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Interleukin-10 negatively modulates extracellular signal-regulated kinase 1 and 2 in aorta from hypertensive mouse induced by angiotensin II infusion

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Abstract

The activation of extracellular signal-regulated kinase 1 and 2 (ERK 1/2) pathway promotes increased vascular contractility in angiotensin II (Ang II)-induced hypertensive mice. Interleukin-10 (IL-10) is an immune-regulatory cytokine with the ability to prevent vascular hypercontractility during hypertension. We hypothesized that IL-10 would downregulate vascular ERK 1/2 activation during Ang II-induced hypertension. Wild-type (WT) or IL-10 knockout (IL-10^{-/-}) mice received Ang II infusion (90 ng.min) or vehicle (saline), via osmotic mini-pumps (0.25 µl/hour for 14 days), whereas another WT group were infused with exogenous IL-10 (0.5 ng/min, 14 days) simultaneously, or not, with Ang II. Aortic rings were mounted in a myograph and concentration-response curves to phenylephrine were evaluated, in the presence or absence of ERK 1/2 inhibitor (PD98059, 10 µM, 40 minutes). Protein expression of vascular ERK 1/2 was determined by Western blot. Ang II infusion increased the maximal contractile response in both WT and IL-10^{-/-} mice. Concomitant infusion of IL-10 and Ang II prevented hypercontractility in the vasculature. Exogenous IL-10 infusion prevented ERK 1/2 activation and hypercontractility, induced by Ang II. These findings suggest that IL-10 negatively modulates ERK 1/2 activation and prevents hypercontractility during Ang II-induced hypertension.

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AUTHOR CONTRIBUTION STATEMENT

AFB and GAF performed the functional and molecular experiments. AFB, VVL and FRG performed the statistical analysis. FRG designed the hypothesis. RCT, VVL, FRG and RCW provided financial support, supervised the study, continuously contributed with ideas and expertise for the project and conducted revisions for the paper.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

Keywords

cytokine; MAP kinase; smooth muscle cell and contraction

INTRODUCTION

Interleukin 10 (IL-10) works as an immunoregulatory cytokine in several pathologies [1]. Its use has been proposed for the treatment of neoplasia, prevention of stroke and in pathological conditions involving chronic inflammation [2,3].

Ang II-induced hypertension, in part, is mediated by an intense inflammatory activity [4]. This experimental model of hypertension has been consistently used to elucidate the effects of the cytokines on the blood pressure regulation, including IL-10, which concentrations are decreased upon Ang II chronic infusion [8,38].

The extracellular signal-regulated kinase 1 and 2 (ERK 1/2) is a member of the mitogen-activated kinases (MAPK) family and can be activated by Ang II in some cells [5]. In the vasculature, ERK 1/2 modulates the activity of caldesmon and calponin, two proteins involved in vascular contraction. When activated, these proteins bind to actin, preventing their interaction with the myosin light chain, favoring relaxation. However, the activation of ERK 1/2 by Ang II results in caldesmon and calponin phosphorylation, allowing the actin-myosin interaction, eliciting vascular contraction [6].

Infusion of IL-10 in animal models of Ang II-induced hypertension was shown to be effective to prevent vascular dysfunction [7] and to confer vascular protection under hypertensive conditions [8]. In addition, antagonism of Ang II promotes simultaneously increases IL-10 levels, blood pressure decreases and vascular function improves [9].

Deregulation of IL-10 has been consistently linked to the pathophysiology of experimental models, as well as in human hypertension and the modulation of IL-10 levels may represent an additional mechanism for the search of new isolated or combined therapies to control blood pressure. Considering the evidence that ERK 1/2 activation contributes to the vascular damage observed in Ang II-induced hypertension along with the improvement of both vascular function and blood pressure levels conferred by exogenous IL-10, we hypothesized that IL-10 negatively modulates vascular ERK 1/2 activation during Ang II-induced hypertension.

METHODS**Animals**

All procedures were performed in accordance with the Guiding Principles in the Care and Use of Animals, approved by Ethics Committee on the Use of Animals of Federal University of Mato Grosso (23108.219143–2017-47).

Male wild-type [(WT); C57BL/6] and IL-10 knockout [(IL-10^{-/-}); B6.129P2-Il10^{tm1Cgn/J} - The Jackson Laboratory, Maine, US] mice, aged from 10 to 12 weeks, were used in this

study. The animals were kept in cages (4 animals per cage), with light and dark cycle of 12–12 h at 24 °C, with free access to a standard feed diet and fresh water.

The animals were anesthetized with isoflurane through a nose cone (initially with 5% and maintenance in 2%, in 100% oxygen) for the implantation of osmotic mini-pumps (0.25 µl per hour – 14 days – model 1002, Alzet Co., Cupertino, CA) in the dorsal region. WT and IL-10^{-/-} mice were infused with vehicle (saline; n= 8 for WT and n=6 for IL-10^{-/-}) or with 90 ng·min⁻¹ of Ang II [(Phoenix Pharmaceutical Inc - Burlingame, CA); n= 8 for WT and n=6 for IL-10^{-/-}], for 14 days. Additionally, another group of WT mice was infused with either vehicle (saline; n=6) or 90 ng·min⁻¹ of Ang II (n=6), or IL-10 0.5 ng·min⁻¹ (BD Bioscience - San Jose, California), or with Ang II and simultaneously infused with recombinant IL-10; all groups were infused for 14 days. Stock solutions were prepared in ultrapure water or saline (Ang II and IL-10).

At the end of 14 days, mice were killed in a CO₂ chamber, and the aorta was isolated for functional and molecular studies. Blood was collected to evaluate the plasmatic levels of IL-10, determined by sandwich enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN).

Vascular function

Thoracic aortas were rapidly excised and placed in ice-cold physiological salt solution [PSS (NaCl 130 mM; NaHCO₃ 14,9 mM; KCl 4,7 mM; KH₂PO₄ 1,18 mM; MgSO₄·7H₂O 1,17 mM; D-Glucose 5,5 mM; CaCl₂·2H₂O 1,56 mM and EDTA 0,026 mM)], and carefully dissected. Endothelium was mechanically removed by rubbing the artery with a metallic pin. Aortas were mounted as ring preparations (4 mm in length) in standard organ chambers for isometric tension recording by a PowerLab 8/SP data acquisition system (AD Instruments Pty Ltd., Colorado Springs, CO). The segments were adjusted to maintain a passive force of 5 mN. Aortas were equilibrated for 60 min in PSS at 37 °C, and continuously bubbled with carbogen (5% CO₂ and 95% O₂). Arterial integrity was assessed by stimulation of arteries with a similar volume of high potassium solution (KCl, 120 mM). After washing and a new stabilization period, the absence of endothelium was assessed by contracting the segments with phenylephrine [PE; 1 µM (Sigma Chemical Co. - St. Louis, MO)] followed by stimulation with acetylcholine [ACh; 10 µM (Sigma Chemical Co. - St. Louis, MO)] The absence of a relaxation-response to ACh stimulation was taken as evidence of endothelium removal. Concentration-response curves to PE (1 nM to 100 µM) were performed in the presence or absence of PD98059 [10 µM, 40 min (Tocris - Ellisville, MO) an ERK 1/2 inhibitor.

Western blot analysis

Proteins (60 µg) from endothelium-denuded aortas were distributed in 10% polyacrylamide gel wells, taken to the electrophoresis, and transferred to a nitrocellulose membrane. Non-specific binding sites were blocked with 5% skim milk. Membranes were incubated with primary antibodies overnight at 10 °C: p44/42 MAP kinase (ERK 1/2; Cell signaling), phospho-p44/42 MAP kinase (ERK 1/2^{Thr202/Tyr204}, Cell signaling). On the next day, the membranes were removed from primary antibodies and washed with a Tris-buffered saline

solution with Tween (TBS-T) and treated with the conjugated secondary antibody (Anti-mouse IgG, Cell signaling). The signals were visualized through chemiluminescence and images were captured using L-Pix Chemi express (LOCUS), and the bands submitted to optical densitometric analysis. All membranes were evaluated for the β -actin (Sigma, 1:2000), control protein to ensure equal loading of protein. The results were normalized to β -actin protein and expressed in arbitrary units.

Statistical analysis

The results are presented as mean $\pm$ standard error of the mean (SEM), where “n” represents the number of animals used for each experiment. Vascular contraction data were performed using one arterial segment per mice, recorded through changes in basal force-displacement (mN), normalized by KCl contraction and are plotted as percent contraction induced by KCl. The concentration-response curves were fitted using a nonlinear interactive fitting program and two pharmacological parameters were obtained: the maximum effect generated by the agonist (EMax) and -log half maximal effective concentration (EC50) [pD2]. Statistical analyses were performed by unpaired Student's t-test or ANOVA one way followed by Tukey post hoc test, as indicated in the legend of the figure. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Ang II infusion in WT mice caused a significant reduction in IL-10 plasma concentrations (pg/mL) compared to control group (4.6 ± 2.2 vs. 10.5 ± 1.4 , respectively; $p = 0.42$; $n = 8$ each group). Exogenous supplementation of IL-10 increased cytokine level in the absence or presence of Ang II (18.0 ± 2.2 vs. 24.7 ± 2.0 , respectively; $p = 0.47$; $n = 6$ each group).

Modulatory role of IL-10 on vascular ERK 1/2 activation

Ang II infusion increased contractile responses to PE (force, % KCl) in aorta from WT mice, compared to the vehicle infused group. Inhibition of ERK 1/2 attenuates the effect of Ang II on vascular contraction, generating greater inhibition of PE-induced contraction if compared to vehicle-infused group (Fig 1).

Ang II infusion in IL-10^{-/-} mice increased the contractile response to PE compared to the respective vehicle infused group. Treatment with an inhibitor of ERK1/2 (PD98059) eliminated the differences between the groups and significantly reduced the contractile response in both groups (Fig 2).

Arteries from mice simultaneously infused with exogenous IL-10 and Ang II showed similar contractile responses to PE if compared to IL-10 infused mice. ERK 1/2 inhibition was similar between the group (Fig 3).

IL-10^{-/-} mice infused with vehicle displayed a similar contractile response to PE in the WT group infused with Ang II. However, when IL-10^{-/-} mice were infused with Ang II, the contractile response to PE was greater than in WT mice infused with Ang II. Additionally, simultaneous infusion of exogenous IL-10 and Ang II reduced contractile response, if compared to the Ang II group. ERK 1/2 inhibition significantly reduced the contractile

response in all groups, compared with their respective curve in the absence of PD98059 (Table 1).

Effects of IL-10 on ERK 1/2 vascular expression

The endogenous absence of IL-10 increased vascular expression (arbitrary units) of total ERK 1/2 compared to WT + vehicle. Ang II infusion in WT mice increased total ERK 1/2 vascular expression, compared to WT + vehicle. IL-10^{-/-} mice infused with Ang II increased the expression of total ERK 1/2 compared to the WT group infused with Ang II (Fig 4A).

The extent of ERK 1/2 phosphorylation has not changed in IL-10^{-/-} mice, compared to WT mice infused with vehicle. Ang II infusion in WT mice increased phosphorylated ERK 1/2 vascular expression compared to WT + vehicle. Ang II infusion in IL-10^{-/-} mice increased phosphorylated ERK 1/2 expression compared to IL-10^{-/-} + vehicle (Fig 4B).

The exogenous infusion of IL-10 has not altered total ERK 1/2 expression in WT mice to WT + vehicle group. IL-10 infused simultaneously with Ang II prevented total ERK 1/2 increase in WT mice vs. WT + Ang II (Fig 5A).

The single infusion of IL-10 has not alter phosphorylated ERK 1/2 expression compared to WT + vehicle group. IL-10 infused simultaneously with Ang II prevented phosphorylated ERK 1/2 increase in WT + Ang II (Fig 5B).

The ratio between phosphorylated and total ERK 1/2 was calculated for WT, IL-10^{-/-} or IL-10^{exog} groups, infused with vehicle or Ang II. IL-10^{-/-} mice, infused or not with Ang II displayed decreased ERK 1/2 activity compared to WT or IL-10^{exog} groups. Ang II infusion increased ERK 1/2 activity only in WT mice (p= 0.039) and IL-10^{-/-} mice (p=0.0003), compared to their respective control infused with vehicle. No differences were observed in ERK 1/2 ratio between IL-10^{exog} groups (Fig 6).

DISCUSSION

The main findings of this study demonstrate that Ang II infusion and the absence of endogenous IL-10 promotes increased vascular contraction, partially mediated by ERK 1/2 activation. Additionally, exogenous IL-10 attenuates the Ang II-induced vascular dysfunction, partially via ERK 1/2 inhibition in the vasculature. Altogether, these findings support our hypothesis, suggesting that the protective effects of IL-10 in the vasculature is partially mediated by ERK 1/2 inactivation and during Ang II-induced hypertension, decreased levels of IL-10 may further contribute to vascular damages.

Interleukin-10 improves vascular function upon chronic pro-inflammatory conditions, including hypertension. This protection reflects in blood pressure regulation, since in mouse, the absence of endogenous production of IL-10 further contribute to blood pressure elevation after Ang II infusion, whereas exogenous administration of IL-10 prevents the pressoric actions of Ang II [10–7,22]. The present data suggest that another mechanism by which IL-10 may modulate blood pressure is via down-regulation of ERK 1/2 pathway, since it

results in decreased vascular contraction and therefore, reducing vascular resistance, since Ang II chronic infusion results in augmented vascular resistance.

Ang II-induced hypertension is an important experimental toll, since chronic infusion of Ang II contributes to cell proliferation, hypertrophy and inflammation, and after three days, the sustained elevation of blood pressure induces several vascular alterations, including vascular hypercontractility, decreased production of vasodilator factors, augmented medial thickness of conductance vessels, among other alterations [11–14]. Ang II modulates blood pressure levels by simultaneously activating various mechanisms, including the generation of a pro-inflammatory environment, directly impacting blood pressure regulation [15]. The predominance of proinflammatory cytokines secreted during hypertension suppress the action of immunoregulatory factors, such as IL-10, favoring an immunological imbalance [16] consequently, contributing to vascular dysfunction [44]. Reduced IL-10 circulating levels, contrasting with augmented Ang II levels are observed during hypertension in humans [17]. The relation of lower IL-10 levels with the development of vascular complications has been proposed in some studies. For example, during the stroke, IL-10 levels are below normal [18]. In preeclamptic women, the reduction of IL-10 receptors, as well as lower IL-10 circulating levels, is observed, suggesting IL-10 as a biomarker of preeclampsia [19, 20].

The absence of endogenous IL-10 results in an intense inflammatory state, [12, 21] leading to hypercontractility, as demonstrated here. Even without a hypertensive stimulus, endogenous IL-10 plays an important regulatory function on vascular contraction. Over time, hypercontractility results in augmented vascular resistance, which directly affects blood pressure regulation. In young adult IL-10^{-/-} mice, blood pressure levels are slightly increased but remains at normal levels [8]. However, when blood pressure is assessed in older IL-10^{-/-} mice, this function is significantly increased, in part, due to reduced vascular relaxation and increased eicosanoid production. In older animals, the absence of IL-10 also results in stiffer vessels and impairs cardiac function, demonstrating that this regulatory cytokine is crucial for maintaining the vascular compliance and endothelial function during the aging process [22].

The present study evaluated the impact of IL-10 modulation on the vascular contractile function. However, it is possible that rather than controlling vascular function, IL-10 may also affect the vascular structure. On this regard, it is known that IL-10 inhibits cardiac remodeling, by decreasing fibrosis [23] in a model of myocardial infarction. Hence, the absence of endogenous IL-10 results in vascular remodeling due to augmented oxidative stress production via Nox 1/NADPH oxidase upregulation, showing that IL-10 is essential for the maintenance of the normal vasculature [24]. Ang II also plays an important role in vascular remodeling, inducing hypertrophic vascular stress via superoxide anion production derived from NADPH oxidase [25]. Therefore, it is possible that the association of the absence of endogenous IL-10 simultaneously with augmented Ang II would synergistically induce vascular remodeling, further contributing to blood pressure elevation, but this possibility remains to be studied.

Ang II activates ERK 1/2 through the AT1 receptor, which stimulates components from the MAP kinase pathway [26–28]. When activated, the ERK 1/2 pathway regulates various processes in the vasculature, including contraction, cell cycle progression, migration, differentiation, proliferation, and increased cell transcription [29]. Our results demonstrated that upon Ang II stimulation, exogenous IL-10 plays an inhibitory role in vascular ERK 1/2 activation. Similarly, exogenous IL-10 inhibits ERK 1/2 activation and other proteins related to cellular remodeling in cardiomyocytes infected with *Trypanosoma cruzi* [30]. IL-10 inhibits the activation of macrophages and granulocytes by decreasing the production of reactive oxygen species, via inactivation of ERK 1/2 and modulation of NADPH oxidase [31].

It is important to mention that because IL-10 plays a regulatory role in several tissues, upon different stimuli, it is possible that this cytokine elicits distinct roles in different cells. For example, in other cell types, ERK 1/2 activation is required to release IL-10. This is the case of the dendritic cells stimulated with TLR4 and TLR2, ligands that enhance IL-10 production by ERK 1/2 activation [32, 33]. Similarly, upon LPS stimulation or *Mycobacterium tuberculosis* heat shock protein 60 (Mtbhsb60) TLR4 receptors are activated in bone marrow-derived macrophages, resulting in increased IL-10 levels, due to ERK1/2 activation in these cells [34].

What seems to be consistently clear is that strategies to improve IL-10 levels may result in vascular protection. Indeed, IL-10 has been frequently shown to confer vascular protection in several studies. It has been shown that IL-10 plays an effective role in vascular inflammation, prevent atheroma formation due to reducing monocyte adhesion, neutralizes endothelin-1 vasoconstricting effects, restores nitric oxide synthase expression and prevent superoxide-mediated endothelial dysfunction upon several pro-inflammatory stimuli. IL-10 can prevent the compromised endothelium-dependent relaxation caused by TNF- α , inhibiting the expression of eNOS [35–39, 45].

The modulatory effect of IL-10 in ERK1/2 activation among different tissues may also be explained by the activation of phosphatases within different tissues. For example, the dual-specificity phosphatase 6 (Dusp6) preferentially dephosphorylates extracellular signal-regulated kinases 1 and 2 (ERK1/2). It was shown in a double-knockout mouse for IL-10 and Dusp6, which display exacerbated spontaneous colitis, that ERK1/2 inhibition was effective to prevent this inflammation [40]. A positive feedback mechanism is also proposed between IL-10 and mitogen-activated protein kinase phosphatase-1 (MKP-1), where IL-10 can, at the transcriptional level, activate MKP-1 [41]. Studies indicate that the increase of IL-10 occurs simultaneously with the increased production of MKP-1 phosphatase in the intestinal mucosa [42]. Indeed, in the intestinal mucosa, double knockout for MKP-1 and IL-10 accelerated the development of colitis, demonstrating the interplay between MKP-1 and this cytokine [43].

CONCLUSIONS

These results demonstrate that the absence of IL-10 favors vascular hypercontractility and activation of ERK 1/2 in animals infused with Ang II. Infusion of exogenous IL-10 prevents

the vascular effects of Ang II on contractility and ERK 1/2 activation, acting as a modulatory molecule.

Considering that therapeutic strategy that inhibits Ang II action in the vasculature are used to treat hypertension, which simultaneously improves IL-10 circulating levels, it is possible that besides IL-10 acts to terminate inflammatory responses. This regulatory cytokine also modulates contractile protein function, limiting hypercontractility during hypertension.

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Figure 1. ERK 1/2 inhibition abolishes hypercontractility in aorta from Ang II-infused mice. Concentration-response curves to PE were conducted in aortas from WT animals infused with vehicle (open symbol/ $n=7$) or Ang II (closed symbol/ $n=7$), in the absence (**A**) or presence (**B**) of PD98059 (10 μ M, 40 min). Values are presented as the mean $\pm$ SEM. Statistical comparison was performed between the E_{Max} by Student's t-test. * $p<0.05$ vs. WT.

Figure 2. ERK 1/2 inhibition abolishes hypercontractility in aorta from IL-10^{-/-} mice after Ang II infusion.

Concentration-response curves to PE in aortas from IL-10^{-/-} animals infused with vehicle (open symbol/ n=6) or Ang II (closed symbol/ n=6), in the absence (**A**) or presence (**B**) of PD98059 (10 μM, 40 min). Values are presented as the mean ± SEM. Statistical comparison was performed between the E_{Max} by Student's t-test. * p<0.05 vs. WT.

Figure 3. IL-10 infusion restores ERK 1/2 activation in aorta from Ang II-infused mice. Concentration-response curves to PE in aortas from WT animals infused with vehicle (open symbol/ n=6) or Ang II + IL-10 (closed symbol/ n=6), in the absence (**A**) or presence (**B**) of PD98059 (10 μ M, 40 min). Values are presented as the mean $\pm$ SEM. Statistical comparison was performed between the E_{Max} by Student's t-test.

Figure 4. The endogenous absence of IL-10 associated with Ang II positively modulates vascular expression of total and phosphorylated ERK 1/2.

Total ERK 1/2 (A) and phosphorylated^{Thr202/Tyr204} ERK 1/2 (B) protein expression was evaluated in aortas from WT and IL-10^(-/-) mice infused or not with Ang II (n= 4–5). Representative images were selected within the same membrane. *P < 0.05 vs. WT + vehicle; [†] p < 0.05 vs. WT + Ang II.

Figure 5. IL-10 negatively modulates vascular expression of total and phosphorylated ERK 1/2. Total ERK 1/2 (**A**) and phosphorylated^{Thr202/Tyr204} ERK 1/2 (**B**) protein expression was evaluated in aortas from WT mice infused or not with the vehicle, Ang II, IL-10 or IL-10 and Ang II. (n= 4–5). Representative images were selected within the same membrane. * P <0.05 vs. WT + vehicle; † p <0.05 vs. WT + Ang II.

Figure 6. IL-10 negatively modulates vascular activity of ERK 1/2.

Ratio between phosphorylated^{Thr202/Tyr204} and total ERK 1/2. Protein expression was evaluated in aortas from WT, IL-10^{-/-} or IL-10^{exog} groups, infused with vehicle (white bar; n= 4–5) or Ang II (black bars; n= 4–5) * P<0.05 vs. respective vehicle group.

TABLE 1.

$E_{\max}$ and pD_2 values obtained from the concentration-response curves performed in aortas from WT and IL-10^{-/-} mice, before or after ERK 1/2 inhibition.

	$E_{\max}$ (%KCl)	pD_2	PD98059	
			$E_{\max}$ (%KCl)	pD_2
WT	160.1±7.8	7.07±0.2	126±6.5 [#]	7.22±0.2
WT + Ang II	218.9±10.4 [*]	7.28±0.2	143.±13.1 [#]	6.68±0.2
IL-10^{-/-}	202.1±9.2 [*]	7.74±0.4 [*]	120.7±5.4 [#]	7.29±0.2
IL-10^{-/-} + Ang II	244.7±5.5 [†]	7.69±0.2	120.7±10.7 [#]	6.95±0.23
IL-10^{exog}	148.3±4.8	7.08±0.1	107.2±6.7 [#]	7.35±0.3
IL-10^{exog} + Ang II	171.1±5.8 [†]	7.09±0.1	130.1±9.7 [#]	6.96±0.2

ERK 1/2 inhibition was performed with PD98059 (10 µM, 40 min) incubation.

^{*} p < 0.05 vs. WT + vehicle;

[†] p < 0.05 vs. WT + Ang II; and

[#] p < 0.05 vs. respective group before PD98059 incubation.

Abbreviations: *Ang II*, angiotensin II; $E_{\max}$, maximum effect generated by the agonist; *IL-10*, interleukin-10; *IL-10^{-/-}*, interleukin-10 knockout mice; *IL-10^{exog}*, exogenous infusion with interleukin-10; pD_2 , -log of half maximal effective concentration; *WT*, wild-type mice;