial infarction and with an increase in the risk of death from cardiovascular causes that had borderline significance (Nissen et al. 2007). Some authors believe that concerns about the cardiovascular safety of thiazolidinediones are unjustified and due to methodologically inappropriate analyses (Mannucci and Monami 2009). There are not definitive conclusions about the cardiovascular risk of rosiglitazone (Home et al. 2009). However the available data do ring an important bell and this subject deserves further investigation not only in a clinical but also in a mechanistic level. The mechanisms underlying the possible cardiovascular effects of rosiglitazone remain unknown (Stulc and Ceska 2008; Psaty and Furberg 2007; Nissen et al. 2007).

On the basis of cellular assays, Benson et al. (2004) demonstrated that telmisartan (and irbesartan in higher concentrations) can act as a partial agonist of PPARγ. Moreover, telmisartan increased the expression of known PPARγ target genes and induced adipogenesis in 3T3-L1 preadipocytes. In order to clarify if, inversely, PPARγ agonists act on renin-angiotensin system, Bastos et al. (2008) and Silva et al. (2009) performed experiments to compare angiotensin II receptor antagonists and rosiglitazone in pre-adipocytes and cardiac nerve terminals. Surprisingly, they found that rosiglitazone and AT₁-receptor antagonists had opposite effects on the noradrenaline release-enhancing effect of angiotensin II. While AT₁-receptor antagonists reduced, rosiglitazone increased the facilitatory action of angiotensin II on noradrenaline release by sympathetic nerve stimulation. This was interpreted as a potential contribution for the adverse effects of rosiglitazone in the cardiovascular system because activation of the sympathetic nervous system is a component of physiopathology of certain diseases like hypertension, cardiac heart failure and acute myocardium infarction.

However there are some questions about this property of rosiglitazone that need to be clarified. Is this effect of rosiglitazone dose-dependent? Is it a general action on the release of noradrenaline or is it specific for the noradrenaline release-enhancing effect of angiotensin II?
Does it depend on the PPARγ, the known target of TZD, or does it depend on another receptor?

Storka et al (2008) reported that in a cell-free assay the binding affinity of the AT₁ receptor antagonists telmisartan and valsartan to the PPARγ is close to that of the PPARγ selective agonist rosiglitazone, with a higher half-maximal effective concentration for the telmisartan. Using western blot analysis of phosphorylated targets of PPARγ and measurement of the release of visfatin they confirmed the agonism of telmisartan and valsartan on PPARγ. AT₁ receptor antagonists block the angiotensin II-enhancing effect on noradrenaline release from sympathetic neurons (Guimarães et al 2001). So we tested whether rosiglitazone would also modify the prejunctional facilitatory effect of angiotensin II. It was also established that angiotensin II requires ongoing α₂-autoinhibition for the full extent of his noradrenaline release-enhancing effect (Trendelenburg et al 2003). Facilitation by α₂-autoreceptor antagonists was also tested in the presence of rosiglitazone.

So the aim of this study was the characterization of sympathetic actions of the TZD rosiglitazone through the study of interaction between rosiglitazone and α₂-antagonists in sympathetic terminals on the prejunctional modulation of noradrenaline release.
Materials and methods

The experiments were carried out in left ventricle slices. Male normotensive Wistar rats weighing 200-250 g were killed by decapitation, the heart was rapidly removed, the left ventricle isolated and cut in slices of about 7x7 mm. The slices were rapidly placed in warmed, aerated (with 95% O₂ and 5% CO₂) modified Krebs-Henseleit solution containing ³H-noradrenaline (0.2 μM) and agitated for 1 hour (incubation period). The referred Krebs-Henseleit solution had the following composition (mM): NaCl 119, CaCl₂ 2.52, KH₂PO₄ 1.18, MgSO₄ 1.23, NaHCO₃ 25.0, glucose 10.0 (Guimarães and Osswald 1969).

After incubation, the slices were mounted in perfusion chambers and perfused with Krebs-Henseleit solution, warmed at 37° C, aerated with 95% O₂ and 5% CO₂, for 1 hour at a flow rate of 0.8 ml/min. Then the perfusing solution was changed to Krebs-Henseleit solution containing cocaine (12 μM), with the other conditions remaining the same, and the tissues were perfused for 20 more minutes.

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

In the experiments using angiotensin II, 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 and phentolamine, two further periods of transmural electric stimulation were applied at min 200 (S3) and 250 (S4). Rauwolscine or phentolamine were added at increasing concentrations before S2, S3 and S4 (Sdrug).

Drugs were added to the perfusion 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 perfusate after addition of 8 ml of scintillation mixture (Wallac OptiPhase “HiSafe” 3; Fischer Chemicals, Loughborough, UK).

The outflow of tritium that spontaneously passed to the perfusion fluid was calculated as a fraction of the amount of tritium in the tissue at the beginning of the respective collection period (fractional rate of loss per min).
In order to calculate 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 pre-stimulation sample. The spontaneous outflow measured in the last pre-stimulation sample was assumed to represent outflow in subsequent samples; it was subtracted from the total outflow determined in stimulation and post-stimulation samples.

The fractional release per shock (FR) was calculated by dividing the stimulation evoked tritium by the tritium present in the tissue at the beginning of the stimulation and by the number of shocks.

The effect of drugs was expressed as the ratio of the FR evoked by \( S_{\text{drug}} \) over that evoked by \( S_{\text{control}} \).

**Statistics**

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-[\(^{2,5,6-}\text{H}\)]-noradrenaline (49.5 Ci/mmol; PerkinElmer, Boston, MA., USA); phentolamine hydrochloride (Sigma); rauwolscine hydrochloride (Tocris, Ellisville, MO., USA); rosiglitazone maleate (maleate of 5-[[4-\(\text{[2-(methyl-2-pyridinylamino)-ethoxy]phenyl[methyl]-2,4-thiazolidinedione}\) (Kemprotec, Middlesbrough, UK). Stock solutions of rosiglitazone (10 mM) were prepared in ethanol. All other solutions were prepared in water.
Results

Noradrenaline release from sympathetic neurones was evoked by electrical stimulation of rat left ventricle slices mounted in perfusion chambers. Sympathetic nerve endings were preloaded with $^3$H-noradrenaline. The tissue content of the $^3$H-noradrenaline in the ventricle slices at the beginning of the second electrical stimulation was 27.0 ± 3.4 nmol/mg of tissue (n = 12). The spontaneous efflux of $^3$H-noradrenaline from the tissue to the perfusion fluid expressed as the fractional rate of loss of the tissue content was constant with a value of $35.3 \times 10^{-4} \pm 3.3 \times 10^{-4}$ per minute (n = 12). The overflow induced by electrical stimulation, expressed as the fractional release per pulse in the control stimulation ($S_{\text{control}}$) was $16.5 \times 10^{-6} \pm 2.9 \times 10^{-6}$ (n = 12). Angiotensin II (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 (Figure 1). The maximal effect of angiotensin II was reached at about 100 nM and had a value of 110 ± 8% (n = 18) increase in relation to controls. Potency values expressed as pEC$_{30\%}$ are shown in Table 1. Rosiglitazone in concentrations from 300 nM to 3 μM had no effect on the release of $^3$H-noradrenaline induced by electrical stimulation. However the concentration-response to angiotensin II was shifted to the left in the presence of rosiglitazone in a concentration-dependent way. Potency values for the prejunctional effects of angiotensin II expressed as pEC$_{30\%}$ values were significantly increased by rosiglitazone at the concentrations of 1 and 3 μM (Table 1).

The α$_2$-adrenoceptor antagonists rauwolscine and phentolamine also increased $^3$H-noradrenaline release induced by electrical stimulation (Figure 2 and Figure 3). Rauwolscine was more potent than phentolamine (Table 1). Rosiglitazone caused no shift of the concentration-response curve of neither rauwolscine nor phentolamine (Figure 2 and Figure 3). The pEC$_{30\%}$ values of rauwolscine and phentolamine in the presence of rosiglitazone in concentrations up to 3 μM were not different from those obtained in its absence (Table 1).
Discussion

Rosiglitazone has been associated with cardiovascular adverse effects. These effects consist in significant increase in the risk of myocardial infarction and an increase in the risk of death from cardiovascular causes with a borderline significance (Nissen et al 2007).

The mechanisms underlying the possible cardiovascular effects of rosiglitazone remain unknown (Stuc et al 2008; Psaty et al 2007; Nissen et al 2007). Some explanations have been made like increased LDL levels, decreased in hemoglobin levels (which could result in physiological stress thereby provoking myocardial ischemia) or the capability of TZDs to precipitate heart failure (Nissen et al 2007). The latter has been associated with fluid retention (Nesto et al 2004) and there are several explanations for this increase in plasma volume like arterial vasodilation, altered intestinal ion transport, alteration in endothelial permeability and increased activity of sympathetic nervous system (Nesto et al 2004).

Bastos et al (2008) and Silva et al (2009) showed that in sympathetic terminals of rat left ventricle rosiglitazone had noradrenaline release-enhancing effect. This finding was interpreted as a potential contribution for the adverse effects of rosiglitazone in the cardiovascular system because activation of the sympathetic nervous system is a component of physiopathology of certain diseases like hypertension, cardiac heart failure and acute myocardium infarction. The results of the present work corroborate these previous findings (Figure 1). The concentration-response curve to angiotensin II in the presence of fixed concentrations of rosiglitazone was shifted to the left with increase of the maximum. Indeed, rosiglitazone increased the angiotensin II noradrenaline release-enhancing effect in a concentration-dependent manner. Again, it can be suggested that this effect of rosiglitazone can contribute to the cardiovascular adverse effects reported.

Angiotensin II increases exocytotic release of noradrenaline from sympathetic neurons (Boehm and Kubista 2002). Angiotensin II acts on presynaptic AT_{1} receptors which in some tissues are different from smooth muscle AT_{1} receptors in their pharmacological properties (Guimarães et al 2001). It was also established that angiotensin II requires ongoing \alpha_{2}-autoinhibition for the full extent of his noradrenaline release-enhancing effect (Trendelenburg et al 2003). Specifically, their experiments indicate that it is the \alpha_{2C}-adrenoreceptor that interacts with AT_{1} receptors and not the \alpha_{2A} adrenoreceptor although \alpha_{2B} adrenoreceptors may also contribute. To test if the noradrenaline release-enhancing effect of rosiglitazone is specific for angiotensin II, it was studied the interactions between rosiglitazone and \alpha_{2}-antagonists on the pre-junctional modulation of noradrenaline release. As we can realize observing the figures 2 and 3, the release-enhancing effects of neither rauwolscine nor
phenolamine (1-300 nM) were changed by rosiglitazone with a great sobreposition of the curves concentration-response in the presence of different concentrations of rosiglitazone (varying from 0.3 μM to 3 μM). So rosiglitazone does not seem alter this alternative mechanism of noradrenaline’s release-enhancing. It should be noted that there are another modes of presynaptic action of angiotensin II. In fact there may be α2-autoinhibition-independent mechanisms (Mota et al 2000).

Our results show that rosiglitazone potentiates the noradrenaline-release enhancing effect of angiotensin II without changing the release-enhancing effect of α2-receptor antagonists. It seems more likely that a positive allosteric modulation of angiotensin II receptors by rosiglitazone may occur in sympathetic terminals. It should be noted that it cannot be proved that this effect specifically depends of AT1 subtype receptor. A specific AT1 receptor antagonist like eprosartan could be used but this would lead to an antagonism of the action of angiotensin II we wish to study. An alternative approach consists in using knock-out mice lacking AT1 receptor.

It cannot be stated whether this property of rosiglitizone depends on its effect on the well known target (PPARγ) or is caused by another action of rosiglitazone, acting on other type of receptor. The PPARγ has been considered as a nuclear receptor. PPARs are nuclear ligand-activated transcription factors that belong to a family of nuclear hormone receptors that also includes the retinoid X receptor, the vitamin D receptor and the thyroid hormone receptor (Chiarelli et al 2008). Briefly, this receptor becomes activated upon ligand binding resulting in an increase or decrease of the target genes transcription which, ultimately, lead to the up-regulation of the insulin-sensitizing factors and down-regulation of the insulin-resistant factors (Guo et al 2006). However, Storka et al (2008) reported that in a cell-free assay the binding affinity of the AT1 receptor antagonists telmisartan and valsartan to the PPARγ is close to that of the PPARγ selective agonist rosiglitazone. This description of a PPARγ out-side the nucleus provide us the possibility that rosiglitazone can exercise its noradrenaline enhancing effect acting on his well known target. Otherwise, it will be hard to explain how in few minutes rosiglitazone is able to modify the release enhancing effect of angiotensin II acting on PPARγ localized in nucleus when its mechanisms supposes modifications in genes transcriptions.

With the results of the present work it cannot be concluded whether this effect of rosiglitazone depends on PPARγ or not. In this setting it will be crucial to realize the experiments in the presence of the PPARγ antagonist GW9662. This subject will be explored in future experiments.

Apart from the question on the importance to study the influence of the antagonism of PPARγ on this noradrenaline release-enhancing effect of rosiglitazone, it is also important to
verify whether pioglitazone has this same type of effect. In fact, pioglitazone does not increase the risk of myocardial infarction and may decrease the risk for stroke and revascularization (Nagajothi et al 2008).

In conclusion, results show that rosiglitazone potentiates the noradrenaline-release enhancing effect of angiotensin II without changing the release-enhancing effect of α2-receptor antagonists suggesting that a positive allosteric modulation of angiotensin II receptors by PPARy agonists may occur in sympathetic terminals.
References


Tables

Table 1: Influence of rosiglitazone on pEC_{50} values for angiotensin II, rauwolscine and phentolamine.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>0.3 µM</th>
<th>1 µM</th>
<th>3 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin II</td>
<td>8.6 ± 0.1</td>
<td>8.8 ± 0.2</td>
<td>9.2 ± 0.2*</td>
<td>9.3 ± 0.3*</td>
</tr>
<tr>
<td>Rauwolscine</td>
<td>8.7 ± 0.3</td>
<td>8.6 ± 0.2</td>
<td>8.7 ± 0.1</td>
<td>8.9 ± 0.3</td>
</tr>
<tr>
<td>Phentolamine</td>
<td>8.5 ±0.1</td>
<td>8.1 ± 0.3</td>
<td>8.3 ± 0.2</td>
<td>8.6 ± 0.3</td>
</tr>
</tbody>
</table>

Results are mean ± SEM (n=4-5)
* p<0.05 vs control
Figure 1

![Graph showing the ratio of drug to control over log M (Angiotensin II) for different concentrations of rosiglitazone.]

- Control
- Rosiglitazone 300 nM
- Rosiglitazone 1 μM
- Rosiglitazone 3 μM
Figure 2

![Graph showing the effect of Rauwolscine on drug binding](image_url)

- control
- rosiglitazone 300 nM
- rosiglitazone 1 μM
- rosiglitazone 3 μM
Figure 3

![Graph showing the ratio of drug to control response as a function of log M [Phentolamine]. The graph includes points for control, rosiglitazone 300 nM, rosiglitazone 1 μM, and rosiglitazone 3 μM, with error bars indicating variability.](image)
Legends

**Fig. 1** Effects of rosiglitazone (300 nM – 3 μM) in the presence of angiotensin II 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 $S_{\text{drug}}$ over that evoked by $S_{\text{control}}$. Shown are means (n = 3-5) and best-fitting curves representing a 2-parameter logistic equation (software GraphpadPrism).

**Fig 2** Effects of rosiglitazone (300 nM – 3 μM) in the presence of rauwolscine 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 $S_{\text{drug}}$ over that evoked by $S_{\text{control}}$. Shown are means (n = 3-5) and best-fitting curves representing a 2-parameter logistic equation (software GraphpadPrism).

**Fig 3** Effects of rosiglitazone (300 nM – 3 μM) in the presence of phentolamine 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 $S_{\text{drug}}$ over that evoked by $S_{\text{control}}$. Shown are means (n = 3-5) and best-fitting curves representing a 2-parameter logistic equation (software GraphpadPrism).