Title: Role of endothelial kinin B_1 receptor on the membrane potential of transgenic rat aorta

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Summary

The kinin receptors are classically involved in inflammation, pain and sepsis. The effects of the kinin B_1 receptor agonist des-Arg^9-bradykinin (DBK) and lipopolysaccharide (LPS) were investigated by comparing the membrane potential responses of aortic rings from transgenic rats overexpressing the kinin B_1 receptor (B1R) in the endothelium (TGR(Tie2B$_1$)) and Sprague Dawley (SD) rats. No difference in the resting membrane potential in the aorta's smooth muscle from the transgenic and SD rats was observed. The aorta rings from SD rats hyperpolarized only to LPS but not to DBK, whereas the aorta rings from TGR(Tie2B$_1$) responded by the administration of both drugs. DBK and LPS responses were inhibited by the B_1 receptor antagonist R715 and by iberiotoxin in both cases. Thapsigargin induced a hyperpolarization in the smooth muscle

of SD rats that was not reversed by R715, but was reversed by iberiotoxin and this hyperpolarization was further augmented by DBK administration. These results show that the model of overexpression of vascular B_1 receptors in the TGR(Tie2B_1) rats represent a good model to study the role of functional B_1 receptors in the absence of any pathological stimulus. The data also show that K_{Ca} channels are the final mediators of the hyperpolarizing responses to DBK and LPS. In addition, we suggest an interaction between the B1R and TLR4, since the hyperpolarization induced by LPS could be abolished in the presence of R715.

Key words

TGR(Tie2B_1) transgenic rat; kinin B_1 receptors; potassium channels; membrane potential; LPS.

Introduction

Kinins are released in injured tissues from kininogens either by tissue kallikreins or by plasma kallikreins in sites of inflammation [1, 2], where they induce smooth muscle contraction or relaxation, vasodilation, increased vascular permeability and pain [3]. The physiological actions of the kinins are consequence of their interaction with two G protein-coupled kinin receptor subtypes: the kinin B_2 receptor (B2R), constitutively expressed in a variety of cells under physiological conditions, mediates the majority of the visceral and vascular actions of bradykinin (BK) in rodents and kallidin (Lys-BK) in humans [2, 4]; and the kinin B_1 receptor (B1R), with high affinity for the metabolites of kinins des-Arg^9-BK (DBK) and des-Arg^{10}-Lys-DBK [2]. The B1R is absent or under expressed in physiological condition [5, 6] but may be expressed in response to specific cytokines and pro-inflammatory agents [7, 8].

One of the most studied bacterial surface molecules is the lipopolysaccharide (LPS), a glycolipid present in gram negative bacteria [9, 10, 11]. LPS is a potent signal molecule involved in the initiation of septic shock, that activates multiple cells to release cytokines, nitric oxide (NO), and kinins [12]. The overproduction of NO is believed to be the most important factor responsible for the systemic vasodilation induced by LPS administration [13, 14] and the nitrovasodilators

are potent activators of K_{Ca} channels in vascular smooth muscle cells, which contribute to endotoxin-induced hypotension [15]. Moreover, the local administration of LPS is able to induce the overexpression of B1R [16], indicating the role of B1R in inflammation and sepsis [5, 17, 18].

Genetically modified rodents are useful models for investigating the events initiated by kinin membrane receptors [19]. In order to better understand the pathological role of the kinin B_1 receptor, we generated a transgenic rat model overexpressing the B1R specifically in endothelial cells, designated TGR(Tie2B$_1$) [5]. B1R agonist induces relaxation of TGR(Tie2B$_1$) isolated aorta, which is mediated by NO and K^+ channels and, after LPS treatment, they present a more pronounced hypotensive response and marked bradycardia associated with increased mortality when compared to non-transgenic control animals [5].

In this paper, we aimed to evaluate the functional role of the endothelial B1R in the maintenance of the membrane potential in smooth muscle cells measured in aortic rings from TGR(Tie2B$_1$) and the control SD rats.

Materials and Methods

Animals

Experiments were carried out using male Sprague Dawley (SD) rats and transgenic rats (TGR(Tie2B$_1$)) overexpressing B1R exclusively in the endothelium [5]. The animals were provided by the Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia (CEDEME) at the Escola Paulista de Medicina/Universidade Federal de São Paulo, Brazil. The rats were 16-30 weeks old and 250-350 g. All transgenic rats were genotyped by polymerase chain reaction (PCR) identifying the presence of the transgenic *Bdkrb1* gene.

The animals were killed by decapitation, and their thoracic aorta was removed. It was cleaned of adherent connective tissue and cut into rings (3-4 mm length) for tension and electrophysiological measurements. Care was taken to ensure that the endothelial layer was not damaged during tissue preparation. In some rings, the endothelium was removed by gentle rubbing of the intimal surface

with a plastic tube wrapped in cotton. All procedures were approved by the Ethic Committee of the Federal University of São Paulo (protocol number: 2011/1403).

Membrane potential

In our study, we used direct measurements of the vascular smooth muscle membrane potential to avoid interference from the effects of those agonists in our measurements exactly as described by Farias *et al.* (2004) [20].

The aortic rings, with or without endothelium, were placed in a 2 ml perfusion chamber containing Krebs-bicarbonate solution (NaCl 122 mM, KCl 5.9 mM, $MgCl_2$ 1.25 mM, $NaHCO_3$ 15 mM, glucose 11 mM, $CaCl_2$ 1.25 mM, pH 7.4) and were kept at rest at a tension of 1g for 2 h, after which the analyses of interest could be started. [20, 21]. They were superfused at a rate of 3 $mL.min^{-1}$ with Krebs solution, bubbled with a 2.5% CO_2-95% O_2 gas mixture and maintained at pH 7.4 and 37°C.

Micropipettes (borosilicate glass capillaries 1B120F-6, World Precision Instruments, WPI) were made by means of a horizontal puller (Model PN-3, Narishige, Tokio, Japan) and filled with 2M KCl (tip resistance 20-40 MΩ and tip potential <6 mV). The microelectrodes were mounted in Ag/AgCl half-cells on a micromanipulator (Leitz, Leica) and connected to an electrometer (Intra 767, WPI).

The impalements of the smooth muscle cells were made from the adventitial side. The electrical signals were continuously monitored on an oscilloscope (Model 54645A, Hewlett Packard) and recorded in a potentiometric chart recorder (Model 2210, LKB-Produkter AB). The successful implantation of the electrode was evidenced by a sharp drop in voltage upon entry into a cell, followed by a stable potential (± 3 mV) for at least 1 min after impalement, being ready to start the registration [25]. In a second time after the registration, there is a sharp return to zero upon exit and minimal change (< 10%) in microelectrode resistance after impalement [20].

Membrane potentials were measured before and after stimulation of the vessels with thapsigargin, a non-competitive inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) (Sigma Chemical Co., THAP, 1 µM); des-Arg^9-bradykinin, a B1R agonist that displays selectivity for B1R over B2R

(Sigma Chemical Co., DBK, 1 μM); bradykinin (Sigma Chemical Co., BK, 10 μM), a kinin B2R agonist; or lipopolysaccharide (LPS), the main component of gram-negative outer bacterial membrane signaling through the toll-like receptor 4 (TLR4) (*Escherichia coli* lipopolysaccharide, 0111:B4, 10 $\mu g.ml^{-1}$); R715, a potent and selective B1R antagonist (Biosyntan GmbH, 10 μM); Hoe140, a selective B2R antagonist (Sigma Chemical Co., 10 μM); iberiotoxin, a Ca^{2+}-activated K^+ channel inhibitor (Sigma Chemical Co., IBTX, 10 nM); N^{ω}-nitro-L-arginine, a nitric oxide synthase inhibitor (Sigma Chemical Co., L-NNA, 50 μM). The time of contact of the drugs with the preparations before the impalements was 10 min.

Before drug incubation, we recorded the resting membrane potential (RMP) in rat thoracic aorta rings of all animals. Then, acetylcholine (ACh) was used and the potential was measured to test endothelial integrity and function [22]. After that, the aorta rings were incubated with DBK, BK or LPS. When a response was observed, the rings were pre-incubated with Hoe140 and R715. To investigate the mechanism involved in the hyperpolarizing response of the B1R, we pre-incubated the aorta rings with IBTX or L-NNA.

More than one impalement was made on the same aortic ring from the same rat after drug incubation, the measurements were averaged and considered as *n*=1.

Expression of mRNA in the aorta

The expression of B1R and TLR4 mRNA in the aorta were measured in SD and TGR(Tie2B$_1$) rats. Total RNA was isolated using TRIzol (Life Technologies). 1 μg of total RNA from thoracic aorta were reverse-transcribed using M-MLV (Invitrogen) to cDNA as suggested by the manufacturer. The reaction product was amplified by Real-Time PCR (7500 Real Time PCR System, Life Technologies) using 1:10 diluted cDNA and primers (50 ng/μl) for SyberGreen reaction (Thermo). Experiments were performed in duplicates for each data point. The mRNAs of interesting genes were quantified as a relative value compared with GAPDH, a reference gene, whose abundance was assumed not to change between the varying experimental conditions. Melting curves were analysed for the presence of nonspecific amplification. Primers used for Real-Time PCR are as follows: rat GAPDH forward primer 5`-

GGAGATTGTTGCCATCAACGACC-3´ and reverse primer 5´-GGTCATGAGCCCTTCCACAATGC-3´; rat B1R forward primer 5´-TGTGTCCAGAGAGCTGCCCAG-3´ and reverse primer 5´-CTTCAGCGAGGCCTGGGACG-3´; rat TLR4 forward primer 5´-AGCAGAGGAGAAAGCATCTATGATGC-3´ and reverse primer 5´-GGTTTAGGCCCCAGAGTTTTTCTCC-3´. The expression levels were obtained from the cycle threshold (Ct) associated with the exponential growth of the PCR products. Quantitative values for mRNA expression were obtained by the parameter 2^{-dCt}, in which dCt represented the subtraction of the GAPDH Ct values from the ones of interested genes.

Statistical analysis

The statistical analyses were carried out with the Graphpad Prism (La Jolla, CA, USA) software package. All data are expressed as means ± S.E.M. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Newman-Keuls test in the case of pairwise comparisons between groups. When the data consisted of repeated observations at successive time points, ANOVA for repeated measurements was applied to determine differences between groups. Differences were considered statistically significant when $P<0.05$.

Results

DBK induces membrane hyperpolarization in TGR(Tie2B$_1$) rats through B1R activation and this effect is K_{Ca} channels dependent, but no NO dependent

Similar resting membrane potentials (RMP) were observed in the aortic rings smooth muscle cells from SD (-52.5 ± 0.9 mV) and TGRTie2B$_1$ (-52.2 ± 1.4 mV) rats (Fig. 1). As expected, DBK did not affect the membrane potential of the smooth muscle cells measured in aortic rings from SD rats (Fig. 1A) and a significant hyperpolarization was induced in TGR(Tie2B$_1$) rat preparations, which was abolished in deendothelized aorta (Fig. 1B). Pre-incubation of the aortic rings from transgenic rats with Hoe140 had no effect on the hyperpolarizing response induced by DBK, but R715 inhibited the hyperpolarizing response induced by DBK (Fig. 1C).

The hyperpolarization induced by DBK in TGR(Tie2B$_1$) smooth muscle cells was completely inhibited by the addition of iberiotoxin (IBTX) (Fig. 2A). Considering the dependence of K_{Ca} channels and the cytoplasmatic calcium concentration (data not shown) for the hyperpolarizing effect induced by DBK in aortic rings from TGR(Tie2B$_1$) rats, we evaluated whether an increase in $[Ca^{2+}]_i$ in the SD aortic rings could turn these vessels sensitive to DBK. For this purpose, we treated the aorta from SD rats with thapsigargin (THAP), which caused hyperpolarization in the aorta's smooth muscle of SD rats and was not reversed by washing the preparation for at least 2 h [27]; this hyperpolarizing effect was inhibited by IBTX (Fig. 2B). During the hyperpolarized state, due to previous treatment with THAP, DBK elicited a further hyperpolarizing response in the SD aortic rings, and that hyperpolarization was reversible upon washout and was blocked by IBTX (Fig. 2B).

To evaluate the NO participation in the response of DBK in the aorta rings of SD and TGR(Tie2B$_1$) rats, stimulation with the agonist was performed in the presence of L-NNA (Fig. 2C, D). Interestingly, L-NNA did not block the hyperpolarization promoted by DBK or the THAP response (Fig. 2C, D).

LPS hyperpolarization is kinin B_1 receptor and K_{Ca} channels dependent, but not NO dependent

Because B1R antagonists block the hyperpolarization response induced by LPS in the rat smooth muscle [23], we evaluated the effects of LPS in the aorta rings from TGR(Tie2B$_1$) rats. LPS induced a hyperpolarizing response in SD (Fig. 3A) and transgenic (Fig. 3B) rat aorta. Pre-incubation of the aortic rings with Hoe140 had no effect on the hyperpolarizing response induced by LPS, which was, however, inhibited by R715.

In aortic rings of SD (Fig. 4A) and TGR(Tie2B$_1$) (Fig. 4B) rats, the hyperpolarization effect induced by LPS was totally inhibited by IBTX, but not by L-NNA (Fig. 4C, D).

B1R and TLR4 mRNA levels before and after electrophysiology

After the thoracic aorta was removed from the animal, it was divided in two parts: one was used for electrophysiology experiments and the other was rapidly frozen for RNA extraction and analysis. The same procedure was

performed in the aortic rings used after the electrophysiology experiments. We measured the basal levels of B1R mRNA in SD rat in both situations (Fig. 5A). The B1R mRNA expression was not altered after electrophysiology assays in TGR(Tie2B$_1$) (Fig. 5A), but we could observe a higher expression in transgenic rat compared to SD rat aorta. The TLR4 mRNA expression increased in the aortic rings of SD rats after electrophysiology experiments, whereas it didn´t change in the preparation of transgenic rats (Fig. 5B).

Discussion

The induction of B1R expression in the transgenic rat overexpressing B1R was shown in endothelial cells *in vitro* and *in vivo* with several methods described in Merino *et al*. (2008) [5]. The overexpression of B1R exclusively in the endothelium did not alter the RMP of TGR(Tie2B$_1$) aortic rings when compared to SD rats. Although the presence of constitutive B1R has been demonstrated in aortic smooth muscle cells of normal Wistar rats (NWR) [24], Farias *et al*. (2004) [20] showed that B1R agonist DBK had no effect on the membrane potential of aortic rings of these animals, indicating that in these vessels, the receptor may be present, but probably not functional or its expression needs to be induced by inflammatory stimuli, as the classical concept indicates.

In this work, we analyzed the *in vitro* effect of LPS and B1R agonists and antagonists in thoracic aortic rings from the TGR(Tie2B$_1$) rats. The B1R agonist DBK caused a significant hyperpolarization in the aortic rings from transgenic rats, confirming that a large number of B1R is present and functional in the endothelium of the aortic rings in these animals, as already shown by Merino et al. (2008) [5]. This response was blocked by R715, a B1R antagonist, whereas Hoe140, a kinin B_2 receptor antagonist, did not block the hyperpolarization induced by DBK, indicating that B2R are not involved in this response.

Many studies analyzing the role of B1R in the cardiovascular system have been performed, but the function of these receptors is still not fully understood [25-28]. In vascular tissues, the classical signaling pathway mediated by activation of B1R is NO dependent [5], however our findings show that the activation of B1R in aortic rings from TGR(Tie2B$_1$) rats is also linked to the activation of Ca^{2+}-dependent K^+ channels, consolidating the results of Farias *et al.* (2004)

[20] and Levy *et al*. (2017) [19]. Thapsigargin hyperpolarized the aortic smooth muscle cell membrane from SD rats, rendering them sensitive to the B1R agonist. This effect could be inhibited by iberiotoxin, indicating that the B1R responses are mediated by K_{Ca} channels, similar to the results obtained in SHR aorta [20] and in microglia [29]. As already shown in the aortas from SHR [20], the increased calcium concentration produced by thapsigargin might activate K_{Ca} channels leading to the smooth muscle cell membrane hyperpolarization by a mechanism independent on NO (in contrast to the effect in the SHR). Interestingly, these results showed that in aortic rings from SD rats, B1R are constitutively expressed, not functional, but can be activated by increasing $[Ca^{2+}]_i$. This increase in $[Ca^{2+}]_i$ causes opening of the Ca^{2+}-sensitive K^+ channels in the smooth muscle cells of SD rats and these channels are the final mediators of the responses to DBK and LPS, similar to aortas from SHR [20, 30, 31].

The kallikrein-kinin system has been demonstrated to be involved in the severe hypotension associated to endotoxic shock and activation of several signaling pathways [8, 12, 32]. The B1R antagonist has been shown to block hyperpolarization response induced by LPS, and the same effect was not observed by the kinin B2R antagonist [23]. The LPS-induced hyperpolarizing effect on the aortic rings was completely inhibited by IBTX. Both the activation of B1R by DBK and TLR4 by LPS induce an increase in intracellular Ca^{2+} [33], which can stimulate K_{Ca} channels present in smooth muscle. The antagonist of kinin B1R, R715, blocks the hyperpolarization induced by LPS, what could indicate a functional interaction between LPS-induced effects and B1R. LPS commonly signals through TLR4 receptors [34], inducing the expression of pro-inflammatory cytokines and initiating immune responses [33, 35, 36]. Although the specificity and mechanism by which a K^+ channel is involved in pro-inflammatory signals are not yet fully understood, Jo *et al*. (2011) [36] showed that the K^+ channel maxi-K (BK channel) is activated by TLR/IL-1 receptor and thereby is involved in the production of pro-inflammatory cytokines, indicating the functional association of the K^+ channel and TLR signaling complexes. Based on our data showing the increased expression of TLR4 after aortic rings incubation, we can hypothesize that this probable relationship between LPS/TLR4 and B1R may occur through a direct contact between both

molecules. On the other hand, this interaction could also be indirect, promoted by the increase in intracellular calcium concentration in endothelial cells, inducing K_{Ca} channels activation in the smooth muscle.

Unlike what is described in the literature in different rat strains [20], our data do not show a relationship between NO and the hyperpolarization caused by DBK and LPS in TGR(Tie2B$_1$) rats. In this transgenic model overexpressing the kinin B1R, we observed that the responses were dependent of intracellular Ca^{2+} concentration and K_{Ca} channels, which are important effectors of NO action for hyperpolarization and relaxation of vascular smooth muscle [37 - 43]. The endothelium-derived hyperpolarizing factor (EDHF) is a NO-independent factor that exerts endothelium-dependent hyperpolarization in vascular smooth muscle cells. EDHF is released by endothelial cells under the stimulation of kinin receptors agonists, for example. EDHF can cause smooth muscle cell hyperpolarization and relaxation by different mechanisms, as pointed out by Velasquez & Wang (2016). In our animal model, EDHF could diffuse through endothelial cells and bind to calcium-activated potassium channels expressed in the vascular smooth muscle cells, leading to potassium efflux. This process, in turn, could induce smooth muscle hyperpolarization through activation of the Na^+/K^+ pump (Fig.6) [44]. Further experiments will be necessary to elucidate this hypothesis.

In summary, our findings show that kinin B1R are expressed on endothelial cells of aortic rings from SD rats, but are not functional. On the other hand, the transgenic TGR(Tie2B$_1$) rats are an appropriate model for the analysis of the mediators and interactions involving the responses of endothelial cells of arteries to B1R agonists, since their aortic rings overexpress B1R and do not require any stimulus to be fully functional. In addition, we also demonstrated that the activation of this receptor becomes evident not only after tissue injury or under pathological conditions [4, 45], but also in the presence of an active mediator that increases intracellular Ca^{2+} concentration and stimulates K_{Ca} channels. Based on our findings, we suggest that K_{Ca} channels are the final mediators of the hyperpolarizing responses to DBK and LPS, and speculate a possible interaction between the B1R and TLR4 (Fig.6). Furthermore, considering the inhibitory action of the B1R antagonist R715 on the hyperpolarizing effect of LPS we can also hypothesize that this B1R/TLR4

interaction could modulate the responses of both LPS and the B1R agonists, as proposed by Sodhi *et al*. (2018) [46] and Munoz-Rodríguez *et al*. (2018) [47].

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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Figures

Fig. 1. DBK induces membrane hyperpolarization in TGR(Tie2B$_1$) rats through B1R activation and its effect is K_{Ca} channels dependent. Membrane potentials measured in aortic rings from SD (**A**) and TGR(Tie2B$_1$) rats (**B**), with or without endothelium, in the absence (RMP) and in the presence of 10 μM ACh, 10 μM Hoe140 or 10 μM R715, in the absence and in the presence of 1 μM DBK. For

each aortic ring obtained from individual rats (the number of which is shown in parentheses below the bars), 5 to 8 cells were impaled, and the averages of the respective measurements were used to obtain the means and s.e.m. RMP, resting membrane potential; ACh, acetylcholine; DBK, des-Arg^{9}-bradykinin – a B1R agonist; Hoe140, a B2R antagonist; R715, a B1R antagonist. (**A**) *P<0.001 versus RMP, ACh, DBK, endothelized RMP and deendothelized RMP, Hoe140, R715 and R715+DBK; ***P<0.05 versus RMP, endothelized RMP and deendothelized RMP, ACh, DBK, Hoe140, R715 and R715+DBK (Newman-Keuls test).

Fig. 2. DBK hyperpolarization effect is K_{Ca} channels dependent. (**B**) RMP and effects of 1 µM DBK on the absence or in the presence of 10 nM IBTX. (**B**) Effects of 1 µM DBK after 1 µM THAP incubation; the DBK effect was observed in the absence or in the presence of 10 nM IBTX. RMP and effects of 1 µM DBK on the absence or in the presence of 50 µM L-NNA from TGR(Tie2B$_1$) (**C**) and SD (**D**) rats. For each aortic ring obtained from individual rats (the number of which is shown in parentheses below the bars), 5 to 8 cells were impaled, and the averages of the respective measurements were used to obtain the means and s.e.m. RMP, resting membrane potential; DBK, des-Arg^{9}-bradykinin - a B1R agonist; IBTX, iberiotoxin - a K_{Ca} inhibitor; THAP, thapsigargin - a SERCA inhibitor; L-NNA, N$^{\omega}$-nitro-L-arginine - a NOS inhibitor. (**A** and **B**) *P<0.001 versus RMP and the response to IBTX and IBTX+DBK. (**B**) ***P<0.05 versus the response to THAP alone. (**C**) *P<0.001 and ***P<0.05 versus RMP and L-NNA. (**D**) *P<0.001 versus RMP and ***P<0.05 versus the response to THAP (Newman-Keuls test).

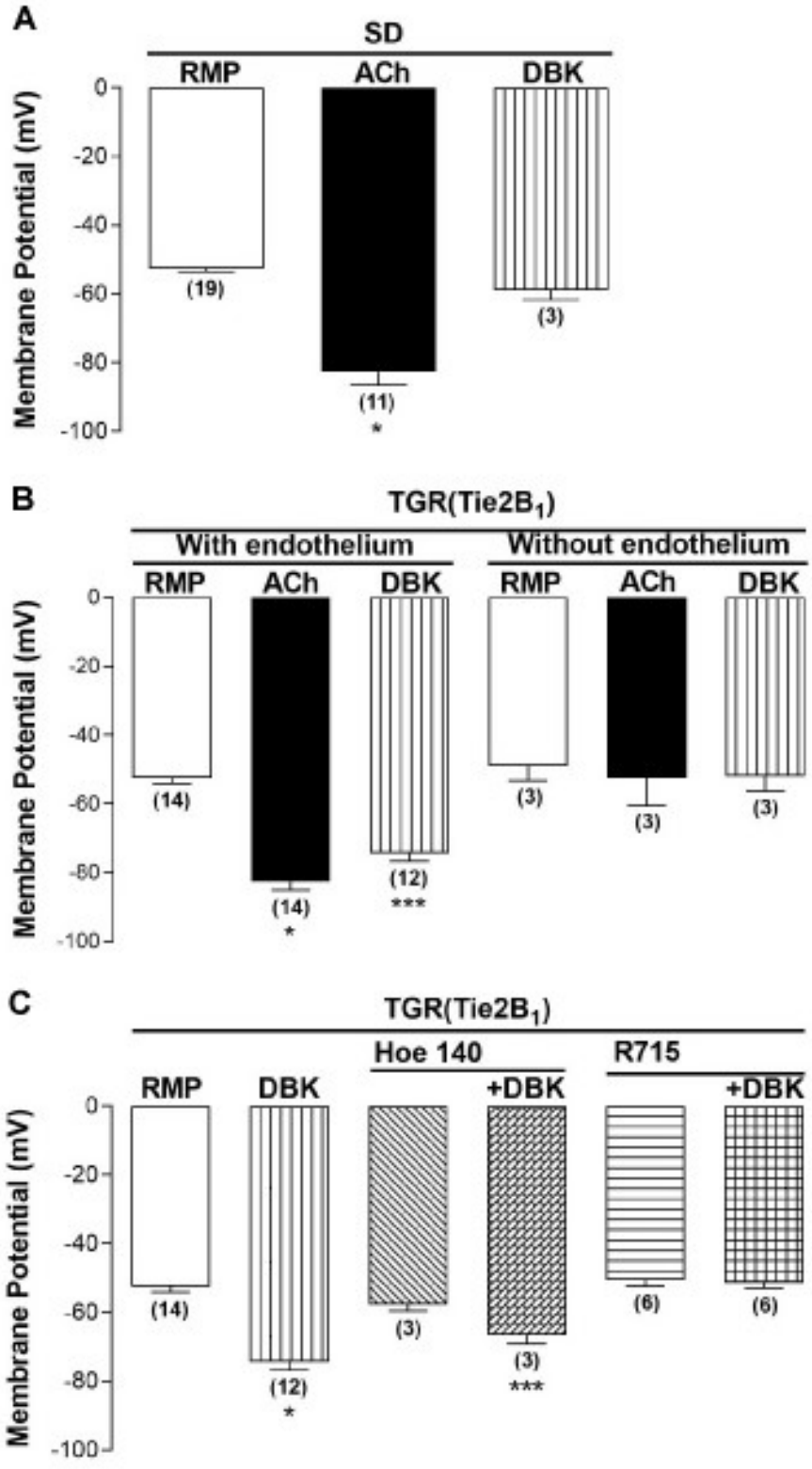
Fig. 3. LPS induces hyperpolarization in the aorta's smooth muscle of SD and TGR(Tie2B$_1$) rat, which is inhibited by the B1R antagonist R715. RMP and effects of 10 µg.ml^{-1} LPS in the absence or in the presence of 10 µM Hoe140 or in the presence of 10 µM R715, from SD (**A**) and TGR(Tie2B$_1$) (**B**) rats. For each aortic ring obtained from individual rats (the number of which is shown in parentheses below the bars), 5 to 8 cells were impaled, and the averages of the respective measurements were used to obtain the means and s.e.m. RMP, resting membrane potential; LPS, lipopolysaccharide; Hoe140, a B2R antagonist; R715, a B1R antagonist. (**A**) ***P<0.05 versus RMP and the response to Hoe140, R715 and R715+LPS. (**B**) *P<0.001 and ***P<0.05 versus RMP and the response to Hoe140, R715 and R715+LPS (Newman-Keuls test).

Fig. 4. LPS hyperpolarization is K_{Ca} channels dependent. RMP and effects of 10 µg.ml^{-1} LPS in the absence or in the presence of 10 nM IBTX (**A** and **B**) or 50 µM L-NNA (**C** and **D**), from SD (**A** and **C**) and TGR(Tie2B$_1$) (**B** and **D**) rats. For each aortic ring obtained from individual rats (the number of which is shown in parentheses below the bars), 5-8 cells were impaled, and the averages of the respective measurements were used to obtain the means and and s.e.m. RMP, resting membrane potential; LPS, lipopolysaccharide; IBTX, iberiotoxin - a K_{Ca} inhibitor; L-NNA, N$^{\omega}$-nitro-L-arginine - a NOS inhibitor. (**A**) *P<0.001versus RMP and the response to IBTX and IBTX+LPS. (**B**) **P<0.001 versus RMP and the response to IBTX and IBTX+LPS. (**C**) ***P<0.05 versus RMP. (**D**) *P<0.001 and ***P<0.05 versus RMP and the response to L-NNA (Newman-Keuls test).

Fig. 5. B1R (**A**) and TLR4 (**B**) mRNA levels before and after aortic rings electrophysiology. Number of aortas analysed = 7 in each of situations. (**A**) *P<0.001 versus SD values. (**B**) *P<0.001 versus SD before electrophysiology (Newman-Keuls test).

Fig. 6. Schematic review of the B1R effects in SD and TGR(Tie2B$_1$) rat aorta. (**A**) The increase in intracellular calcium concentration in endothelium turns a non-functional B1R into a receptor able to induce hyperpolarizing responses via stimulation of Ca^{2+}-dependent K^+ channels in smooth muscle cells. (**B**) The transgenic TGR(Tie2B$_1$) rats are a model to analyze the mediators and interactions involved in the responses of arterial smooth muscles to B1R agonists and to LPS; Ca^{2+}-sensitive K^+ channels in the smooth muscle are the final mediators of the hyperpolarizing responses to DBK and LPS and B1R antagonist inhibit the LPS effect suggesting a putative interaction between the B1R and LPS receptors. EDHF could diffuse through endothelial cells and bind to calcium-activated potassium channels expressed in the vascular smooth muscle cells, leading to potassium efflux. This process, in turn, could induce smooth muscle hyperpolarization through activation of the Na^+/K^+ pump.

Fig. 1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SD
RMP
ACh
DBK
Membrane Potential (mV)
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-20
-40
-60
-80
-100
(19)
(11)
*
(3)
B
TGR(Tie2B1)
With endothelium
Without endothelium
RMP
ACh
DBK
RMP
ACh
DBK
(14)
(14)
*
(12)
***
(3)
(3)
(3)
C
TGR(Tie2B1)
Hoe 140
R715
RMP
DBK
+DBK
+DBK
(14)
(12)
*
(3)
(3)
***
(6)
(6)

Fig. 2

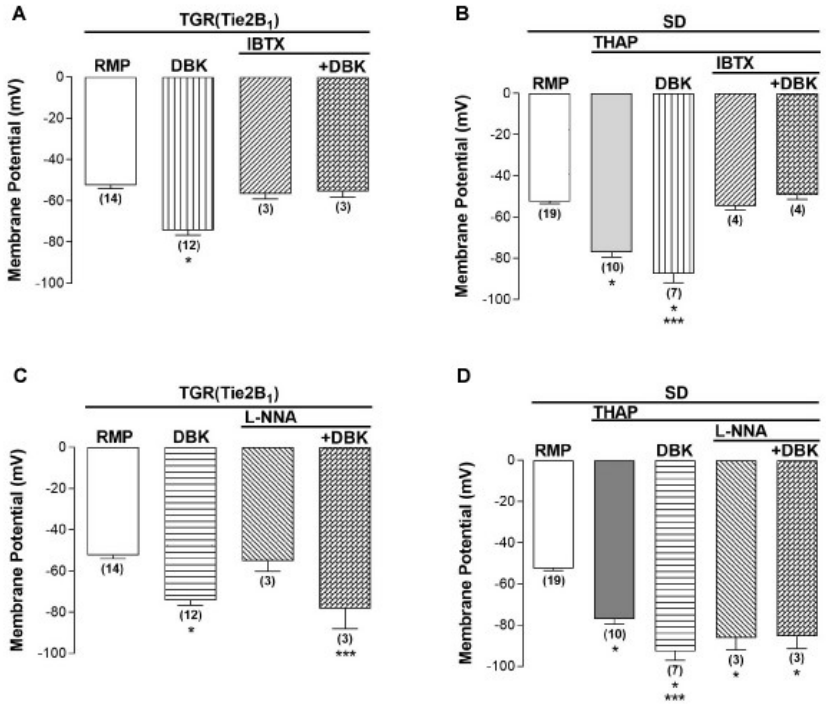

A
TGR(Tie2B1)
IBTX
RMP
DBK
+DBK
Membrane Potential (mV)
0
-20
-40
-60
-80
-100
(14)
(12)
*
(3)
(3)
B
SD
THAP
IBTX
RMP
DBK
+DBK
Membrane Potential (mV)
(19)
(10)
*
(7)
*
***
(4)
(4)
C
TGR(Tie2B1)
L-NNA
RMP
DBK
+DBK
Membrane Potential (mV)
(14)
(12)
*
(3)
(3)
***
D
SD
THAP
L-NNA
RMP
DBK
+DBK
Membrane Potential (mV)
(19)
(10)
*
(7)
*
***
(3)
*
(3)
*

Fig. 3

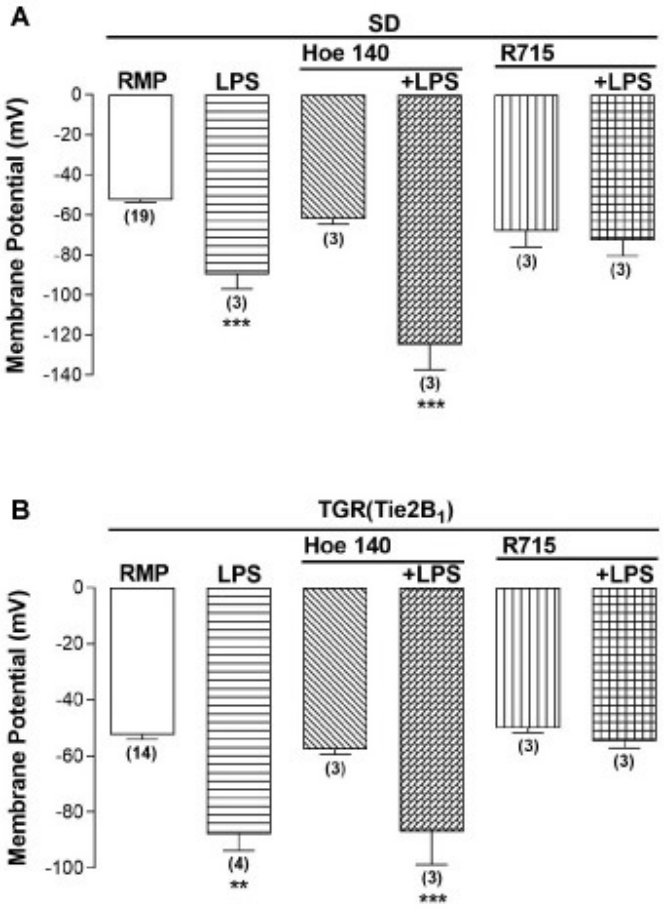

A
SD
Hoe 140
R715
RMP
LPS
+LPS
+LPS
Membrane Potential (mV)
0
-20
-40
-60
-80
-100
-120
-140
(19)
(3)
***
(3)
(3)
(3)
***
(3)
B
TGR(Tie2B1)
Hoe 140
R715
RMP
LPS
+LPS
+LPS
Membrane Potential (mV)
0
-20
-40
-60
-80
-100
(14)
(4)
**
(3)
(3)
(3)
***
(3)

Fig. 4

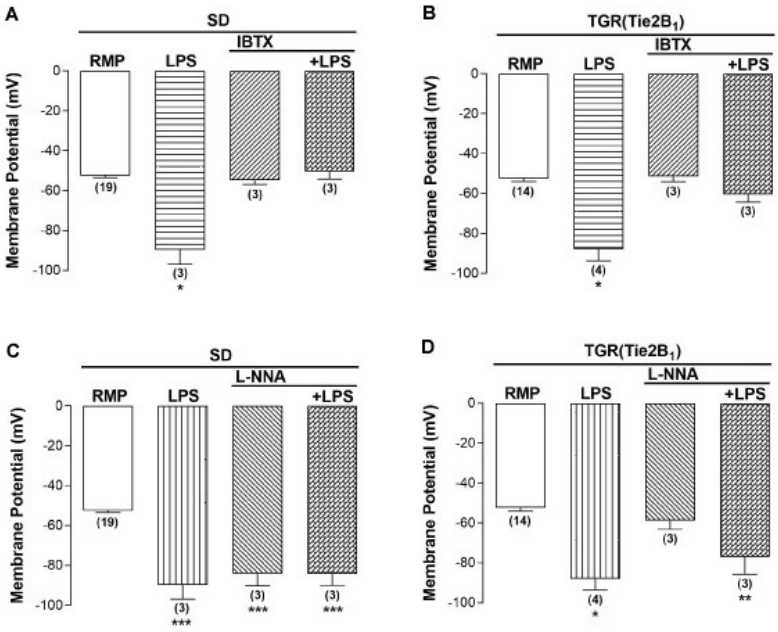

A
SD
IBTX
RMP
LPS
+LPS
Membrane Potential (mV)
0
-20
-40
-60
-80
-100
(19)
(3)
(3)
(3)
*
B
TGR(Tie2B1)
IBTX
RMP
LPS
+LPS
Membrane Potential (mV)
(14)
(3)
(3)
(4)
*
C
SD
L-NNA
RMP
LPS
+LPS
Membrane Potential (mV)
(19)
(3)
(3)
(3)
***
***
***
D
TGR(Tie2B1)
L-NNA
RMP
LPS
+LPS
Membrane Potential (mV)
(14)
(3)
(3)
(4)
*
**

Fig. 5

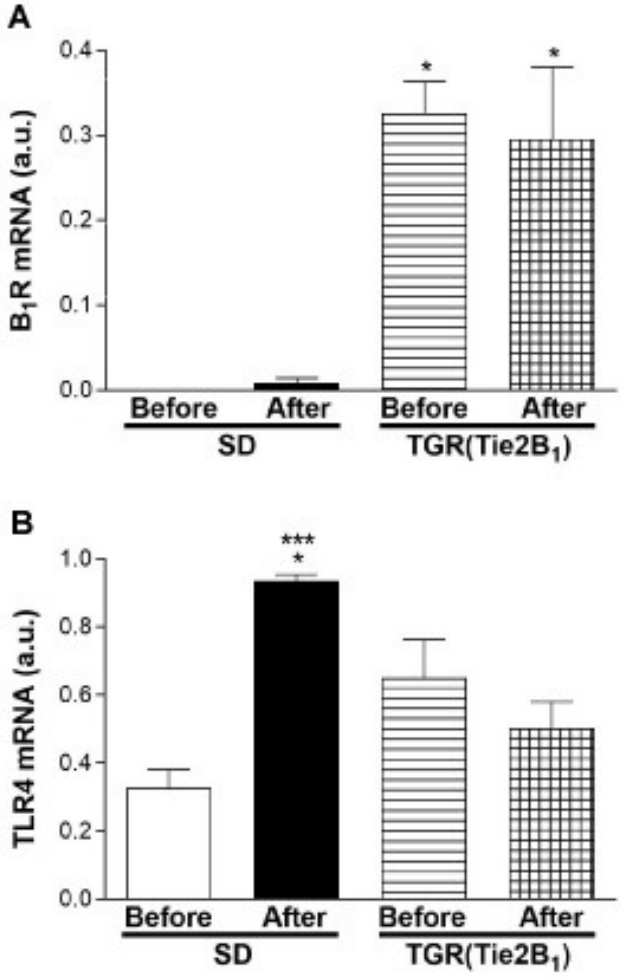
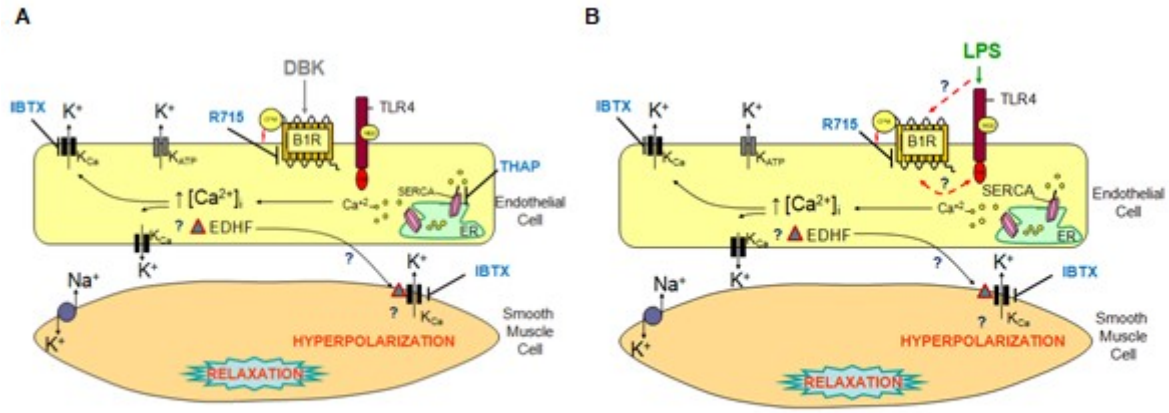

A
B1R mRNA (a.u.)
0.4
0.3
0.2
0.1
0.0
*
*
Before
After
Before
After
SD
TGR(Tie2B1)
B
TLR4 mRNA (a.u.)
1.0
0.8
0.6
0.4
0.2
0.0
***
*
Before
After
Before
After
SD
TGR(Tie2B1)

Fig. 6


A
DBK
IBTX
K+
K+
R715
B1R
TLR4
THAP
↑ [Ca2+]i
SERCA
Endothelial Cell
? EDHF
ER
Na+
K+
IBTX
Smooth Muscle Cell
HYPERPOLARIZATION
RELAXATION
B
LPS
?
TLR4
IBTX
K+
K+
R715
B1R
SERCA
Endothelial Cell
↑ [Ca2+]i
? EDHF
ER
Na+
K+
IBTX
Smooth Muscle Cell
HYPERPOLARIZATION
RELAXATION