Direct stimulation of angiotensin II type 2 receptor reduces nitric oxide production in lipopolysaccharide treated mouse macrophages

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ABSTRACT
The angiotensin II type 2 receptor (AT2) is upregulated after tissue damage and mediates protective functions in the renin-angiotensin-aldosterone system (RAAS). One of these is to inhibit inducible nitric oxide synthase (iNOS) in activated macrophages. In the present study, we assessed the effect of AT2 receptor ligands on nitric oxide production in murine macrophages as a potential assay to determine the functional activity of an AT2 receptor ligand. Mouse macrophage J744.2 and RAW264.7 were cultivated in lipopolysaccharide (LPS) to induce M1 differentiation and increase iNOS expression. Using Griess reagent and spectrophotometric analysis, the nitric oxide levels were determined, while employing Western blot and immunocytochemistry to determine basal protein expression.

Using the first reported selective non-peptide AT2 receptor agonist, compound C21, we conclude that activation of AT2 receptor reduces nitric oxide production in M1 macrophages. Furthermore, the AT2 receptor selective ligand compound C38, a regioisomer of C21, reported as a selective AT2 receptor antagonist exhibits a similar effect on nitric oxide production. Thus, we propose C38 acts as a partial agonist in the macrophage system. Monitoring nitric oxide attenuation in M1 J744.1 and RAW264.7 macrophages provides a new method for characterizing functional activity of AT2 receptor ligands, foreseen to be valuable in future drug discovery programs.

1. Introduction
The octa-peptide angiotensin II (AngII) is the major effector of the renin-angiotensin-aldosterone system (RAAS). RAAS is well known for its role in blood pressure regulation and fluid-electrolyte balance, an effect exerted by the AngII type 1 receptor (AT1) (Raizada et al., 1993). AngII also binds to the AngII type 2 receptor (AT2), identified in the late 1980s (Chiu et al., 1989; Whitebread et al., 1989). In adults, the AT2 receptor is mainly expressed in specific tissues such as uterus, adrenal gland, smooth muscle, heart, and kidney (De Gasparo et al., 2000; The Human Protein Atlas, 2018). During tissue damage the AT2 receptor is upregulated (Altarche-Xifro et al., 2009; Busche et al., 2009; Gallinat et al., 1998; Li et al., 2005; Nakajima et al., 1995; Nio et al., 1995), and it has been shown to inhibit cell proliferation, cause vasodilatation, exhibit neuronal protective and regenerative properties, be involved in apoptosis, and modulate inflammation (Lu et al., 2004; Ruiz-Ortega et al., 2000; Steckelings et al., 2010; Sumners et al., 2015; Suzuki et al., 2003; Uhal et al., 1998; Unger et al., 2015; Wang et al., 1998; Yamada et al., 1996). In recent years, the AT2 receptor has emerged as a promising new drug target (Foulquier et al., 2013; Padia and Carey, 2013; Unger et al., 2015).

The first selective non-peptide agonist of the AT2 receptor, compound C21 (Wan et al., 2004) (Fig. 1) has been extensively studied in vivo (Hallberg M. et al., 2018; Larhed et al., 2016) and recently entered phase II clinical trials for the indication idiopathic pulmonary fibrosis (Sumners et al., 2019). The AT2 receptor antagonist EMA401 (Fig. 1), has been evaluated clinically as a potential treatment for neuropathic pain (Smith et al., 2013; Rice et al., 2014). These varied clinical indications, related to the ligands’ functional activity, highlight the importance of determining the functional response of ligands binding to the AT2 receptor.

The agonistic property of C21 was initially shown in an in vivo...
bicarbonate secretion assay in rat duodenum (Johansson et al., 2001; Wan et al., 2004). Subsequently, the agonistic function of C21 was confirmed using a neurite outgrowth assay in NG108-15 cells (Buisson et al., 1992; Gendron et al., 2003; Laflamme et al., 1996; Wan et al., 2004). Due to the time- and cost-consuming nature of both these assays only a few compounds have since been evaluated (Hallberg M. et al., 2017). The AT2 receptor selective compound C38 (Murugaiah et al., 2012) (Fig. 1) exerted antagonistic properties in the neurite outgrowth assay. In contrast to C21, that stimulated the mitogen activated protein kinases p42/p44 \textbf{mapk}, C38 inhibited the AngII activation of p42/p44 \textbf{mapk} and blocked AngII-induced neurite outgrowth. Thus, C38 acted similar to the established AT2 receptor antagonist PD123,319 in this model (Blankley et al., 1991) (Fig. 1), but at a lower dose (Guimond et al., 2013; Murugaiah et al., 2012).

The focus of this article was to identify a bioassay with potential to allow a higher throughput of AT2 receptor ligands with agonistic properties. The protective function of AT2 receptor has been well established (Unger et al., 2015) and its anti-inflammatory effect have been investigated by several groups. Monocytes diffuse into damaged tissue and differentiate to form macrophages that, depending on the micro-environmental stimuli, will polarize to either phenotype M1 or M2 (Wynn et al., 2013). The former phenotype secretes inflammatory cytokines and produce free radicals (e.g. nitric oxide (NO)) by the inducible NO synthase (iNOS), and the latter anti-inflammatory cytokines. However, direct stimulation of AT2 receptor in lipopolysaccharide (LPS)-triggered monocytes has been shown to inhibit nuclear factor kappa\textbeta\text (NF-\textkappa\textbeta) and attenuate cytokines interleukin (IL)-6 and IL-10 as well as tumor necrosis factor-\alpha (TNF-\alpha) (Menk et al., 2015; Rompe et al., 2010). Thus, we hypothesized that a decreased in NO-production via the iNOS pathway in macrophages indicates agonistic properties of AT2 receptor ligands (Fig. 2). In aerobic solution NO is quickly converted into nitrite (Ignarro et al., 1993), which simplifies the bioassay for evaluate functional activity of AT2 receptor ligands in an early drug discovery program.

2. Materials and methods

2.1. Cell culture

Mouse macrophage cell line J774.2 and RAW264.7 was purchased from SigmaAldrich (Stockholm, Sweden; cat. no. 85011428 and 91062702) and the cells were cultured in Dulbecco’s Modified Eagle’s Medium with 10% fetal bovine serum, 1% non-essential amino acids, and 100 IU/ml penicillin-streptomycin. All cell culture products were purchased from Life Technologies Invitrogen AB, Lidingö, Sweden. Cells were grown in a humidified atmosphere at 5% CO2 and 37 °C.

2.2. Treatment and stimulation experiments

The macrophages were grown to a confluence near but below 95%, after which the cells were scraped loose and seeded onto 24-well cell culture plates at a density of 0.5 × 10⁶ cells/well. Before initiating the experiments, the macrophages were validated with LPS. When the cells had grown for 30 h, they were triggered to differentiate into M1 macrophages with 0–100 ng/ml of LPS (SigmaAldrich) and 0.2 mM L-arginine (SigmaAldrich) during 16 h. The nitrite concentration was determined in the supernatant using the Griess reagent described below. Selected compounds were subsequently evaluated over 16 h with simultaneous LPS-activation to M1 differentiation. Time-matched vehicle-treated samples served as control. Experiments were performed in triplicates, and each experiment was repeated twice or more. This means that the minimum number of analyses for each substance is n ≥ 6. After 16 h-treatments, the cell count and the viability were estimated using trypan blue staining and a TC20™ Automated Cell Counter (BioRad Laboratories, Hercules CA, USA).

2.3. Quantification of nitrite release

The nitrite (NO2⁻) concentration in the supernatant was measured as an indicator of NO production using Griess reagent (SigmaAldrich). After treating the cells according to the outlined protocols, 50 μl of the supernatant was mixed with 50 μl Griess reagent in a 96-well plate. A 6-
point sodium nitrate standard curve for correlation of nitrite levels was prepared with concentration ranging from 1.25 to 100 μM. The absorbance was measured at λ 540 nm using a microplate reader (Epoch, BioTek, Winooski VT, USA). All samples were run as triplicates.

2.4. Western blot analysis

After treatment, the semi-adherent J774.2 and RAW264.7 cells were scraped into 75 μl lysis buffer in protein kinase blocking solution [1% Triton X-100 and 1 M ethylenediaminetetraacetic acid (EDTA) in deionized water] containing a protein kinase inhibitor buffer [10 mM potassium phosphate buffer (pH 6.8), 10 mM 3-([3-cholamidopropyl]-dimethylammonio)-1-propanesulphonic acid (CHAPS), and protease inhibitor cocktail tablet Complete]. After extraction, the protein levels were determined using the Bradford method. The protein concentration was used as a measure of cell viability (i.e. the number of cells correspond to a certain amount of protein regardless of treatment). The proteins were separated with NuPage 10% Bis-Tris gel electrophoresis using MOPS SDS buffer (Invitrogen AB). The proteins were transferred to a polyvinyldifluoride transfer membrane (Hybond, 0.45 μm, Amersham Buckinghamshire, UK) using the iBlot dry blotting system (Invitrogen AB). The presence of AT1 and AT2 receptors, iNOS, NF-κB, and GAPDH was determined using antibodies for AT1 receptor (rabbit polyclonal Ab; Santa Cruz Biotechnology, cat. no. SC-1173), AT2 receptor (rabbit monoclonal Ab; Abcam, cat. no. 7074), was applied for 1 h at room temperature and visualization was carried out using the WesternBright Quantum reagents (K-12042, Advanta Corporation, Menlo Park CA, USA). The signal intensities of specific bands were detected and analyzed using a Chemidex XRS cooled charge-couple device camera and the Quantity One software (BioRad Laboratories, Hercules CA, USA).

2.5. Immunocytochemistry

To visualize the presence of AT1 and AT2 receptors in the macrophages, the cells were seeded onto 8-well chamber slides (BioRad Laboratories, Solna, Sweden) at a density of 0.1 × 106 cells/well. The cells were allowed to grow for 24 h after which they were gently washed with medium and treated with 100 ng/ml LPS and 0.2 mM L-arginine for 16 h. The cells were fixed with phosphate-buffered saline (PBS) containing 4% formaldehyde for 20 min. The fixed cells were then permeabilized for 10 min using 0.3% Triton X-100 in PBS, and subsequently unspecific binding of antibodies was blocked by incubating the cells for 1.5 h with 5% goat serum (Invitrogen, Rockford IL, USA; cat. no. A11001) in PBS with 0.1% Triton X-100. The cells were incubated with primary antibodies AT1 receptor (rabbit polyclonal Ab, Santa Cruz Biotechnology) or AT2 receptor (rabbit monoclonal Ab, Abcam) or 5% goat serum (as negative control) overnight at 4 °C. The incubation with secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen; cat. no. A11008) was performed at room temperature for 1.5 h. The nucleus was stained for 10 min using Hoechst nucleus stain (Sigma Aldrich). Between each step outlined above, the cells were washed with 0.1% Triton X-100 in PBS. The slides were sealed with anti-fade reagent ProLong Gold (Invitrogen) and analyzed at 40× magnification using a fluorescence microscope (Leica Microsystems, Wetzlar, Germany) equipped with DAPI and FITC filters.

2.6. Compounds evaluated

To evaluate the proposed assay, four suitable selective AT2 receptor ligands were used (Fig. 1). Using the confirmed AT2 receptor agonist C21 (Syntagon, Södertälje, Sweden) the effect of AT2 receptor activation on nitric oxide levels in cells were studied with or without the established prototype antagonist PD123,319 (SigmaAldrich) or the AT2 receptor antagonist EMA400 (AstaTech Inc., Bristol PA, USA). The clinically evaluated EM401 is the S-enantiomer of EMA400. To confirm AT2 receptor mediated the observed effect of C21, the cells were treated with or without the selective AT1 receptor antagonist Losartan (Sigma Aldrich) or compound A779 (Sigma Aldrich). A779 is an antagonist to the Ang (1–7)-ligand sensitive Mas receptor. In addition, the proposed AT2 receptor antagonist C38 was evaluated. Ligand C38 was synthesized according to previously published methods (Murugaiah et al., 2012; Wannberg et al., 2018) and evaluated with or without the prototype antagonist PD123,319 and antagonist EMA400.

2.7. Statistical analysis

Vertical lines in figures indicate standard error of mean (S.E.M.), and data is described in text as “mean ± S.E.M.”. Ordinary one-way variance analysis ANOVA and two-tailed Student’s t-test was used for comparing group mean, as indicated in figures. Statistical analysis was performed using the GraphPad Prism software (GraphPad Software, San Diego CA, USA), and statistical significance was assumed at P < 0.05. 3. Results

3.1. Cell validation and experimental set-up

The J774.2 and RAW264.7 macrophages were validated for nitrite production using LPS. Cells in a passage between 5 and 25 were confirmed to react dose-dependently to 16 h LPS stimuli (Fig. 3A and B), producing stable and repetitive nitrite levels. The calculated cell number, the cell viability, and the protein concentration in the wells remained constant after varied LPS treatment (Supplementary Table 1). Cells with passages below 5 and above 25 had a lower nitrite response after stimulation (see Supplementary Fig. 1). Fig. 3C shows the macrophages at the end of the experiment, after LPS pretreatment for 16 h, nearing complete confluence.

3.2. Expression of AT1, AT2 receptors, iNOS, and NF-κB

The basal expression of important proteins was determined. Both J774.2 and RAW264.7 macrophages expressed all the desired proteins (AT1, AT2 receptors, iNOS, and NF-κB, Fig. 3D). Using immunocytochemistry, the presence of AT1 and AT2 receptors after LPS stimulation was visualized (Fig. 3E). Although the protein levels were not measured, a notable difference is observed with a higher detection of AT1 compared to AT2 receptor. This is likely due to the efficacy of the antibody used. Although a different basal expression of these two proteins could also account for the difference. Key proteins after treatment with LPS were measured. This is likely due to the efficacy of the antibody used. Although a different basal expression of these two proteins could also account for the difference. Key proteins after treatment were evaluated by Western blot in relation to LPS-treated control. The expression of AT2 receptor was significantly up-regulated while iNOS expression was significantly down-regulated after LPS and C21-treatment. None of these receptors were changed in the macrophages after addition of PD123,319, suggesting an AT2 receptor involvement. The AT1 receptor expression was stably independent of treatment (Fig. 3F).

3.3. Effect of C21 on the nitric oxide production

Using the selective AT2 receptor agonist C21 the effect of AT2 receptor activation on NO production in J774.2 macrophages could be studied. Comparing the measured nitrite levels to a time-control on each plate, set to 100%, enabled comparison between experiments. Treating the cells with 50 ng/ml LPS and C21 at doses 1, 10, and 100 μM resulted in a linear dose-dependent attenuation of nitrite (Fig. 4A). At 1 μM of C21 the nitrite levels were reduced to 79 ± 2%, 10 μM C21 reduced to 48 ± 2% and 100 μM almost completely
Fig. 3. Dose-dependent effect of LPS on nitrite levels (indirect measure of NO release) in J774.2 (A) and RAW264.7 (B) macrophages stimulated for 16 h. RAW264.7 macrophages nearing confluence were observed through contrast microscope at 10 × magnification (C). Immuno-blot analysis of basal expression of iNOS, AT₁ and AT₂ receptors, and NF-κB, in untreated and LPS-treated (50 ng/ml) J774.2 and RAW264.7 macrophages (D), detection of GAPDH served as a loading control. Presence of AT₁ or AT₂ receptors visualized in LPS-treated RAW264.7 macrophages using immunocytochemistry (E). Expression analysis of AT₁ and AT₂ receptors, and iNOS in LPS-treated (50 ng/ml), in LPS and C21-treated (10 μM), in LPS, C21 and PD123,319-treated (10 μM) macrophages (F). Data from five independent experiments were normalized to LPS-treated (set to 100%). *P < 0.05; data is presented as mean ± S.E.M.

Fig. 4. The effect of AT₂ receptor agonist C21 (1–100 μM) on nitrite as compared to control. The J774.2 macrophages (A) and the RAW264.7 macrophages (B) were incubated with 50 ng/ml LPS and C21 at varying doses for 16 h after which the nitrite levels were measured. The effect of C21 1 μM and 10 μM could be blocked by treatment with 10 μM PD123,319 (C) or 10 μM EMA400 (D) in J774.2 cells. The effect of C21 1 μM could not be blocked by simultaneous treatment with neither the AT₁ receptor antagonist Losartan receptor nor Mas antagonist A779 in J774.2 cells (E). ***P < 0.001; **P < 0.01; *P < 0.05; ns = no significance (P > 0.05); data is presented as mean ± S.E.M., two-tailed Student’s t-test.
blocked the nitrite level to 10 ± 1%. A similar reductive effect on the nitrite levels was observed when treating RAW264.7 macrophages with varying doses of C21 (Fig. 4B). The effect of C21 in J774.2 cells could be significantly blocked by 10 μM PD123,319 at the two lowest doses tested (Fig. 4C). Simultaneous treatment with 10 μM EMA400 in J774.2 cells also had the ability to block the nitrite level at the two lowest concentrations of C21 (Fig. 4D). To confirm the effect of C21 was not linked to AT1 receptor or Mas receptor, the J774.2 macrophages were treated with 10 μM of AT1 receptor antagonist Losartan or 10 μM of Mas receptor antagonist A779 in the presence of C21 1 μM. Neither pretreatment with Losartan nor A779 could prevent the C21-triggered AT2 receptor derived attenuation of nitrite (Fig. 4E). Despite major changes in NO-production, the calculated cell number, the cell viability, and the protein concentration in the wells was the same after various treatments (Supplementary Table 1).

3.4. Effect of C38 on the nitric oxide production

The proposed AT2 receptor antagonist C38 was investigated next (Fig. 5A). Surprisingly, treating the cells with C38 reduced the nitrite levels, similar to the effect of C21, indicating an agonistic function of the ligand in this assay. At the lowest concentration of C38 (1 μM) the nitrate levels were reduced to 69 ± 5%, 10 μM of C38 reduced nitrite levels to 43 ± 4%, and 100 μM brought the levels to 14 ± 1%. Treating RAW264.7 cells with C38 resulted in a similar dose-dependent reduction of nitrite (Fig. 5B). The effect of C38 in J774.2 cells could be significantly blocked by both PD123,319 and EMA400 (10 μM of both) at the two lowest doses tested (Fig. 5C and D respectively). Despite the large shifts in NO-production, the calculated cell number, the cell viability, and the protein concentration in the wells was the same after various treatments (Supplementary Table 1).

4. Discussion

Stimulating macrophages with LPS is known to result in NO release and production of pro-inflammatory cytokines. This effect results from LPS activation of toll-like receptor 4 (TLR4), causing stimulation of NF-κB that in turn is linked to regulation of inflammatory cytokines, as well as TNF-α. It is further well established that NF-κB regulate cell expression of iNOS, which converts L-arginine to L-citrulline while producing NO. Direct stimulation of AT2 receptor has been shown to inhibit NF-κB and attenuate IL-6, IL-10, and TNF-α, while direct AT1 receptor stimulation has displayed the opposite effect and upregulated the same proteins (Dhande et al., 2015; Guo et al., 2011; Menk et al., 2015; Rompe et al., 2010).

The present study, performed in J774.2 and RAW264.7 mouse macrophages, revealed that direct AT2 receptor stimulation with the selective agonist C21 decreases NO release in LPS-activated cells. Western blot analysis confirmed that direct AT2 receptor stimulation with C21 increased the AT2 receptor levels. The simultaneously decreased iNOS expression may, at least in part, explain the reduced NO output. The present study further revealed that the proposed AT2 receptor antagonist C38 display a similar attenuating effect on NO release, indicating this ligand acts as an agonist in this assay.

This contrasts to the C21 activating AT2 receptor causing vasodilating in human aortic endothelial cells (Peluso et al., 2018), and its anti-fibrotic effect in pulmonary fibrosis, both mechanisms via eNOS resulting in NO release (Sumners et al., 2019). While the eNOS (via Akt signaling) and iNOS (via protein phosphatases inhibiting NF-κB) pathways are different, it is interesting that the selective AT2 receptor agonist C21 can have such opposing effects on NO release in different cells (Rompe et al., 2010). However, the precise cellular mechanism underlaying the anti-inflammatory effects of AT2 receptor are not fully understood. Using NO release in J774.2 and RAW264.7 macrophages,
measured with Griess reagent and spectrophotometric analysis, to test AT2 receptor ligands functional activity presents as a feasible biological assay, allowing a higher throughput of ligands than those previously presented.

The initial experiments were designed to confirm that the macrophages had differentiated to the M1 phenotype, produced NO, and that the cells in this phenotypic state express desired proteins (i.e. AT1 and AT2 receptors, NF-κB, and iNOS). Adding LPS to the medium resulted in phenotypic differentiation, giving dose-dependent NO production, and the macrophages expressed the proteins needed to perform the subsequent experiments. Cells in passage 5–25 were identified as phenotypically stable, a trend that was also observed by Taciak et al. (2018). The doses applied herein are in the micro-molar range, and although loss of viability could explain the ability of C21 and C38 to attenuate the NO production, the calculated cell number, the cell viability measurements, as well as the stable protein concentrations seen across protocols make this an unlikely explanation.

Treating the cells simultaneously with selective agonist C21 and LPS to trigger M1 phenotypic differentiation produced linear dose-response attenuation of NO. The linearity in dose-response confirm that while LPS continuously activate TLR4 and NF-κB it will be continuously expressed, which in turn results in AT1 and AT2 receptors remaining at (or near) the cell surface. The high attenuation at the highest doses of C21 (100 μM) is likely related to the surface (or near surface) expression of AT2 receptor resulting from the constant signaling from TLR4 to NF-κB. Blocking the effect of C21 with simultaneous treatment with 10 μM of PD123,319 was successful at the two lowest doses of C21 (1 and 10 μM) in J774.2 cells. The reported racemic antagonist EMA400 (10 μM), structurally related to PD123,319, could also block the effect of C21. This confirms the antagonistic character of this ligand in the J774.2 macrophages. The observed effect was confirmed to derive from AT2 receptor activation as treatment with an AT1 receptor or Mas blocker did not affect the reduced NO expression.

The proposed AT2 receptor antagonist C38 displayed similar, agonistically linked, attenuation of NO as agonist C21. The agonistic character of C38 in this assay was confirmed with simultaneous treatment with 10 μM of PD123,319 or EMA400 that resulted in a complete recovery of NO release for the two lower doses in J774.2 macrophages. It is possible that agonistic properties of C38 could not be discerned in the neurite outgrowth assay used previously due to assay limitations and thus, C38 seemingly displayed antagonistic properties. Another possible explanation could be different signaling pathways in the two assays (neurite outgrowth cf. nitrite production), resulting in varied functional activity of the same ligand between the two.

While several authors conclude that activating AT2 receptor will give an anti-inflammatory response (Dhanda et al., 2015; Menk et al., 2015; Rome et al., 2010; Sampson et al., 2016), in correlation with our presented findings, it should be noted that contrasting findings have recently been presented. C21 was recently reported to be without effect on aortic aneurysm in a mouse model of Marfan Syndrome (Bruggev et al., 2018). Moreover, in 2018 Shepherd et al. concluded that AT2 receptor activation in macrophages trigger production of reactive oxygen/nitrogen species (ROS/RNS, i.e. NO), in contrast with previous research and data presented in this article. Shepard et al. (2018) investigated the effect of the clinical candidate AT2 receptor antagonist EMA401, the S-enantiomer of EMA400 (Blankley et al., 1991; VanArten et al., 1993), and found its effect to be mediated via macrophages infiltrating the injured nerves, and not a direct interaction of EMA401 with AT2 receptor in the damaged nerves (Shepherd et al., 2018; Shepherd et al., 2018). Shepard et al. (2018) found that increased levels of ROS/RNS cause cytokine modification of the transient receptor potential ankyrin 1 (TRPA1) channel in the dorsal-root ganglia (DRG) sensory neurons, resulting in increased hypersensitivity. Blocking AT2 receptor activation reduced ROS/RNS and in turn reduced hypersensitivity (Shepherd et al., 2018). This is in contrast with our findings that AT2 receptor activation in macrophages decreases the levels of NO.
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