The Citrulline Recycling Pathway is Incapable of Supplying Arginine for NO Production in RAW 264.7 Macrophages

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BACKGROUND

Arginine (ARG) is conditionally essential in sepsis and its depletion is thought to contribute to multiorgan injury[1]. Nitric oxide synthase (NOS) catalyzes ARG into nitric oxide (NO), an important vasodilator and immune modulator, and citrulline (CIT). Argininosuccinate synthase (ASS1) and lyase (ASL), key enzymes of the CIT recycling pathway, can potentially regenerate the intracellular pool of ARG from CIT, though data are conflicting[2,3]. Supplementation of ARG in sepsis remains controversial, though CIT supplementation remains a therapeutic alternative to restore ARG concentrations. NOS in macrophages is highly active, therefore it is not clear to what extent the CIT recycling pathway can sustain NO production.

PURPOSE

We aimed to determine the capability of ASS1 and ASL to restore NO production in an activated, widely used macrophage cell line.

METHODS

- RAW 264.7 murine macrophages (ATCC, TIB-71) were grown in complete media (DMEM containing 10% FBS) and activated with LPS (1 µg/mL, InvivoGen, San Diego) for 6-24 hours.
- Total protein extracts were analyzed by Western blotting for inducible iNOS, ASS1, ASL and arginase (ARG1).
- A second set of cells were deprived of ARG (ARG-free DMEM containing 10% FBS [dialyzed]) and supplemented with various concentrations of ARG (0-1000 µM) or CIT (0-1000 µM) and subjected to 24 hours of LPS (1 µg/mL) treatment.
- NO release was assessed by analyzing nitrite concentration of macrophage culture supernatants by Griess assay.
- Data were analyzed using ANOVA with Dunnett's post-hoc test or a 4-parameter logistic regression model (to determine EC_{50}). Significance was set at $p<0.05$.

Fig 1: Utilization of ARG by NOS to produce NO and CIT, which can be recycled back to ARG via the CIT recycling pathway (red).

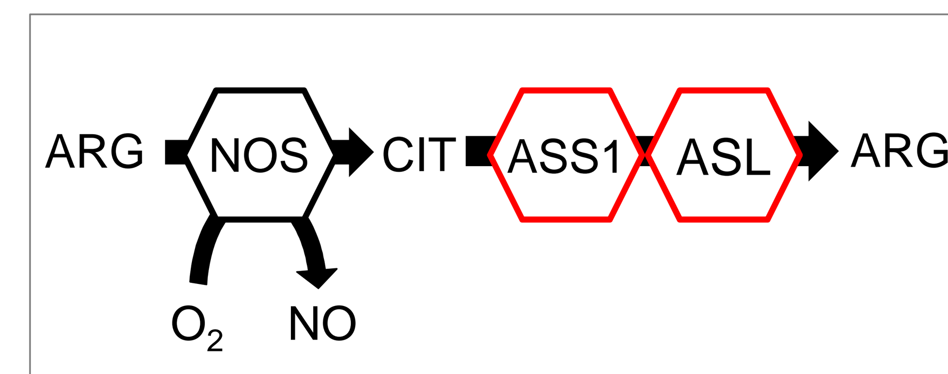
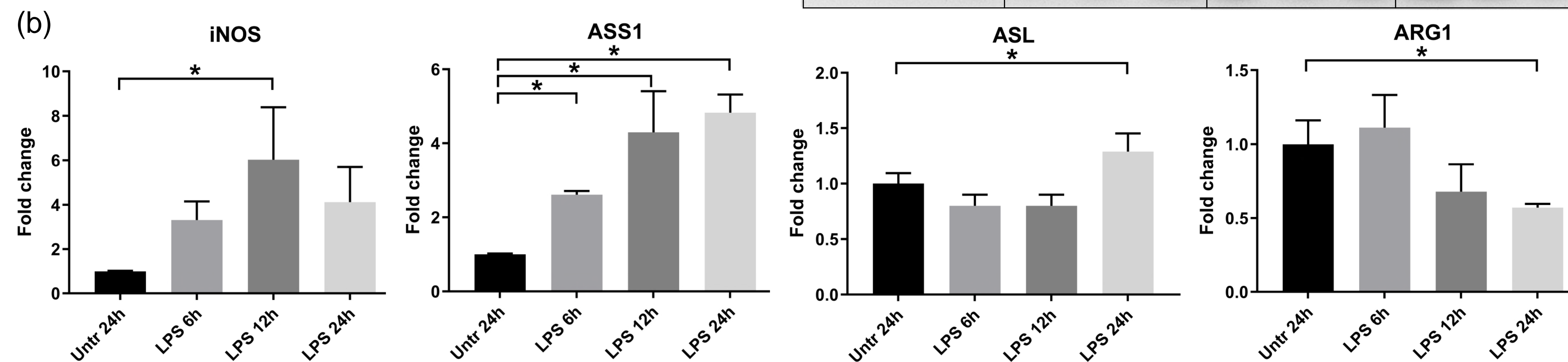


Fig 2: LPS induced iNOS, ASS1, and ASL protein expression in RAW 264.7 macrophages. Western blot (a) and fold change (b) of LPS treatment effect on induction of enzymes involved in ARG metabolism. *p<0.05; n = 3/group

(a)

24h Untr | 6h LPS | 12h LPS | 24h LPS

iNOS
ASS1
ASL
ARG1
β-actin



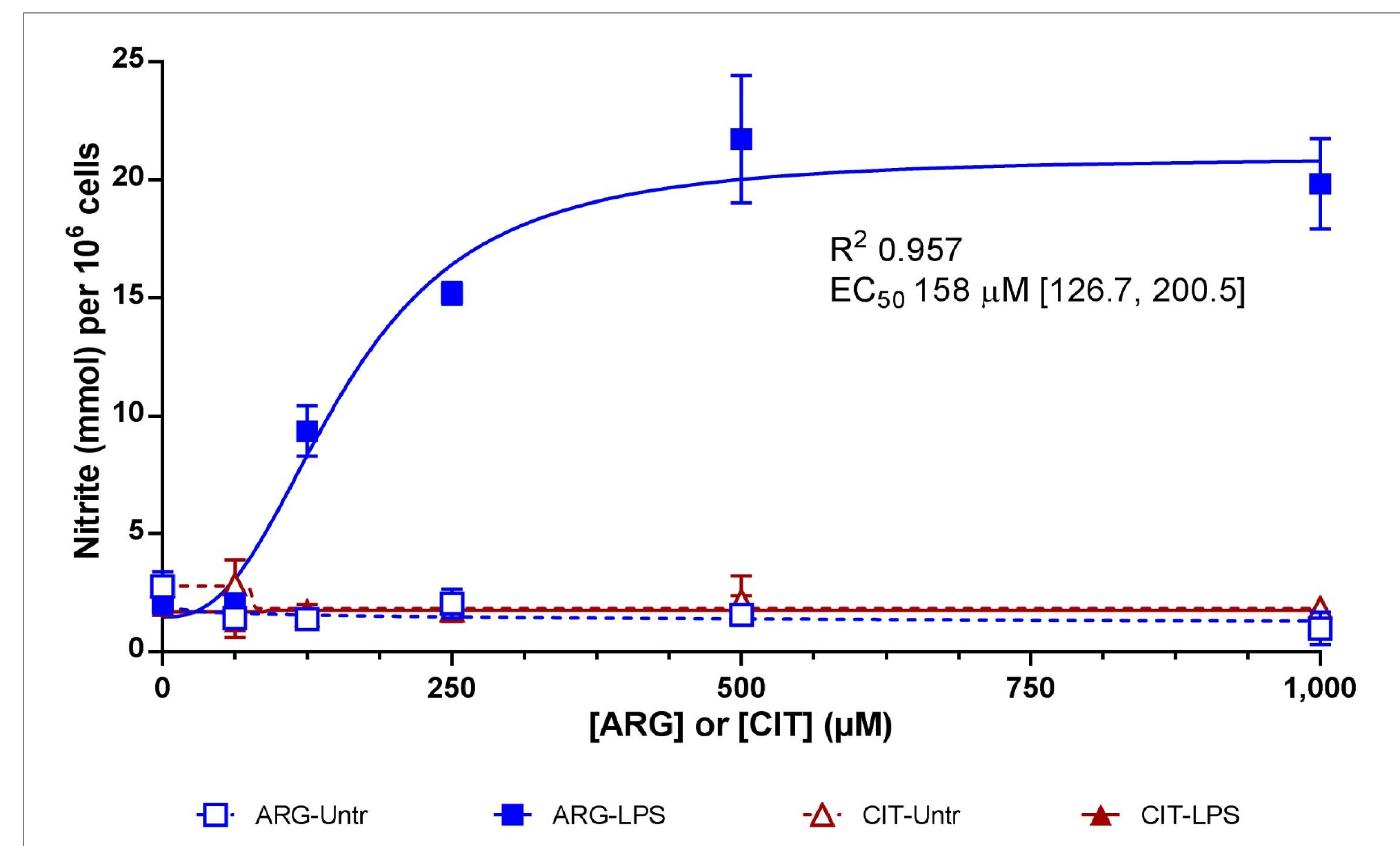


Fig 3: ARG supplementation is necessary to induce NO production in macrophages. Amount of nitrite produced by untreated and LPS-treated (24h) RAW 264.7 murine macrophages supplemented with varied concentrations of ARG or CIT after ARG-deprivation. Supplementation of ARG was sufficient for nitrite production in LPS-treated macrophages, while CIT supplementation alone was insufficient for nitrite production.

RESULTS

- LPS induced iNOS, ASS1, and ASL protein expression. **(Fig. 2b)**
- ARG1 protein expression was decreased after 24h LPS treatment. **(Fig. 2b)**
- ARG supplementation decreased NO production by unstimulated macrophages. **(Fig. 3)**
- LPS-stimulated macrophages were unable to increase NO production in the absence of ARG. **(Fig. 3)**
- LPS-stimulated macrophages produced significantly more NO with increasing ARG supplementation compared to unstimulated macrophages **(Fig. 3)**
- CIT supplementation after ARG deprivation did **not** permit NO production in unstimulated or LPS-stimulated macrophages **(Fig. 3)**

CONCLUSIONS

LPS-mediated induction of the enzymes of ARG metabolism support the hypothesis of ARG restoration via the CIT recycling pathway. However, CIT supplementation was inadequate to restore NO production in activated RAW 264.7 macrophages despite intact protein expression of ASS1 and ASL, suggesting an apparent dependency of these cells on exogenous ARG. Our findings highlight cell-specific regulation of CIT recycling and potential post-translational regulation of ASS1 and ASL in RAW 264.7 murine macrophages and confirm similar findings reported in rat alveolar macrophages[3]. Future studies will validate these findings in human THP-1 monocyte derived macrophages.

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