Angiotensin II modulates CD40 expression in vascular smooth muscle cells

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ABSTRACT

The signalling pathway CD40/CD40L (CD40 ligand) plays an important role in atherosclerotic plaque formation and rupture. AngII (angiotensin II), which induces oxidative stress and inflammation, is also implicated in the progression of atherosclerosis. In the present study, we tested the hypothesis that AngII increases CD40/CD40L activity in vascular cells and that ROS (reactive oxygen species) are part of the signalling cascade that controls CD40/CD40L expression. Human CASMCs (coronary artery smooth muscle cells) in culture exposed to IL (interleukin)-1β or TNF-α (tumour necrosis factor-α) had increased superoxide generation and enhanced CD40 expression, detected by EPR (electron paramagnetic resonance) and immunoblotting respectively. Both phenomena were abolished by previous incubation with membrane-permeant antioxidants or cell transfection with p22phox antisense. AngII (50–200 nmol/l) induced an early and sustained increase in CD40 mRNA and protein expression in CASMCs, which was blocked by treatment with antioxidants. Increased CD40 expression led to enhanced activity of the pathway, as AngII-treated cells stimulated with recombinant CD40L released higher amounts of IL-8 and had increased COX-2 (cyclo-oxygenase-2) expression. We conclude that AngII stimulation of vascular cells leads to a ROS-dependent increase in CD40/CD40L signalling pathway activity. This phenomenon may be an important mechanism modulating the arterial injury observed in atherosclerosis-related vasculopathy.

INTRODUCTION

AngII (angiotensin II) is the primary effector molecule of the RAS (renin–angiotensin system). It is an octapeptide that may affect physiological processes in cells from virtually all organs, acting as a hormone as well as a local paracrine and autocrine signalling molecule [1]. The relevance of AngII is established better in the cardiovascular system, where it is crucial for homoeostasis maintenance in VSMCs (vascular smooth muscle cells), endothelial cells and cardiac fibroblasts.

AngII signalling in vascular and cardiac cells includes ROS (reactive oxygen species) production mediated by NADPH oxidase activation. Although ROS production is important in normal vascular function, they are widely implicated in inflammation, altered vascular reactivity,
growth, migration and fibrosis, all of which combine to ultimately cause cardiovascular diseases [1].

A cardinal feature appears to be the ability of AngII to induce vascular inflammation [2]. Binding of AngII to AT1 receptors (AngII type 1 receptors) triggers a pro-inflammatory response in monocytes, macrophages, VSMCs and endothelial cells through NF-κB (nuclear factor κB) activation [3–5]. NF-κB induces the production of cell adhesion molecules such as VCAM-1 (vascular cell adhesion molecule-1), ICAM-1 (intercellular adhesion molecule-1) and E-selectin, and chemokines, including MCP-1 (monocyte chemotactic protein-1), IL (interleukin)-6 and IL-8 [1, 6–8].

Noteworthy is the fact that ACE (angiotensin-converting enzyme) inhibitors or ARBs (AngII receptor blockers) have been described to reduce cardiovascular risk, both in experimental [9] and clinical studies [10]. This effect may be related to blunting of the pro-inflammatory effects of AngII in the vessel wall.

The signalling pathways and subcellular mechanisms whereby AngII mediates its physiological and pathophysiological effects in the vessel wall are not yet completely understood. However, experimental findings indicate that the production of ROS and activation of redox-dependent signalling cascades are integral to AngII-induced events [11]. Redox signalling mediated mainly by superoxide and H2O2 is described in all vascular cells, and appears to be relevant to the development of diseases such as hypertension and atherosclerosis. The major source of ROS in the vascular wall are non-phagocytic NADPH oxidases [12], which are regulated by vasoactive agents [AngII, ET-1 (endothelin-1), thrombin and serotonin], cytokines [IL-1 and TNF-α (tumour necrosis factor-α)], growth factors [PDGF (platelet-derived growth factor), IGF-1 (insulin-like growth factor-1) and EGF (epidermal growth factor)] and mechanical forces (cyclic stretch, and laminar and oscillatory shear stress) [13].

The membrane receptor CD40 and its ligand, CD40L, represent one of the most important inflammatory mediators in the formation and development of atherosclerotic plaques [14]. Vascular cell activation, via CD40/CD40L interaction, induces expression of adhesion molecules, secretion of pro-inflammatory cytokines, MMPs (matrix metalloproteinases), tissue factor and chemokines, all relevant processes in atherogenesis [15, 16]. More interesting, from a clinical point of view, is the fact that CD40/CD40L signalling may activate processes that culminate in plaque instability and rupture, triggering acute coronary syndromes [16–18]. Furthermore, elevated plasma levels of sCD40L (soluble CD40L) were found in patients with unstable angina but not in control subjects [19, 20]. Elevated sCD40L is also a marker in patients with acute coronary syndromes at heightened risk of death and recurrent myocardial infarction, independent of other predictive variables [21].

It is interesting to note that CD40/CD40L expression in different cell types can be up-regulated by enhanced ROS generation caused, for example, by UV radiation [22], growth factors [23], phorbol esters [24] or cytokines [25]. The occurrence of similar phenomena and its relevance in vessels has not been investigated.

Therefore, as AngII and CD40/CD40L are two of the most important pathways in vascular pathophysiology, the main aim of the present study was to determine whether there were interactions between these two pathways. More specifically, we tested the hypothesis that AngII increases CD40/CD40L activity in vascular cells and that ROS are part of the signalling cascade that controls CD40/CD40L expression. Initially, we determined whether CD40 or CD40L expression might be redox-mediated in human CASMCs (coronary artery smooth muscle cells). Secondly, considering that AngII induces ROS generation in vascular cells, we sought to determine whether AngII was capable of increasing CD40/CD40L activity in CASMCs in culture.

MATERIALS AND METHODS

Cell culture
CASMCS were purchased from Clonetics. Cells were grown in Dulbecco's modified medium containing supplements, according to the supplier's instructions, and were used after reaching 80–90 % confluence, between passages 6 and 12.

ROS generation and CD40/CD40L expression in CASMCs
Initially, we investigated whether cytokine exposure induced increased ROS generation in CASMCs and its relationship with CD40/CD40L expression. Cells were treated for 12 h with human recombinant IL-1β (10 ng/ml) or TNF-α (5 ng/ml), both obtained from Calbiochem. After incubation, cells were collected to determine ROS generation or CD40/CD40L expression, as described below.

In some experiments, 6 h before exposure to cytokines, cells were treated with the antioxidant NAC (N-acetylcysteine; 1 mmol/l) or the flavoprotein inhibitor DPI (diphenyleneiodonium; 10 μmol/l) in order to blunt intracellular ROS.

In another set of experiments, CASMCs were transfected with phosphorothioate-modified oligonucleotides against the p22phox subunit of NADPH oxidase [26]. The oligonucleotides were labelled with fluorescein and transiently transfected into CASMCs by liposomes using the Superfect Transfection Reagent kit (Qiagen), following the manufacturer's instructions. At 6 h after transfection, cells were observed under fluorescent light. Transfection efficiency was determined to be 50 % on the basis of the number of cells that had intracellular fluorescence.
divided by the total cell number. Transfected cells were then exposed to IL-1β or TNF-α for an additional 12 h, harvested and EPR (electron paramagnetic resonance) measurements or immunoblotting were performed.

Treatments described above were not toxic to CASMCs, as no change was observed in cell viability assays. Cell viability was determined by the conversion of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] into formazan utilizing NADH and NADPH pyridine nucleotide cofactors. MTT was added to a final concentration of 0.5 mg/ml, incubated for 4 h and solubilized for 24 h at 37 °C. Absorbance was read using a Tecan Genius plate reader at 550–690 nm.

**EPR experiments**

After the treatments described above, superoxide detection was performed by EPR, using the spin trap DMPO (5,5-dimethylpyrroline-N-oxide; Dojindo Laboratories). A suspension containing 1 x 10⁶ cells was incubated with the spin trap DMPO (50 mmol/l) at 37 °C for 30 min, after which cells were transferred to a quartz flat cell and EPR spectra were acquired at room temperature (23 °C) with a Bruker ER 300 spectrometer. The spectrometer operated at X-band with a TM110 cavity using a modulation frequency of 100 kHz, modulation amplitude of 0.5 G, microwave power of 20 mW and a microwave frequency of 9.78 GHz, as described previously [27]. A total of ten serial 60-s acquisitions were accumulated to obtain the final spectrum. Incubation of the buffer plus the spin trap alone at 37 °C for 30 min provided no EPR signal. In some experiments, cells were incubated previously with the membrane-permeant SODm (superoxide dismutase mimetic) M40403 (MetaPhore Pharmaceuticals) for 30 min before and during incubation with the spin trap. Spin adducts were quantified using specific procedures as described elsewhere [27].

**Western blotting and immunoprecipitation assays**

After the treatments described above, CD40 and CD40L were detected by immunoblotting and immunoprecipitation. CASMC-extracted proteins were separated by SDS/PAGE, blotted on to nitrocellulose membranes and exposed to monoclonal antibodies against CD40 or CD40L (BD Pharmingen). As a loading control, blots were stripped and exposed to α-actin antibody.

For immunoprecipitation analyses, cell extracts were incubated with the primary monoclonal antibody against CD40 or CD40L (BD Pharmingen) and precipitated by agarose–Protein G, before immunoblotting was performed.

In another set of experiments, designed to confirm the role of ROS in CD40/CD40L expression, we initially analysed superoxide generation by CASMCs in response to cytokines.

After the treatments described above, superoxide detection by EPR was performed by incubating the buffer plus the spin trap alone at 37 °C for 30 min provided no EPR signal. In some experiments, cells were incubated previously with the membrane-permeant SODm (superoxide dismutase mimetic) M40403 (MetaPhore Pharmaceuticals) for 30 min before and during incubation with the spin trap. Spin adducts were quantified using specific procedures as described elsewhere [27].

**CD40/CD40L activity assay**

After stimulation with AngII for 12 h, CASMCs were exposed to 1 mg/ml rhCD40L (recombinant human trimeric CD40L; Peprotech). After 4 h, the supernatant was collected and IL-8 concentrations were measured by ELISA (R&D Systems), following the manufacturer’s instructions. Attached cells were harvested and the expression of COX-2 (cyclo-oxygenase-2) was detected by immunoprecipitation (using a monoclonal antibody from BD Pharmingen), using the protocol as described above.

**Statistical analysis**

All results are expressed as means ± S.E.M. Comparisons among groups were performed by Student’s t test or one-way ANOVA, with a Tukey–Kramer test for post-hoc analysis. Values of P < 0.05 were considered statistically significant. The Primer of Biostatistics computer program was used (version 3.01).

**RESULTS**

**Superoxide detection by EPR**

In order to determine whether ROS act as second messengers for CD40 expression, we initially analysed superoxide generation by CASMCs in response to cytokines.
Superoxide generation by CASMCs

Increased CD40 expression induced by cytokines

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human CASMCs were exposed to IL-1β (DMPO-hydroxyl) adduct was detected by EPR when incubated with the spin trap DMPO (50 mmol/l). Exposure of these cells to IL-1β or TNF-α (left-hand panel) triggered the appearance of a stronger DMPO-OH adduct. These adducts disappeared when cells had been treated previously with a membrane-permeant mSOD (results not shown), suggesting that they derived from decay of a DMPO-superoxide adduct. Transfection of CASMCs with antisense against the p22phox subunit of the vascular oxidase did not affect the basal superoxide production (right-hand panel), but blunted the increase in superoxide generation observed after exposure of these cells to IL-1β or TNF-α (right-hand panels).

CASMCs were exposed to the pro-inflammatory cytokines IL-1β or TNF-α and superoxide generation was detected by EPR, using the spin trap DMPO.

A quartet signal, characteristic of a DMPO-OH (DMPO-hydroxyl) adduct was detected by EPR when human CASMCs were exposed to IL-1β or TNF-α (Figure 1). Using a specific computational method [27], the rate of superoxide generation by these cells was calculated to be 0.18 ± 0.03 and 0.16 ± 0.04 nmol·mg of protein⁻¹·min⁻¹ after exposure to IL-1β or TNF-α respectively. These values represent 167 ± 25 and 151 ± 14 % of those obtained from cells under basal conditions. The DMPO-OH signals were derived from superoxide radicals trapped by DMPO, as they were completely abolished by previous incubation with the membrane-permeant mSOD M40403.

Superoxide was derived from a p22phox-containing oxidase, as transfection of CASMCs with p22phox antisense oligonucleotides completely abolished IL-1β- or TNF-α-induced superoxide generation (Figure 1). Antisense transfection did not affect the basal generation of superoxide. Transfection with scrambled oligonucleotides did not affect either basal or cytokine-induced superoxide generation.

These results indicate that human CASMCs in culture have an increase in superoxide generation, derived from a p22phox-containing oxidase, when exposed to cytokines such as IL-1β or TNF-α.

Modulation of cytokine-induced CD40/CD40L up-regulation by antioxidants

Stimulation of CASMCs with IL-1β or TNF-α increased CD40 expression by 262 ± 18 and 345 ± 11 % respectively, a finding similar to other studies [25]. As these cytokines were shown above to increase superoxide generation in CASMCs in culture, experiments were performed to determine whether cytokine-induced up-regulation of CD40/CD40L expression was dependent on ROS generation. Previous treatment of these cells with the antioxidant NAC (1 mmol/l) or the flavoenzyme inhibitor DPI (10 μmol/l) did not affect the basal expression of CD40, but decreased its up-regulation induced by cytokines. *P < 0.05 compared with no stimulation; † P < 0.05 compared with control+IL-1β; ‡ P < 0.05 compared with control+TNF-α.

Figure 1 Superoxide generation by CASMCs

Non-stimulated CASMCs generated a small DMPO-OH adduct (Basal; left-hand panel) when incubated with the spin trap DMPO (50 mmol/l). Exposure of these cells to IL-1β or TNF-α (left-hand panel) triggered the appearance of a stronger DMPO-OH adduct. These adducts disappeared when cells had been treated previously with a membrane-permeant mSOD (results not shown), suggesting that they derived from decay of a DMPO-superoxide adduct. Transfection of CASMCs with antisense against the p22phox subunit of the vascular oxidase did not affect the basal superoxide production (right-hand panel), but blunted the increase in superoxide generation observed after exposure of these cells to IL-1β or TNF-α (right-hand panels).

Figure 2 Increased CD40 expression induced by cytokines is blocked by antioxidants

Upper panel, densitometric quantitative assessment of CD40 expression, detected by immunoblotting. Values are means ± S.E.M. of at least four experiments in each group. Lower panel, a representative CD40 immunoblot. Exposure of CASMCs to the pro-inflammatory cytokines IL-1β or TNF-α triggered an increased expression of CD40. Previous treatment with antioxidant NAC (1 mmol/l) or the flavoenzyme inhibitor DPI (10 μmol/l) did not affect the basal expression of CD40, but decreased its up-regulation induced by cytokines. *P < 0.05 compared with no stimulation; † P < 0.05 compared with control+IL-1β; ‡ P < 0.05 compared with control+TNF-α.
Angiotensin II and CD40 expression

Angiotensin II and CD40 expression

Figure 3 CD40 expression is decreased in cells transfected with antisense against the p22phox subunit of the vascular NADPH oxidase

Upper panel, densitometric quantitative assessment of CD40 expression detected by immunoprecipitation. Values are means ± S.E.M. of at least four experiments in each group. Lower panel, a representative CD40 immunoblot. CD40 expression in scrambled antisense transfected cells was low, but increased when cells were exposed to IL-1β or TNF-α. Cells transfected with an antisense against the p22phox subunit of the vascular NADPH oxidase, which inactivated the oxidase, did not have any increase in CD40 expression induced by cytokines. ∗P < 0.05 compared with the control/scrambled DNA. IP, immunoprecipitated.

AngII increases CD40 expression in CASMCs

In view of the results above and the fact that AngII exerts most of its effects in VSMCs by enhancing intracellular ROS generation [11], we investigated CD40 expression in AngII-stimulated cells. At 2 h after CASMCs were treated with AngII, there was a rapid increase in CD40 mRNA, which remained elevated for at least 6 h (Figure 5A). This effect of AngII on CD40 mRNA was concentration-dependent in the range of 50–200 nmol/l (Figure 5B).

Human CASMCs treated with AngII for 12 h also had increased CD40 protein expression, as detected by immunoblotting (Figure 6). This up-regulation was dependent on intracellular ROS generation, as it was blunted by treatment with the antioxidant NAC. CD40L mRNA and protein were not affected by AngII exposure (results not shown).

We also determined whether increased CD40 expression translated into enhanced activity of this signalling pathway. CASMCs exposed to rhCD40L (1 mg/ml) for 4 h had increased IL-8 production and COX-2 expression (Figure 7). In cells treated previously with AngII (200 nmol/l), both phenomena were more evident, suggesting that higher CD40 expression in these cells represents a more active and functional CD40/CD40L signalling pathway.

DISCUSSION

In the present study, we have shown that CD40 expression and activity is up-regulated by AngII in human CASMCs in culture. We have also demonstrated that ROS, derived from a p22phox-containing oxidase, act as second messengers in CD40 up-regulation induced by AngII or by the pro-inflammatory cytokines IL-1β and TNF-α. As CD40/CD40L is a pivotal signalling pathway in atherosclerotic plaque development and rupture, these findings provide a new mechanism linking AngII, redox signalling and the inflammatory process characteristic of atherosclerosis.

AngII and CD40L trigger pro-inflammatory processes in the vascular wall when bound to their receptors, AT1 and CD40 respectively [2,16]. As AngII is known to induce increased ROS production in target cells

Figure 4 Expression of CD40, but not CD40L, is increased by exogenously applied H2O2

Upper panels, relative expression of proteins after normalization to α-actin expression. Values are means ± S.E.M. of at least three experiments in each group. Lower panels, representative CD40 and CD40L immunoblots. In all experiments, CD40L was detected as a two-band pattern. CASMCs have a low expression of CD40 under basal conditions; however, H2O2 exposure (100 μmol/l) triggered a marked increase in CD40 expression (left-hand panel). CD40L expression was measurable under basal conditions, but it was not affected when CASMCs were exposed to H2O2 (right-hand panel). ∗P < 0.05 compared with no stimulation.
and some stimuli recognized as CD40 up-regulators are identified to affect redox signalling, we hypothesized that a cross-talk between AngII and CD40/CD40L signalling pathways could occur.

To determine whether ROS act as second messengers in CASMCs, we quantified superoxide production by these cells. We used EPR with the spin trap DMPO and a specific quantification method developed in our laboratory [27]. The high specificity of this methodology to detect free radicals allows the measurement of very small differences. CASMCs in culture produced negligible amounts of superoxide; however, when exposed to TNF-α or IL-1β, a small, but significant, increase in superoxide generation was triggered.

Furthermore, we have demonstrated that increased superoxide generation was responsible, at least in part, for the up-regulation of CD40 expression in these cells. Oxidases from the Nox family, which contain a p22phox subunit, appear to be the main source of superoxide in CASMCs, as described in other vascular cells [12,29,30]. As no specific pharmacological inhibitor(s) of oxidase(s) is (are) known, different approaches were used to determine the role of oxidase-derived ROS in CD40 expression. We blunted ROS availability by using a non-specific oxidase inhibitor (DPI), a ROS scavenger (NAC) and a p22phox antisense that inactivates enzyme. All three approaches decreased superoxide generation by CASMCs and, concomitantly, CD40 expression. We did not investigate the precise characterization of the Nox isoform involved in this phenomenon because it was beyond the objective of the present study.

Increased CD40 expression in response to IL-1β and TNF-α has been reported previously in VSMCs [25]. We expanded these findings to show that ROS are required for cytokine-induced CD40 up-regulation. It is necessary to note that the mechanisms underlying CD40 expression appear to involve more than one signalling pathway. In rat VSMCs, TNF-α induces CD40 expression through the transcription factor NF-κB, whereas IFN-γ (interferon-γ) activates STAT-1 (signal transducer and activator of transcription-1) [31]. Cross-talk appears to exist between these signalling cascades, as IFN-γ-induced CD40 expression also requires TNF-α production and NF-κB activation [32]. In our present study, modulating
Exposure to AngII increases CD40L-induced expression of pro-inflammatory molecules

After treatment with AngII (200 nmol/l) for 12 h, CASMCs were exposed to rhCD40L to investigate IL-8 (A) and COX-2 (B) expression, molecules dependent on CD40/CD40L signalling. (A) IL-8 levels in the cell culture supernatant were determined by ELISA. Values are means ± S.E.M. of at least three experiments in each group. (B) Densitometric quantitative assessment of COX2 expression detected by immunoblotting. Values are means ± S.E.M. of at least three experiments in each group. Lower panel, a representative COX-2 immunoblot. Cells treated with AngII had an increased production of IL-8, as well as an up-regulation of COX2 expression. These results show that AngII-induced increased expression of CD40 makes the CD40/CD40L signalling pathway more active. *P < 0.05 compared with control rhCD40L; and **P < 0.05 compared with AngII.

ROS generation in CASMCs only partially inhibited the cytokine-induced CD40 up-regulation, suggesting that this phenomenon is controlled by, but not exclusively dependent on, redox signalling, confirming further the complexity of the regulation of CD40 expression.

These findings were confirmed by exposure of CASMCs to H2O2. H2O2 is suggested to be the main ROS involved in redox signalling, due to its lower reactivity and higher membrane permeability compared with other ROS[33]. Interestingly, exposure of CASMCs to a single bolus of H2O2 was sufficient to increase CD40 expression, suggesting that a mechanism exists that is quickly switched on by oxidative stress leading to the up-regulation of this membrane receptor. This effect was not detailed in the present study; however, it has been shown previously that transcription factors such as NF-κB can be activated by a transient burst of ROS[34,35].

On the other hand, CD40L expression was not affected either by H2O2 exposure or by antioxidant treatment when cells were stimulated with IL-1β and TNF-α, suggesting that different signalling pathways control CD40 and CD40L expression. In fact, CD40 expression is controlled by transcription factors NF-κB and STAT-1[32,36,37], whereas CD40L expression is regulated by the calcineurin-dependent transcription factor NF-AT (nuclear factor of activated T-cells)[38]. Although several studies suggest NF-κB activation by ROS[35,39], DNA-binding activity of NF-AT was reported to be abolished by oxidative stress[40]. Distinct control mechanisms for CD40 and CD40L expression can be interpreted as a redundant mechanism to control the function of the dyad CD40/CD40L, as unrequired activation of this signalling pathway can have deleterious effects on the coagulation system[18] or in vascular integrity[17].

Given the experimental evidence of ROS acting as second messengers in CD40 expression, we investigated whether AngII modulates CD40 expression. Intracellular superoxide generation triggered by AngII binding to AT1 receptors is widely acknowledged in the literature[11]. In the present study, we have shown that exposure of CASMCs to AngII rapidly triggers CD40 mRNA transcription, leading to an increase in CD40 protein expression. This phenomenon is dependent on intracellular ROS generation, as it was blocked by NAC. Although pharmacological experiments were not performed, it is reasonable to suppose that AT1 is the receptor involved in this phenomenon, because AngII-dependent superoxide generation is triggered by signalling through this receptor.

AngII-stimulated CD40 expression was not detected by other authors[41]; however, they used smooth muscle cells from human saphenous veins, whereas we used cells from coronary arteries. Distinct vascular beds could, therefore, explain the differences observed in these studies.

AngII-induced increased CD40 expression appears to be biologically relevant, as exposure of AngII-treated cells to CD40L resulted in enhanced activity of this signalling pathway, as shown by the augmented IL-8 secretion and COX-2 expression. Both effects are significant in atherosclerotic plaque formation, development and rupture[42,43]. Consistent with the results obtained with cytokine stimulation, AngII did not affect CD40L expression, re-inforcing the suggestion that different mechanisms regulate the expression of the two components of this signalling pathway in smooth muscle cells.

In summary, the present study shows that CD40 expression and activity are up-regulated by AngII, in a manner dependent on ROS generation by a p22phox-containing oxidase. Cellular CD40L expression is subjected to a distinct control mechanism. These findings might be important in explaining some inflammatory vascular complications observed in patients with cardiovascular disease.
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Received 14 May 2008/17 July 2008; accepted 11 September 2008
Published as Immediate Publication 11 September 2008, doi:10.1042/CS20080155