Neuroinflammation and copper in Alzheimer's disease: the role of mitochondrial oxidative stress

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INTRODUCTION

Changes in copper (Cu) homeostasis have been consistently linked to Alzheimer's disease (AD). Cu can bind to amyloid beta (Aβ) and enhance neurotoxicity by generating reactive oxygen species (ROS)[1,2]. Furthermore, mixed evidence indicates that Cu possesses both pro- and anti-inflammatory properties that may be partly mediated by its spatial proximity to amyloid plaques[3]. A key pro-inflammatory pathway reported induced in AD is the NLRP3 inflammasome[4] that leads to the activation of inflammatory caspases, mainly caspase 1, and the processing of interleukin-1β (IL-1β) into its active form. Whether increased Cu levels can regulate Aβ-induced inflammasome and contribute to the aberrant activation of microglia is not fully known.

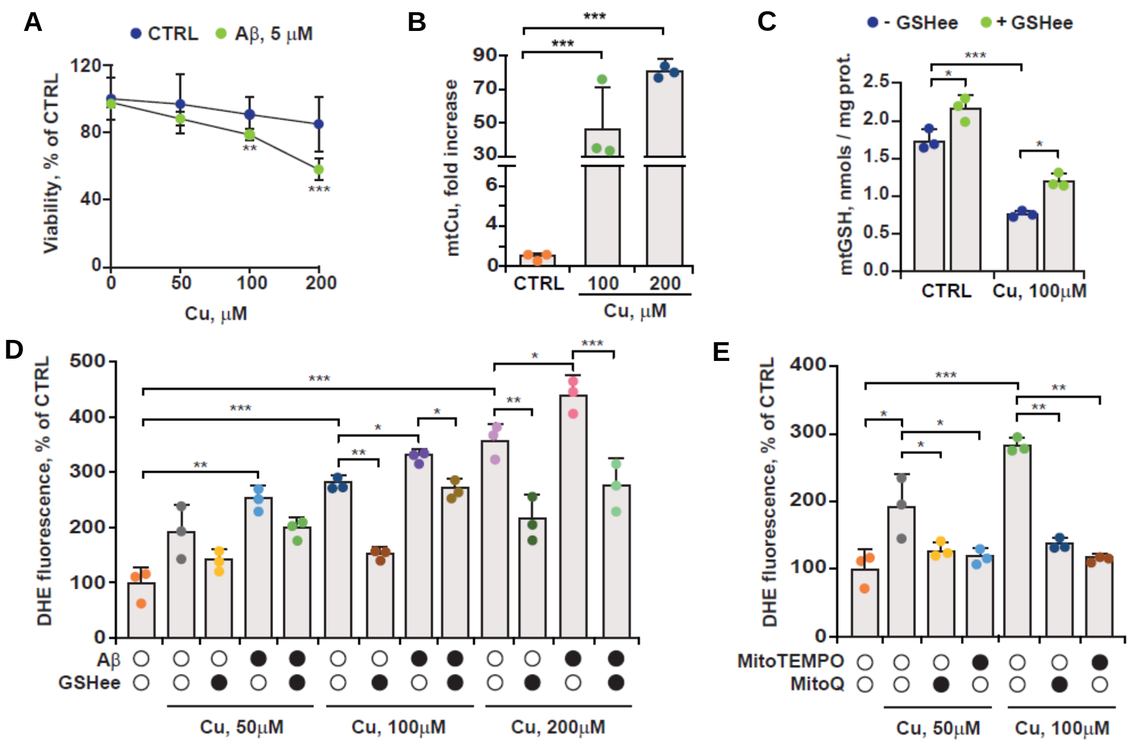
This work aimed to assess the synergistic interaction between Cu overload and the Aβ-induced inflammatory response on microglia and evaluate the underlying mechanisms.

METHODS

Immortalized mouse microglia cells (SIM-A9) were exposed to Aβ and sub-toxic doses of Cu for 24 h. ROS production was measured by fluorometry using dihydroethidium (DHE, 10 µM) for 30 min at 37°C. The expression levels of inflammasome-related proteins were analysed by western blot and RT-PCR, while the formation of intracellular ASC specks, as an indicator of inflammasome assembly, was assessed by confocal microscopy in cells transiently transfected with the pLEX-MSC-ASC-GFP vector. Microglial phagocytosis was evaluated by confocal microscopy using HiLyte™ Fluor 488-labeled Aβ. Cholesterol levels in total homogenates and mitochondrial fractions—isolated using digitonin—were quantified with the Amplex Red Cholesterol Assay Kit. Statistical significance was determined by one-way ANOVA followed by Bonferroni post-hoc test, considering $p < 0.05$ statistically significant.

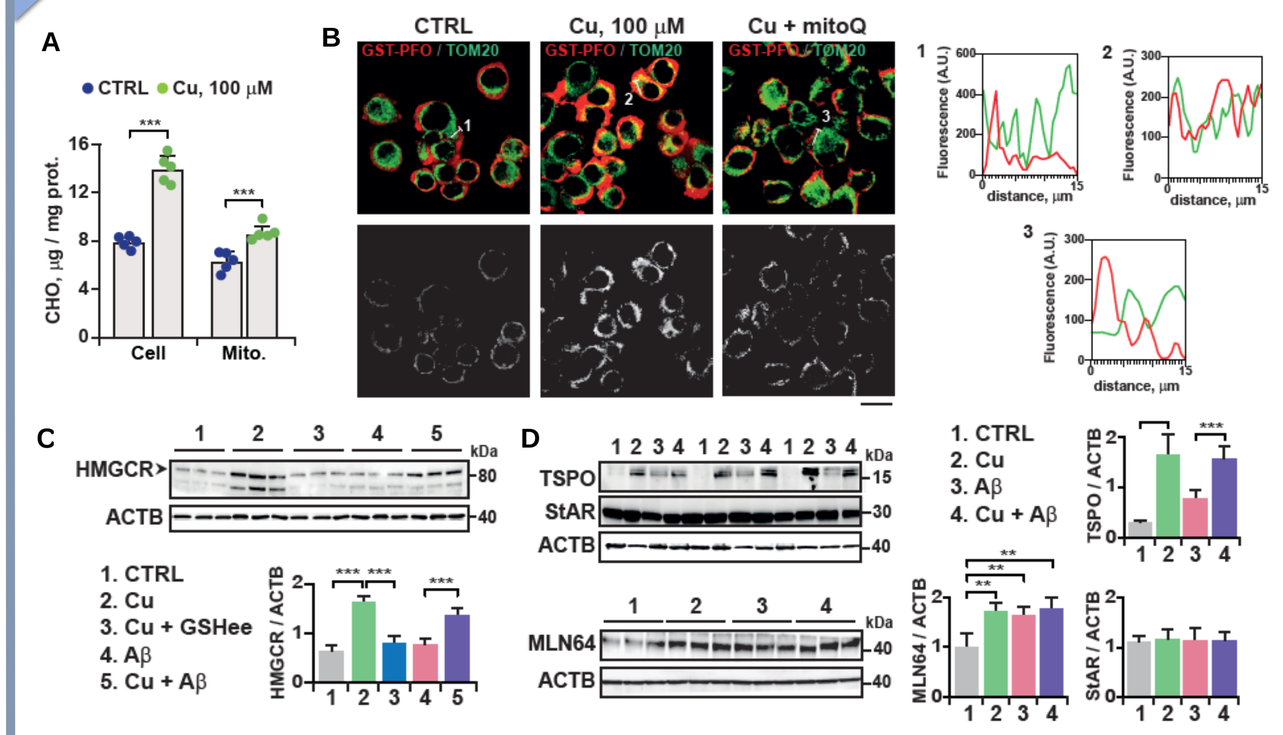
RESULTS

1 Cu treatment enhances the mitochondrial oxidative stress elicited by Aβ



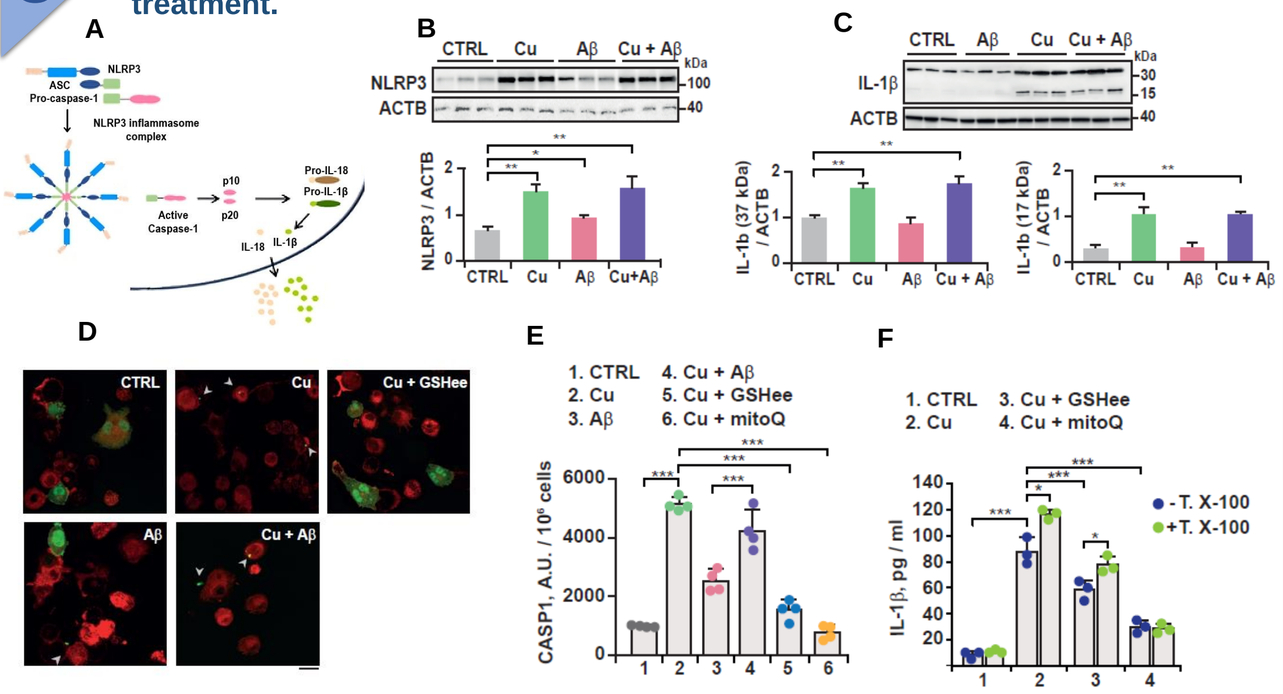
(A) Cell viability after exposure to different copper concentrations (50 µM, 100 µM, and 200 µM) with or without Aβ (5 µM) for 24 h. Results are expressed as a percentage relative to control (CTRL).
(B) Mitochondrial Cu levels measured as fold increase relative to control after copper exposure (100 µM and 200 µM).
(C) Changes in mitochondrial glutathione (mtGSH) levels after exposure to copper (100 µM) compared to control conditions (CTRL).
(D) Intracellular fluorescence measurements (DHE) showing ROS levels following copper treatments at varying concentrations (50 µM, 100 µM, and 200 µM).
(E) Protective effects of MitoQ and MitoTEMPO on oxidative stress markers (DHE fluorescence) during copper exposure (50 µM and 100 µM). Statistical significance is indicated as *p < 0.05, **p < 0.01, ***p < 0.001.

2 Impact of Cu exposure on cholesterol metabolism and mitochondrial cholesterol transport



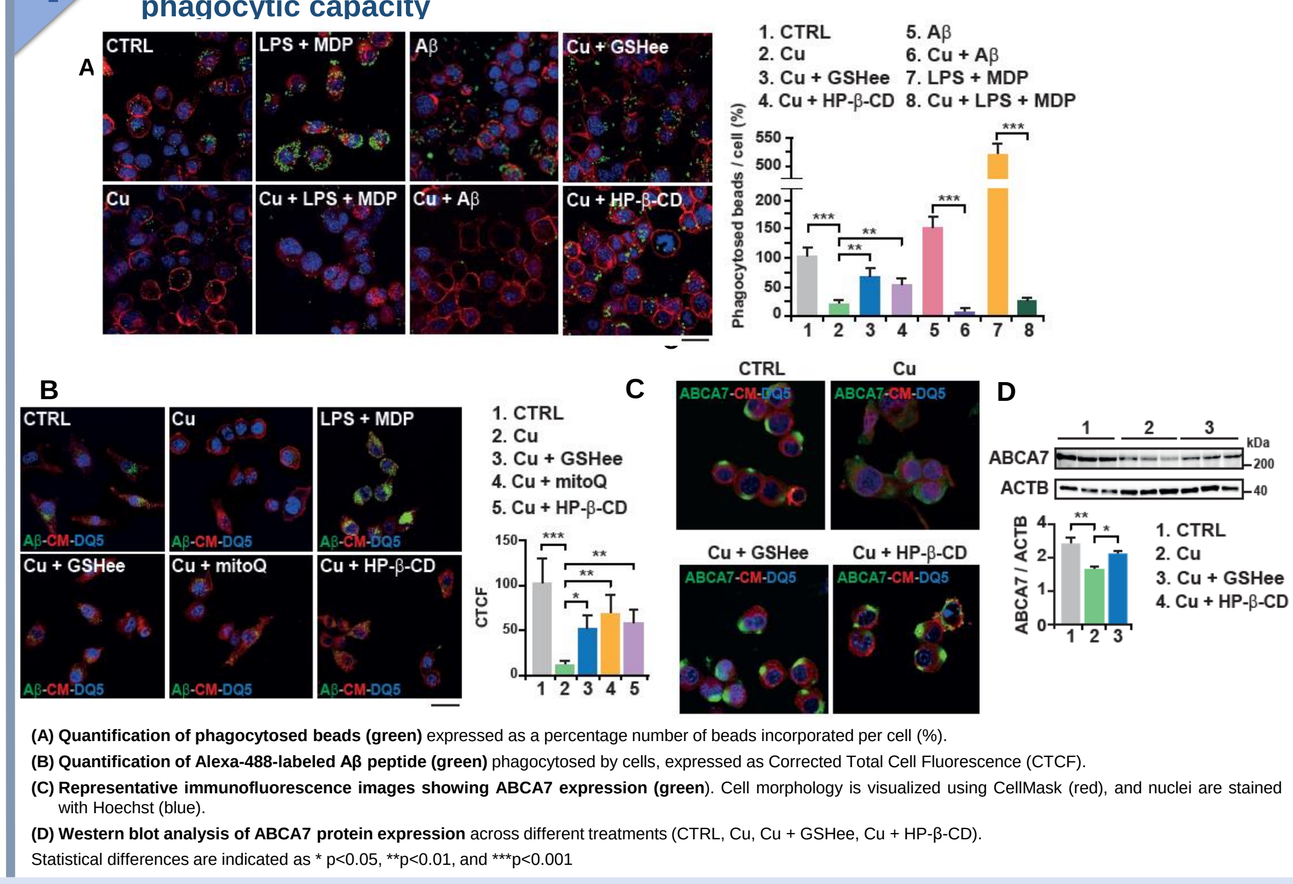
(A) Total cholesterol levels (CHO) in cell extracts and mitochondrial fractions after exposure to copper (100 µM) compared to control (CTRL).
(B) Cholesterol distribution in mitochondria is assessed by colocalisation of the GST-PFO probe, which binds cholesterol, and TOM20, a mitochondrial marker. The grey images represent the colocalisation mask, highlighting the overlap between the two signals.
(C) Western blot analysis of HMGCR protein levels normalised to ACTB.
(D) Western blot analysis of TSPO, MLN64, and Star proteins levels normalized to ACTB.
Statistical significance is indicated as *p < 0.05, **p < 0.01, ***p < 0.001.

3 NLRP3 inflammasome activation after Cu exposure, prevented by GSH treatment.



(A) Inflammasome pathway.
(B) NLRP3 and (C) IL-1β protein levels assessed by Western blot, normalised with ACTB**.**
(D) ASC-GFP fluorescence (green) in cells transfected with pLEX-MSC-ASC-GFP plasmid, indicating inflammasome activation (ASC speckles).
(**E) Caspase-1 activity** measured by Caspase-Glo® 1 Inflammasome Assay kit (A.U./million cells).
(D) IL-1β release quantified by ELISA, distinguishing free vs. compartmentalised IL-1β with or without Triton X-100.
Statistical differences are indicated as * p<0.05, **p<0.01, and ***p<0.001

4 Activation of SIMA9 cells after Cu overload is accompanied by impaired phagocytic capacity



(A) Quantification of phagocytosed beads (green) expressed as a percentage number of beads incorporated per cell (%).
(B) Quantification of Alexa-488-labeled Aβ peptide (green) phagocytosed by cells, expressed as Corrected Total Cell Fluorescence (CTCF).
(C) Representative immunofluorescence images showing ABCA7 expression (green). Cell morphology is visualized using CellMask (red), and nuclei are stained with Hoechst (blue).
(D) Western blot analysis of ABCA7 protein expression across different treatments (CTRL, Cu, Cu + GSHee, Cu + HP-β-CD).
Statistical differences are indicated as * p<0.05, **p<0.01, and ***p<0.001

CONCLUSIONS

Elevated Cu levels regulate the microglial response to Aβ by increasing mitochondrial oxidative stress, leading to inflammasome activation and the release of proinflammatory cytokines, such as IL-1β. This process is aggravated by a Cu-induced increase in cholesterol levels, which reduces ABCA7 in the membrane, affecting phagocytosis. This decreases the ability to clear Aβ, contributing to forming Aβ deposits. Overall, our findings provide new insights into the role of Cu in the pathogenesis of Alzheimer's disease (AD) and suggest possible therapeutic targets to modulate microglial function, cholesterol metabolism, and Aβ phagocytosis.

Acknowledgements

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