Calcimycin inhibits intracellular mycobacterial growth by regulating autophagy

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ABSTRACT

Mycobacterium tuberculosis (*M. tb*), the causative agent of tuberculosis resides and multiplies in macrophages by inhibiting various host defensive pathways like autophagy. Increased autophagy by different inducers like adenosine triphosphate (ATP) has been shown to be antimycobacterial through calcium-dependent mechanism that decreased *M. bovis* BCG viability. Our preliminary screening experiment using whole cell based approach has identified Calcimycin, which increases intracellular calcium as a potent inhibitor of *M. tb* growth. We observed that Calcimycin was bactericidal in action and inhibited *M. tb* growth *in vitro* by 99% at 1.25 µM. So, present study was undertaken to decipher the role of Calcimycin on intracellular mycobacteria in THP-1 cells. We found by MTT assay that 0.4 µM of Calcimycin is non-toxic even after 72 h of treatment. Viability findings were further corroborated by Trypan blue dye exclusion assay where Calcimycin treatment for 72 h reduced cell viability to 86.4 ± 1.8%, 73.0 ± 11.9% and 62.2 ± 10.2% at 0.6 µM, 0.8 µM and 1 µM respectively. Time kinetic experiment was performed with 0.4 µM of Calcimycin to study its effect on the autophagy in THP-1 cells. We found optimum up-regulation of different autophagy markers like Beclin-1, Atg 7 and Atg 3 after 12 h of treatment through western blotting. Increased conversion of LC3-I to LC3-II, widely used marker of autophagy induction was also observed in treated cells suggesting increased autophagy. Addition of 3-methyl adenine (3-MA), an autophagy inhibitor, abrogated the effect of Calcimycin on autophagy. We found down-regulation of Beclin-1, Atg 7, Atg 3 and LC3-II expression in Calcimycin treated cells in the presence of 3-MA. Increased autophagy in Calcimycin treated cells led to decrease in intracellular *M.* smegmatis viability and addition of 3-MA reversed this effect. Studies are currently underway to determine the mechanism of how Calcimycin induced autophagy exerts its antimycobacterial effect.

INTRODUCTION

Tuberculosis (TB) is a major reason of deaths worldwide caused by obligate human pathogen, *Mycobacterium tuberculosis* (*M. tb*). Infection with *M. tb* begins with inhalation of tubercle bacilli, which are phagocytosed by alveolar macrophages. The macrophages most often destroy the pathogen by employing various defence mechanisms like generation of reactive oxygen and nitrogen intermediates, production of lysosomal enzymes and toxic peptides, increased phagosome-lysosome fusion, apoptosis and increased autophagy of the infected cells. Autophagy during infection acts as an innate defence mechanism. Autophagy machinery on immune responses show a complex reciprocal relationship, as it can regulate the immune and inflammatory responses and on another hand, immune or inflammatory responses can suppress or induce autophagy. There are reports supporting the role of autophagy in modulating intracellular mycobacterial growth. The recent finding of calcium-dependent autophagy induction in an adenosine triphosphate (ATP)-dependent pathway is associated with the reduction of this intracellular pathogen. Therefore, we intended to study the effect of novel calcium-inducing compounds like Calcimycin, which is an ionophorous, polyether antibiotic from *Streptomyces chartreusensis* on autophagy of mycobacteria infected macrophages and intracellular viability of mycobacteria. Strategies aimed at stimulation of autophagy through pharmacological agents like Calcimycin that induces the increase in calcium level may have therapeutic implications in treating TB.

METHODS

THP-1 cells were differentiated with 50 nM of PMA for overnight and treated with different concentration of Calcimycin. The effect of Calcimycin on the viability of THP-1 cells was studied using MTT cell proliferation and Trypan blue dye exclusion assay. Time-kinetic studies were then performed to see the autophagic potential of selected non-toxic concentration of Calcimycin in inducing autophagy by studying expression of various autophagic protein markers through western blotting. To check the specificity of autophagy, in some experiments, well-characterized autophagy inhibitor like 3-MA was also used. Calcimycin-induced autophagy was also validated by studying LC3 puncta formation in treated samples using confocal microscope. Effect of autophagy induction on the viability of mycobacteria was also studied by CFU plating the lysates of control and treated samples on 7H10 agar plates.

RESULTS

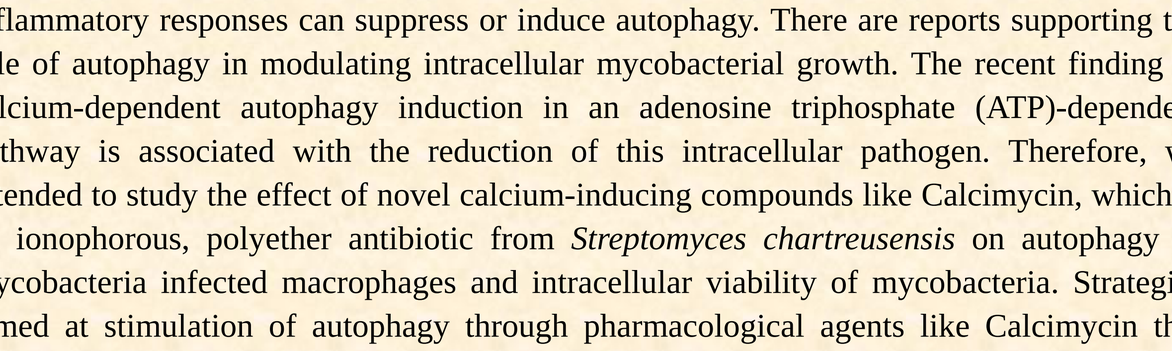

Fig. 1. Effect of varying concentration of Calcimycin on cumulative % cell viability of THP-1 cells after 24, 48 and 72 hr of treatment . (A) **MTT assay.** Differentiated THP-1 cells were treated with Calcimycin for 24, 48 and 72 h. At indicated time points, MTT reagent was added and plate was incubated for 3 h and then lysis buffer was added. OD was taken at 562 nm. Data are expressed as percentage cell viability over control cells. (B) **Trypan blue dye exclusion assay.** Differentiated THP-1 cells were treated with Calcimycin for 24, 48 and 72 h. At indicated time points, cells were mixed in equal proportions with trypan blue dye. Cells were then counted under tissue culture inverted microscope. Data are expressed as percentage cell viability over control cells. Values are expressed as Mean±SEM. ** signifies $p < 0.005$ and * signifies $p < 0.05$.

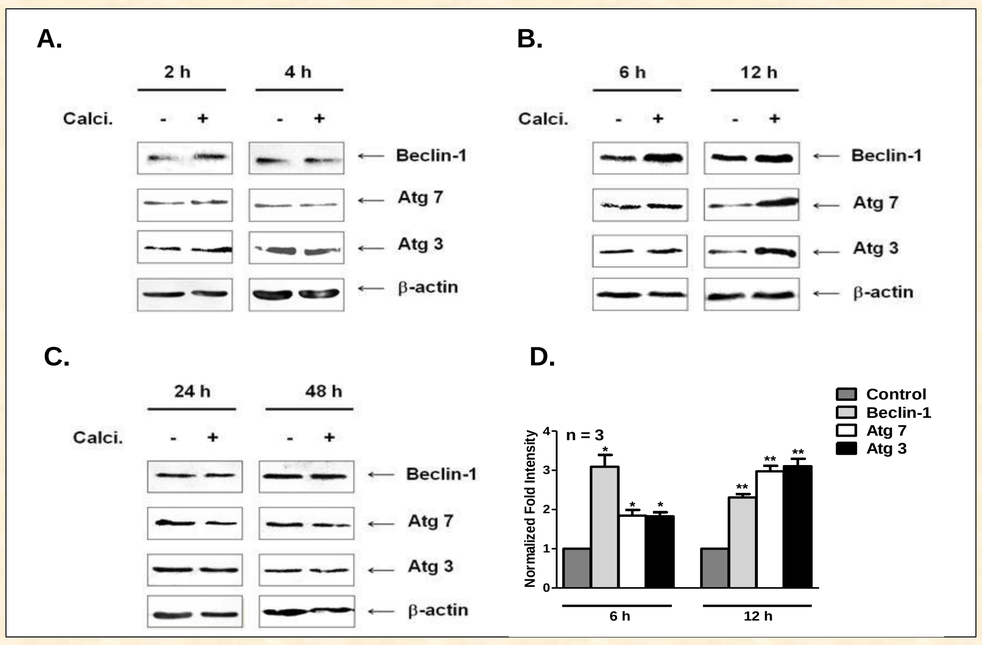

Fig. 2. Effect of Calcimycin (0.4 µM) on autophagy in differentiated THP-1 cells. (A-C) THP-1 cells were treated with Calcimycin and at indicated time points, whole-cell lysates were subjected to Western blotting. Data shown are picture of one experiment performed in triplicate. (D) Statistical analysis of western blotting replicates showing normalized fold intensity of proteins after 6 and 12 h of Calcimycin treatment. Values are expressed as Mean±SEM. ** signifies $p < 0.005$ and * signifies $p < 0.05$.

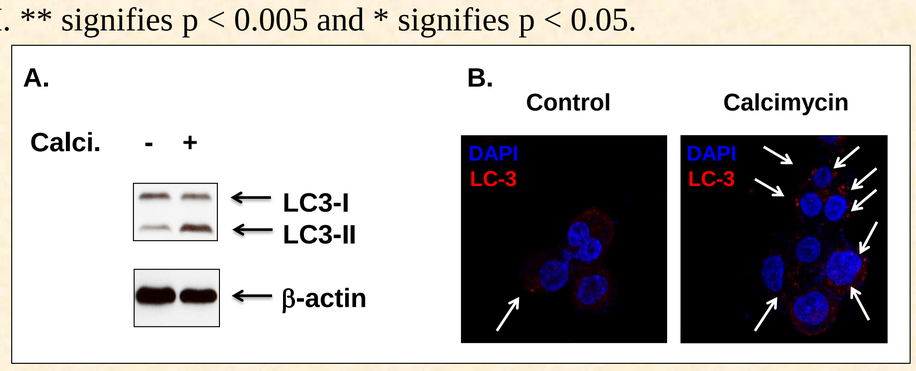

Fig. 3. Effect of Calcimycin on LC3 conversion in differentiated THP-1 cells. (A) THP-1 cells were treated with Calcimycin for 12 h and then whole-cell lysates were subjected to Western blotting. (B) Treated THP-1 cells were stained with anti-LC3 antibody and DAPI. Cells were then visualized under confocal microscope.

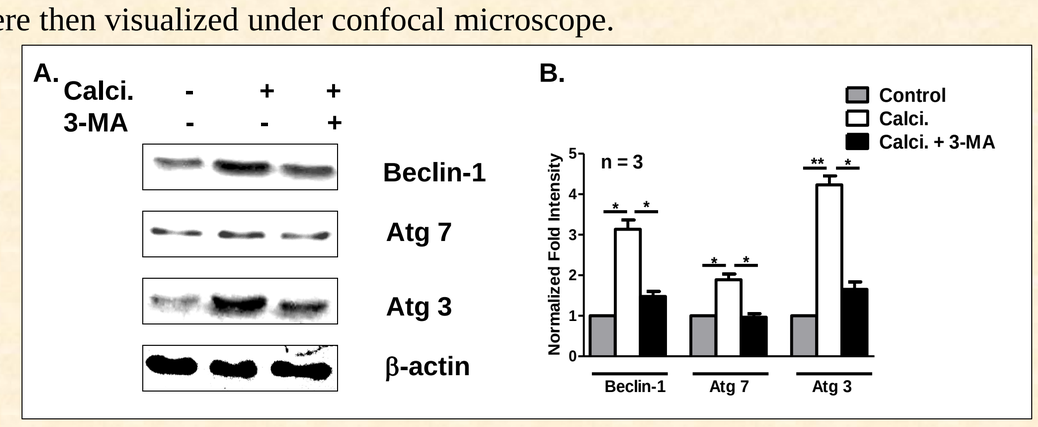

Fig. 4. Effect of 3-MA on autophagy in Calcimycin treated THP-1 cells. (A) THP-1 cells were pretreated 1 h with 3-MA before Calcimycin addition. After 12 h of addition, whole-cell lysates were prepared and subjected to western blotting. Data shown are the pictures of one experiment performed in triplicate. (B) Statistical analysis of western blotting replicates showing normalized fold intensity of proteins in different combinations. Values are expressed as Mean±SEM. ** signifies $p < 0.005$ and * signifies $p < 0.05$.

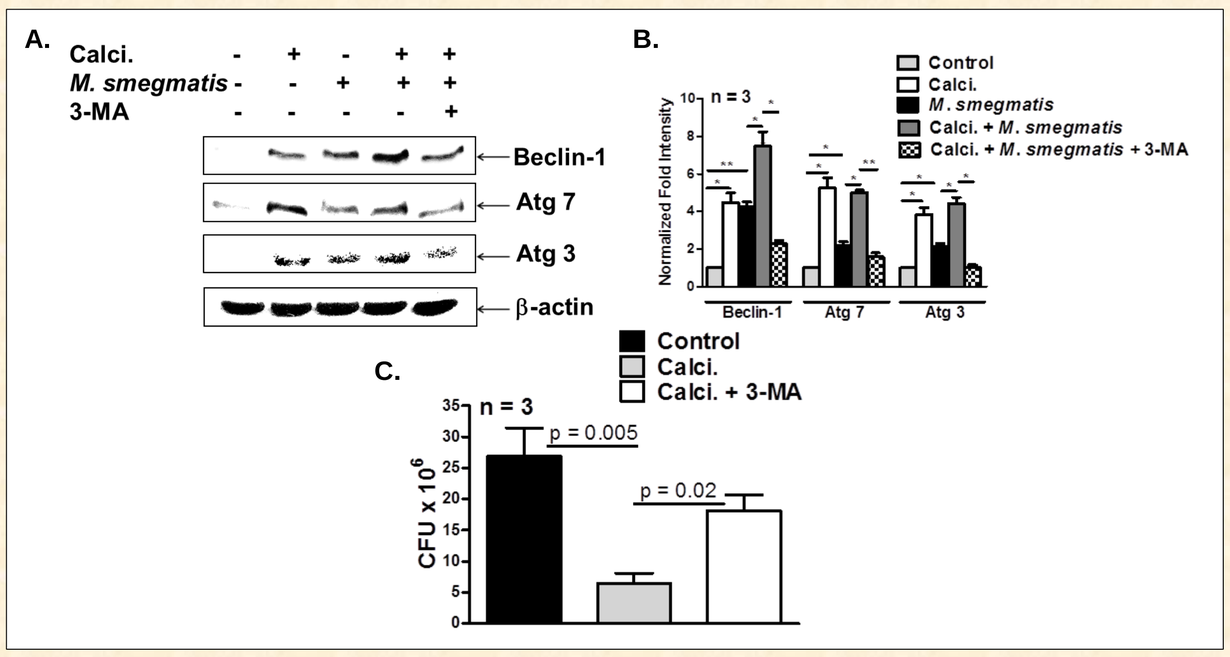

Fig. 5. Effect of Calcimycin on autophagy and intracellular mycobacterial viability in infected macrophages. (A) Infected THP-1 cells were treated with Calcimycin. After 12 h of treatment, whole-cell lysates were subjected to Western blotting. Data shown are picture of one experiment performed in triplicate. (B) Statistical analysis of western blotting replicates showing normalized fold intensity of proteins. Values are expressed as Mean±SEM. ** signifies $p < 0.005$ and * signifies $p < 0.05$. (C) Infected THP-1 cells were treated with Calcimycin. After 2 days of treatment, cells were lysed and lysates were log diluted and plated on 7H10 agar plates.

CONCLUSIONS

1. Calcimycin above 0.4 µM conc. is cytotoxic to PMA differentiated THP-1 cells.
2. Non-toxic concentration of Calcimycin induced autophagy in differentiated THP-1 cells
3. 3-MA abrogated the autophagy induction potential of Calcimycin.
4. Autophagy induction by Calcimycin decreased intracellular mycobacterial viability.

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