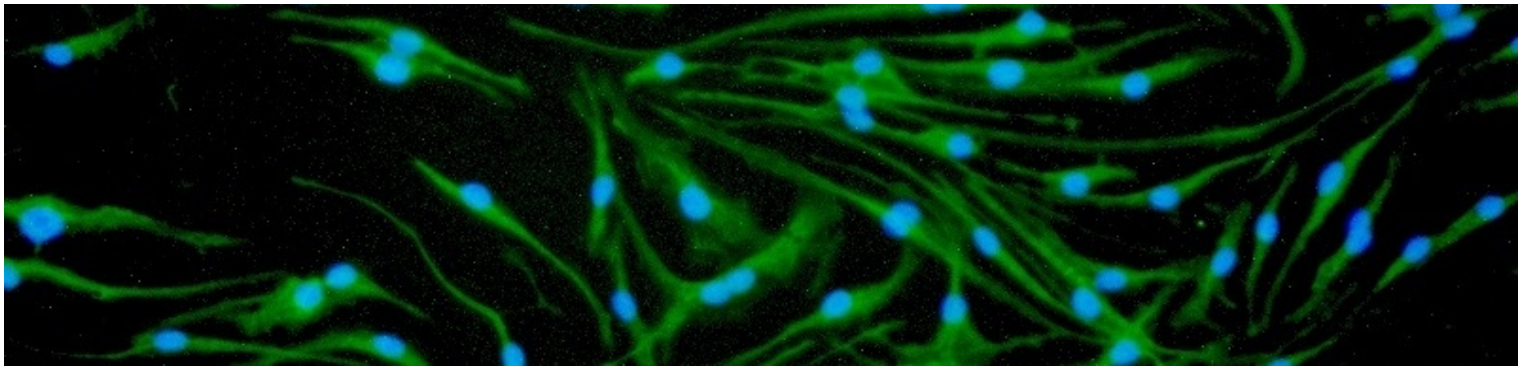
The link between sterol degradation and virulence

Insight into the pathogenicity of *Mycobacterium tuberculosis* and *Rhodococcus equi*



Sytske Bekker s1964437

06-03-2014

Supervisor: Dr. J.M. Petrusma

rijksuniversiteit groningen

Abstract

Mycobacterium tuberculosis (*Mtb*) is still a great cause of death in third world countries and for people with a attenuated immune system. *Rhodococcus equi* is also a great cause of disease and death, however these occur mostly in foals. Both bacteria can cause pneumonia and granuloma formation. Because of their similarities a lot of research about sterol degradation is done in both organisms. It is thought that the ability of both bacteria to degrade sterols is related to its pathogenicity. A few of the enzymes involved in the cholesterol degradation in *Mtb* and *R. equi* are discussed. A closer look is taken at the relevance of these enzymes in the pathogenicity of *Mtb* and *R. equi*. It is found that many enzymes indeed play crucial roles in the cholesterol degradation. Mutants of kstD, kshA/kshB and hsaC were not able to utilize cholesterol. Also, FadA5 is essential for the growth of *Mtb* on cholesterol. An acyl-CoA synthase (FadD3) occurs in the reaction were HIP is transformed to HIP-CoA, it initiates the catabolism of the steroid rings C and D in actinobacteria. The role of ChoD isn't clear yet. ChoD seemed to play a role in the first step of sterol degradation. Yet, the choD mutant of *Mtb* was able to grow on cholesterol. The differences in the results of the studies might be due to different use of methods in the experiments (different strains and a different experiment time). The exact process of cholesterol degradation still isn't clear yet although there is a very likely reaction pathway of the cholesterol catabolism. It is of great importance that more insight is obtained about cholesterol metabolism. Many people would benefit from new insights into this process.

Cover picture: mouse macrophage
http://www.sciencellonline.com/site/productInformation.php?keyword=R1920

Introduction

Many experiments indicated that cholesterol plays a crucial role in the pathogenisis of *Mycobacterium tuberculosis* (*Mtb*). A suite of genes critical for survival of *Mtb* in the macrophage was discovered to be involved in cholesterol degradation. However the precise role of this metabolism remains unclear. Nowadays *Mtb* still is one of the greatest killers worldwide. A lot of people in third world countries have to deal on a daily basis with Tuberculosis. Besides that, the growing multi-drug-resistance of numerous bacteria, including *Mtb*, is becoming a threat. Therefore it is of great importance that the process of sterol degradation in *Mtb* is investigated.

Another bacterial infectious disease is caused by *Rhodocuccus equi*. This bacterium has a lot in common with *Mtb*, in particular its sterol degradation mechanism. *R. equi* isn't in the first place infectious for humans, rather in foals. Because of the similarities between *Mtb* and *R. equi* and because a lot of research about sterol degradation is done in *R. equi*, both organisms will be discussed in this thesis.

The main goal of this thesis is to give an overview of the effect of sterol degradation on the virulence of *Mtb* and *R equi*. The differences and similarities between *Mtb* and *R. equi* will be briefly explained. Then the function of sterols and the mechanism of sterol degradation are discussed. Eventually the link between sterol degradation and pathogenis in *Mtb* and *R. equi* will be made. Because the great number of types of enzymes involved in the sterol degradation process and because of the limited size of this thesis, it is not possible to discuss the whole process. Sterol degradation will be briefly discussed, subsequently seven enzymes involved in the sterol catabolism of *Mtb* and *R. equi* will be treated. Finally the results are discussed.

Mycobacterium tuberculosis and Rhodococcus equi

Tuberculosis is second only to HIV as the greatest killer worldwide. In 2012 an estimated 0.94 million people died from Tuberculosis worldwide and 8.6 million people got infected.[21] Another serious problem is the continuing multi-drug-resistance of Mtb. Mtb requires high levels of oxygen and therefore is aerobic. For this reason *Mtb* is almost always found in the lungs of mammals. It is a large rod-shaped bacterium that belongs to the Actinomycetes. *Mtb* is a facultative intracellular parasite with a slow generation time (15-20h). The macrophage is the main replication niche of *Mtb*. It has developed several strategies for surviving in the hostile environment of the macrophage. By interacting through several different receptors with the macrophage, phagocytosis of *Mtb* will take place.[1] Once *Mtb* has entered the macrophage it inhibits several aspects of phagosomal maturation. When *Mtb* starts to multiply, the bacilli spread to regional lymph nodes in the host. In the end granulomas will start to form.

Rhodococcus equi (previously known as Mycobacterium equi) is a common cause of pneumonia in foals. Pneumonia is a major cause of disease and death in foals between 3 and 24 weeks of life.[7] In addition, *R. equi* can infect certain risk groups in humans, such as AIDS patients. It is a coccobacillus bacterium that has a lot in common with *Mtb*. Because of the mycolic acids and because of the route of infection it is often compared with *Mtb*. Inhalation is the main route of infection in *Mtb* and *R. equi*. Once inhaled, *R. equi* is taken up by macrophages through the same process as in *Mtb*, receptor-mediated phagocytosis. *R. equi* is able to multiply within the phagosome, where it is shielded from the immune system. The bacterium contains a large plasmid that has been shown to be essential for infection of foals.

R. equi and *Mtb* are members of the mycolata. This group is known for their lipid-rich cell envelope that contains mycolic acids.[16] Mycolic acids are linked to the peptidoglycanarabinogalactan cell wall polysaccharide, and to glycolipids. The mycolic acids form a barrier to hydrophilic compounds. The ability of *R. equi* and *Mtb* to survive in hostile environments such as the macrophage is linked to this mycolic barrier. Also, both bacteria are able to degrade certain sterols. These sterols are used as a carbon and energy source within the macrophage. Cholesterol is present in the macrophage cell membrane.

Sterol degradation in Actinobacteria

Sterols are hydrophobic molecules that are characteristic because of their four cycloalkane rings. Ring A, B and C are six membered rings and ring D is a five membered ring (fig. 1). Sterols and their metabolites are frequently used as regulatory molecules in eukaryotes. Sterols also function as components of cell membranes, e.g. cholesterol decreases the membrane fluidity. Mostly, sterols are absent in bacteria, however they are degraded by some bacteria. The degradation of sterols in bacteria is a step-by-step process in which many enzymes are involved. Bacteria use steroids as a carbon and energy source. A lot of *Actinobacteria* are known for their capability to use sterols. They are able to degrade the steroid ring and the steroid side chain. The process of sterol degradation roughly involves two steps: the elimination of the alkyl side chain and the opening of the polycyclic steroid nucleus.[10] Either, first the elimination of the alkyl side chain will occur or the opening of the polycyclic steroid nucleus. This order depends on the bacterial genera and the genus. [17]

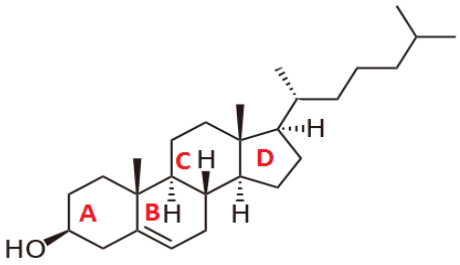


Fig 1. A Cholesterol molecule. Cholesterol is a sterol with four cycloalkane rings. Ring A, B and C are six membered rings and ring D is a five membered ring. It is an essential component in animal cell membranes. Sterols and their metabolites are used as regulatory molecules in eukaryotes and function as components of cell membranes.

First, before degradation takes place, sterols are transported into actinobacterial cells by the Mce4 steroid transporter. The mce4 gene encodes a ABC-like transport system.[11] In *Mtb*, cholesterol is a substrate for the Mce4 transporter. The Mce4 transporter is the major cholesterol import system of *Mtb*.[14] Strains that lacked the Mce4 proteins were not able to use cholesterol as a carbon source. However, the ability to import cholesterol was not completely turned off. Under *in vitro* conditions the mce4 mutants of *Mtb* were slightly able to use cholesterol.[14] This shows that there might be another, less efficient, import system involved in cholesterol uptake.

In *Mtb*, first the side-chain is degraded and then the ring opening will occur (fig. 2). Cholesterol degradation is both initiated form the 4- and 26- carbons of the molecule.[11] The 4 carbon is cleaved from ring A and the 26 carbon from the side chain is removed from cholesterol as a propionyl-CoA unit. In macrophages infected with *Mtb,* propionyl-CoA accumulates. Propionyl-CoA is then converted into branched chain lipids.

Figure 2 shows the catabolic pathway of cholesterol in *Mtb*. The whole process is catalyzed by many different enzymes, e.g. ChoD, FadA5, KSTD, KSH and HsaC. During microbial degradation pathway intermediates are formed. In *Mtb*, Sterols like 4-androstene-3, 17-dione (AD) or 1,4-androstadiene-3,17-dione (ADD) are formed as intermediates.

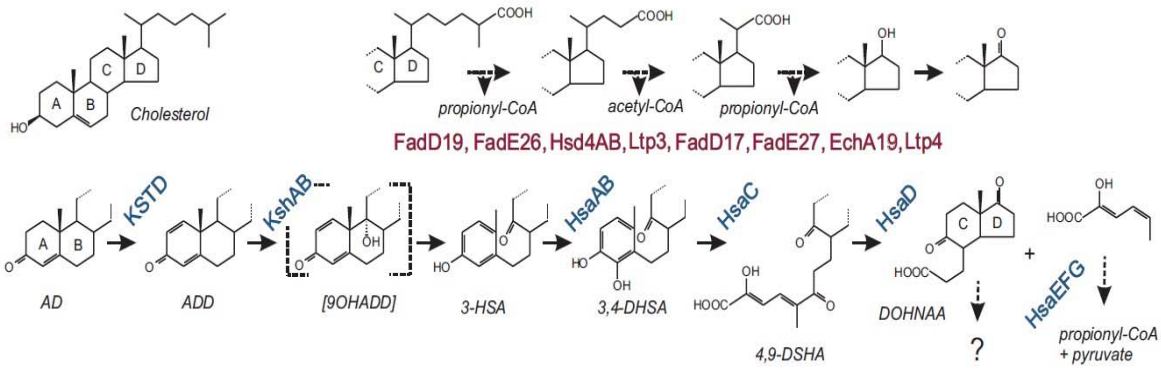


Figure 2. Cholesterol catabolic pathway of *Mtb* and *R. equi*.[18] First side-chain degradations occurs, then the ring will be opened by several enzymes. Many enzymes play crucial roles in this catabolic pathway e.g. ChoD, FadA5, KSTD, KSH, HsaC and FadD3.

In *R. equi* the catabolic pathway of cholesterol degradation is practically the same. Also, AD and ADD are intermediates, however other enzymes catalyze the process. HIP and 50-HIP are intermediates that play a crucial role in *R. equi,* but also in a *Mtb* infection (fig. 3)[19]. Mce4 is an important cholesterol transporter in *Mtb*. Nevertheless, experiments showed that Mce4 does not play an essential role in the cholesterol catabolism of R. equi.[19]

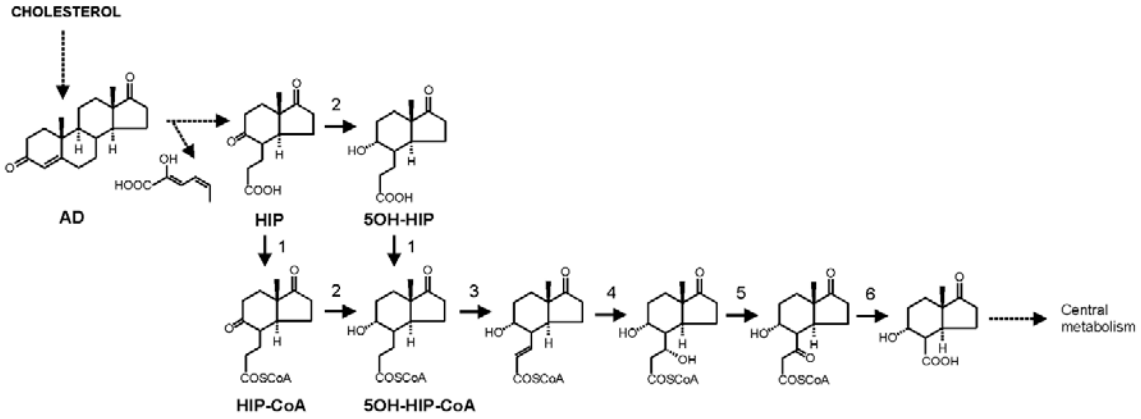


Figure 3. Pathway of AD degradation via ß-oxidation by R. equi[19]. AD and ADD are intermediates in this degradation pathway. HIP and 50-HIP are intermediates that are found in *R. equi* and *Mtb*.

Importance of sterol catabolism in pathogenicity of *Mtb* and *R. equi*

A mechanism that is not clear yet is the ability of *Mtb* and *R. equi* to survive in macrophages. It is well known that these bacteria are able to survive for long periods of time and even replicate within the macrophage, whereas the environment of the macrophage should be very hostile. The mainstream theory these days is that the ability of *Mtb* and *R. equi* to degrade sterols is one of the main reasons they are able to survive within the macrophage. In many articles ([11,20,14]) it is assumed that the degradation of Cholesterol has something to do with the pathogenicity of these bacteria. However, it still remains unclear what the exact process is and how it contributes to it's pathogenicity. By using transposon hybridization, already 126 genes were identified that are necessary for the survival of *Mtb* in macrophages. Several of these genes were also involved in sterol catabolism.[18] Figure 2 shows the pathway of cholesterol catabolism in *Mtb*. It now is clear that the opening of the ring and the degradation of the side-chain is very important for the survival of *Mtb* in the macrophage.

As mentioned before, multidrug resistance of (pathogenic) bacteria is becoming a huge problem. Therefore, clarifying the mechanisms of sterol degradation would be of great interest while they are obvious targets for novel antibiotics. Because of the great number of enzymes involved in sterol catabolism, it is not possible to discuss them all in this thesis. Six important enzymes will be discussed in the next subparagraphs. The order of the enzymes is as shown in figure 2 (ChoD, ChoE, FadA5, KSTD, KSH, HsaC).

The first reaction in the aerobic metabolism of cholesterol is the transformation of cholesterol into cholestenone. The oxidation of cholesterol to cholestenone is a two step process. First cholesterol is oxidized to cholest-5-en-3-one, then isomerisation will occur. There are two classes of enzymes responsible for these two steps: cholesterol oxidases (ChOX) and 3ß-hydroxysteroid dehydrogenases (HSDs).[6]

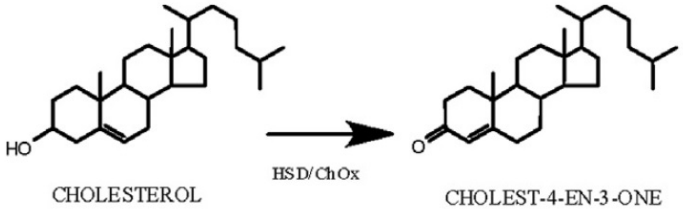


Fig 4. HSD and ChOx in the first step of cholesterol degradation. Cholesterol is oxidized to cholest-5-en-3-one, then isomerisation follows.[6] First the cholesterol is oxidized, then isomerisation occurs.

An enzyme that is very important in the process of the transformation of cholesterol is ChoD (cholesterol oxidase). ChoD was found in *Mtb* and in *R. equi* where it has been identified as an important cytosolic factor.[3] ChoD (a cholesterol oxidase) catalyzes the first step of the cholesterol degradation process, the oxidation of cholesterol to cholestenone (fig 4, fig 5).

After the oxidation process, cholestenone is further catabolised in the cholesterol degradation process. The enzymes involved in the latter process will be discusses in the next subparagraph. In mice some experiments that involved ChoD where done, where it showed the significance of ChoD in the pathogenisis of *Mtb*. The mice were mutant and had a non-functional copy of the choD gene. When these mice got infected with *Mtb*, their macrophage activity was attenuated. Wild-type mice had no problems attacking the *Mtb*.

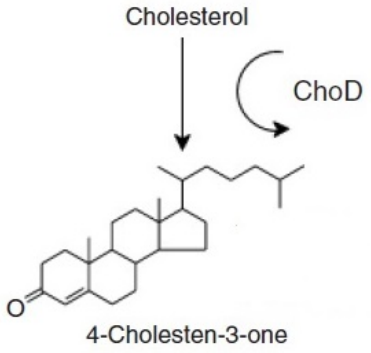


Fig 5. Pathway for cholesterol degradation.[3] ChoD catalyzes the first step in the cholesterol degradation pathway. It oxidises cholesterol to cholestenone. Cholestenone is further catabolised by a number of enzymes to final inorganic compounds.

ChoE has great similarities with the cholesterol oxidase (ChoD) encoded in *Mtb*. However, ChoE is found in *R equi* instead of *Mtb*. ChoE is, just like ChoD, a cholesterol oxidase.[12] The gene encoding this enzyme has been found and several experiments to prove it's role in pathogenicity of *R. equi* were done. The experiments showed that cholesterol oxidase is a major cytotoxic factor that is involved in macrophage destruction.[12] The ß-oxidation shows that cholesterol is a crucial source of carbon for *R. equi* during the growth in macrophages.[15] Also it was suggested that the enzyme might be a major membrane-damaging factor of the organism during infection.

The next enzyme involved in the catabolic pathway of *Mtb* is FadA5. FadA5 is a lipid-metabolizing thiolase. It catalyzes the thiolysis of acetoacetyl-coenzyme A (CoA). This activity is required for the production of androsterones. It is shown that fadA5 mutants grow normally in mouse lungs but when the cellular immune response is induced, they do not.[3] This shows that cholesterol is not required as a primary carbon source during the growth phase of the infection. In wild-type strains of *Mtb* cholesterol is metabolised to androst-4-ene-3,17-dion (AD) and androsta-1,4-diene-3,17-dion (ADD). After cholesterol has been metabolised these metabolites (AD and ADD) are exported into the medium. The fadA5 mutant strain is defective for this activity. FadA5 is required for the production of AD and ADD. It is concluded that cholesterol metabolism is only essential in the persistent stage of *Mtb* infection.

Another important enzyme that catalyzes the opening of ring B and the aromatization of ring A is 3-ketosteroid Δ1-dehydrogenase (KSTD). The *Mtb* kstD mutant, lacking functional KstD, accumulates non-toxic cholesterol. The strain is unable to grow on minimal medium with cholesterol as a carbon and energy source. Moreover, it was observed that the intracellular replication of the kstD mutant was attenuated in both resting and activated macrophages compared to the wild-type strain. These data suggest that cholesterol catabolism is important for *Mtb* at multiple stages of the infection. However it remains unclear whether the only reason of attenuation of cholesterol degradation mutants in macrophages is due to their inability to use cholesterol as a source of carbon and energy. It shows that de degradation of cholesterol is required for *Mtb* to survive during infection in macrophage. It is indicated that there is a relationship between the degradation of cholesterol by *Mtb* and the survival of *Mtb* in macrophages.[2]

3-Ketosteroid 9α-hydroxylase (KSH) plays a crucial role in the opening of the steroid ring structure. There are two genes that encode for KSH, kshA and kshB. Both genes are required for KSH to proper activity.[9] Just as like KSTD, KSH is involved in the opening of ring B and the aromatization of ring A. KSH consists of two components, a KshA oxygenase and a KshB reductase. Both components are located in the catabolic gene cluster of *Mtb*. The roles of KshA and KshB in *Mtb* were explored by making deletion mutants of KshA and KshB. It was found that the KshA and KshB mutants weren't able to use cholesterol. Even so, the KshB deletion mutant had a changed cell wall. Both mutant strains were unable to survive in resting and in activated macrophages. It is speculated that KshA and KshB have additional functions in the metabolism of *Mtb*. However, the probable roles of these enzymes are unclear and need further investigation. The deletion of both of these genes lead to rapid death of *Mtb* in macrophages. This shows that KshA and KshB play essential roles in the pathogenicity of *Mtb.*

HsaC is en iron-dependent extradiol dioxygenase that cleaves catechols. Catechols are compounds with the molecular formula C6H4(OH)2 (fig 6). For a long time it was accepted that cholesterol was of great importance in the chronic stage of *Mtb* infection. Nowadays, studies have shown that the cholesterol uptake starts in an earlier stage of *Mtb* infection.[22] Cholesterol may even play a role in the distribution of the pathogen within the host.[22] HsaC catalyzes the meta-cleavage of DHSA (fig 6). By making a deletion mutant of hsaC in *Mtb* the role of HsaC in cholesterol catabolism was investigated. The wild-type of *Mtb* was able to grow on a medium with cholesterol, confirming that *Mtb* can utilize cholesterol as a growth substrate. However, the hsaC deletion mutant failed to grow on a medium with cholesterol. The hsaC mutant was tested in two animal models (mice and guinea pigs). Mice infected with the hsaC mutant survived substantially longer than those infected with the wild-type *Mtb*. The guinea pigs infected with the hsaC mutant had significantly fewer organisms in the lung compared to the wild-type infected guinea pigs. This shows that cholesterol metabolism contributes to the survival of *Mtb* in the host.

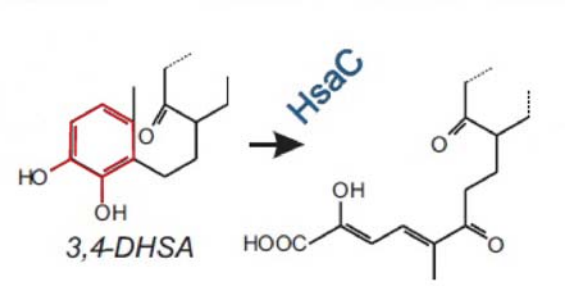


Figure 6. HsaC in the cholesterol catabolic pathway of *Mtb*. The catechol is coloured red. HsaC catalyzes the meta-cleavage of DHSA.

In the last steps of cholesterol ring degradation, FadD3 is found. Both in *Mtb* an *R. equi* it plays a crucial role in cholesterol metabolism. FadD3 is a acyl-CoA synthase that catalyzes the reaction were HIP is transformed to HIP-CoA (fig 7)[5]. Acyl-CoA synthases use ATP and Coenzyme to thioesterify substrates. The process is a two step mechanism that has a acyl-adenylate intermediate. To investigate the role of FadD3 in actinbacteria, a *Rhodococcus jostii* (RHA1) mutant was made. The fadD3 gene was deleted. It was very clear that the wild-type strain grew significantly better on cholesterol then the fadD3 mutant. Also, the mutant was complemented with a multi-copy plasmid carrying the wild-type fadD3 gene from *Mtb*. It showed that HIP did not accumulate during growth on cholesterol[5]. Thus, the mutant phenotypes are able to degrade cholesterol again. The fact that the fadD3 of *Mtb* is able to regain the mutant's phenotype makes it very plausible that the *Mtb* orthologue plays the same role. These experiments show that FadD3 initiates the catabolism of steroid rings C and D in actinobacteria and thus probably also occurs in *Mtb* and *R. equi*.

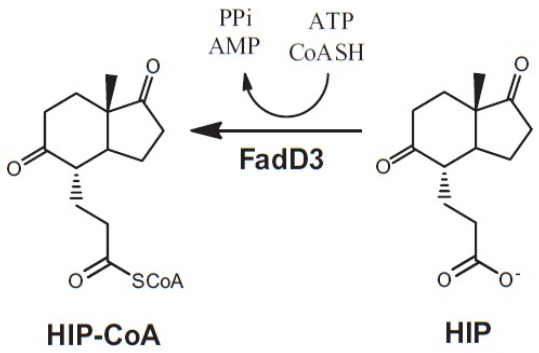


Fig 7. FadD3 catalyzes the reaction from HIP to HIP-CoA.[5] It is a acyl-CoA synthase that uses ATP and Coenzyme to thioesterify it's substrates. It's a two step process that has a acyl-adenylate intermediate.

Discussion

Many experiments have shown that *Mtb* metabolizes cholesterol, though the role of this metabolism in pathogenicity remains unclear. Various *Mtb* mutants defective in the ability to transport or degrade cholesterol have been investigated. It is clear that cholesterol plays an essential role in the uptake of mycobacteria by macrophages. Cholesterol is important for infections of macrophages by *Mtb*. *Mtb* uses the cholesterol as a carbon and energy source. Nowadays the genes involved in cholesterol degradation in the *Mtb* genome are identified. Also, the ABC-like transporter that mediates the uptake of cholesterol has been discovered. When the ABC-like transporter was inactivated, it affected the cholesterol degradation cascade.[11] Several experiments with key enzymes involved in the opening of the steroid ring structure showed the same effects. Mutants of kstD, kshA/kshB and hsaC were not able to utilize cholesterol. Also, the importance of FadA5 in the degradation of the side chain of cholesterol was tested. It showed that FadA5 is essential for the growth of tubercle bacilli on cholesterol.[10] However, some other experiments showed that the effect of ChoD and HsdD were not as black and white as expected. ChoD seemed to play a crucial role in the first step of sterol ring degradation, however this effect was never confirmed *in vitro*. The choD mutant was less virulent in mouse models. Moreover, the choD mutant of *Mtb* was able to grow on cholesterol. These findings contradict the findings of the other experiments.[4] Even the double mutant ChoD/HsdD of *Mtb* can use cholesterol as a carbon and energy source. Mutants were constructed by replacement of the genes that encode ChoD and HsdD. The mutants were able to grow on minimal medium supplemented with cholesterol. Also a choD, hsdD kstD mutant of *Mtb* was produced and was grown on minimal medium with cholesterol. No accumulation of intermediates was observed.

Another article is very firm about the role of cholesterol during infection of *Mtb*.[21] It states that cholesterol is not an essential source of nutrition during infection. In vitro tests were done to decide whether 3ß-hydroxysteroid dehydrogenase (HSD) or ChoD play crucial roles during growth on cholesterol. ChoD is, as discussed before, an cholesterol oxidase that plays a role in the transformation of cholesterol. HSD is responsible for the 3β-hydroxysterol oxidation in *Mtb*, which is the first step in the catabolic pathway of cholesterol degradation.[22] During *in vitro* growth it was tested if either HSD or ChoD was required. It was found that HSD is indeed necessary for growth on cholesterol as a carbon source. However, both the ChoD mutant and the wild-type of *Mtb* grew on cholesterol medium. Furthermore, the role of HSD in the growth of *Mtb* in macrophages was tested. Both wild-types and mutants were used to infect macrophages. There were no differences detected in the growth rate of *Mtb* which indicates that HSD does not limit *Mtb* in its replication in macrophages. In the end, guinea pigs were infected with the HSD mutant and the wild-type. The growth rate was determined over a 6-week time course. Some differences were explained because of different immune responses. However, there were no significant differences between the HSD mutant and the wild-type.

The differences in results of the above named studies might be due to different use of methods in the experiments. The results from Yang et al.[23], were obtained during a relatively short time, 5h after cholesterol was added. In the experiments of Brzostek et al.[4], strains were analyzed during 72 hours. Also different strains were used in both studies. There already have been some reports of the differences between these two strains (H37Rv and CDC1551) in cholesterol degradation.

There was also an article that stated the direct opposite of the importance of ChoE in the virulence of *R. equi*.[15] It claims that ChoE is not important in the virulence of R. equi. The experiments that showed that ChoE might be a major membrane damaging factor, were based on theoretical grounds. In this study, several experiments were done on mice. A ChoE mutant was reconstructed and injected in mice. No significant differences between the wild-type and the mutant were fount. Also foals were infected with the choE mutant. After infection and death, in both groups (control and mutant) the classic *R. equi* lesions were found. There was no significant difference in the mean number of bacteria found in the lungs of foals.[15]

It is clear that more research has to be done to gain insight into the effect of sterol degradation on the virulence of *Mtb* and *R. equi.* There is a link between sterol degradation and the virulence of *Mtb* and *R. equi.* As was shown in several of the above-mentioned experiments. Cholesterol is an important carbon and energy source for *Mtb* in the infection of macrophages. Mutants of kstD, kshA/kshB and hsaC were not able to utilize cholesterol and therefore probably play a role in cholesterol degradation. Also FadA5 showed to be essential for the growth of *Mtb* on cholesterol. Yet, the role of ChoD isn't clear. ChoD seemed to play a role in the first step of sterol degradation. Nevertheless, the choD mutant of *Mtb* was able to grow of cholesterol. The process of sterol degradation isn't completely clear yet. Figure 1 shows a very likely reaction pathway of the cholesterol catabolism in *Mtb*. Many of the enzymes in this figure can be used as targets for novel antibiotics. Furthermore obviously, insight in the cholesterol catabolism will benefit numerous people in third-world countries. In short, although significant insight has been gained, it is of great importance that we gain more insight into the pathogenicity mechanism of *Mtb* and *R. equi*.

References

[1]Amanda Welin , Survival strategies of *Mycobacterium tuberculosis* inside the human macrophage

[2]Brzezinska et al., The role of 3-Ketosteroid 1(2)-dehydrogenase in the pathogenicity of *Mycobacterium tuberculosis*

[3]Brzostek et al., Cholesterol oxidase is required for virulence of *Mycobacterium tuberculosis*

[4]Brzostek et al., ChoD and HsdD can be dispensable for cholesterol degradation in mycobacteria

[5]Casabon et al., FadD3 is an acyl CoA synthetase that initiates catabolism of cholesterol rings C and D in actinobacteria

[6]Garcia et al., Catabolism and Biotechnological applications of cholesterol degrading bacteria

[7]Giguere S, Slovis N M, Rhodococcus equi: clinical manifestations, virulence, and immunity

[8]Griffin et al, High-Resolution phenotypic profiling defines genes not essential for mycobacterial growth and cholesterol catabolism

[9]Hu et al., 3-Ketosteroid 9alpha-hydroxylase is an essential factor in the pathogenesis of *Mycobacterium tuberculosis*

[10]Marscheck et al., Microbial degradation of sterols

[11]Miner M, Sherman D, Role of cholesterol in *Mycobacterium tuberculosis* infection

[12] Navas et al., Identification and Mutagenesis by Allelic Exchange of choE, Encoding a Cholesterol Oxidase from the Intracellular Pathogen *Rhodococcus equi*

[13]Nesbitt et al., A thiolase of *Mycobacterium tuberculosis* is required for virulence and production of androstenedione and androstadienedione from cholesterol

[14]Pandey et al., Mycobacterial persistence requires the utilization of host cholesterol

[15] Pei et al., Cholesterol oxidase (ChoE) is not important in the virulence of Rhodococcus equi

[16]Sellon, Long, Equine infectious diseases, ISBN: 978-1-4557-0891-8

[17]van der Geize R, FadD19 of *Rhodococcus rhodochrous* DSM43269, a steroid-coenzyme a ligase essential for degradation of C-24 branched sterol side chains

[18] van der Geize et al., A gene cluster encoding cholesterol catabolism in a soil *Actinomycetes* provides insight into *Mycobacterium tuberculosis* survival in macrophages

[19]van der Geize et al., The steroid catabloic pathway of the intracellular pathogen *Rhodococcus equi* is important for pathogenesis and a target for vaccine development

[20]Wilbrink M , Microbial sterol side chain degradation in Actinobacteria, ISBN: 978-90-367-4807-0

[21]World Health Organisation, www.who.int/gho/tb/epidemic/cases_deaths/en/

[22]Yam et al., Study of a ring-cleaving dioxygenase illuminate the role of cholesterol metabolism in the pathogenesis of *Mycobacterium tuberculosis*

[23]Yang et al, Cholesterol is not an essential source of nutrition for *Mycobacterium tuberculosis* during infection

[24]Yang et al., Rv1106c from *Mycobacterium tuberculosis* is a 3ß-hydroxysteroid dehydrogenase