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Physiological regulation of pro-inflammatory cytokines expression in rat cardiovascular tissues by sympathetic nervous system and angiotensin II

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Abstract. Pro-inflammatory cytokines regulation by sympathetic nervous system (SNS) and angiotensin II (ANG II) was widely described in cardiovascular system, but the role of such neuro-humoral interaction needs further investigation in this context.

We tested SNS-ANG II interaction on IL-6 and TNF-α mRNA expression in left ventricle (LV) and aorta from normotensive rats by sympathectomy with guanethidine and blockade of the ANG II AT1 receptors (AT1R) antagonist with losartan. mRNA synthesis of IL-6 and TNF-α were performed by Q-RT-PCR.

In the LV, IL-6 mRNA increased by 63% ($p < 0.01$) after sympathectomy, still unchanged after losartan treatment and decreased by 38% ($p < 0.05$) after combined treatment. TNF-α mRNA decreased by 44% ($p < 0.01$), only after combined treatment. In the aorta, IL-6 mRNA increased equally by 65% ($p < 0.05$) after sympathectomy or losartan treatment. TNF-α mRNA decreased by 28, 41, and 42% ($p < 0.05$) after sympathectomy, losartan and combined treatments, respectively. Our data suggest that ANG II stimulates directly (*via* AT1R) and indirectly (*via* SNS) IL-6 mRNA synthesis in LV and aorta and TNF-α mRNA in LV. ANG II seems unable to influence directly TNF-α mRNA synthesis in the aorta but can stimulate this cytokine *via* SNS. The results are relevant to prevent or reduce proinflammatory cytokines overexpression seen in cardiovascular diseases.

Key words: Sympathectomy — Angiotensin II AT1 receptor antagonist — IL-6 — TNF-α — Left ventricle — Aorta

Introduction

Proinflammatory cytokines, such as interleukin IL-6 and tumor necrosis factor α (TNF-α) played a crucial role in the inflammation response (Papanicolaou et al. 1998). Higher systemic concentrations of IL-6 and TNF-α are largely implicated in the development and progression of chronic heart failure (Levine et al. 1990) and atherosclerosis (Kher and Marsh 2004).

Angiotensin II (ANG II) and sympathetic nervous system (SNS) activities were described in inflammatory process in post-infarcts situations and atherosclerosis. ANG II is a potent proinflammatory mediator which is able to promote atherosclerosis development (Brosnan et al. 2000) by controlling expression of cytokines genes, chemokins and adhesion molecules after activation of transcriptional factor NF-kB (Han et al. 1999; Ruiz-Ortega et al. 2001). Blockage of ANG II biosynthesis by inhibition of angiotensin converting enzyme is largely used to prevent degradation of post-infarctus cardiac function due to overexpression of proinflammatory cytokines (Wei et al. 2002). However, there is some contradictory results and interpretation about implication of SNS on proinflammatory cytokines regulation in cardiovascular disease. A positive correlation between circulating catecholamine and cytokine levels was associated with either reduce (Parthenakis et al. 2003; Diakakis et al. 2008) or hyperactivity of SNS (Petretta et al. 2000). Norepinephrine stimulates the production of

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proinflammatory cytokines in cardiomyocyte (Kan et al. 1999) which is prevented by the use of β1 adrenergic receptors antagonists (Prabhu et al. 2000).

Interaction between SNS and ANG II was demonstrated by several functional studies which showed that ANG II stimulates sympathetic neurotransmitter (NE) release by activation of its specific AT1 receptor (AT1R) located on presynaptic SNS terminal axon reaching cardiovascular tissues (Saxena, 1992; Cox et al. 1995; Dendorfer et al. 1998; Wang and Ma 2000). Moreover, the blockage of ANG II receptor AT1R with losartan reduces NE release and enhances its reuptake by SNS terminal axon (Raasch et al. 2004). However, little is known about the SNS-ANG II interaction on the proinflammatory cytokines expression in cardiovascular system.

Therefore, we advanced an experimental approach by performing chemical sympathectomy with guanethidine and AT1R blockade with losartan that conducted separately or in combination in normotensive rats. The levels of IL-6 and TNF-α transcription were determined by RT-PCR in the left ventricle and aorta. Systolic blood pressure (SBP) was measured to check the efficacy of simple or combined treatment, since losartan and chemical sympathectomy are well known to reduce SBP (Dwyer et al. 2004; Raasch et al. 2004).

Materials and Methods

Animals

The animal protocols used for this study were approved by the University Animal Care and Use Committee of University of Claude Bernard (Lyon 1, France) and were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Male Wistar-Kyoto rats ($n = 32$), were equally divided in four groups: a guanethidine-treated (Gua), a losartan-treated (Los), a combined-treated (Gua+Los) and a control (CTRL) group. Rats were treated as previously described (Dab et al. 2009) with guanethidine sulfate (50 mg/kg *i.p.*, 5 days/week for 5 weeks) (Sigma). The success of guanethidine injections was attested by the development of ptosis by the second day of treatment and during the experiment (Dwyer et al. 2004). Losartan (20 mg/kg/day) was administered in drinking water for 5 weeks. The water consumption and rat body weight were measured daily and the concentration of losartan was adjusted each day according to water consumption and animal body weight as previously described (Dab et al. 2009). The combined-treated group received guanethidine and losartan as described above for 5 weeks. The CTRL group received injections of saline for the same time period.

At the end of the treatment, rats were deeply anesthetized with pentobarbital (50 mg/kg *i.p.*). The left ventricle and aorta were removed, washed thoroughly from blood and then frozen immediately in liquid nitrogen, and stored at −80°C.

Blood pressure measurement

SBP was measured weekly during the 5 weeks of treatment *via* a tail-cuff method (Cerutti et al. 2006).

Total RNA extraction and RT-PCR analysis

The protocol of RNA extraction and RT-PCR analysis is described in detail in our previous study (Dab et al. 2009). Briefly, total RNA was extracted from LV and aorta (100 mg) using Trizol Reagent (Invitrogen, Carlsbad, CA). After DNase treatment, 5 μg of total RNA was reverse-transcripted with superscript II transcriptase (Invitrogen, France) using random hexamers as primers (pdN6; Amersham Biosciences, Piscataway, NJ). Real-time RT-PCR was performed with a MyiQ thermal cycler (Bio-Rad Laboratories, Hercules, CA). Real-time RT-PCR was performed using the resulting cDNA as template, iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA), and the appropriate set of primers (Invitrogen, France) specific to IL-6 (sense: GAAACGGAACTCCAGAAGACC; anti-sense: GACTGCCTTCCCTACTTCACA) and TNF-α (sense: TGTTCATCCGTTCTCTACCC; anti-sense: TTGCACCCTGAGCCACAA). For each sample, PCR was performed in duplicate. Cycle threshold values were calculated for the different products with Optical System Software v1.0 (Bio-Rad Laboratories). Cycle threshold values were calculated for the different products with Optical System Software v1.0 (Bio-Rad Laboratories, Hercules, CA). Expression levels obtained from the standard curves were normalized against 18S rRNA (sense: AGTCGGCATCGTTTATGGTC; antisense: TGAGGCCATGATTAAGAGGG). Relative amount of each studied gene (ng/μg 18S RNA) was calculated with Optical System Software v1.0 and results were expressed as fold changes to control group.

Statistical analysis

Statistical comparisons between treated and control groups were performed using ANOVA test. Subsequent pair-wise comparisons were performed with the HSD Tukey test using statistica software (Statsoft, France). Results are expressed as mean ± SEM. $p < 0.05$ is taken as a significant probability and n values indicate the number of replicates.

Results

The body and LV weights were not affected by individual or combined treatments. SBP was significantly decreased

after individual treatment and the two treatments exercised together a synergic effect (Table 1).

Expression of IL-6 and TNF-α in LV

mRNA of IL-6 was significantly increased by 1.6-folds ($p < 0.01$) in the Gua group, decreased by 1.2-fold ($p < 0.05$) in the combined treatment group and remained similar to control in the Los group ($p = 0.32$) (Fig. 1A). IL-6 was significantly higher in Gua than in Los group by 1.36-fold ($p < 0.05$). mRNA expression of TNF-α was only affected after combined treatment and was significantly decreased by about a half compared to control ($p < 0.01$) (Fig. 1B).

Expression of IL-6 and TNF-α in aorta

mRNA of IL-6 was significantly increased by about 1.6-fold ($p < 0.05$) in the sympathectomized and Los groups and remained similar to control in the Gua+Los group (Fig. 2A). mRNA expression of TNF-α was significantly lower in the three treated groups by 72, 59, and 60% after chemical sympathectomy, Los and Gua+Los, respectively (Fig. 2B).

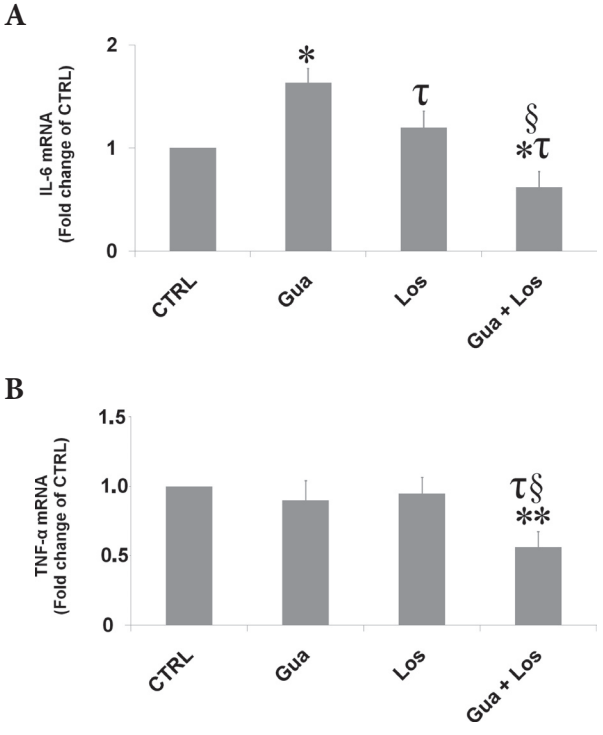

Figure 1. Real-time RT-PCR analysis of IL-6 (**A**) and TNF-α (**B**) mRNA in the LV from control and treated rats. Values are presented as fold change of mRNA to CTRL group. Data are mean ± SEM, $n = 8$ in each group. * $p < 0.05$, ** $p < 0.01$ *vs.* CTRL; $^{\tau}$ $p < 0.05$ *vs.* Gua group; § $p < 0.05$ *vs.* Los group. CTRL, control group; Gua, guanethidine-treated group; Los, losartan-treated group; Gua+Los, combined-treated group.

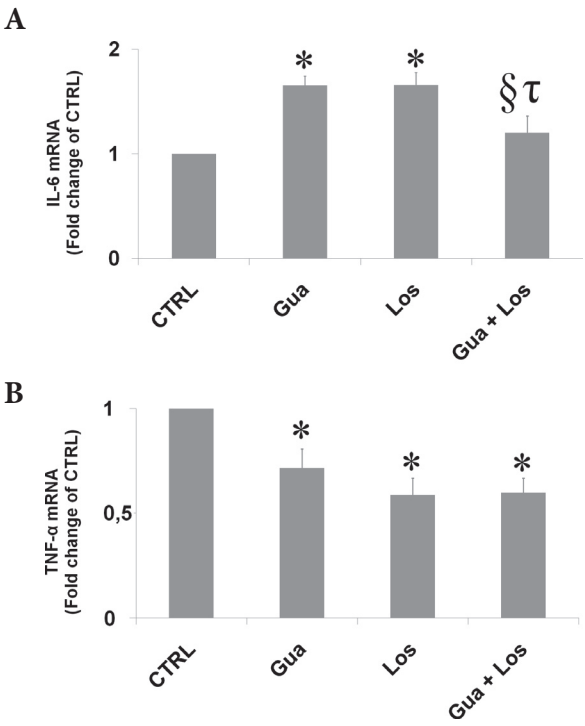

Figure 2. Real-time RT-PCR analysis of IL-6 (**A**) and TNF-α (**B**) mRNA in the aorta from control and treated rats. Values are presented as ratio fold change of mRNA to control group. Data are mean ± SEM, $n = 8$ in e ach group. * $p < 0.05$, ** $p < 0.01$ *vs.* CTRL; $^{\tau}$ $p < 0.05$ *vs.* Gua group; § $p < 0.05$ *vs.* Los group. CTRL, control group; Gua, guanethidine-treated group; Los, losartan-treated group; Gua+Los, combined-treated group.

Discussion

In the present study, we investigated the interaction between SNS and ANG II on the mRNA expression of proinfammatory cytokines IL-6 and TNF-α in rat LV and aorta, by inhibition of one or two systems simultaneously.

AT1R of ANG II is expressed in cardiovascular cells but also in the SNS axons that innervate cardiovascular tissue. Thus, we hypothesise that ANG II could mediate its action directly or indirectly through SNS. In our experimental approach, the direct effect of ANG II could be deducted by comparison of the Gua and Gua+Los groups, since AT1R could be either free (in the Gua group) or blocked (in the Gua+Los group). The influence of SNS could be deducted by comparison of Los group (presence of SNS) and Gua+Los group (absence of SNS) (Fig. 3).

Table 1. Body weight, LV weight index and blood pressure records at the end of the experiment

Group	Body weight (g)	Systolic blood pressure (mmHg)
CTRL	462 ± 25	136.36 ± 3.48
Gua	441 ± 24	121.42 ± 2.87**
Los	468 ± 22	118.47 ± 2.43 **
Gua+Los	455 ± 22	105.79 ± 1.17*** $^{\tau\tau}$ §§

Values are means ± SD. ** $p < 0.01$, *** $p < 0.001$ *vs.* CTRL; $^{\tau\tau}$ $p < 0.01$ *vs.* Gua; §§ $p < 0.01$ *vs.* Los. CTRL, control group; Gua, guanethidine-treated group; Los, losartan-treated group; Gua+Los, combined-treated group.

It is well known that guanethidine removes selectively sympathetic fibers from all tissues and induces a dramatic loss of catecholamine in the circulation and tissues (Aberdeen et al. 1990; Villanueva et al. 2003) including blood vessels and heart (Heath and Burnstock 1977) except adrenal gland and brain (Johnson et al. 1977). Also, chronic administrations of guanethidine or AT1R inhibitor losartan reduce SBP (Balt et al. 2001; Supowit et al. 2005). In this study, efficacies of guanethidine and losartan treatments were inspected by the decrease of SBP.

Proinflammatory cytokines such as TNF-α and IL-6 are expressed in heart by myocardial macrophages, mast cells and cardiac myocytes (Meldrum et al. 1989; Shiota et al. 2003; Agrawal et al. 2010) and in vascular system by endothelial cells and vascular smooth muscle cells (Loppnow and Libby 1989; Sironi et al. 1989). The major investigations of SNS and ANG II implication in cytokines regulation are mostly made in circulation and in pathological conditions or *in vitro*. The present study has examined for the first time the interaction between SNS and ANG II on IL-6 and TNF-α synthesis at transcriptional level in the cardiovascular tissue *in vivo* in normotensive rat.

It has been demonstrated that the renin angiotensin system enhances expression of TNF-α and IL-6 in aortic stenosis (Côté et al. 2010) and ANG II stimulates TNF-α- and IL-1β synthesis and secretion in hypertrophic cardiomyocytes (Zhou et al. 2007). Also, *in vitro* studies showed that ANG II stimulates mRNA expression of IL-6 in cultured vascular smooth muscle cell *via* activation of NF-kβ (Han et al. 1999; Kranzhöfer et al. 1999).

Our results suggest that this stimulation is mediated *in vivo* directly *via* AT1R and indirectly through SNS for IL-6 in both LV and aorta and for TNF-α in LV. Indeed, mRNA synthesis decreased in the Gua+Los groups (absence of SNS and AT1R were blocked) when compared either to the sympathectomy group (absence of SNS and AT1R were free) or to the Los group (presence of SNS and AT1R were blocked).

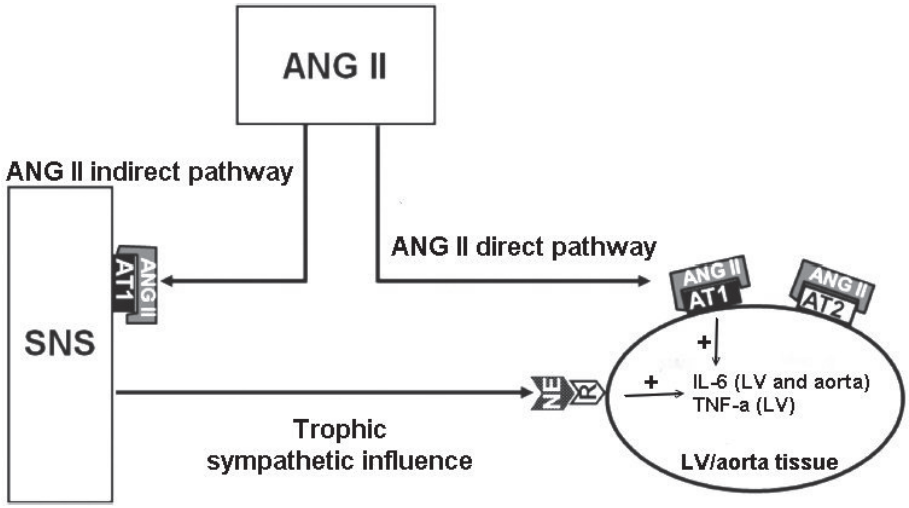

Figure 3. Schematic pathways of hypothetic neurohumoral regulation of cytokine expression in heart and aorta. Both AT1 and AT2 receptors of ANG II are expressed in sympathetic fibers coursing the heart and vessels (presynaptic receptors) and in the tissue (Timmermans and Smith 1994). Based on the literature, we hypothesize that ANG II from circulation or locally synthesized acts through two pathways to influence the local synthesis IL-6 and TNF-α: 1) Sympathostimulator pathway where ANG II incites neurotransmitters release from sympathetic fibers including norepinephrine *via* AT1R (Gironacci et al. 1994; Dendorfer et al. 1998; Balt et al. 2001; Raasch et al. 2004). Norepinephrine (NE) assure its action through noradrenergic receptors (R) expressed on cardiac cells; 2) direct pathway where ANG II mediates directly its action on cardiac tissue *via* AT1R essentially but can also access to AT2 receptor. Empty arrows: ANG II-mediated action indirectly *via* SNS or directly *via* AT1 receptors on MMPs expression. +, stimulation.

However, ANG II seems enable to influence directly TNF-α synthesis in aorta, but can stimulate this cytokine through SNS, since TNF-α mRNA is equally decreased in absence of SNS when AT1R were free (sympathectomy group) or blocked (Gua+Los group). The reduced TNF-α noted in the Los group could be explained by a reduction of SNS activity under blockage of AT1R (Rocha et al. 2007). Another explication is that the fall in TNF-α expression seen in aorta from the three treated groups could be resulted from inhibition exerted by ANG II *via* its AT2 receptors remaining accessible to ANG II.

The autonomic unbalance was associated with increased systemic inflammation in old patients (Guinjoan et al. 2004). Contradictory data and interpretation was found about the role of SNS on cytokines expression. A reduced cardiac sympathetic innervation was correlated with higher circulating proinflammatory cytokine levels of IL-6 and TNF-α from patients with heart failure (Parthenakis et al. 2003; Diakakis et al. 2008). In contrast, enhanced cytokine production was associated with hyperactivity of SNS (Petretta et al. 2000) and high plasma norepinephrine levels (Kinugawa et al. 2003). Furthermore, it has been shown *in vivo* and *in vitro* that cytokine production by the heart may be regulated by SNS through β-adrenergic receptors (Guirao et al. 1997; Kan et al. 1999; Prabhu et al. 2000). Chronic β-adrenergic stimulation with isoproterenol enhances gene expression and protein production of TNF-α, IL-1 β, and IL-6 in the heart (Murray et al. 2000) while β-adrenergic receptors antagonists inhibit these productions (Kan et al. 1999). It emerges from our experimental approach that SNS stimulates mRNA cytokines synthesis and this is despite of apparent variable responses of cytokines to chemical sympathectomy. To explain the paradox findings seen here and elsewhere, we hypothesize that the increase or absent change of cytokines expression observed after sympathectomy could result from ANG II stimulation through direct pathway. Also, our data suggests that the stimulation effect of ANG II is more important than that of the SNS on the synthesis of the IL-6 in aorta to explain the strong increase in IL-6 observed in the sympathectomy group compared to the Los group.

Our data show that treatments induce the fall of blood pressure with a synergic relation but without a correlation with cytokines changes. This is making improbable the implication of hemodynamic factors on the modulation of cytokine expression seen here.

Conclusions

Although our data corroborate previous investigations made in other tissue showing an enhanced IL-1β, IL-2, and IL-6 gene expression after central ANG II administration in splenic-intact rats (Ganta et al. 2005) and an ANG II modulation of the immune system through activation of efferent sympathetic nerve outflow, a mutual interactions between ANG II and cytokines exist (Wang et al. 2012) and cytokines are also able to influence ANG II syntheis in tissues (Suski et al. 2013). Inhibition of SNS or ANG II activity may be beneficial to prevent cardiovascular disorders by cytokine overexpression. Our results indicate that inhibition of both systems is more relevant to prevent an increase or to reduce cytokines synthesis in cardiovascular diseases.

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Declaration of interest: The authors report no declarations of interest.

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