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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 β 2-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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1 Title:

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

4

5

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28

1 **Abstract**

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

16
17 **Key words:** Lung; Inflammation; Chemokines; Polycyclic Aromatic Hydrocarbons; Calcium;
18 Adrenergic Receptors.

1

2 **Introduction**

3 Nitro-polycyclic aromatic hydrocarbons (nitro-PAHs) are ubiquitous air pollutants associated
4 with combustion particles, in particular diesel exhaust particles. Nitro-PAHs have long been
5 considered among the main contributors to the mutagenic effects of DEP (Hayakawa et al.,
6 1997; Scheepers et al., 1995). Nitro-PAHs may also exhibit considerable pro-inflammatory
7 potential, by inducing cytokine and chemokine responses in epithelial lung cells (Øvrevik et
8 al., 2010; Øvrevik et al., 2009; Park and Park, 2009; Pei et al., 2002).

9

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

18

19 Of notice, it has recently been shown that certain PAHs may induce calcium signaling in
20 endothelial cells through an aryl-hydrocarbon receptor (AhR)-independent mechanism
21 (Mayati et al., 2011). The effects were reported to be due to direct PAH-mediated activation
22 of β 2-adrenergic receptors (β 2AR), leading to a G protein/adenylyl cyclase/cyclic-AMP-
23 mediated calcium release from the endoplasmic reticulum (Mayati et al., 2012). Pyrene
24 seemed to be a particular potent inducer of this pathway and caused considerably stronger
25 calcium response in endothelial cells compared to other PAHs including B[*a*]P, chrysene and

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

12

13

14 **Materials and methods**

15 *Reagents*

16 B[*a*]P, 1-NP, dimethyl sulphoxide (DMSO), ICI 118551 and carazolol were purchased from
17 Sigma-Aldrich (St. Louis, MO, USA). LHC-9 cell culture medium and fura-2 acetoxymethyl
18 ester (Fura-2-AM) were from Invitrogen (Carlsbad, CA, USA). Cytokine ELISA assay for
19 CXCL8 (Human IL-8 Cytoset) was purchased from Biosource International (Camarillo, CA,
20 USA). Antibodies against β 2AR (sc-569) were from Santa Cruz Biotechnology (Santa Cruz,
21 CA, USA), whereas antibodies against β -actin were from Sigma-Aldrich. Short interfering
22 RNA (siRNA) against β 2AR (sc-35147) with corresponding non-targeting control siRNA (sc-
23 37007) were from Santa Cruz Biotechnology (CA, USA). All other chemicals used were
24 purchased from commercial sources at the highest purity available.

25

1 *Cell cultures and exposures*

2 The BEAS-2B cell line, a SV40 hybrid (Ad12SV40) transformed human bronchial epithelial
3 cell line, was from European Collection of Cell Cultures (ECACC, Salisbury, UK). Cells
4 were maintained in LHC-9 medium in collagen-coated (PureCol™, Inamed Biomaterials,
5 Fremont, CA, USA) flasks in a humidified atmosphere at 37°C with 5% CO₂, and passaged
6 twice per week. Prior to exposure, cells were plated in 12-well culture dishes, grown to near
7 confluence in serum-free LHC-9 medium and exposed to PAHs and inhibitors as described
8 elsewhere. Controls for 1-NP -exposed cells were treated with vehicle (DMSO) only. DMSO
9 concentrations in all samples were below 0.5%.

10

11 *Chemokine release*

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

16

17 *Gene silencing by siRNA*

18 Cells were reverse-transfected with siRNAs against β 2AR or non-targeting siRNAs, using
19 HiPerFect transfection reagent as recommended by the manufacturer (Qiagen, Germany: Fast-
20 Forward protocol for adherent cells). SiRNAs and HiPerFect were mixed by vortexing in
21 LHC-9 medium, incubated at room temperature (5-10 min) to form transfection complexes,
22 and added drop-wise to the cell cultures (100 μ l/well) immediately after seeding (at a final
23 siRNA concentration of 10 nM and 2.75 μ l of HiPerFect in a total of 1 ml growth medium).
24 Gene silencing was monitored by measuring β 2AR protein levels by Western blotting.

25

1 *Calcium measurements*

2 Variations in intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) were analyzed by
3 microspectrofluorimetry using the acetoxymethyl ester form of the Ca^{2+} -sensitive probe Fura-
4 2, , as previously reported (Le et al., 2002; N'Diaye et al., 2006). Cells were then submitted
5 alternatively to 340 and 380 nm excitation wavelengths and the fluorescence from the trapped
6 dye was measured at 510 nm. The F340/F380 ratio, i.e., ratio of fluorescence intensities after
7 excitation at 340 nm and 380 nm, respectively, was used to estimate $[\text{Ca}^{2+}]_i$. To avoid
8 potential problems with autofluorescence, PAH-concentrations were restricted to 10 μM in
9 the $[\text{Ca}^{2+}]_i$ -assay.

10

11 *Statistical analysis*

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

14

15

16 **Results**

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

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

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

3

4 We next assessed whether 1-NP could affect intracellular Ca^{2+} -concentrations ($[\text{Ca}^{2+}]_i$) in
5 BEAS-2B cells. Cells were loaded with the Ca^{2+} -sensitive fluorescent probe Fura-2 prior to 1-
6 NP exposure, and changes in $[\text{Ca}^{2+}]_i$ were assessed by microspectrofluorimetry. As seen from
7 Fig 2A, 10 μM 1-NP induced a time-dependent increase in $[\text{Ca}^{2+}]_i$ in BEAS-2B cells. The
8 kinetics of 1-NP-induced $[\text{Ca}^{2+}]_i$ in BEAS-2B cells resembled that reported for B[a]P in
9 endothelial HMEC cells (Mayati et al., 2011; Mayati et al., 2012) with a small, transient peak
10 around 5 min after exposure, followed by a gradual increase that reached maximum after
11 about half an hour exposure. Moreover, it has recently been reported that PAHs may induce
12 increases in $[\text{Ca}^{2+}]_i$ in HMEC cells through direct activation of $\beta 2\text{AR}$ (Mayati et al., 2012).
13 Thus, to assess whether similar mechanisms could be involved in the present PAH-induced
14 Ca^{2+} response, BEAS-2B cells were pre-incubated with the β -blocker carazolol for 30 min
15 prior to exposure with 10 μM 1-NP. Carazolol almost completely blocked the early phases of
16 1-NP induced increases in $[\text{Ca}^{2+}]_i$ (Fig. 2B). However, 1-NP induced a sustained increase in
17 $[\text{Ca}^{2+}]_i$ lasting at least up to 6 h (Fig. S1A, online supplementary materials). The later phases
18 of 1-NP-induced (from 45 min to 6 h) seemed unaffected by Carazolol treatment (Fig. 2B).

19

20 Of notice, previous studies have shown that PAHs such as B[a]P and un-substituted pyrene
21 may stimulate $\beta 2\text{AR}$ -mediated $[\text{Ca}^{2+}]_i$ already at 1 μM concentration (Mayati et al., 2011;
22 Mayati et al., 2012). In line with this we observed that 1 μM B[a]P was able to induce
23 increased $[\text{Ca}^{2+}]_i$ in BEAS-2B cells. However, 1-NP failed to affect Ca^{2+} signaling at this low
24 concentration. Moreover, at 10 μM B[a]P appeared to induce considerably higher effects on
25 $[\text{Ca}^{2+}]_i$ compared to 1-NP (Fig S1B and C, online supplementary materials). Thus, although 1-

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

3

4 *Role of β 2AR in 1-NP-induced CXCL8 responses in BEAS-2B cells*

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

15

16

17 **Discussion**

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

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

8
9 Interference with β 2AR-signalling by use of blocking antibodies, a pharmacological beta-
10 blocker, or by silencing β 2AR-expression by siRNA-transfection, led to a suppression of 1-
11 NP-induced CXCL8 responses. Thus 1-NP-induced CXCL8 release in BEAS-2B cells
12 seemed at least partly dependent on β 2AR-activation. In line with this, the β 2AR agonists
13 salbutamol and salmeterol have been found to enhance CXCL8 and interleukin-6 (IL-6)
14 responses by IL-1 β or virus infections in BEAS-2B cells and primary human bronchial
15 epithelial cells (Edwards et al., 2007; Holden et al., 2010). However, salbutamol and
16 salmeterol had no effect on CXCL8 or IL-6 responses alone, suggesting that β 2AR-signalling
17 alone may be insufficient for activation of cytokine/chemokine responses. Indeed, B[a]P
18 appeared to be a more potent inducer of β 2AR-dependent $[Ca^{2+}]_i$ -responses than 1-NP, but is
19 nevertheless unable to induce CXCL8 or other chemokines in BEAS-2B cells (Øvrevik et al.,
20 2010; Øvrevik et al., 2013). Thus, additional 1-NP-induced mechanisms are likely required
21 for the response. This notion is supported by previous findings suggesting that 1-NP-induced
22 CXCL8 responses also involve metabolic activation by CYP-enzymes and possibly ROS
23 (Øvrevik et al., 2013).

24

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

9
10 The strong CXCL8-induction by 1-NP seems predominately to occur at high concentrations
11 ($\geq 10 \mu\text{M}$) in BEAS-2B cells (Øvrevik et al., 2013). However, we recently observed that low
12 concentrations of 1-NP ($1 \mu\text{M}$; giving no cytokine release alone) potentiated CXCL8-
13 responses induced by priming the cells with a Toll-like receptor 3-agonist. Similar effects
14 were observed with low concentrations of 1-AP and un-substituted pyrene (Øvrevik et al.,
15 2013). Since 1-NP may exacerbate CXCL8 responses at concentrations that are insufficient to
16 induce β 2AR-dependent $[\text{Ca}^{2+}]_i$, other cellular targets seem to exist that are more sensitive
17 towards 1-NP than the β 2AR. Such low-concentration targets may be more important
18 scenarios of real-life exposure to pyrene and pyrene-derivatives. Other PAHs may have
19 considerably higher affinity for β 2AR. As reported in the present study and elsewhere (Mayati
20 et al., 2012), B[a]P may induce β 2AR-dependent $[\text{Ca}^{2+}]_i$ -responses already at $1 \mu\text{M}$. In fact,
21 the affinity of B[a]P ($K_d = 10 \text{ nM}$) appears to be among the highest reported for β 2AR
22 ligands (Mayati et al., 2012). However, it should also be noted that 1-NP concentrations in
23 diesel exhaust particles may be up to 10-fold higher than the concentration of B[a]P
24 (Totlandsdal et al., 2012; Totlandsdal et al., 2014).

25

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

10

11

12 **Acknowledgements**

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

15

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10
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1

2 **Figure legends**

3

4 **FIGURE 1. CXCL8 gene expression and protein release in 1-NP-exposed BEAS-2B cells,**
5 **and role of Ca²⁺-chelation by BAPTA-AM.** Cells were exposed to 20 μ M 1-NP or vehicle
6 (DMSO) alone. CXCL8 gene expression was measured after 6 h by real-time PCR (A). Cells
7 were pre-incubated with 10 μ M of the cell-permeable Ca²⁺-chelator BAPTA-AM for 30 min
8 prior to exposure to 20 μ M 1-NP or vehicle (DMSO) alone for 18 h (B). The figure depicts
9 mean \pm SEM of three independent experiments. *Significantly different from unexposed
10 controls ($P < 0.05$); †Significant down-regulation by chelator-treatment ($P < 0.05$).

11

12 **FIGURE 2. Intracellular Ca²⁺-levels and role of β 2AR-signaling in 1-NP exposed BEAS-**
13 **2B cells.** Cells were pre-incubated with 10 μ M of the β -blocker carazolol (Cara) prior to
14 incubation with 10 μ M 1-NP or vehicle (DMSO) alone (A and B). Intracellular Ca²⁺-
15 concentrations ($[Ca^{2+}]_i$) were measured by incubation with the Ca²⁺-sensitive probe Fura-2
16 and the ratio of fluorescence intensities after excitation at 340 nm and 380 nm, respectively,
17 was used to estimate $[Ca^{2+}]_i$. Figure A depicts the mean of normalized $[Ca^{2+}]_i$ from two
18 independent continuous recordings, while figure B depicts the mean \pm SEM of three
19 independent experiments after 6 h (B) exposure.

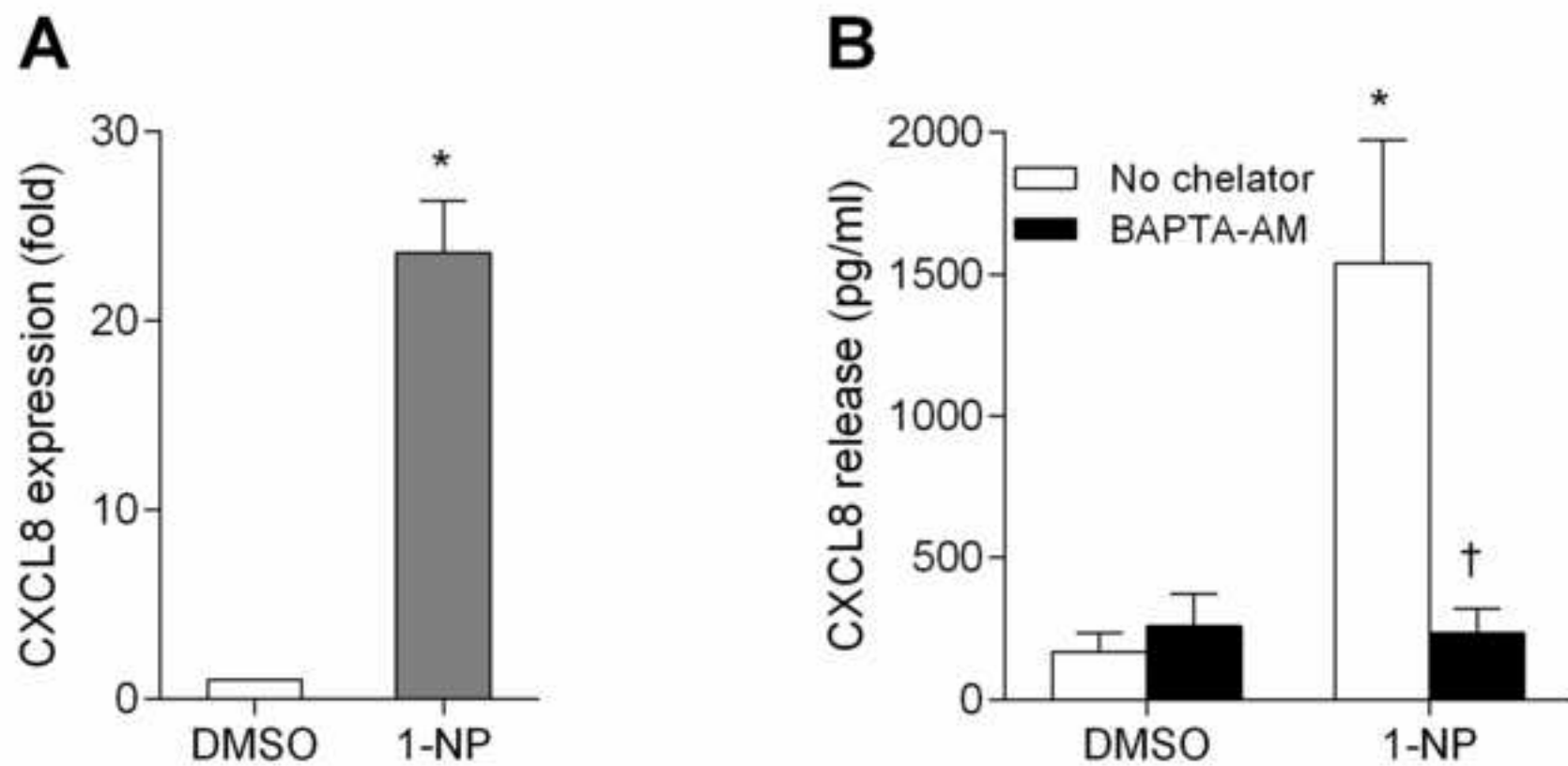
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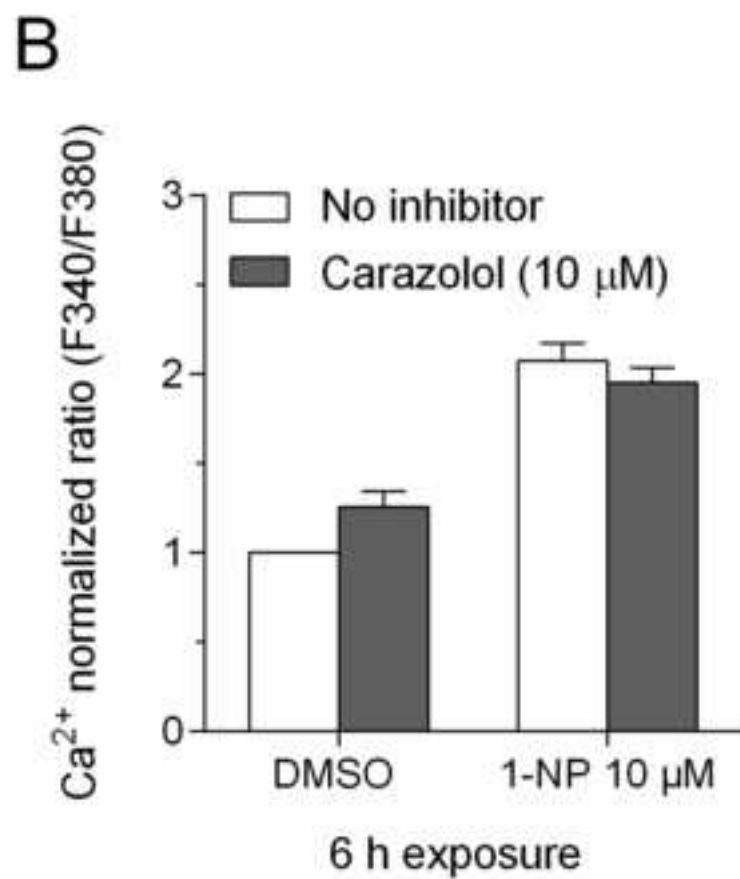
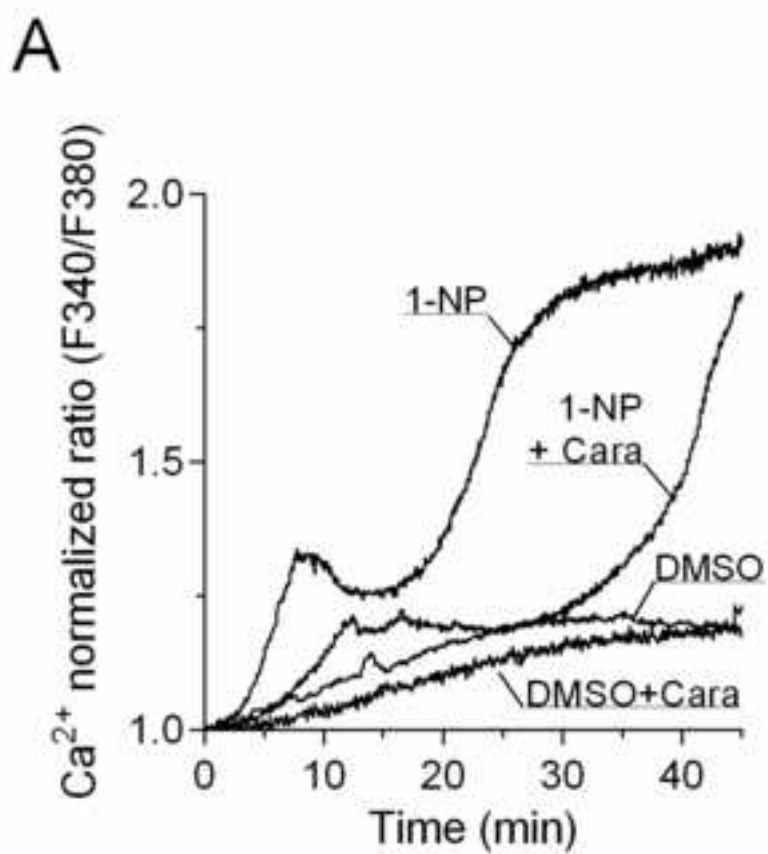
21 **FIGURE 3. Role of β 2AR-signaling in CXCL8-responses in 1-NP-exposed BEAS-2B**
22 **cells.** Cells were pre-incubated for 30 min with a β 2AR-blocking antibody (A) or the β -
23 blocker ICI 118551 (B), or transfected with siRNA against β 2AR (si β 2AR) or non-targeting
24 control siRNA (siNT) (C), prior to exposure with 20 μ M 1-NP or vehicle (DMSO) alone for
25 18 h. CXCL8 release were measured by ELISA. Efficiency of β 2AR knock-down by siRNA

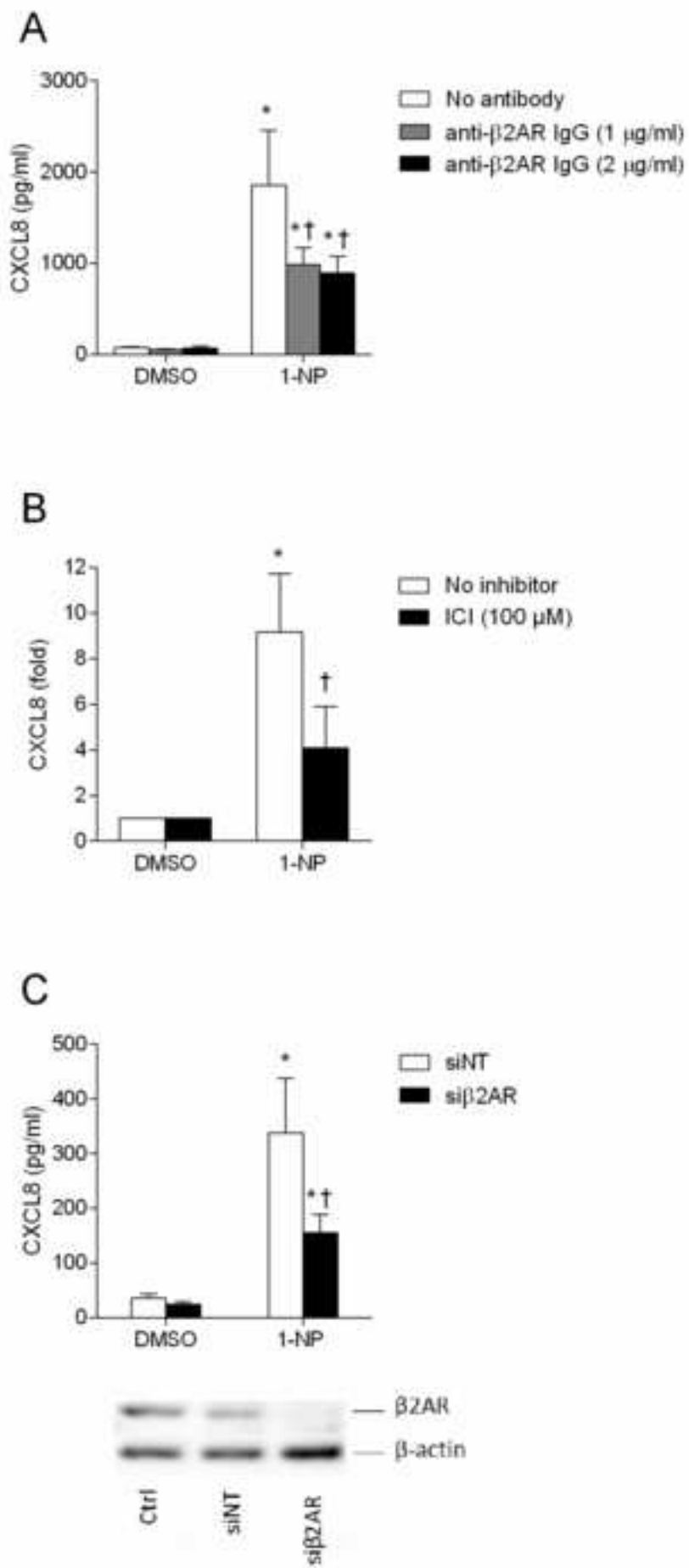
1 was assessed by Western blotting (C). The figures depict mean \pm SEM of three or more
2 independent experiments. *Significantly different from unexposed controls ($P < 0.05$);
3 †Significant down-regulation by inhibitor/antibody/siRNA treatment ($P < 0.05$).

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2 **Highlights**

- 3
- 4 • We examined mechanisms of 1-NP induced CXCL8 responses in BEAS-2B cells
 - 5 • Treatment with a Ca²⁺-chelator abrogated the 1-NP-induced CXCL8 response
 - 6 • 1-NP induced a rapid increase in intracellular Ca²⁺-levels
 - 7 • Beta-blocker treatment suppressed the 1-NP-induced Ca²⁺-responses
 - 8 • Inhibition of β 2AR suppressed 1-NP-induced CXCL8 responses

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