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Protein kinase D, ubiquitin and proteasome pathways are involved in adenosine receptor-stimulated NR4A expression in myeloid cells☆

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ABSTRACT

Adenosine is a purine nucleoside pivotal for homeostasis in cells and tissues. Stimulation of the adenosine receptors (AR) has been shown to regulate the *nuclear orphan receptor 4A* (*NR4A1-3*) family, resulting in attenuation of hyper-inflammatory responses in myeloid cells. The *NR4A1-3* orphan receptors are early immediate response genes and transcriptional regulators of cell and tissue homeostasis. The signal transduction and transcriptional mechanism(s) of how AR-stimulation promotes *NR4A* expression in myeloid cells is unknown and is the focus of this study. We confirm that adenosine and the stable analogue, 5′-N-Ethylcarboxamidoadenosine (NECA), enhance *NR4A1-3* expression in THP-1 cells. Pharmacological approaches identified that protein kinase D (PKD) mediates AR-stimulated *NR4A* expression in myeloid cells and reveals no involvement of PKA nor PKC. The role of NF-κB, a principal regulator of *NR4A* expression in myeloid cells, was examined as a possible transcriptional regulator downstream of PKD. Utilising BAY11-7082 and MG-132, inhibitors of the respective ubiquitin and proteasome pathways essential for NF-κB activation, suggested a prospective role for NF-κB, or more specifically signalling via IKKα/β. However, biological interventional studies using overexpression of IκBα in myeloid cells and MEF cells lacking IKKα and IKKβ (IKKα/β$^{-/-}$) revealed the NF-κB pathway is not utilised in mediating AR-stimulated *NR4A* expression. Thus, this study contributes mechanistic insight into how AR signalling modulates the expression of *NR4A* receptors, pivotal regulators of inflammatory responses in myeloid cells.

1. Introduction

The purine nucleoside, adenosine, plays multiple roles in physiological and pathological disease states [1]. Once accumulated, adenosine binds with varied affinities to one of four target G-protein coupled adenosine receptors (AR): A1, A2a, A2b and A3. When stimulated they then act through secondary intracellular messenger systems including cAMP and calcium [1]. The downstream kinases identified as being important for carrying out the functions of adenosine are primarily protein kinase A (PKA) and protein kinase C (PKC), along with some studies demonstrating a role for protein kinase D (PKD) [2]. Finally, beyond kinase activation, adenosine has non-genomic and genomic effects, such as regulating ion transport directly or regulating major transcription factors such as CREB and NF-κB [1,3,4].

The *nuclear receptor family 4A* (*NR4A*) receptors have emerged over the past decade as pivotal regulators of inflammatory responses [5]. Two of the main transcriptional regulators upstream of *NR4A* receptor activation are CREB and NF-κB [5–7]. AR stimulation has been shown to enhance the expression of all three *NR4A* family members [8–13]. Studies have uncovered the roles of PKA, PKC, PKD, MAPK/ERK, CREB and NF-κB in mediating stimulus- and cell-specific *NR4A* regulation [5–7,14–17]. However, few studies have examined the signalling molecules/pathways following AR-stimulation. Therefore, within this study we examined the factors

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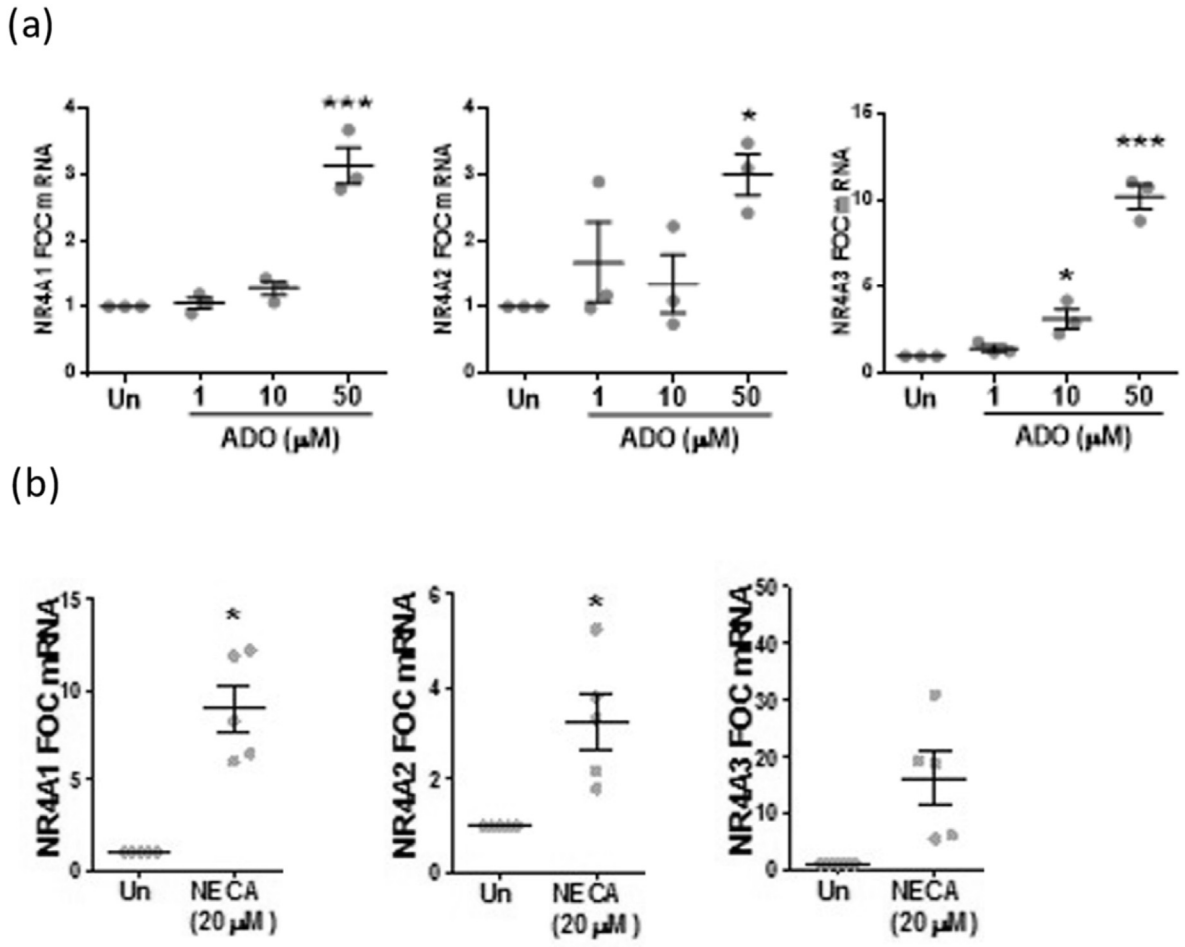

Fig. 1. Adenosine receptor stimulation enhances NR4A expression in THP-1 cells. (a, b) THP-1 cells were exposed to adenosine (ADO) (1, 10 and 50 μM) (n = 3) (a) and NECA (20 μM) (n = 5) (b) for 2 hs followed by RNA isolation. Analysis: (a, b) Following RNA isolation, qRT-PCR was performed for NR4A1-3 and the housekeeping gene GAPDH. For statistical analysis of qRT-PCR experiments, a one-way ANOVA with a Tukeys post-hoc test was used. *$p < 0.05$, ***$p < 0.001$ treatments compared to untreated control (Un).

involved in AR-stimulated *NR4A* expression at both the signalling and transcriptional levels in myeloid cell line THP-1.

Here, we showed that concentration-dependent adenosine and 5′-N-Ethylcarboxamidoadenosine (NECA), a stable adenosine analogue, control of *NR4A1-3* expression in THP-1 cells. Following this, we investigated for the first time which protein kinase was involved downstream of AR stimulation in myeloid cells. Pharmacological approaches reveal that PKD, and not PKA or PKC, is the kinase involved in AR-driven *NR4A1-3* expression in monocytes. We then assessed if a major *NR4A* regulator in myeloid cells, NF-κB, was involved in AR-driven NR4A expression. Using a combination of pharmacological and biological interventions we provide evidence that while NF-κB is not involved in AR-driven *NR4A* expression, ubiquitin and proteasome pathways are involved in AR-mediated control. Thus, this study contributes mechanistic insight into how AR signalling modulates the expression of *NR4A* receptors, pivotal regulators of inflammatory responses in myeloid cells.

2. Methods

2.1. Cell culture

Human monocyte THP-1 cells were obtained from American type culture collection (ATCC® TIB-202™) and were cultured in RPMI-1640—GlutaMax™ media (Life Technologies™). Mouse embryonic fibroblast cells lacking IKKα and IKKβ (Ikkα/β$^{-/-}$) and their wild-type controls (WT) were a generous gift from Dr Inder Verma (Salk Institute, La Jolla, CA). All media was supplemented with 10% (v/v) FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml streptomycin. All cells were cultured at 21% O_2, 5% CO_2 and maintained in a humidified tissue culture incubator at 37 °C, balanced with nitrogen. Reagents/chemicals used throughout experiments were adenosine (Sigma-Aldrich,#A9251-1G), NECA (Tocris Bioscience,#1691), BAY11-7082 (Sigma-Aldrich,#BM5556-10 MG), MG-132 (Enzo Life Sciences,#BML-P1102-0005), H-89 (Santa Cruz Biotechnology,#sc-3537), GO6983 (Tocris Bioscience,#2285), phorbol myristate acetate (PMA) (Sant Cruz Biotechnology,#sc-3576), CID-755673 (Sigma-Aldrich, #SML003-5 MG) Lipopolysaccharide (LPS) (Sigma-Aldrich, #L6529-1 MG).

2.2. Real-time quantitative reverse transcription PCR (qRT-PCR)

RNA extraction, cDNA synthesis and qRT-PCR were performed as previously described [8]. Primer sequences used are detailed in Supplemental Table 1.

2.3. Western blotting

Whole cell lysis, and western blotting for *NR4A2* (PP—N1404-00)

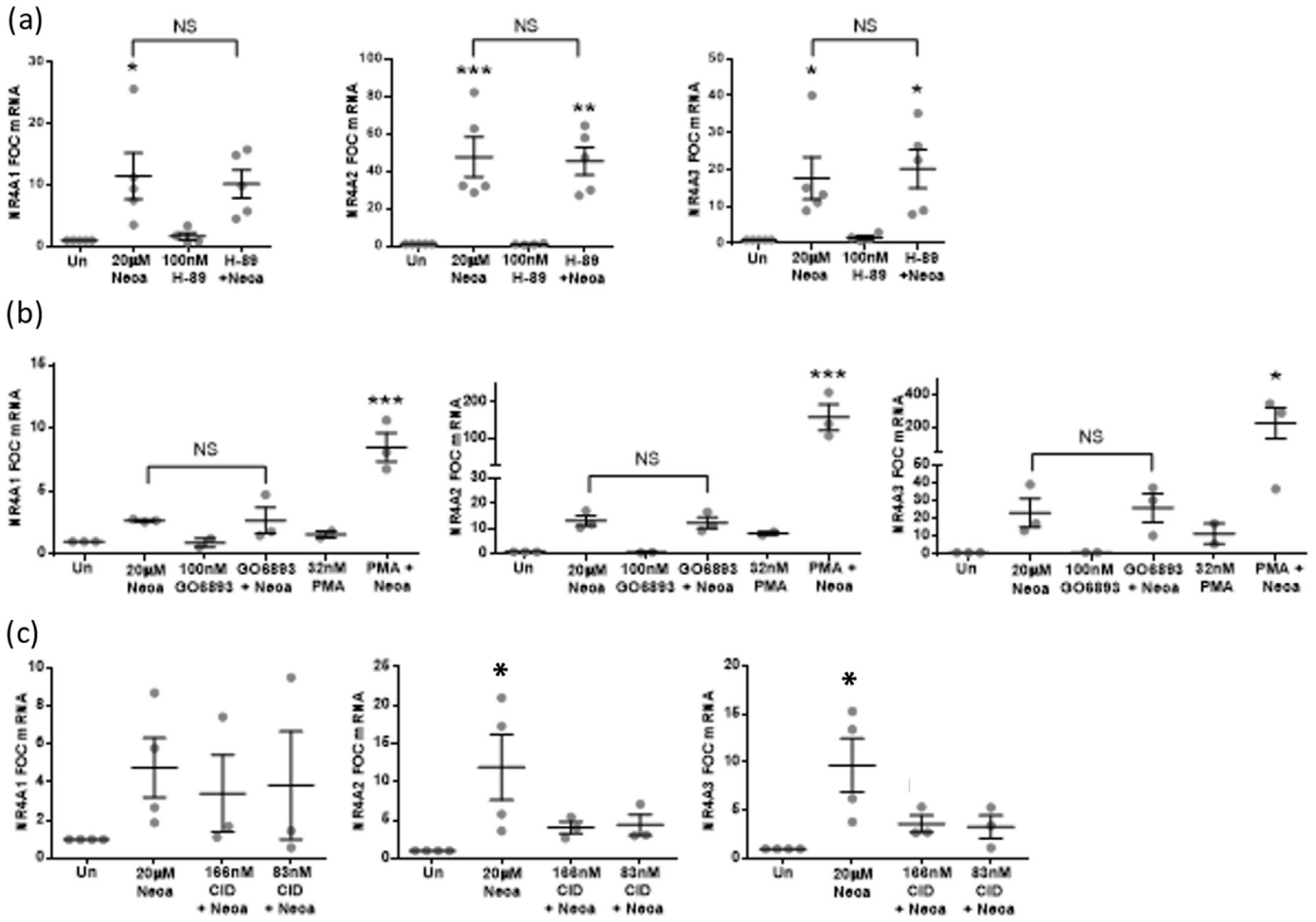

Fig. 2. Adenosine upregulation of NR4A's in THP-1 cells does requires protein kinase D. (a) THP-1 cells were exposed to NECA (20 μM) for 2.5 hs alone, H-89 dihydrochloride (100 nM) for 2.5hs alone or NECA (20 μM) for 2 hs following a 30 min pre-treatment with H-89 dihydrochloride (100 nM) followed by RNA isolation (n = 5). (b) THP-1 cells were exposed to NECA (20 μM), GO6893 (100 nM) and PMA (32 nM) (EC50 or Ki) for 2.5 hs alone or NECA (20 μM) for 2 hs following 30 min pre-treatment with GO6893 (100 nM) or PMA (32 nM) followed by RNA isolation (n = 3). (c) THP-1 cells were exposed to NECA (20 μM) for 2.5 hs alone and for 2 h after a 30 min pre-treatment with CID 755673 (83 and 166 nM), followed by RNA isolation (n = 4). Analysis: (a,b,c) Following RNA isolation, qRT-PCR was performed for targets genes NR4A1-3 and housekeeping gene GAPDH. For statistical analysis of qRT-PCR experiments, a one-way ANOVA with a Tukeys post-hoc test was used. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, treatments compared to untreated control (Un). NS=Not significant. Comparison between treatments is shown using a bar attachment.

(RnD systems) and TATA-binding protein (TBP) (Abcam, ab51841) was performed as previously described [8].

2.4. Transient transfection of THP-1 cells

THP-1 cells were transiently transfected with IκBα over-expression constructs and plasmid backbone controls using the TransIT-X2® (Madison WI, USA) protocol as per manufacturer's instructions. Briefly, transient transfections were performed in 12 well plates with 500,000 cells in each well in 900 μL of cell culture media. A master mix was then prepared for the IkappaBalpha (IκBα) plasmid (pIκBα-EGFP) and backbone control plasmid (EGFP, Clontech) consisting of Optimem:TransIT-X2:plasmid (100 μl:3 μl:1 μg), reagents mixed and allowed to stand for 20 min followed by addition to the cells. pIκBα-EGFP was a gift from Prof. Michael White [18].

2.5. Data presentation and statistical analysis

All data are presented as fold over control (FOC) ± standard error of the mean (±SEM) (minimum n = 3 independent biological replicates), and representative western blots shown. Statistical significance was performed using a one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test, or a paired Student's t-test as appropriate (Prism-6® software, GraphPad, San Diego, CA, USA) and this is specified in figure legends.

3. Results

3.1. Adenosine receptor (AR) stimulation enhances NR4A expression in THP-1 cells

Initially, we established *NR4A1-3* gene expression in THP-1 cells stimulated with adenosine or NECA. There was a concentration-dependent induction of *NR4A1-3* expression in cells treated with adenosine (1, 10 and 50 μM), and a significant increase with NECA (20 μM) for 2 hs (Fig. 1a and b). Throughout the rest of the study NECA (20 μM) was primarily used during 2 h exposures (unless stated otherwise), as it is more stable than adenosine and gave a similar pattern of *NR4A* expression in THP-1 cells. Taken together, adenosine and NECA enhance *NR4A* expression in THP-1 cells.

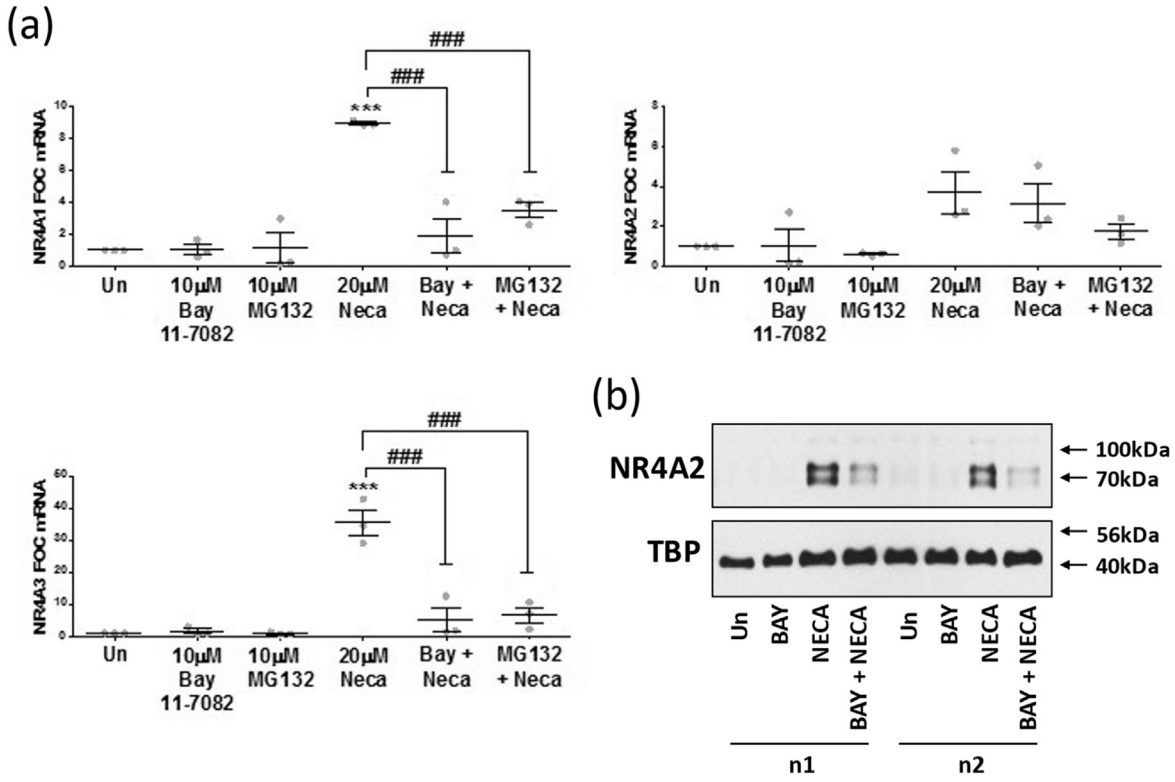

Fig. 3. Ubiquitin and proteasome pathways are involved in adenosine receptor driven NR4A expression. (a) THP-1 cells were exposed to NECA (20 μM), BAY11-7802 (10 μM) and MG-132 (10 μM) alone for 2hr or NECA (20 μM) for 2 hs following 30 min pre-treatment with BAY11-7082 (10 μM) or MG132 (10 μM), followed by RNA isolation (n = 3). (b) THP-1 cells were exposed to NECA (20 μM) and BAY 11–7802 (10 μM) alone for 4 hs or NECA (20 μM) for 4 h s following 30 min pre-treatment with BAY 11–7082 (10 μM), followed by whole cell lysis (n = 2). Analysis: (a) Following RNA isolation, qRT-PCR was performed for targets genes NR4A1-3 and housekeeping gene GAPDH. (b) Following whole cell lysis Western blot analysis was performed for NR4A2 and the housekeeping protein TATA-binding-protein (TBP). For statistical analysis, a one-way ANOVA with a Tukeys post-hoc test was used. ***$p < 0.001$ treatments compared to untreated control (Un). $^{\#\#\#}p < 0.001$ specific treatments compared shown using a bar attachment.

3.2. Protein kinase D is involved in AR-mediated NR4A expression in THP-1 cells

Next, we examined kinases known to act downstream of AR stimulation. Fig. 2a and b shows pre-treatment of THP-1 cells for 30 min with the PKA inhibitor, H-89 (100 nM) (IC_{50}-135 nM), followed by 2 hs with NECA or a pan-PKC inhibitor, GO6983 (100 nM) (IC50 = 7, 7, 6, 10, 60 and 20000 nM for PKCα, PKCβ, PKCγ, PKCδ, PKCζ and PKCμ (PKD) respectively) did not reduce NECA-enhanced *NR4A1-3* expression in THP-1 cells.

To further explore PKC signalling THP-1 cells were pre-treated for 30 min with phorbyl mysterate acetate (PMA) (32 nM) (EC_{50}-14 nM), a known PKC activator, can enhance *NR4A1-3* expression alone (2 hs incubation) and has a significant synergistic effect when combined with NECA (Fig. 2b). On further analysis, we demonstrate that PMA, in addition to PKC activation, activates PKD (also known as PKCμ). Following this, we used a specific inhibitor of PKD, CID-755673 (83 and 166 nM) (IC_{50}: 0.182, 0.280, 0.227, >10 μM at PKD1, PKD2, PKD3, PKC respectively), on cells 30 min prior to stimulation with NECA in order to establish if PKD was involved. CID-755673 at both concentrations attenuated the significant induction of *NR4A2-3* expression by NECA (Fig. 2c). In contrast, CID-755673 pre-treatment did not alter NECA-enhanced *NR4A1* expression (Fig. 2c). Taken together, PKD activity is required for AR-stimulated *NR4A2* and *NR4A3* gene regulation with no involvement in AR-mediated *NR4A1* expression in THP-1 cells.

3.3. NF-κB pathway does not play a role in AR-induced NR4A expression in THP-1 cells

We next investigated which transcription factor(s) downstream of PKD were responsible for the enhanced *NR4A* gene expression observed in THP-1 cells following AR stimulation. The two main transcriptional regulators upstream of *NR4A* genes are CREB and NF-κB. Moreover, PKD plays roles in activating both CREB and NF-κB [19–24]. During AR stimulation, when CREB is involved, it is established this is typically a PKA-mediated event [3,6]. Since we have already shown in inhibitor studies that PKA is not involved in AR-driven *NR4A* expression in THP-1 cells, CREB is also unlikely to play a role (Fig. 2a). Therefore, we set out to examine the role, if any, of NF-κB in this pathway.

The combination of inhibitors, BAY11-7082 and MG132, are useful tools to investigate a putative role for NF-κB within biological pathways. These inhibitors interfere with ubiquitin and proteasome pathways respectively, which are essential for NF-κB activation [25–28]. Pre-treatment (30 min s) separately with BAY11-7082 (10 μM) (IC_{50}: 10 μM for E2 ubiquitin (Ub) conjugating enzyme) and MG132 (10 μM) (IC_{50}: 100 nM for proteasome and 1.2 μM for calpain) inhibited NECA-induced *NR4A1* and *NR4A3* gene expression. While modest effects of both inhibitors on NECA-induced *NR4A2* gene expression in THP-1 cells were measured (Fig. 3a), BAY11-7082 (10 μM) inhibits NECA-induced *NR4A2* protein expression in THP-1 cells over 4 hs (Fig. 3b).

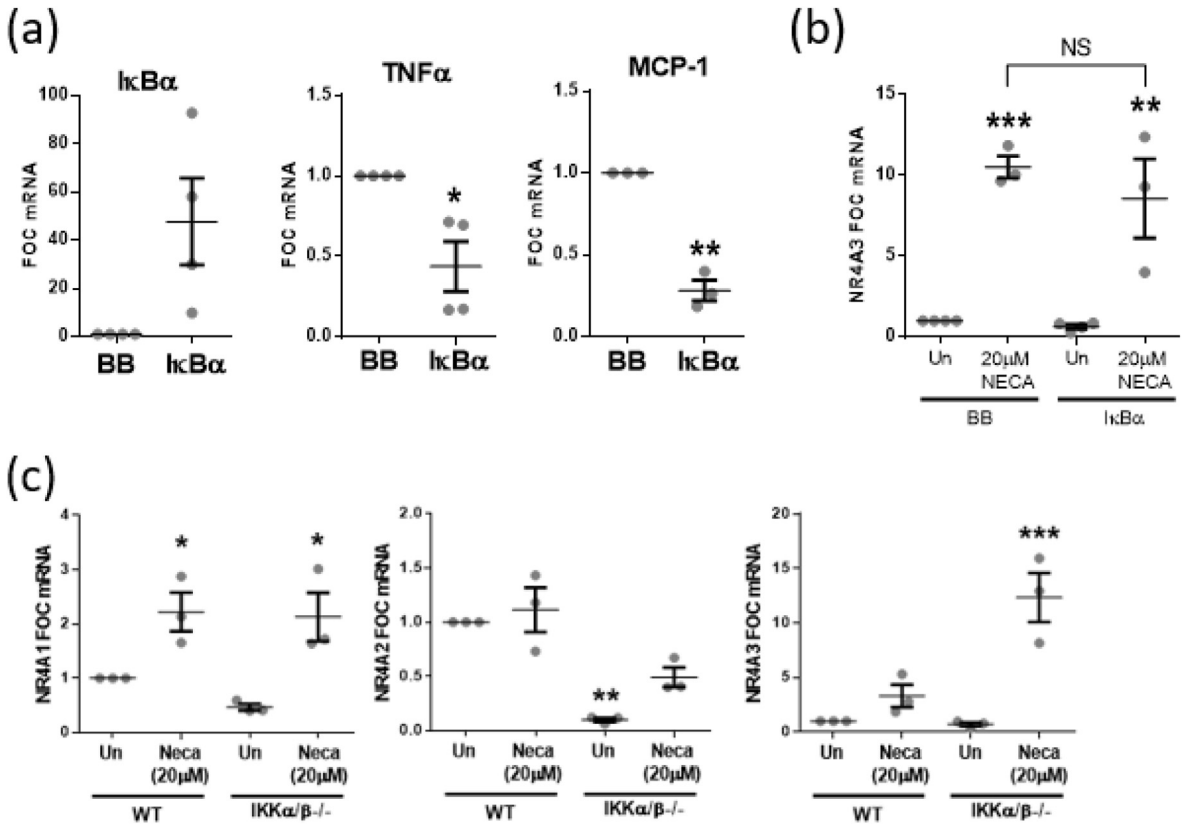

Fig. 4. NF-kB and its specific family members IKKα and IKKβ are not involved in adenosine receptor stimulated NR4A expression. (a) THP-1 cells were transfected with an IκBα plasmid and its backbone (BB) control plasmid for 48 hs followed by RNA isolation. (b) THP-1 cells were transfected with an IκBα plasmid and a backbone (BB) control plasmid for 48 hs followed by treatment with NECA (20 μM) for 2 hs, followed by RNA isolation (n = 4). (b) Wild type (WT) and IKKα/β knockout (IKKα/β−/−) mouse embryonic fibroblast (MEF) cells were exposed to (NECA (20 μM) for 2 hs followed by RNA isolation (n = 3). (c) WT and IKKα/β−/− MEF cells were exposed to LPS (1 μg/mL) for 2 h s followed by RNA isolation. Analysis: (a,b,c) Following RNA isolation, qRT-PCR was performed for targets genes IκBα, TNFα, MCP-1, NR4A1-3, TNFα and housekeeping gene GADPH. For statistical analysis of (a,b) a paired T-test was used to compare against BB untreated, and (c) a one-way ANOVA with a Tukeys Post-hoc test was used. *p < 0.05, **p < 0.01, ***p < 0.001, treatments compared to untreated BB control or untreated control (Un). NS=Not significant, specific treatments shown using a bar attachment.

Subsequently specific biological interventional approaches were utilised to confirm the role of NF-κB in this pathway. We overexpressed IκBα, an endogenous inhibitor of NF-κB activation, in THP-1 cells. Following transient transfection, we examined levels of IκBα in cells and also that of two established NF-κB target genes, TNFα and MCP-1. Increased IκBα mRNA expression was detectable in THP-1 transfected for 48 hs with the IκBα plasmid compared to backbone (BB) control (Fig. 4a). Furthermore, we observed significant attenuation of the endogenous expression of TNFα and MCP-1 following transient transfection with the IκBα plasmid compared to the BB control (Fig. 4a). This indicates an appropriate transfection efficiency, with IκBα functionally inhibiting the NF-κB pathway. Following this, we treated the IκBα expressing THP-1 cells with NECA and found that overexpression of IκBα does not prevent AR-induced expression of *NR4A3*. As shown in Fig. 4b, the most highly AR-induced *NR4A* family member, *NR4A3*, both basal and NECA-induced mRNA levels remained unchanged with co-expression of IκBα. This suggests that the inhibitors, namely BAY11-7082 and MG-132, are altering AR-stimulated *NR4A* expression independent of the NF-κB pathway downstream of IκBα.

BAY11-7082 can modify the IKK complex, which can function independently of NF-κB [25,29,30]. Additionally, IKKβ itself can be activated by PKD [19,29,30]. Therefore, we tested mouse embryonic fibroblast (MEF) cells lacking both IKKα and IKKβ (IKKα/β$^{-/-}$) to further examine the role of IKK. While we did observe some basal level differences in *NR4A* gene expression in WT versus IKKα/β$^{-/-}$ cells, no attenuation of NECA-induced *NR4A* genes was observed in the cells lacking IKKα/β$^{-/-}$ (Fig. 4c). This data reinforces the conclusion that the NF-κB family is not involved in AR-stimulated *NR4A* gene expression.

4. Discussion

Studies have demonstrated that adenosine receptor activation can enhance the expression of *NR4A1-3* genes, leading to attenuation of hyper-inflammatory responses in distinct cell types [8–11,13]. However, the signalling factors downstream of AR-stimulation that lead to enhanced expression of *NR4A* genes in myeloid cells is unknown and were the primary focus of this study.

Following establishing that adenosine and its stable analogue NECA can enhance *NR4A* expression in THP-1 cells, we set out to examine the kinase(s) involved downstream of AR stimulation leading to *NR4A* expression. Multiple kinases such as PKA, PKC and PKD are known to act downstream of AR stimulation, and they have also been shown to regulate *NR4A* gene expression in other contexts [16,31,32]. The PKA inhibitor, H-89, did not inhibit AR-stimulated *NR4A* expression in the THP-1 cells, which has also been reported in mast cells [10], whereas adenosine-induced *NR4A* expression has been shown to be PKA-dependent in neutrophils [13].

Using the pan-PKC inhibitor, GO6983, we provide evidence showing that PKC is not likely to be involved in AR stimulated *NR4A* expression in THP-1 cells, whilst it has been shown that PKC was involved in AR-stimulated *NR4A* expression in mast cells [10].

Intriguingly however, exposure of THP-1 cells to PMA, a classical PKC activator, alone enhanced *NR4A* expression and potentiated its expression when combined with NECA, a result that initially cast doubt on the PKC inhibition data. However, PMA can also activate PKD at concentrations used in this study [32]. Additionally, PKD1 and PKD2 have been shown to be essential for PMA and gastrin-induced expression of *NR4A1* in DO11.10 and AGS-B cells respectively [32,33]. Our results provide evidence that PKD inhibition using CID-75573, which inhibits all three PKD isoforms at concentrations used, blocks the capacity of AR stimulation to enhance *NR4A* expression in THP-1 cells. This data supports a role for PKD activation in driving AR-mediated *NR4A* expression (Which is independent of PKC, a phenomena that has been previously reported [34–36]).

Next, we set out to examine which transcription factor was involved downstream of this AR-PKD pathway identified leading to enhanced *NR4A* gene expression, with a focus on NFκB, a principal regulator of *NR4As* in myeloid cells [5]. Pharmacological approaches using BAY 11–7082 and MG-132, inhibitors of ubiquitin and proteasome pathways essential for NF-κB activation, indicated a possible role for NF-κB as a regulator of *NR4A* genes downstream of AR-stimulation [25]. Overexpressed of IκBα (an endogenous inhibitor of NF-κB) in THP-1 cells significantly attenuated TNFα and MCP-1 expression with no effects on AR-induced *NR4A* expression. At this point, we focused on the fact that BAY11-7082 could still inhibit AR-driven *NR4A* expression, but not acting through NF-κB. BAY11-7082 can affect ubiquitin systems, which in turn lead to the inactivation of the IKK complex [25]. IKK itself can have multiple roles independent of NF-κB, for example by modulating cellular regulators including p53, IRF7, FOXO3a, LC3 and, as such, BAY11-7082 could highlight these independent roles [29,30]. Furthermore, it has been shown that PKD can phosphorylate IKKβ promoting its activation [19]. Therefore, here we used MEFs that lack both IKKα and IKKβ (IKKα/β$^{-/-}$) and results indicate that IKKs do not play a role in AR-PKD-driven *NR4A* expression in our cell system.

In summary, this study develops our understanding on the mechanisms involved in AR-mediated *NR4A* expression in myeloid cells. AR stimulation by adenosine-based agonists modulate *NR4A* receptor expression in THP-1 myeloid cells, and our studies identify a central role for PKD in mediating this control. Further investigations exclude NF-κB activity and specific family members, IKKα/β, in AR-PKD-stimulated *NR4A* pathway, while pharmacological evidence shows ubiquitin and proteasome pathways are involved. Thus, the results presented here contribute mechanistic insight into how AR signalling modulates the expression of the *NR4A* receptors, key regulators of inflammatory responses in myeloid cells.

Declaration of competing interest

The authors declare no conflicts of interest associated with this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2021.03.082.

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