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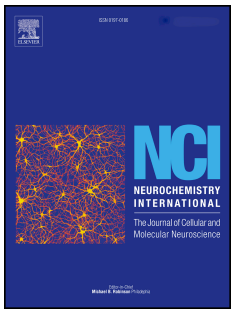

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Iron chelators inhibit amyloid-β-induced production of Lipocalin 2 in cultured astrocytes

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Abstract

Lipocalin 2 (Lcn2) has been implicated to play a role in various neurodegenerative diseases, and normalizing its overexpression may be of therapeutic potential. Iron chelators were found to reduce Lcn2 levels in certain animal models of CNS injury. Focusing on Alzheimer's disease (AD), we found that the iron chelators deferoxamine and deferiprone inhibited amyloid-β (Aβ)-induced Lcn2 production in cultured primary astrocytes. Accordingly, Aβ-exposure increased astrocytic ferritin production, indicating the possibility that Aβ induces iron accumulation in astrocytes. This effect was not significantly modulated by Lcn2. Known neuroprotective effects of iron chelators may rely in part on normalization of Lcn2 levels.

Keywords

Neutrophil gelatinase-associated lipocalin (NGAL); iron metabolism; neuroinflammation; ferritin; deferoxamine; deferiprone

1. Introduction

Lipocalin 2 (Lcn2, also known as neutrophil gelatinase-associated lipocalin (NGAL)) is involved in several physiological processes including inflammation, iron metabolism, cell death and cell survival. Increased Lcn2 levels were found in the central nervous system (CNS) of patients with neurodegenerative diseases, including Alzheimer's disease (AD) and Parkinson's disease. Moreover, mechanistic studies showed that Lcn2 may contribute to their pathophysiology (Kim et al., 2016; Mesquita et al., 2014; Naudé et al., 2012). Regarding AD, it was shown that amyloid-β (Aβ) induces Lcn2 production in cultured primary astrocytes, and that Lcn2 sensitizes primary neurons and astrocytes to Aβ-induced cell death (Mesquita et al., 2014; Naudé et al., 2012). Astrocytes appear to be the major producers of Lcn2 in the brain (Kim et al., 2016; Mesquita et al., 2014). The reported neurotoxic effects of Lcn2 indicate that inhibition of Lcn2 overexpression may be a promising therapeutic strategy for different CNS conditions.

Iron chelators such as deferoxamine and deferiprone have been shown to exert neuroprotective effects (Belaidi and Bush, 2016), maybe partly via reducing the brain iron accumulation that characterizes many CNS conditions. Interestingly, deferoxamine was found to decrease Lcn2 levels in certain animal models of CNS injury (Dong et al., 2013; Zhao et al., 2016). However, it is still unknown if iron chelators may reduce Lcn2 production in the context of AD.

The aim of this study was to explore (1) whether the iron chelators deferoxamine and deferiprone are able to inhibit $A\beta_{1-42}$-induced Lcn2 production in cultured astrocytes, and (2) whether Aβ may affect astrocytic iron metabolism, and the potential effect of Lcn2 hereon by comparing Aβ-treated wild-type (WT) and Lcn2 knock-out (Lcn2 KO) astrocytes.

2. Methods

Primary astrocytes were obtained from newborn (P0-P3) WT and Lcn2 KO (Berger et al., 2006) mouse pups, according to a protocol approved by the local and national animal ethics committees (DEC6659A and CCD-AVD105002016630). Astrocytes were cultured as described previously (Naudé et al., 2012). Six hours before treatment, medium was exchanged for medium containing 5% fetal bovine serum. Human recombinant $A\beta_{1-42}$ (A-1002-1, rPeptide) was prepared as described previously (Granic et al., 2010). Before use, the Aβ stock solution (100 µM in DMEM) was allowed to oligomerize for 6h at 4 °C (Ahmed et al., 2010). The oligomeric state of Aβ was confirmed with non-reducing SDS-PAGE Western blotting. Astrocytes were treated with 1 µM Aβ, 10 ng/ml interleukin 1 beta (IL-1β) or 100 ng/ml lipopolysaccharide (LPS), or were co-treated with 1 µM Aβ and either 0-150 µM deferoxamine (D9533, Sigma-Aldrich), 0-500 µM deferiprone (S4067, SelleckChem), 0-200 µM bathocuproine disulfonic acid (B1125, Sigma-Aldrich) or 0-25 µM tetrathiomolybdate (323446, Sigma-Aldrich) for the indicated periods of time. Collection of proteins and Western blotting were performed as described previously (Naudé et al., 2012). Primary antibodies used include anti-Lcn2 (ab63929, Abcam, 1:1000), anti-ferritin (ab75973, Abcam, 1:1000) and anti-actin (691002, MP Biomedicals, 1:500.000). All treatments were performed three times in duplicate or triplicate.

3. Results

Firstly, it was confirmed that $A\beta_{1-42}$ induced Lcn2 production and secretion by astrocytes (Fig. 1a-b). Intracellular Lcn2 levels peaked 36h after $A\beta_{1-42}$ treatment ($p < 0.0001$). This corresponds to kinetics of Lcn2-induction upon TNF-α, IL-1β and LPS-stimulation ((Naudé et al., 2012) and Suppl. Fig. 1a-b). Secondly, deferoxamine significantly reduced Aβ-induced Lcn2 production, after 36h co-incubation ($p < 0.0001$, Fig. 1c-d). The inhibitory effect of deferoxamine on Aβ-induced Lcn2 production was confirmed with another iron chelator; deferiprone (Suppl. Fig. 1c-d).

- Include Figure 1 around here -

The finding that the Lcn2-inducing effects of Aβ can be suppressed by iron chelators, points to the possibilities that (1) Aβ may provoke iron accumulation in astrocytes, and (2) this disturbance in iron metabolism correlates with the induction of Lcn2 expression. As shown in Fig. 1e, Aβ indeed increased ferritin protein levels in WT and Lcn2 KO astrocytes ($p < 0.05$ at 36h, compared to control), indicating an increase in astrocytic iron accumulation upon Aβ exposure, independent of endogenous Lcn2 production. Although increased astrocytic iron levels might be an important co-factor in the induction of Lcn2, it appeared that iron alone is not sufficient to induce Lcn2 upregulation (Suppl. Fig. 1e).

4. Discussion

Results from this study suggest that iron chelators are potent inhibitors of Aβ-induced Lcn2 production in astrocytes, which may contribute to their reported neuroprotective effects. Interestingly, it was proposed that iron-loaded deferiprone (unlike deferoxamine) may bind to Lcn2, after which the iron-deferiprone-Lcn2 complex is excreted from the body (Zughaier et al., 2014). Certain iron chelators, i.e. deferiprone, might thus not only affect Lcn2 production but also its removal from the body.

The modulation of Aβ-induced Lcn2 production by iron chelators further suggests that Aβ may act in part via increasing iron levels in astrocytes (also illustrated in Fig. 1f). This is supported by our result showing that Aβ causes an increase in astrocytic ferritin levels. This is the first study to our best knowledge that indicates iron accumulation in astrocytes upon direct Aβ-stimulation. This is in accordance with the previously reported Aβ-induced iron accumulation in microglia ((McCarthy et al., 2018) and Suppl. Fig. 1f) and a neuronal cell line (Wan et al., 2011). Future experiments are required to confirm the finding in astrocytes, including direct read-outs of iron accumulation. Moreover, further investigations are needed to elucidate the role of disturbed iron metabolism in Aβ-induced

astrocyte activation and Lcn2 production. Namely, while the current results may suggest a potential involvement of disturbed iron metabolism in Aβ-induced Lcn2 production, it is possible that iron is not essential, and that other factors and pathways are also involved. In addition, more work is required to determine whether deferoxamine and deferiprone inhibit Aβ-induced Lcn2 production by chelating iron, or also via alternative pathways. For example, it is known that deferoxamine and deferiprone are not entirely specific for iron but are also able to chelate copper, suggesting that their effects might partly rely on chelation of copper. Interestingly, we found that Aβ-induced Lcn2 production can be modulated by certain copper chelators: while bathocuproine disulfonic acid (a membrane impermeable copper chelator) did not affect Lcn2 protein levels, tetrathiomolybdate (a membrane permeable copper chelator) was shown to significantly reduce intracellular Lcn2 levels (Suppl. Fig. 1g-j). The observed inhibitory effect of tetrathiomolybdate on Lcn2 production may be explained by a previous finding from Spisni et al. (2009), showing that copper treatment results in increased Lcn2 secretion from cultured neurons. It thus appears that deferoxamine and deferiprone are not the only chelators that can affect Lcn2 production, and that possibly different biometals might influence Lcn2 production.

Finally, although Lcn2 is known to play a role in iron regulation and is able to mediate both cellular iron import and export, no effect of Lcn2 was found on Aβ-induced ferritin protein production in astrocytes when comparing Aβ-treated WT and Lcn2 KO astrocytes (despite a previously reported effect of Lcn2 on ferritin mRNA expression (Mesquita et al., 2014)). This finding indicates that Lcn2 may not significantly affect Aβ-mediated changes in iron metabolism in astrocyte cultures. Interestingly however, Lcn2 appeared to significantly aggravate brain iron accumulation in mouse models of hemorrhagic stroke and AD (Dekens et al., 2018; Ni et al., 2015). In a mouse model of AD, Lcn2 promoted iron accumulation in Aβ plaques and neuronal layers of the hippocampus (Dekens et al., 2018). However, the exact cellular localization of accumulated iron remains to be determined in more detail. For instance, previous studies suggested that also microglia tend to accumulate high

levels of iron under inflammatory conditions (Holland et al., 2018; McCarthy et al., 2018; Thomsen et al., 2015; Urrutia et al., 2013). As such, iron accumulation in AD (which is in part mediated by Lcn2) might occur mostly in specific cell types and structures, including plaques, neurons and microglia. Astrocytes might be less prone to (Lcn2-mediated) iron accumulation (Rathore et al., 2012; Urrutia et al., 2013), which would be in line with the similar ferritin levels in Aβ-treated WT vs. Lcn2 KO astrocyte cultures that were found here. It should be emphasized that the current study is a short report, warranting further investigation of Lcn2-mediated brain iron regulation in various other experimental conditions. For example, effects of Lcn2 on astrocytic iron metabolism might surface when more ferric and/or ferrous iron would be supplemented to the cell culture medium. Moreover, it is important to recognize that brain iron metabolism depends on intricate communication between different brain cell types (You et al., 2017). Therefore, it would be of great relevance to study iron metabolism in co-/slice-cultures and animals, rather than in single cell type cultures.

Iron chelators are promising therapeutic possibilities for various neurodegenerative diseases and CNS conditions. Their beneficial effects might depend in part on normalization of Lcn2 protein levels.

Abbreviations

Aβ; amyloid-β, AD; Alzheimer's disease, Def; deferiprone, DFO; deferoxamine, DMEM; Dulbecco's Modified Eagle Medium, IL-1β; interleukin 1 beta, Lcn2; lipocalin 2, Lcn2 KO; lipocalin 2 knock-out, LPS; lipopolysaccharide, NGAL; neutrophil gelatinase-associated lipocalin, WT; wild type.

Declarations of interest

Declarations of interest: none.

Ethical approval

All applicable international, national, and institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with EU regulations (EU Directive 2010/63/EU for animal experiments), and were approved by the local (University of Groningen, DEC6659A) and Dutch national (CCD-AVD105002016630) animal ethics committees. This work does not contain any studies with human participants performed by any of the authors.

Appendix A. Supplementary data

One Appendix A – Supplementary data file is available. This Supplementary data file contains Suppl. Fig. 1.

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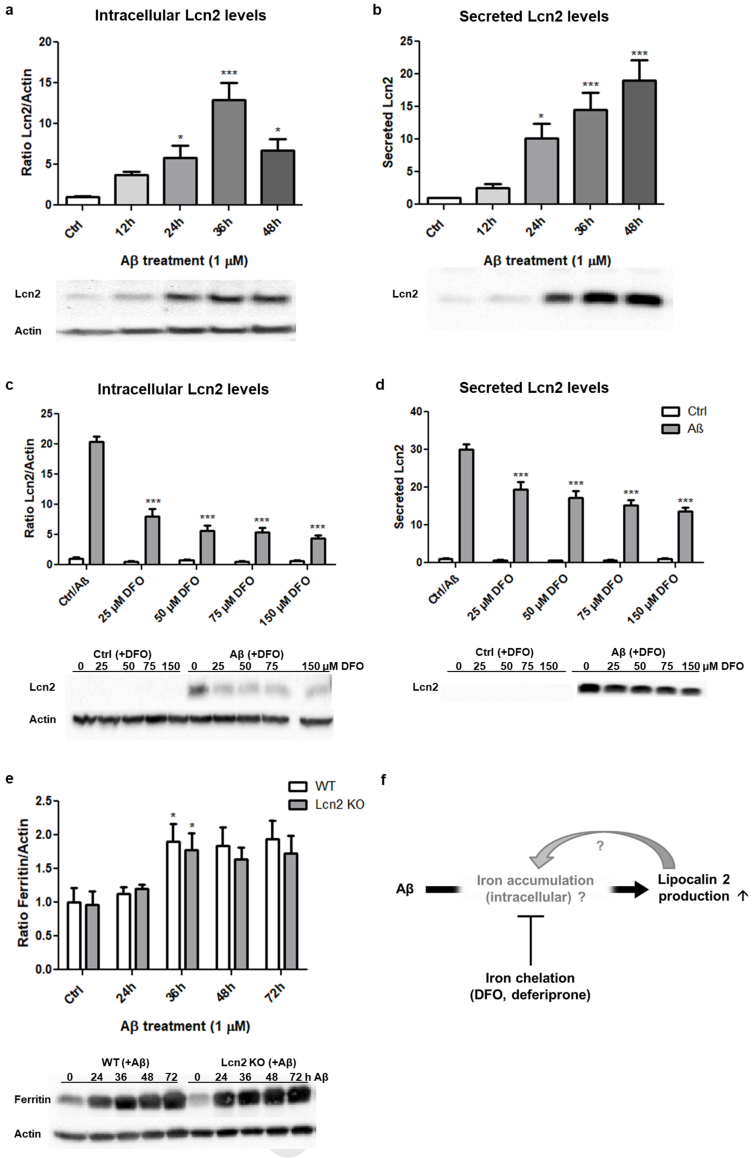
Figure legend

Fig. 1 The iron chelator deferoxamine blocks Aβ-induced astrocytic Lcn2 production, and indicates that Aβ induces a disturbance in astrocytic iron metabolism. **a-b** Intracellular (**a**, controlled for actin) and secreted (**b**) Lcn2 protein levels in primary WT astrocytes treated with 1 µM Aβ for 0-48h. **c-d** Intracellular (**c**, controlled for actin) and secreted (**d**) Lcn2 protein levels in primary WT astrocytes treated with 1 µM Aβ and 0-150 µM deferoxamine (DFO) for 36h. **e** Intracellular ferritin protein levels (controlled for actin) in primary WT and Lcn2 KO astrocytes treated with 1 µM Aβ for 0-72h. **f** Proposed connection between Aβ, iron and Lcn2, with uncertain points indicated in grey. Bars depict the mean and standard error of the mean (SEM). Representative blots are shown below graphs. Tested with one-way ANOVA with Dunnett's multiple comparison post-hoc test to compare conditions to their respective control condition. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.0001$ compared to the respective control conditions.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Ratio Lcn2/Actin
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Ferritin
Actin
f
?
Aβ
Iron accumulation
(intracellular) ?
Lipocalin 2
production ↑
Iron chelation
(DFO, deferiprone)

Highlights

- Amyloid-β (Aβ) induces Lipocalin 2 (Lcn2) production in primary cultured astrocytes.
- Iron chelators deferoxamine and deferiprone abrogate Aβ-induced Lcn2 production.
- Aβ affects iron homeostasis in primary astrocyte cultures.
- Lcn2 is not essential for Aβ-induced disturbance of astrocytic iron homeostasis.