

Long-term exposure to CXCL2 has cytotoxic effects on HEK293T cells stably expressing TRPV1

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Abstract

CXCL2 chemokine was associated with neurodegeneration and cellular death, but little is known about its mechanism of action. To test whether CXCL2 could induce its cytotoxic effects by increased activation of TRPV1 receptors, in the present study we investigated the effect of 24 h incubation with 0.4, 4 and 40 nM CXCL2 on the growth rate of HEK293T cells stably transfected with TRPV1. The results showed that CXCL2 has cytotoxic effects irrespective of the concentration, supporting a TRPV1 involvement in mediating its action.

Keywords: CXCL2, HEK293T-TRPV1 cells, cytotoxicity, viability

1. Introduction

Chemokines are chemoattractant cytokines involved in inflammation by attracting immune cells to the site of injury (Boddeke [3]). CXCL2 (C-X-C motif chemokine 2), a member of the ELR (+) CXC-chemokine family also known as MIP-2 (macrophage inflammatory protein 2), Scyb2 (small inducible cytokine subfamily member 2) or GRO β (growth - regulated protein β) (Pawson et al. [19]), is secreted by activated macrophages, neutrophils, microglia, astrocytes or endothelial cells as response to inflammatory signals (Call et al. [4], Semple et al. [23]). It acts via CXCR2 receptor (Vinader et al. [26]) and it has pleiotropic effects (Bhardwaj et al. [2], Cao et al. [5], Kiguchi et al. [14], Lax et al. [17], Raman et al. [20], Semple et al. [23]), including increased apoptosis and neurodegeneration (de Paola et al. [10], Kolehua et al. [13]).

TRPV1 (transient receptor potential vanilloid type 1) is a polymodal nociceptor acting as a non-selective cation channel with a preference for calcium ions that is highly expressed in a subset of somatosensory neurons located in the dorsal root and trigeminal ganglia. It is directly activated by capsaicin and also by protons or heat which have been shown to cause pain *in vivo* (Caterina et al. [6], Tominaga et al. [25]). In addition, TRPV1 can be activated by other endogenous or exogenous agonists of natural, semisynthetic and synthetic origin (Cevikbas et al. [7], Vriens et al. [27]). When over-activated, TRPV1 is inducing an increase in intracellular calcium concentration which leads to mitochondrial damage and eventually apoptosis due to calcium-dependent apoptosis (Ho et al. [12]). In this paper we investigated the effects of long-term incubation with CXCL2 on HEK293T cells stably expressing TRPV1. We previously found that CXCL1, a chemokine with 78 % sequence homology to CXCL2 (Semple et al. [23]), activates TRPV1 receptor via CXCR2 when acutely applied (Deftu et al, [9]). Assuming that CXCL2 might have similar effects and considering that over activation of TRPV1 alters cellular activity, the main hypothesis of the study was that CXCL2 could increase TRPV1 activation, which consequently would alter the viability, growth rate and possibly cell morphology.

2. Materials and methods

Cell culture. Human embryonic kidney cells (HEK293T) wild type or stably expressing TRPV1 receptors were seeded at 5×10^3 /ml in 24-well plates coated with poly-D-lysine (0.1 mg/ml for 30 min) for growth rate studies or at 3×10^3 /ml in a 96-well plate for MTT (3-(4, 5- dimethylthiazolyl-2)-2, 5- diphenyltetrazolium bromide) assay as previously described (Ristoiu et al. [22]). When necessary, the cells were transiently transfected with $1 \mu\text{g}/\mu\text{l}$ of huCXCR2 (kindly donated by Dr. Ann Richmond, Vanderbilt University, TN, USA) using Lipofectamine 2000. After 1 day in culture, the cells were treated for another 24 hours with 0.4, 4 and 40 nM CXCL2 (PromoKine, Germany), following which CXCL2 containing medium was removed and the cells were maintained for 1 to 5 days in regular medium. We chose these concentrations because in preliminary studies we identified 4 nM as the EC50 concentration at which CXCL1 can have an effect on TRPV1 desensitization (Deftu et al, [9]). Knowing that CXCL2 is 78 % similar to CXCL1, we decided to test a similar concentration, together with concentrations below or above EC50 to examine a putative dose-dependent effect of CXCL2. All experiments were carried out in triplicate. If not otherwise mentioned, all the reagents were from Sigma. *Morphology and growth rate evaluation.* The cells were evaluated for morphology changes and growth rate 1 to 5 days after CXCL2 treatment. The cells that displayed an apoptotic morphology according to The Nomenclature Committee on Cell Death (Kroemer et al. [15], Kroemer et al. [16]) were excluded from further analysis. A population doubling time (PDT) was calculated as

well, according to the formula
$$PDT = \frac{t_2 - t_1}{3.32 * (\log n_2 - \log n_1)}$$
 (where t is time and n is

the number of cells at time t_n), considering as the reference time point 48 h in culture when the cells were at the beginning of their logarithmic growth phase. *MTT*

assay. Cell viability was evaluated 1 to 5 days after CXCL2 treatment, using MTT assay (Galluzzi et al. [11]). For this test the cells were incubated with a freshly made 1 mg/ml MTT in serum-free DMEM medium, for 4 h at 37°C in the dark, triturated for 20 min in DMSO (dimethyl sulfoxide) until the formazan crystals were dissolved and quantified at

570 nm on a FlexStation 3 Multi-Mode Microplate Reader (Luminex, USA). *Western blot analysis.* HEK293T and HEK293T-TRPV1 cells were lysed for 30 min on ice in RIPA (1% NP40) buffer as previously described (Marin et al. [18]). Equal amount of proteins were separated by SDS-PAGE (10% acrylamide following Molecular Cloning 3rd edition recipe, mini gel system from BioRad) in reducing conditions at constant 30mA/gel with a limitation of 200V, transferred in a semidry system on nitrocellulose for 1h at 1.5 mA/cm². Next, the membranes were blocked in a solution of 5% milk in PBS-1% Tween 20 and subsequently probed with rabbit anti-CXCR2 (1:300, Santa-Cruz Biotechnology, Germany), mouse anti-TRPV1 (1:500, Abcam, UK) or goat anti-actin (1:300, Santa-Cruz Biotechnology, Germany) antibodies for 2h at room temperature in blocking solution. The secondary antibodies goat anti-mouse and goat anti-rabbit coupled with HRP (horse radish peroxidase) (1:10000, Santa-Cruz Biotechnology, CA) were added for 1h at room temperature in blocking solution. Visualization was made with Pierce ECL Western blotting substrate according to the manufacturer's instructions (Thermo Pierce) and exposed to chemiluminescent sensitive films (Santa Cruz). *Immunocytochemistry analysis.* Immunostaining of cultured cells was performed as previously described (Ristoiu et al. [22]). The slides were visualized under an AxioObserver D1 Zeiss (Carl Zeiss, Germany) fluorescence microscope and processed with Image-J software 1.37v (National Institutes of Health, USA). The primary antibody was rabbit polyclonal anti-CXCR2 (1:200, Santa-Cruz Biotechnology, Germany) and the secondary antibody was goat anti-rabbit Alexa Fluor 488 (1:1500, Life Technologies, USA).

Statistics. Data were analyzed using Prism5 (GraphPad Software, Inc.) software and were expressed as mean \pm SEM; statistical significance was tested using two-way ANOVA with Bonferonni post-test. A value of $P < 0.05$ was considered to be statistically significant.

3. Results and Discussion

For the beginning we assessed the morphology of cells treated with different concentrations of CXCL2 and did not observe any significant changes for all the conditions investigated over a 5 days period (Figure 1).

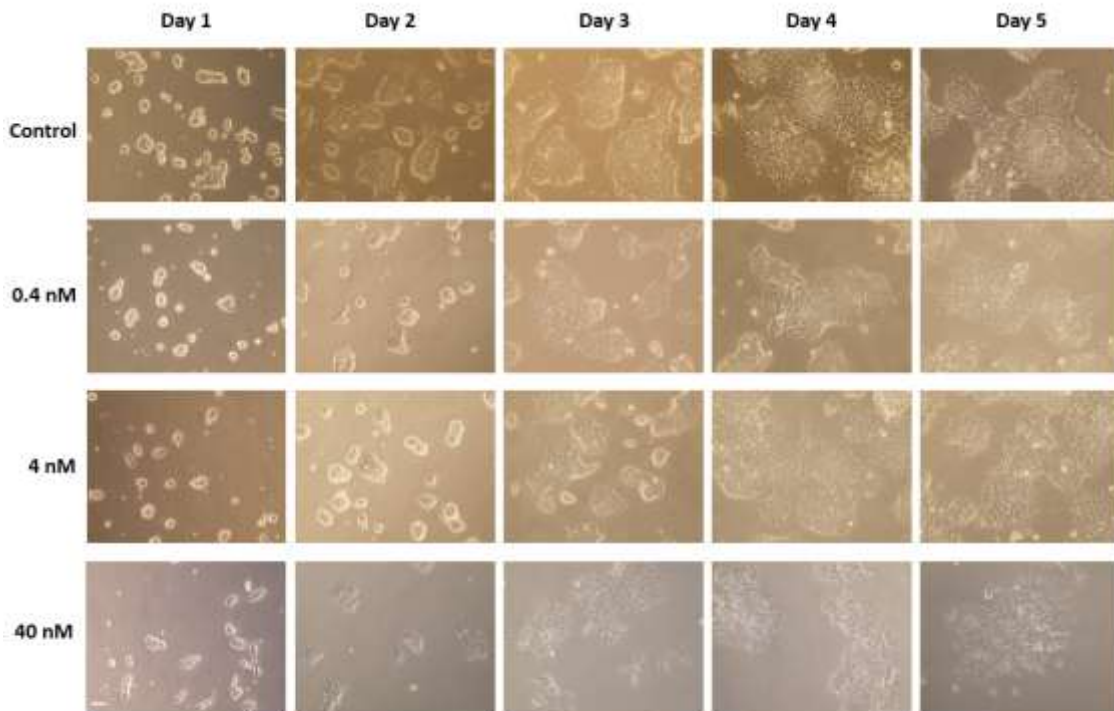


Figure 1. Cell morphology was not affected by 24 hours exposure to CXCL2. Incubation of HEK293T cells stably expressing TRPV1 with different concentrations of CXCL2 did not induce any visible morphological changes at 1 to 5 days after treatment (scale bar = 15 μ m).

Under the same conditions, growth rates were reduced compared to control, with significant values for the 3rd and 4th day when the cells were in their logarithmic growth phase ($P < 0.05$ for all the concentrations, Figure 2a). Table 1 indicates the cells number in different experimental conditions *vs* control. The growth rate reduction was associated with increased population doubling times as well (PDT for control = 24.01 h, for CXCL2 0.4 nM = 30.24 h, for CXCL2 4 nM = 32.57 h and for CXCL2 40 nM = 42.62 h).

To assess whether the growth rate reduction was due to a cell viability decrease, an MTT test was performed. The results showed that indeed cell viability was reduced for all concentrations, with significant values for the 2nd and 3rd day corresponding to the first part of cell growth phase ($P < 0.05$ for all the concentrations, Figure 2b).

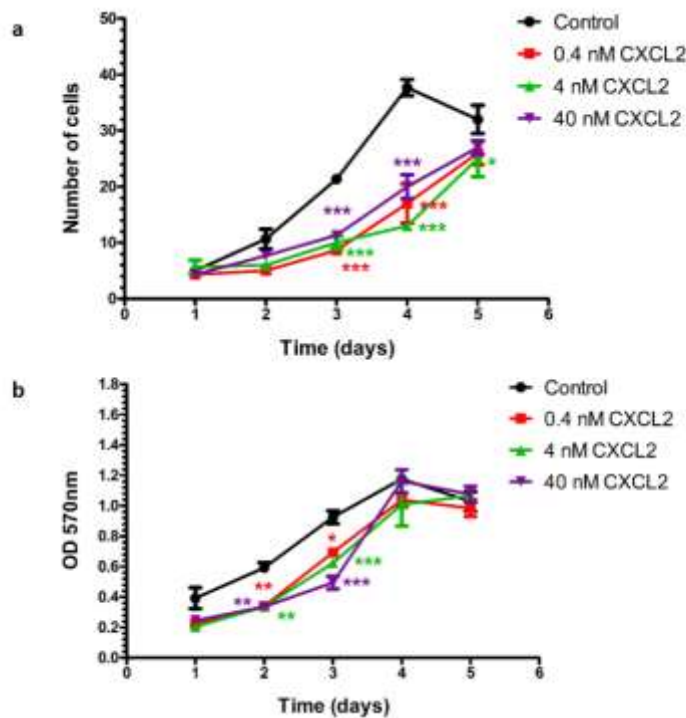


Figure 2. CXCL2 has cytotoxic effects. (a) Growth rate for 24 h incubation with 0.4, 4 and 40 nM CXCL2, 1 to 5 days after treatment. All CXCL2 concentrations have significant cytotoxic effect during logarithmic growth phase corresponding to the 3rd and 4th day (***P < 0.0001 for 0.4 nM, 4 nM and 40 nM). (b) The viability test after 24 h incubation with different concentrations of CXCL2. In the 2nd and 3rd day after treatment, the absorbance values were significantly lower than those in the control in day 2 and 3 after the treatment, indicating a cytotoxic effect of CXCL2 (for days 2, **P < 0.01 for 0.4, 4 and 40 nM, and for day 3, *P < 0.05 for 0.4 nM and ***P < 0.001 for 4 and 40 nM).

Table 1. Number of HEK293T-TRPV1 cells in control conditions and after treatment with different concentrations of CXCL2.

Days after treatment	1	2	3	4	5
Control	n = 5	n = 11	n = 21	n = 38	n = 32
CXCL2 0.4 nM	n = 4 P > 0.05	n = 5 P > 0.05	n = 9 *** P < 0.05	n = 17 *** P < 0.05	n = 26 P > 0.05
CXCL2 4 nM	n = 6 P > 0.05	n = 6 P > 0.05	n = 10 *** P < 0.05	n = 13 *** P < 0.05	n = 25 * P < 0.05
CXCL2 40 nM	n = 4 P > 0.05	n = 8 P > 0.05	n = 11 *** P < 0.05	n = 20 *** P < 0.05	n = 27 P > 0.05

As mentioned in the introduction, CXCL2 binds to CXCR2 receptors. Since we did not transiently transfected CXCR2 receptors in these cells, we assumed that

HEK293T-TRPV1 cells have a certain endogenous level of CXCR2 which could explain the effects mentioned above. To test this hypothesis, we performed a Western blotting analysis of total cell lysates of HEK293T wild-type cells (HEK293T-WT) in comparison with the same cell line overexpressing TRPV1V (HEK293T-TRPV1). As shown in Figure 3a-upper panel, a prominent band corresponding to CXCR2 MW was detected in HEK293T-TRPV1 cells, whilst a very faint band can be detected for the wild type cell line. We also confirmed by Western blotting the overexpression of TRPV1 receptors in HEK293T-TRPV1 cell line as observed in Figure 3a-middle panel.

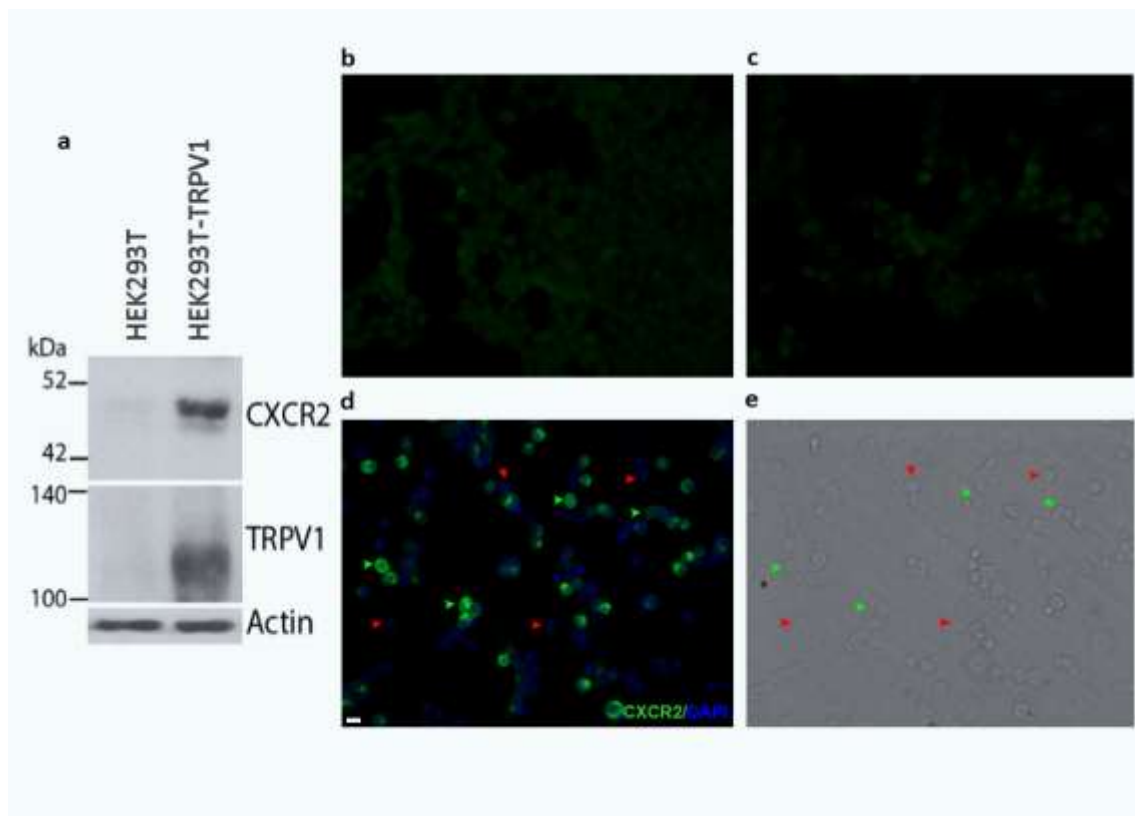


Figure 3. HEK293T-TRPV1 cells endogenously express CXCR2 receptors. (a) CXCR2 and TRPV1 expression assessed by Western blotting for cell lysates from HEK293T-TRPV1 and HEK293T-WT lysates. Only HEK293T-TRPV1 express CXCR2 receptors, as well as TRPV1. Representative images of CXCR2 immunofluorescence in HEK293T-TRPV1 cells with (b) and without (c) permeabilization, and in HEK293T-WT cells transiently transfected with huCXCR2 plasmid (d). Green arrows indicate transfected cells and red arrows indicate non-transfected cells. (e) Bright field image corresponding to the fluorescence image in (d) (scale bar = 8 μ m).

This study showed that long-term incubation of HEK293T-TRPV1 cells with different concentrations of CXCL2 in nM range has significant cytotoxic effects during the logarithmic phase of growth. Since these cells proved to endogenously express CXCR2 receptors compared with HEK293T-WT, one possible explanation for CXCL2

induced cytotoxicity could be the over-activation of TRPV1 receptors via CXCR2 signaling. Previous studies reported no CXCR2 expression in HEK293 cells (Atwood et al. [1]), and we also confirmed this aspect by Western Blot analysis in HEK293T wild-type cells. In contrast, the clone HEK293T-TRPV1 which stably express TRPV1, did express CXCR2. Our results suggest that CXCR2-TRPV1 combination in HEK293 cells which originally are adrenal cells (Shaw et al. [24]), make the cells very sensitive even to low concentrations of CXCL2 and reduces their viability, but is not critical for their survival. In another study from our group we showed that 24 h incubation with 1.5 nM CXCL2 inhibited TRPV1 in DRG (dorsal root ganglia) neurons (Deftu et al. [8]). These two apparent antagonist observations could be explained by the fact that CXCR2 is a very complex receptor that forms dynamic and temporal assembly with other adaptor signaling proteins through which it can initiate different intracellular pathways (Raman et al. [21]), raising the possibility that CXCL2 can have on long-term different effects depending on the cell type and on the signaling pathway that is activated downstream CXCR2. If the observations from this study are valid for native adrenal cells as well, remains to be established in a future study.

4. Conclusions

Long-term incubation with CXCL2 has cytotoxic effects irrespective of the concentration during the logarithmic growth phase of HEK293T cells stably transfected with TRPV1 which endogenously express CXCR2.

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