Role for malonyl coenzyme A:acyl carrier protein transacylase (MCAT) in the growth-inhibitory effect of the calmodulin antagonist trifluoperazine in Mycobacterium bovis BCG

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Received 27 January 2004; returned 17 February 2004; revised 8 March 2004; accepted 18 March 2004

Objectives: To determine whether the fatty acid synthesis enzyme malonyl coenzyme A:acyl carrier protein transacylase (MCAT) is involved in the growth-inhibitory effect of trifluoperazine in the tubercle bacillus Mycobacterium bovis BCG.

Methods: BCG was grown in liquid culture with various concentrations of trifluoperazine and growth was monitored by OD measurement. To determine the effect of trifluoperazine on MCAT protein level, total protein was extracted from BCG cultures and was analysed by 2D gel electrophoresis and western blot. To confirm trifluoperazine-dependent reduction in the MCAT protein level, two BCG strains overexpressing MCAT at a low and high constitutive level were similarly tested. The synergistic effect of trifluoperazine and isoniazid was tested at sub-MIC levels in liquid cultures.

Results: Trifluoperazine inhibition of growth correlates with reduction in the steady-state level of MCAT protein. Overexpression of MCAT confers resistance to trifluoperazine. Trifluoperazine acts synergistically (albeit weakly) with isoniazid and no resistance towards isoniazid alone was observed due to overexpression of MCAT. This suggests MCAT to be a specific target of trifluoperazine.

Conclusion: These results indicate MCAT as a target of trifluoperazine and provide an explanation for the inhibitory effect of trifluoperazine on mycobacterial lipid synthesis observed earlier. This makes MCAT a potential target for new antimycobacterials.

Keywords: fatty acid synthesis, M. bovis, antimycobacterials

Introduction
Trifluoperazine, an antagonist of mammalian calmodulin, inhibits growth of Mycobacterium tuberculosis at MICs of 8–32 mg/L. Its mechanism of action is unknown. Ratnakar & Murthy showed that trifluoperazine exposure inhibits incorporation of [14C]acetate into lipids, suggesting fatty acid synthesis as a possible target of the compound. Interestingly, genetic work carried out earlier in Escherichia coli mapped spontaneous trifluoperazine resistance mutations to the fabD gene which encodes malonyl coenzyme A:acyl carrier protein transacylase (MCAT), an essential component of the fatty acid biosynthetic pathway (FASII). The molecular nature of the mutations was not determined. Here, we asked whether MCAT is involved in the growth-inhibitory effect of trifluoperazine in the tubercle bacillus Mycobacterium bovis BCG.

Materials and methods
Strain, media, cultivation and monitoring of growth
All experiments were conducted with M. bovis BCG Pasteur ATCC 35734 at 37°C. Liquid medium was Dubos Tween-albumin broth (Difco); solid medium was Dubos oleic-albumin agar (Difco). Trifluoperazine, isoniazid and kanamycin were obtained from Sigma. Liquid cultures (100 mL) were grown in 10 × 14 cm plastic roller bottles at 1 rpm. and with a starting OD600 of 0.05. The containers were opened daily to
allow exchange of air. Growth was determined by turbidity measurement in an Ultraspec 3000 photometer (Pharmacia Biotech).

Preparation of protein extracts, sodium dodecyl sulphate polyacrylamide gel electrophoresis, 2D gels and western-blot analysis

Protein extracts were prepared as previously described. The cells were washed twice in cold PBS, resuspended in lysis buffer (9 M urea, 4% CHAPS, 50 mM DTT, 1 g/L Pefabloc, 1 mg/L pepstatin, 1 mg/L leupeptin) and disrupted in a Mini-Bead beater (Bio-Spec, Bartlesville, OK, USA) at 4°C for a total of 2 min using 0.5 mm glass beads. Protein concentrations were determined using the Bio-Rad protein assay reagents and protocols; 10 μg of total protein was electrophoresed on 10% SDS–PAGE using Bio-Rad Mini-Protein electrophoresis cells. For 2D gels, 60 μg of total protein was subjected to isoelectric focusing using Immobiline Dry Strips of pH range 4.5–5.5 in an IPGphor isoelectric focusing unit (Amersham) and subsequently separated on SDS–polyacrylamide gels using the Bio-Rad Protein IIxi system. For immunodetection of phosphoproteins, the proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad) at 110 V for 1 h in a Bio-Rad transblot apparatus. The membranes were blocked in PBS buffer containing 1% bovine serum albumin (BSA-Fraction V, Sigma) and 0.1% Tween 20 for 45 min at 37°C. The blots were probed overnight at 4°C with anti-histidine monoclonal antibody (1:1000 dilution; Roche Diagnostic GmbH, Mannheim, Germany), diluted in blocking buffer. Then the blots were subjected to three washes (10 min each) in PBS containing 0.1% Tween 20 and incubated with horseradish peroxidase conjugated goat anti-mouse IgG (1:1000; Sigma) for 1 h at room temperature. After three washes, the blots were developed using the enhanced chemiluminescence (ECL) kit (Amersham) as described by the manufacturer.

Strong overexpression of MCAT in BCG

To generate a BCG strain that strongly overexpresses MCAT, the MCAT coding sequence (Rv2243) was PCR-amplified using the primers AAG-GGCCCATTGGTTGTCGACCCCGGAACAGGTT [containing an Apal site (underlined)] and CGGGATCTTAGGTGGGTGGGTGGTTG-GGTGTAGTTGGACGCTGTC [containing a BamHI site (underlined), a TAA stop codon (italics) and six histidine codons (underlined); Rv number and primer sequences according to http://genolist.pasteur.fr/Tuberculist/] digested with Apal and BamHI and ligated into the shuttle vectorpJEM15-physp60 containing a strong hsp60 expression cassette. High-level expression of MCAT-His in the resulting recombinant BCG strain (BCG MCAT-His-HIGH) was confirmed by SDS–PAGE/Coomassie Blue staining and western-blot analysis with anti-histidine antibody (data not shown). There is no effect of MCAT overexpression on growth within the period tested.

Synergetic activities of trifluoperazine and isoniazid

Various combinations of trifluoperazine and isoniazid were tested to see their effect on growth of BCG wild-type, BCG MCAT-His-LOW and BCG MCAT-His-HIGH strains to underscore the relationship between the degree of sensitivity to drugs and the level of expression of MCAT. All the combinations tested were at sub-MIC levels and the individual values were chosen on the basis of individual MICs for each drug. Sub-MICs (in mg/L) of trifluoperazine and isoniazid were 1.25, 2.5 and 5.0; and 0.015, 0.03, 0.06 and 0.12, respectively.

Results and discussion

BCG was grown at various trifluoperazine concentrations. Figure 1(a) shows that 5 mg/L trifluoperazine strongly reduced growth of the culture. The MIC was found to be 10 mg/L trifluoperazine. To determine the effect of trifluoperazine on MCAT, total protein was extracted from BCG cultures grown for 1 day either under drug-free conditions or in the presence of 5 mg/L trifluoperazine. The proteins were separated via two-dimensional gel electrophoresis and visualized using silver staining. Figure 2(a) shows that, surprisingly, trifluoperazine treatment resulted in a marked reduction in the steady-state level of the MCAT protein. Comparison of the two-dimensional gels from drug-free and trifluoperazine-containing cultures did not reveal any other obvious differences in the protein pattern suggesting that the observed effect of trifluoperazine on MCAT was rather specific. To confirm the trifluoperazine-dependent reduction in MCAT protein level, we made use of a previously constructed recombinant BCG strain expressing a histidine-tagged version of MCAT at a low constitutive level (‘BCG MCAT-His-LOW’). Figure 1(b) shows that trifluoperazine had similar effects on growth of this low-level MCAT-His expressing strain when compared to the effects of the compound on the wild-type strain shown in Figure 1(a). The western blots depicted in Figure 2(b) show the effect of various trifluoperazine concentrations and exposure times on the MCAT-His level. The effect of trifluoperazine on MCAT-His protein level correlated with its effect on growth; marked reduction in MCAT-His level appears to be associated with a severe reduction in the growth rate (compare Figure 2b with Figure 1b). Taken together, these results show that exposure of BCG to trifluoperazine at concentrations that affect growth is associated with a reduction in the MCAT steady-state level.

If the observed trifluoperazine-dependent reduction in MCAT level is indeed the cause for growth inhibition, strong overexpression of MCAT should increase the MIC of trifluoperazine. Figure 1(c) shows that growth of a recombinant BCG strain expressing MCAT at a high constitutive level (BCG MCAT-His-HIGH, see Materials and methods for details) was not affected by 10 mg/L trifluoperazine, i.e. the MIC for wild-type BCG. Furthermore, the MIC for BCG

Figure 1. Effect of trifluoperazine on the growth of wild-type BCG and recombinant BCG strains expressing a histidine-tagged version of MCAT. (a) Wild-type BCG. (b) BCG MCAT-His-LOW, expressing MCAT-His at a low constitutive level. (c) BCG MCAT-His-HIGH, expressing MCAT-His at a high constitutive level. Wild-type and recombinant BCG strains were grown for 2 days in roller bottles without trifluoperazine and with 5, 10 and 20 [and for (c) 40] mg/L trifluoperazine. Growth was monitored by OD₆₀₀ measurement. The experiment was repeated three times with independent cultures. Standard deviations are indicated.
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MCAT-His-HIGH was increased to 40 mg/L trifluoperazine; overexpression of MCAT confers resistance to trifluoperazine. Our data indicate that trifluoperazine inhibits growth via reduction in the steady-state level of MCAT protein. This enzyme catalyses the transacylation of malonate from malonyl coenzyme A to acyl carrier protein, to generate malonyl acyl carrier protein, which is an elongation step in fatty acid and mycolic acid synthesis. Isoniazid inhibits synthesis of mycolic acids downstream of MCAT via interference with KasA and InhA. Hence one might expect a synergetic effect of trifluoperazine and isoniazid on growth. The MIC of isoniazid for wild-type BCG was determined to be 0.24 mg/L. The effect of the combination of the two drugs was evaluated and growth inhibition of BCG at sub-MIC concentrations of trifluoperazine was observed at sub-MIC concentrations of isoniazid (data not shown). Although trifluoperazine acts synergetically (albeit weakly) with isoniazid, no resistance towards isoniazid alone is observed due to overexpression of MCAT (data not shown). This suggests MCAT to be a specific target of trifluoperazine.

In conclusion, we have demonstrated that (i) exposure of BCG to trifluoperazine at concentrations that exert antimicrobial activity is associated with a marked reduction in MCAT protein level; (ii) overexpression of MCAT causes an increase in trifluoperazine MIC; and (iii) no resistance to isoniazid is observed in MCAT overexpressed strains suggesting specificity of trifluoperazine towards MCAT. These results indicate MCAT as a target of trifluoperazine and provide an explanation for the inhibitory effect of trifluoperazine on mycobacterial lipid synthesis observed earlier by eliminating the essential fatty acid biosynthesis enzyme MCAT, lipid synthesis is blocked and the bacilli cannot grow. Hence, this work provides the first molecular target for trifluoperazine in tubercle bacilli and indicates the potential of MCAT as a novel target for antimycobacterials.

It is interesting to note that several attempts to isolate spontaneous trifluoperazine-resistant BCG mutants failed. From these experiments, we could conclude that the spontaneous mutation frequency that confers resistance to 5×MIC (50 mg/L trifluoperazine) is less than 10−10 per cfu (selection at lower trifluoperazine concentrations was leaky). The possibility that the observed resistance towards trifluoperazine in MCAT overexpressing strains may be due to altered lipid composition of the mycobacterial cell wall resulting in an altered penetration of trifluoperazine into the cell, cannot be excluded. To disprove the possibility of such secondary effects as opposed to a direct effect, gene array technology could be potentially useful in confirming target specificity.

The molecular mechanism by which trifluoperazine causes a reduction in MCAT protein level remains to be elucidated. In mammals, trifluoperazine acts via calmodulin. Some evidence points to the existence of calmodulin-like activities in Mycobacteria. Recently, Murthy and co-workers identified the first calmodulin-like protein from Mycobacterium smegmatis and demonstrated trifluoperazine-dependent inhibition of its activity. Does trifluoperazine exert its antibacterial activity via inhibition of calmodulin-like proteins in mycobacteria? If that were to be the case, what then, could be the link between inhibition of calmodulin-like proteins and MCAT? In this context, it is interesting to note that MCAT appears to be threonine-phosphorylated. Could this possibly point to a link between trifluoperazine, a calmodulin-like protein, a Ser/Thr protein kinase and MCAT? Intriguingly, some recent biochemical evidence indicates the presence of a calmodulin-dependent kinase activity in mycobacteria.

Acknowledgements

We thank Brigitte Gicquel for the pJEM15 vector. We are grateful to Robin J. Philp, Bioprocessing Technology Centre, Singapore, for his help and suggestions. T.D. is an adjunct staff member of the Department of Microbiology, National University of Singapore. This work was supported by the Agency for Science, Technology and Research (A*STAR), Singapore.

References

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