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# Angiotensin II promotes the inflammatory response to CD40 ligation via TRAF-2

Johannes Ruef, Michael Browatzki, Caroline AH Pfeiffer, Joachim Schmidt and Roger Kranzhöfer

**Abstract:** A plethora of evidence supports a link between inflammation and atherogenesis. Both the vasoactive peptide angiotensin II (ANG II) as well as the CD40/CD154 signaling pathway exhibit proinflammatory properties with a direct influence on atherogenesis. We therefore tested the hypothesis that ANG II interacts with CD40/CD154 in human vascular smooth muscle cells (SMC). ANG II did not increase expression of CD40 in human SMC. However, when SMC were prestimulated with ANG II and thereafter stimulated with CD154, the ligand for CD40, the release of IL-6 as a marker of inflammatory activation was augmented compared to cells not primed with ANG II. TNF receptor-associated factor 2 (TRAF-2), an important adaptor protein involved in CD40 signaling, but not TRAF-5 or -6, was increased by ANG II via activation of the angiotensin II type 1 (AT1) receptor subtype. These results suggest that a signaling pathway downstream of CD40 may be altered by ANG II prestimulation. Thus, ANG II can also indirectly cause inflammatory activation of vascular SMC. The data show a novel link between the proatherogenic vasoactive peptide ANG II and cell–cell contact-mediated inflammatory pathways and implicate options for the prevention and therapy of atherosclerotic disease.

**Key words:** angiotensin II; atherosclerosis; CD40; smooth muscle cell; TRAF

## Introduction

A body of evidence supports a link between inflammation and atherogenesis. The vasoactive peptide angiotensin II (ANG II) is known as a proatherogenic factor not only because of its vasoconstrictive and mitogenic properties but also through its capability to induce an inflammatory response in human vascular smooth muscle cells (SMC).<sup>1,2</sup>

CD40 is a 45–50-kDa membrane glycoprotein belonging to the tumor necrosis factor (TNF) superfamily. In addition to monocytes and macrophages, human vascular endothelial cells and SMC in atherosclerotic plaques also express CD40.<sup>3</sup> CD154 (formerly CD40L), the ligand for CD40, is expressed on T cells. Ligation of CD154 with its receptor CD40 results in a strong inflammatory response mediated via cell–cell contact. More recently, activated platelets were described as another major source of

CD154 triggering inflammatory reactions of endothelial cells<sup>4,5</sup> and elevated levels of soluble CD154 could be found in patients with acute coronary syndromes.<sup>6,7</sup>

Today, CD40/CD154 interactions are recognized as important in the pathogenesis of atherosclerosis.<sup>1</sup> Inhibition of CD40 signaling in mouse models of progressive atherosclerosis resulted in a significantly reduced development of atherosclerotic lesions or yielded a more stable plaque phenotype.<sup>8–10</sup>

Interactions between the ANG II signaling cascade and the CD40-system via activation of the ligand CD154 (extracellular link) have been described.<sup>1</sup> The present study investigated whether additional forms of interactions exist. The following hypothesis was postulated and confirmed: crosstalk between the CD40 and the ANG II signal transduction pathways occurs on the cytoplasmatic (intracellular level) via TNF receptor-associated factor 2 (TRAF-2). This represents a potentially novel link between different inflammatory pathways in human vascular SMC.

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## Materials and methods

### Cell preparation and culture

SMC were cultured by explant outgrowth as described from unused portions of human saphenous veins

harvested for coronary bypass surgery.<sup>11</sup> For experiments, confluent cells from passages 2 to 5 were growth-arrested in serum-free medium.<sup>12</sup>

### Determination of CD40 and TRAF expression by Western blot

Briefly, cell extracts were separated on SDS-PAGE minigels and electrophoretically transferred to Hybond-nitrocellulose membrane (Amersham, Munich, Germany). Equal protein loading was ascertained by Ponceau staining of membranes after transfer. Incubation with monoclonal antibodies for CD40, TRAF-2, TRAF-5, and TRAF-6 (all from Santa Cruz) was followed by incubation with secondary antibodies coupled to horseradish peroxidase. Antibody binding was visualized using the ECL chemiluminescence system (Amersham). Densitometry was performed using the NIH Image software.

### Determination of IL-6 release by ELISA

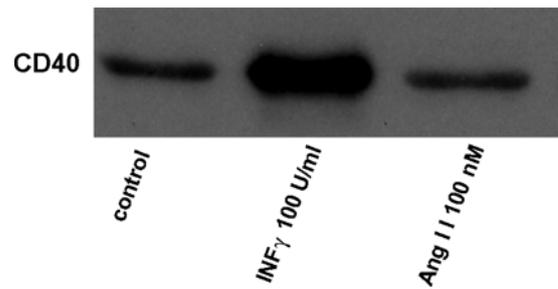
Human vascular SMC were plated on gelatin-coated 96-well tissue culture dishes (CoStar, Cambridge, MA, USA) and incubated at 37°C until confluency. They were kept in insulin-transferrin medium for 2 days before the experiment. For the experiments some of the cells were preincubated with ANG II for 24 h. Then supernatants were removed, replaced by fresh medium and cells were stimulated with recombinant CD154 for another 24 h. Afterwards supernatants were collected and assayed for IL-6 with an ELISA kit (Endogen) according to the manufacturer's instructions. The assay selectively recognizes IL-6 with a limit of detection of <1 pg/ml.

### Statistical analysis

Data are expressed as mean  $\pm$  SEM. Standard computing software (SigmaStat; Jandel Scientific) was applied. Statistical significance was accepted with  $p < 0.05$ .

## Results

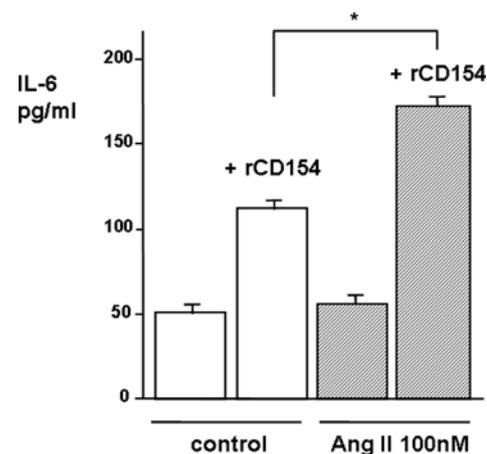
Unlike  $\gamma$ -interferon, ANG II did not increase expression of CD40 protein in human SMC (Figure 1). Compared to controls, densitometric analysis indicated a consistent fivefold increase of CD40 expression after stimulation with  $\gamma$ -interferon and a onefold increase after stimulation with ANG II (data not shown). This is in contrast to other findings of increased release of soluble mediators of inflammation such as IL-6 or monocyte chemoattractant protein-1 (MCP-1) in response to stimulation with ANG II.<sup>13,14</sup> Also, ANG II stimulation did not result in any detectable release of soluble CD40 into the tissue culture medium (data not shown). These data clearly demonstrate that ANG II does not change the amount of CD40 produced by SMC.



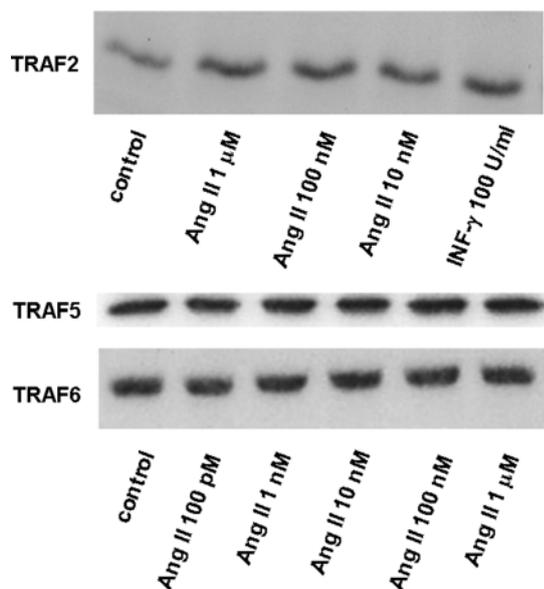
**Figure 1** ANG II does not influence CD40 expression. Human SMC were stimulated with ANG II for 24 h. CD40 protein expression was assessed by Western blot from cell lysates. ( $n = 3$ )

In costimulation experiments, we sought to determine whether angiotensin II and CD154 in concert would lead to an increase in the inflammatory response. When SMC were prestimulated with ANG II (100 nM) and thereafter stimulated with CD154, the ligand for CD40, the release of IL-6 as a marker of inflammatory activation was about 50% higher than from cells not primed with ANG II (Figure 2). Thus, prestimulation with ANG II renders SMC more susceptible to CD40/CD154 ligation. The most likely explanation for this observation is that a signaling pathway downstream of CD40 may be altered by ANG II prestimulation.

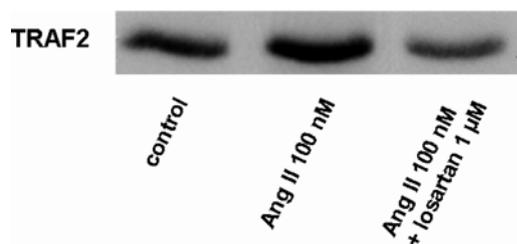
Since the adaptor proteins TNF receptor-associated factors (TRAF) are key mediators of CD40 signaling, we investigated the effect of ANG II on expression of TRAF-2, -5, and -6. TRAF-2 was upregulated by ANG II over the whole concentration range, whereas TRAF-5 and -6 expression was not influenced (Figure 3). Densitometric analysis indicated a consistent 2.0-fold, 1.8-fold and 1.6-fold increase of TRAF-2 after



**Figure 2** ANG II increases CD154 response in SMC. SMC prestimulated with ANG II for 24 h released significantly more IL-6 into the culture medium after stimulation with recombinant CD154 for an additional 24 h. IL-6 release in cell culture supernatants was assessed by ELISA. ( $n = 3$ ;  $*p < 0.05$ )



**Figure 3** ANG II increases TRAF-2 but not TRAF-5 and -6 in SMC. SMC were stimulated with ANG II for 24 h. TRAF expression was assessed by Western blot from cell lysates. ( $n = 3$ )



**Figure 4** ANG II increases TRAF-2 expression via AT1 receptors. SMC were stimulated with ANG II for 24 h in the presence or absence of losartan. TRAF-2 expression was assessed by Western blot from cell lysates. ( $n = 3$ )

stimulation with 1  $\mu$ M, 100 nM and 10 nM ANG II, respectively (data not shown). Thus, ANG II may pre-activate SMC for CD40/CD154-mediated effects by an increase of available TRAF-2. This effect was mediated by the ANG II receptor subtype 1, since losartan blocked upregulation of TRAF-2 (Figure 4). Compared with the controls, densitometric analysis indicated a consistent twofold upregulation of TRAF-2 after stimulation with ANG II (100 nM) and a 0.8-fold upregulation when losartan (1  $\mu$ M) was also present (data not shown).

In summary, these results are in keeping with a crosstalk between angiotensin II type 1 (AT1) receptors and TRAF-2.

## Discussion

The present study indicates that the vasoactive peptide ANG II promotes and augments the inflammatory activation induced by CD40/CD154 ligation in human

vascular SMC. This effect is independent of expression of CD40, the receptor for CD154, since CD40 protein was not altered by ANG II in SMC. A crosstalk between the signal transduction pathways of CD40 and ANG II downstream of CD40 appears to be responsible. This novel mechanism through which ANG II promotes IL-6 release has not previously been reported. Only extracellular interactions between the ANG II and the CD40 signaling transduction pathways have been described, which occur through induction of CD154 expression by ANG II (e.g. in platelets) with subsequent ligation to CD40 expressed for example on endothelial cells.<sup>1</sup>

Both ANG II and CD40 have been well described as activators or mediators of inflammatory responses.<sup>1</sup> Among these is the induction of IL-6 release which can be elicited by both CD40 and ANG II.<sup>15,16</sup> One common downstream signaling event for both appears to be the recruitment of nuclear factor- $\kappa$ B (NF- $\kappa$ B), which then triggers gene transcription of proteins involved in inflammation.<sup>17-19</sup> For ANG II, different activation pathways leading to NF- $\kappa$ B activation and subsequent IL-6 release appear to exist: (a) ANG II, by activating the NADPH-oxidase system, induces the generation of reactive oxygen species<sup>20</sup> which are known activators of transcription factors such as AP-1 and NF- $\kappa$ B; (b) ANG II-induced expression of CD154 with subsequent activation of the CD40 signaling cascade<sup>1</sup>; and (c) activation of the CD40 signaling pathway on the cytoplasmic level as documented in the present study. There also appears to be a NF- $\kappa$ B-independent mechanism of ANG II-induced IL-6 release involving inflammatory p38 kinase.<sup>21</sup>

As for other members of the TNF receptor family, TRAF are important in CD40 signaling since they associate with the CD40 cytoplasmic domain and initiate downstream signaling.<sup>22,23</sup> Interestingly, our study indicates ANG II-induced expression of TRAF-2 but not of TRAF-5 and -6 in SMC. This is in keeping with other findings supporting a role of TRAF-2. In vitro experiments with CD40 mutants incapable of binding TRAF-2 demonstrated that the TRAF-2 binding site of the CD40 cytoplasmic tail is critical for NF- $\kappa$ B, as well as the upregulation of the intercellular adhesion molecule-1 gene.<sup>24</sup> Since NF- $\kappa$ B activation is essential for IL-6 production in human vascular SMC<sup>11</sup>, upregulation of TRAF-2 by ANG II is a very likely mechanism of enhanced IL-6 release after CD154 stimulation of ANG II-primed cells in our experiments. The relevance of TRAF-2 for CD40-induced cytokine production was confirmed recently by the observation that the TRAF-2 binding site in CD40 is required for CD40-stimulated IL-6 production in dendritic cells.<sup>25</sup> However, in a mouse B-cell line transfected with human CD40, IL-6 production induced by CD154 was dependent of TRAF-6 but not of TRAF-2.<sup>26</sup> This discrepancy may reflect species differences in CD40 signaling.

Today, scant data exist on the role of TRAF in vascular cells. Upregulation of TRAF-3 appears to be involved in inhibition of MCP-1 expression by shear stress in human endothelial cells.<sup>27</sup> In rabbit endothelial cells, low shear stress facilitated the association of TNF receptor-1 with TRAF-2 whereas normal shear stress prevented this.<sup>28</sup> Since subsequent stimulation of endothelial cells exposed to low shear stress with TNF $\alpha$  enhanced expression of the adhesion molecule VCAM-1, a phenomenon in early atherogenesis, physiological shear stress may be atheroprotective through inhibition of the interaction of TNF receptors with TRAF-2.

The present data extend previous work by us and others that demonstrated ANG II to be a potent inducer of inflammation by direct activation of vascular SMC.<sup>13,14,29</sup> To our knowledge this is the first study to show that ANG II can cause an inflammatory response in SMC indirectly by activating crosstalk to the CD40 signaling pathway on the cytoplasmic level downstream of CD40. In addition, our experimental data can in part explain the beneficial effects of ACE inhibitors and AT1 receptor blockers on atherosclerotic disease. It is now well established that these drugs exert effects on the vasculature beyond blood pressure lowering (pleiotropic effects). One of these effects is the suppression of soluble mediators of inflammation in patients treated with ACE inhibitors or AT1 receptor blockers for hypertension.<sup>30–32</sup> Whether they also interfere with the CD40/CD154 system in humans remains to be determined.

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