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To cite this version:

Abdullah Mayati, E. Le Ferrec, Jørn A Holme, Olivier Fardel, Dominique Lagadic-Gossmann, et al.. Calcium signaling and β2-adrenergic receptors regulate 1-nitropyrene induced CXCL8 responses in BEAS-2B cells.. Toxicology in Vitro, 2014, 28 (6), pp.1153-7. 10.1016/j.tiv.2014.05.012 . hal-01058607

HAL Id: hal-01058607
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Submitted on 7 Oct 2014

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Title:

Calcium Signaling and β2-Adrenergic Receptors Regulate 1-Nitropyrene Induced CXCL8 Responses in BEAS-2B Cells

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Abstract

Nitro-polycyclic aromatic hydrocarbons (nitro-PAHs) are widespread environmental pollutants, generated from reactions between PAHs and nitrogen oxides during combustion processes. In the present study we have investigated the mechanisms of CXCL8 (IL-8) responses induced by 1-nitropyrene (1-NP) in human bronchial epithelial BEAS-2B cells, with focus on the possible importance of Ca^{2+}-signaling and activation of β2-adrenergic receptors (β2AR). Ca^{2+}-chelator treatment obliterated 1-NP-induced CXCL8 (IL-8) responses. 1-NP at 10 μM (but not 1 μM) induced a rapid and sustained increase in intracellular Ca^{2+}-levels ($[Ca^{2+}]_i$). The early but not the later, sustained phase of 1-NP-induced $[Ca^{2+}]_i$ was suppressed by beta-blocker treatment (carazolol). Moreover, inhibition of β2AR by blocking-antibody, beta-blocker treatment (ICI 118551) or siRNA transfection attenuated CXCL8 responses induced by 1-NP. The results confirm that PAHs may induce Ca^{2+}-signaling also in BEAS-2B cells, at least partly through activation of β2AR, and suggest that both β2AR- and Ca^{2+}-signaling may be involved in 1-NP-induced CXCL8 responses in bronchial epithelial cells.

Key words: Lung; Inflammation; Chemokines; Polycyclic Aromatic Hydrocarbons; Calcium; Adrenergic Receptors.

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Introduction

Nitro-polycyclic aromatic hydrocarbons (nitro-PAHs) are ubiquitous air pollutants associated with combustion particles, in particular diesel exhaust particles. Nitro-PAHs have long been considered among the main contributors to the mutagenic effects of DEP (Hayakawa et al., 1997; Scheepers et al., 1995). Nitro-PAHs may also exhibit considerable pro-inflammatory potential, by inducing cytokine and chemokine responses in epithelial lung cells (Øvrevik et al., 2010; Øvrevik et al., 2009; Park and Park, 2009; Pei et al., 2002).

Previous studies have shown that 1-nitropyrene (1-NP) and 3-nitrofluoranthene (3-NF) are particularly potent inducers of the neutrophil attracting chemokine CXCL8 (IL-8), compared to their amine counterparts (1-AP and 3-AF), un-substituted pyrene or benzo[*a*]pyrene (B[a]P) (Øvrevik et al., 2010; Øvrevik et al., 2009; Øvrevik et al., 2013). This marked effect of 1-NP and 3-NF on CXCL8 induction seems to be independent of aryl-hydrocarbon receptor (AhR) activation, although it appears to involve formation of reactive oxygen species (ROS) and/ or reactive electrophilic metabolites at least partly formed via CYP-mediated metabolism (Øvrevik et al., 2010; Øvrevik et al., 2013).

Of notice, it has recently been shown that certain PAHs may induce calcium signaling in endothelial cells through an aryl-hydrocarbon receptor (AhR)-independent mechanism (Mayati et al., 2011). The effects were reported to be due to direct PAH-mediated activation of β2-adrenergic receptors (β2AR), leading to a G protein/adenylyl cyclase/cyclic-AMP-mediated calcium release from the endoplasmic reticulum (Mayati et al., 2012). Pyrene seemed to be a particular potent inducer of this pathway and caused considerably stronger calcium response in endothelial cells compared to other PAHs including B[*a*]P, chrysene and

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benzo[*e*]pyrene (Mayati et al., 2011). Besides, we previously showed that the cell-permeable calcium chelator BAPTA-AM attenuated CXCL8 responses in BEAS-2B cells by a panel of environmental pollutants, including 1-NP (Øvrevik et al., 2011). Thus, in the present study we have investigated the 1-NP-induced effects on intracellular calcium levels in relation to CXCL8 responses in BEAS-2B cells, in order to test the hypothesis that β2AR-mediated calcium-signaling may be a central mechanism for the pro-inflammatory effects of nitro-PAHs. Our results suggest that 1-NP is able to induce β2AR-mediated calcium responses in BEAS-2B cells, and that both β2AR- and Ca^{2+}-signaling are involved in 1-NP-induced CXCL8 responses. However, 1-NP also appeared to affect intracellular calcium levels through additional mechanisms, and β2AR-mediated Ca^{2+}-signaling alone may not be sufficient for CXCL8-induction.

Materials and methods

Reagents

B[*a*]P, 1-NP, dimethyl sulphoxide (DMSO), ICI 118551 and carazolol were purchased from Sigma-Aldrich (St. Louis, MO, USA). LHC-9 cell culture medium and fura-2 acetoxymethyl ester (Fura-2-AM) were from Invitrogen (Carlsbad, CA, USA). Cytokine ELISA assay for CXCL8 (Human IL-8 Cytoset) was purchased from Biosource International (Camarillo, CA, USA). Antibodies against β2AR (sc-569) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), whereas antibodies against β-actin were from Sigma-Aldrich. Short interfering RNA (siRNA) against β2AR (sc-35147) with corresponding non-targeting control siRNA (sc-37007) were from Santa Cruz Biotechnology (CA, USA). All other chemicals used were purchased from commercial sources at the highest purity available.

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Cell cultures and exposures

The BEAS-2B cell line, a SV40 hybrid (Ad12SV40) transformed human bronchial epithelial cell line, was from European Collection of Cell Cultures (ECACC, Salisbury, UK). Cells were maintained in LHC-9 medium in collagen-coated (PureColTM, Inamed Biomaterials, Fremont, CA, USA) flasks in a humidified atmosphere at 37ºC with 5% CO_2, and passaged twice per week. Prior to exposure, cells were plated in 12-well culture dishes, grown to near confluence in serum-free LHC-9 medium and exposed to PAHs and inhibitors as described elsewhere. Controls for 1-NP -exposed cells were treated with vehicle (DMSO) only. DMSO concentrations in all samples were below 0.5%.

Chemokine release

CXCL8 protein levels in cell-supernatants were determined by ELISA (Biosource International, Camarillo, CA, USA), as described elsewhere (Øvrevik et al., 2010). Absorbance was measured using a plate reader (TECAN Sunrise, Phoenix Research Products, Hayward, CA, USA) complete with software (Magellan V 1.10).

Gene silencing by siRNA

Cells were reverse-transfected with siRNAs against β2AR or non-targeting siRNAs, using HiPerFect transfection reagent as recommended by the manufacturer (Qiagen, Germany: Fast-Forward protocol for adherent cells). SiRNAs and HiPerFect were mixed by vortexing in LHC-9 medium, incubated at room temperature (5-10 min) to form transfection complexes, and added drop-wise to the cell cultures (100 µl/well) immediately after seeding (at a final siRNA concentration of 10 nM and 2.75 µl of HiPerFect in a total of 1 ml growth medium). Gene silencing was monitored by measuring β2AR protein levels by Western blotting.

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Calcium measurements

Variations in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were analyzed by microspectrofluorimetry using the acetoxymethyl ester form of the Ca^{2+}-sensitive probe Fura-2, , as previously reported (Le et al., 2002; N'Diaye et al., 2006). Cells were then submitted alternatively to 340 and 380 nm excitation wavelengths and the fluorescence from the trapped dye was measured at 510 nm. The F340/F380 ratio, i.e., ratio of fluorescence intensities after excitation at 340 nm and 380 nm, respectively, was used to estimate $[Ca^{2+}]_i$. To avoid potential problems with autofluorescence, PAH-concentrations were restricted to 10 µM in the $[Ca^{2+}]_i$ -assay.

Statistical analysis

Statistical significance was evaluated by GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA), using analysis of variance (ANOVA) with Bonferroni post-test.

Results

Role of calcium signaling in 1-NP-induced CXCL8 responses in BEAS-2B cells

We have previously shown that 1-NP induces a concentration-dependent increase in CXCL8 release from BEAS-2B cells from 10 µM (Øvrevik et al., 2010; Øvrevik et al., 2013). In line with this, we observed that 1-NP induced a strong increase in CXCL8 gene expression and protein release in BEAS-2B cells (Fig 1 A and B). To assess the role of Ca^{2+}-signaling in 1-NP-induced CXCL8 responses, BEAS-2B cell were pre-treated with the cell permeable Ca^{2+}-chelator BAPTA-AM for 30 min prior to 1-NP exposure for 18 h. BAPTA-AM completely blocked the 1-NP-induced CXCL8-response in BEAS-2B cells without affecting basal

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CXCL8 levels (Fig. 1B). Thus, 1-NP-induced CXCL8 responses seemed to depend on intracellular Ca^{2+}-levels in BEAS-2B cells.

We next assessed whether 1-NP could affect intracellular Ca^{2+}-concentrations ($[Ca^{2+}]_i$) in BEAS-2B cells. Cells were loaded with the Ca^{2+}-sensitive fluorescent probe Fura-2 prior to 1-NP exposure, and changes in $[Ca^{2+}]_i$ were assessed by microspectrofluorimetry. As seen from Fig 2A, 10 µM 1-NP induced a time-dependent increase in $[Ca^{2+}]_i$ in BEAS-2B cells. The kinetics of 1-NP-induced $[Ca^{2+}]_i$ in BEAS-2B cells resembled that reported for B[*a*]P in endothelial HMEC cells (Mayati et al., 2011; Mayati et al., 2012) with a small, transient peak around 5 min after exposure, followed by a gradual increase that reached maximum after about half an hour exposure. Moreover, it has recently been reported that PAHs may induce increases in $[Ca^{2+}]_i$ in HMEC cells through direct activation of β2AR (Mayati et al., 2012). Thus, to assess whether similar mechanisms could be involved in the present PAH-induced Ca^{2+} response, BEAS-2B cells were pre-incubated with the β-blocker carazolol for 30 min prior to exposure with 10 µM 1-NP. Carazolol almost completely blocked the early phases of 1-NP induced increases in $[Ca^{2+}]_i$ (Fig. 2B). However, 1-NP induced a sustained increase in $[Ca^{2+}]_i$ lasting at least up to 6 h (Fig. S1A, online supplementary materials). The later phases of 1-NP-induced (from 45 min to 6 h) seemed unaffected by Carazolol treatment (Fig. 2B).

Of notice, previous studies have shown that PAHs such as B[*a*]P and un-substituted pyrene may stimulate β2AR-mediated $[Ca^{2+}]_i$ already at 1 µM concentration (Mayati et al., 2011; Mayati et al., 2012). In line with this we observed that 1 µM B[*a*]P was able to induce increased $[Ca^{2+}]_i$ in BEAS-2B cells. However, 1-NP failed to affect Ca^{2+} signaling at this low concentration. Moreover, at 10 µM B[*a*]P appeared to induce considerably higher effects on $[Ca^{2+}]_i$ compared to 1-NP (Fig S1B and C, online supplementary materials). Thus, although 1-

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NP was able to stimulate β2AR-induced $[Ca^{2+}]_i$, it appeared to be a less potent activator compared to other PAHs.

Role of β2AR in 1-NP-induced CXCL8 responses in BEAS-2B cells

Next we wanted to assess whether β2AR could be involved in nitro-PAH-induced CXCL8 responses in BEAS-2B cells. Therefore we pre-incubated the cells with a β2AR-blocking antibody (Mayati et al., 2012) for 30 min prior to exposure with 1-NP. The β2AR-blocking antibody suppressed 1-NP-induced CXCL8 responses by approximately 50% at both tested concentrations (Fig 3A). To further examine the role of β2AR in 1-NP-induced CXCL8 responses, we assess the effects of the selective β2AR-antagonist ICI 118551 and of silencing β2AR by siRNA (siβ2AR). Both ICI treatment and transfection with siβ2AR resulted in a partial, but statistically significant suppression of 1-NP-induced CXCL8 (Fig 3B and C), comparable to the effect obtained with the β2AR-blocking antibody. Thus β2AR-signaling seemed to be involved in 1-NP-induced chemokine responses.

Discussion

In the present study we have assessed the mechanisms of the 1-NP induced CXCL8 response with emphasis on the importance of Ca^{2+}-signaling and the β2AR-receptor. Previously, Ca^{2+}-chelation was found to suppress CXCL8 in BEAS-2B cells by a variety of different air pollution constituents including 1-NP (Øvrevik et al., 2011). In coherence with this, the present results confirmed that the cell-permeable Ca^{2+}-chelator BAPTA-AM almost completely block 1-NP-induced CXCL8. Of interest, PAHs may increase $[Ca^{2+}]_i$ in endothelial cells through AhR-independent mechanisms (Mayati et al., 2011). Recent findings suggest that this effect may be regulated through PAH-mediated binding and activation of the

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β2AR (Mayati et al., 2012). In support of this, we observed that 1-NP (10 μM) exposure induced an increase in $[Ca^{2+}]_i$ in BEAS-2B cells that could be suppressed by beta-blocker treatment. Thus, PAH-induced Ca^{2+}-signaling through β2AR-activation may extend to several cell types. However, only the early phase of the Ca^{2+}-response seemed to depend on signaling through β-adrenergic receptors, as beta-blocker treatment had no effect on the 1-NP induced increase in $[Ca^{2+}]_i$ after 40 min exposure. Thus it is conceivable that 1-NP may induce Ca^{2+}-signaling through multiple mechanisms.

Interference with β2AR-signalling by use of blocking antibodies, a pharmacological beta-blocker, or by silencing β2AR-expression by siRNA-transfection, led to a suppression of 1-NP-induced CXCL8 responses. Thus 1-NP-induced CXCL8 release in BEAS-2B cells seemed at least partly dependent on β2AR-activation. In line with this, the β2AR agonists salbutamol and salmeterol have been found to enhance CXCL8 and interleukin-6 (IL-6) responses by IL-1β or virus infections in BEAS-2B cells and primary human bronchial epithelial cells (Edwards et al., 2007; Holden et al., 2010). However, salbutamol and salmeterol had no effect on CXCL8 or IL-6 responses alone, suggesting that β2AR-signalling alone may be insufficient for activation of cytokine/chemokine responses. Indeed, B[*a*]P appeared to be a more potent inducer of β2AR-dependent $[Ca^{2+}]_i$-responses than 1-NP, but is nevertheless unable to induce CXCL8 or other chemokines in BEAS-2B cells (Øvrevik et al., 2010; Øvrevik et al., 2013). Thus, additional 1-NP-induced mechanisms are likely required for the response. This notion is supported by previous findings suggesting that 1-NP-induced CXCL8 responses also involve metabolic activation by CYP-enzymes and possibly ROS (Øvrevik et al., 2013).

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While β2AR-interference only partly attenuated 1-NP-induced CXCL8 release, Ca^{2+}-chelation by BAPTA-AM completely abrogated the CXCL8 response. It should therefore be considered that the role of β2AR in 1-NP-induced CXCL8-release could be linked to other signaling mechanisms such as cAMP or β-arrestin. Indeed, salbutamol-induced exacerbation of virus-induced IL-6 responses in BEAS-2B cells seemed to be mediated through a cAMP-dependent mechanism (Edwards et al., 2007). If so, the effects of BAPTA-AM on 1-NP-induced CXCL8 may be also related to interference with the later, sustained, β2AR-independent $[Ca^{2+}]_i$-response.

The strong CXCL8-induction by 1-NP seems predominately to occur at high concentrations (≥10 µM) in BEAS-2B cells (Øvrevik et al., 2013). However, we recently observed that low concentrations of 1-NP (1 µM; giving no cytokine release alone) potentiated CXCL8-responses induced by priming the cells with a Toll-like receptor 3-agonist. Similar effects were observed with low concentrations of 1-AP and un-substituted pyrene (Øvrevik et al., 2013). Since 1-NP may exacerbate CXCL8 responses at concentrations that are insufficient to induce β2AR-dependent $[Ca^{2+}]_i$, other cellular targets seem to exist that are more sensitive towards 1-NP than the β2AR. Such low-concentration targets may be more important scenarios of real-life exposure to pyrene and pyrene-derivatives. Other PAHs may have considerably higher affinity for β2AR. As reported in the present study and elsewhere (Mayati et al., 2012), B[*a*]P may induce β2AR-dependent $[Ca^{2+}]_i$-responses already at 1 µM. In fact, the affinity of B[*a*]P (Kd = 10 nM) appears to be among the highest reported for β2AR ligands (Mayati et al., 2012). However, it should also be noted that 1-NP concentrations in diesel exhaust particles may be up to 10-fold higher than the concentration of B[a]P (Totlandsdal et al., 2012; Totlandsdal et al., 2014).

In extension of previous observations from endothelial cells (Mayati et al., 2011; Mayati et al., 2012), the present results suggest that PAHs may induce increased $[Ca^{2+}]_i$ through β2AR-activation also in epithelial lung cells. Moreover, β2AR-signalling and $[Ca^{2+}]_i$ appeared to be involved in the regulation of 1-NP-induced CXCL8 in the BEAS-2B cells. Although other yet unidentified cellular targets may be more sensitive to 1-NP exposure, β2AR appears to be a highly sensitive for other PAHs (Mayati et al., 2012). PAHs have also been reported to impair β2AR-function and interfere with asthma treatment (Factor et al., 2011). Thus, further studies are warranted to clarify the role of β2AR in PAH-induced responses and its possible implications in lung toxicity.

Acknowledgements

The work was supported by the Research Council of Norway, through the Environmental Exposures and Health Outcomes-program (grants no. 185620 and 228143).

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Figure legends

FIGURE 1. **CXCL8 gene expression and protein release in 1-NP-exposed BEAS-2B cells, and role of Ca^{2+}-chelation by BAPTA-AM.** Cells were exposed to 20 µM 1-NP or vehicle (DMSO) alone. CXCL8 gene expression was measured after 6 h by real-time PCR (A). Cells were pre-incubated with 10 µM of the cell-permeable Ca^{2+}-chelator BAPTA-AM for 30 min prior to exposure to 20 µM 1-NP or vehicle (DMSO) alone for 18 h (B). The figure depicts mean ± SEM of three independent experiments. *Significantly different from unexposed controls ($P < 0.05$); †Significant down-regulation by chelator-treatment ($P < 0.05$)..

FIGURE 2. **Intracellular Ca^{2+}-levels and role of β2AR-signaling in 1-NP exposed BEAS-2B cells.** Cells were pre-incubated with 10 µM of the β-blocker carazolol (Cara) prior to incubation with 10 µM 1-NP or vehicle (DMSO) alone (A and B). Intracellular Ca^{2+}-concentrations ($[Ca^{2+}]_i$) were measured by incubation with the Ca^{2+}-sensitive probe Fura-2 and the ratio of fluorescence intensities after excitation at 340 nm and 380 nm, respectively, was used to estimate $[Ca^{2+}]_i$. Figure A depicts the mean of normalized $[Ca^{2+}]_i$ from two independent continuous recordings, while figure B depicts the mean ± SEM of three independent experiments after 6 h (B) exposure.

FIGURE 3. **Role of β2AR-signaling in CXCL8-responses in 1-NP-exposed BEAS-2B cells.** Cells were pre-incubated for 30 min with a β2AR-blocking antibody (A) or the β-blocker ICI 118551 (B), or transfected with siRNA against β2AR (siβ2AR) or non-targeting control siRNA (siNT) (C), prior to exposure with 20 µM 1-NP or vehicle (DMSO) alone for 18 h. CXCL8 release were measured by ELISA. Efficiency of β2AR knock-down by siRNA

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was assessed by Western blotting (C). The figures depict mean ± SEM of three or more independent experiments. *Significantly different from unexposed controls ($P < 0.05$); †Significant down-regulation by inhibitor/antibody/siRNA treatment ($P < 0.05$).

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Figure 1

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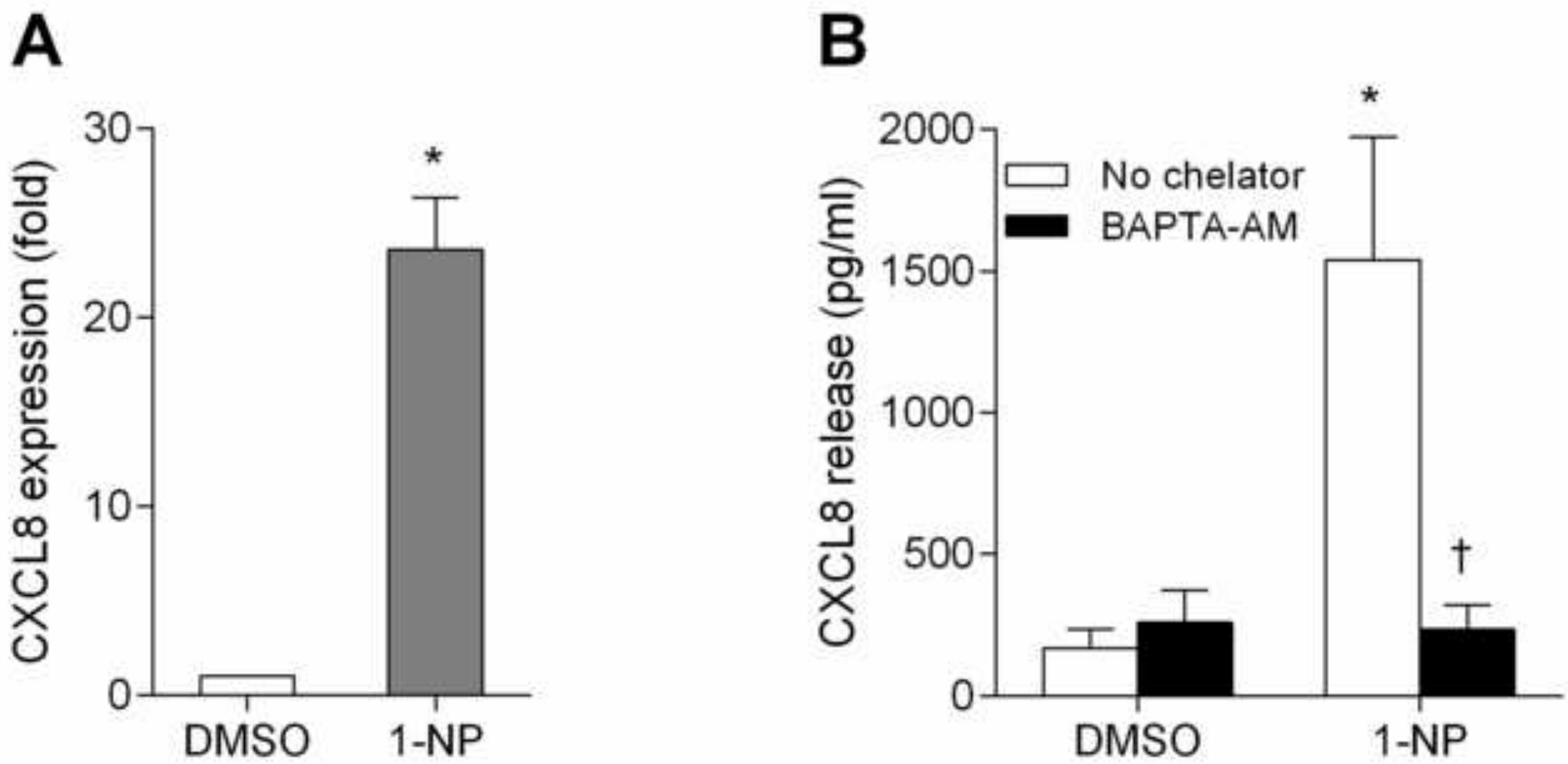

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Figure 2

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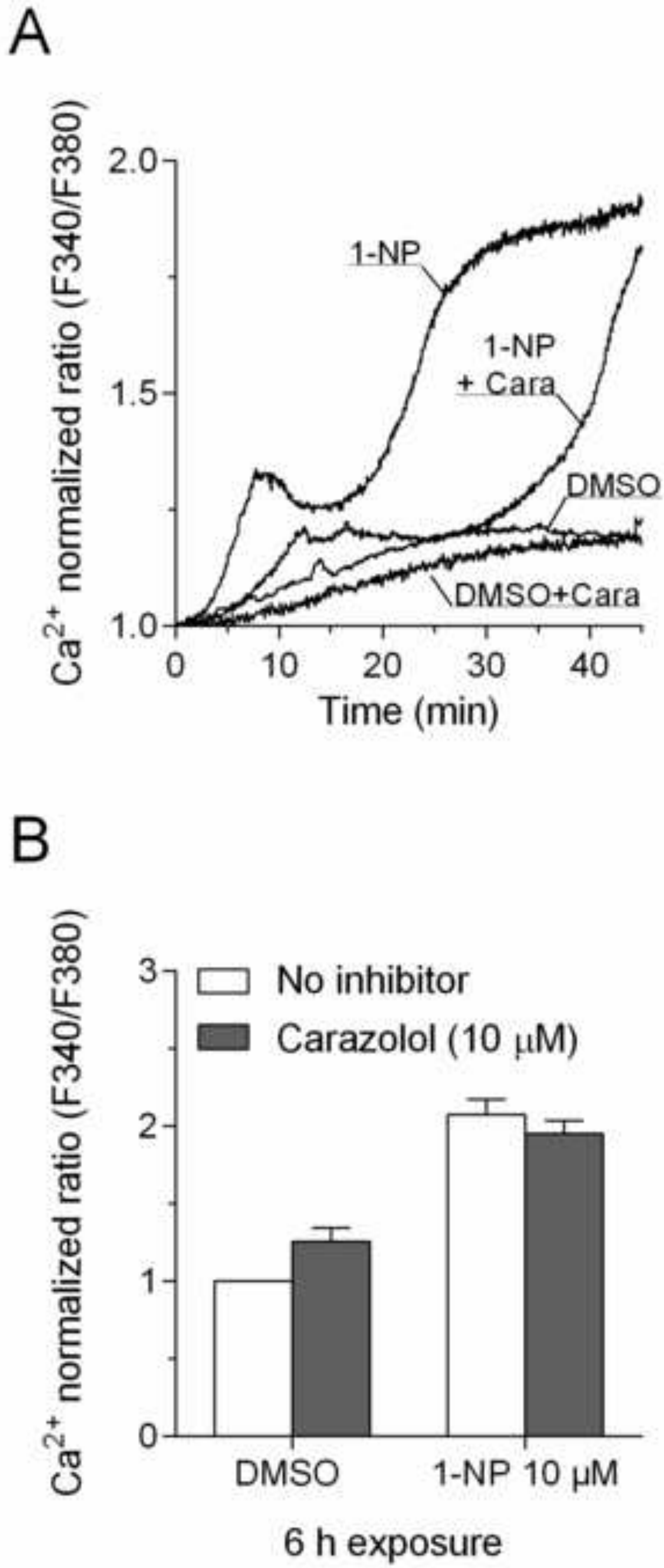

Figure 3

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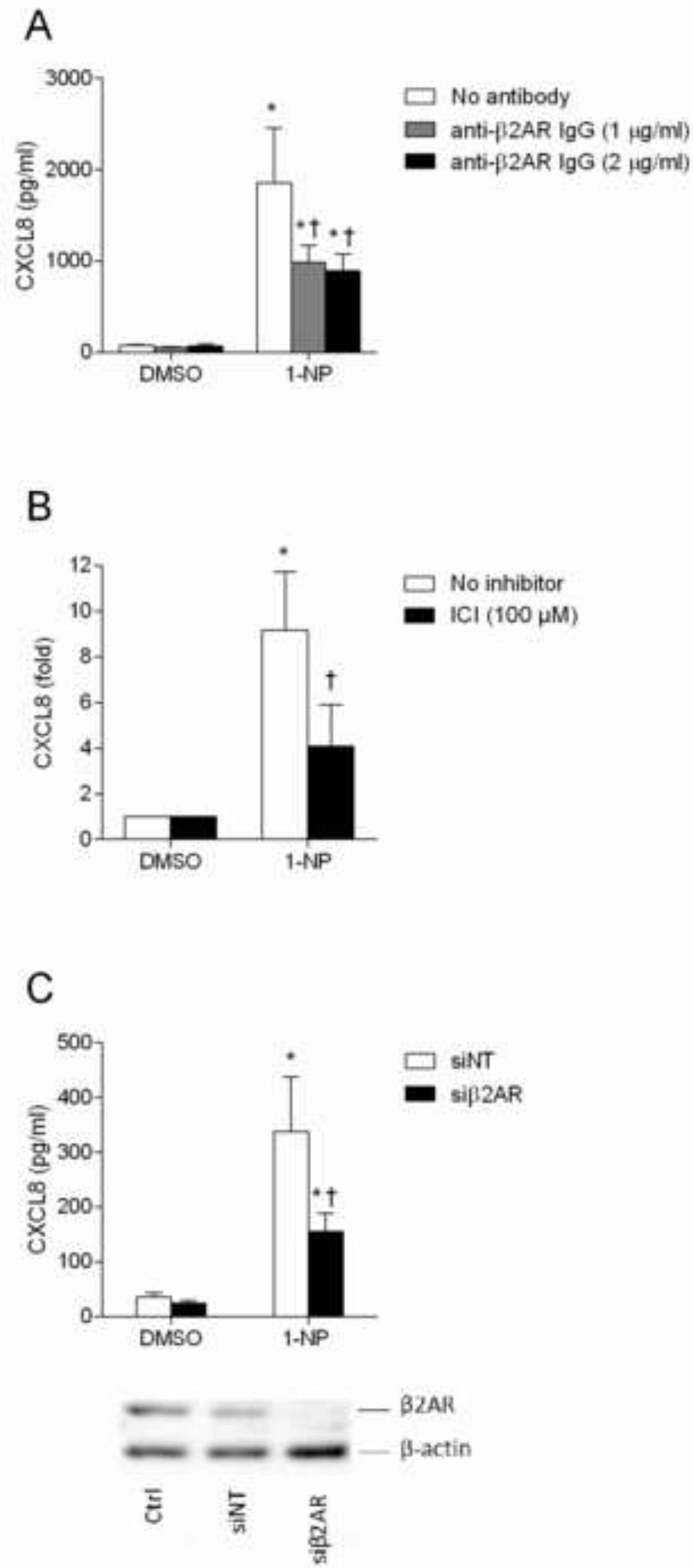

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Highlights

- We examined mechanisms of 1-NP induced CXCL8 responses in BEAS-2B cells
- Treatment with a Ca^{2+}-chelator abrogated the 1-NP-induced CXCL8 response
- 1-NP induced a rapid increase in intracellular Ca^{2+}-levels
- Beta-blocker treatment suppressed the 1-NP-induced Ca^{2+}-responses
- Inhibition of β2AR suppressed 1-NP-induced CXCL8 responses

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