Title: Tyrosinase, could it be a missing link in ochronosis in alkaptonuria?

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Funding: Dr Taylor's work is kindly supported by the Rosetrees Trust.

ABSTRACT

The hypothesis that is proposed is that tyrosinase, an enzyme widely found within the human body is implicated in the ochronosis that occurs in alkaptonuria; an autosomal recessive condition first used by Archibald Garrod to describe the theory of "Inborn Errors of Metabolism." The disease results from the absence of a single enzyme in the liver that breaks down homogentisic acid; this molecule becomes systemically elevated in sufferers. The

condition is characterised by a clinical triad of symptoms; homogentisic aciduria from birth, ochronosis (darkening) of collagenous tissues (from ~30years of age) and ochronotic osteoarthropathy in weight bearing joints due to long term ochronosis in them (from ~40years of age). Tyrosinase, a polyphenol oxidase has been shown in many species to contribute to the darkening of tissues in many organisms; including humans in the production of melanin. Tyrosinase under the right conditions shows alterations in its substrate specificity and may contribute to the darkening seen in AKU where it moves away from polymerising tyrosine but also homogentisic acid, the causative molecule in alkaptonuria, that is present in excess.

INTRODUCTION

Alkaptonuria (AKU) is a rare autosomal recessive condition caused by a mutation in the gene coding for the enzyme Homogentisate 1,2 Dioxygenase (HGD), which breaks down homogentisic acid (HGA) in the tyrosine (TYR) metabolic pathway (1). The incidence of AKU worldwide is stated as being 1:250,000 individuals, however there are pockets of the world that show a much higher incidence with the Dominican Republic and Slovakia having an incidence of around 1:20,000 (2). There are at least 129 mutations reported in the HGD gene and all produce the AKU phenotype. There appears to be no correlation, currently, between the type of mutation and severity of the phenotype (3). The condition was first used by Archibald Garrod in 1908 to demonstrate the concept of "inborn errors of metabolism." The condition displays a triad of clinical features; homogentisic aciduria which is present from birth, ochronosis of collagenous tissues which is believed to commence around the 3rd decade and progresses to cause the final feature which is ochronotic osteoarthropathy of weight bearing joints; due to the build-up of a polymeric derivative of HGA (1). The disorder sits on the TYR metabolic pathway with other conditions such as tyrosinaemia type-I which is fatal (4). However, AKU is not fatal and isn't associated with a shortened life span but sufferers display a much reduced quality of life as a consequence of the numerous co-

morbidities that arise, mainly due to the deposition of the ochronotic pigment in collagenous tissues, such as articular cartilages, heart valves and arterial vessel walls (5-7). In AKU the defective gene has been identified and located to chromosome 3q2 (8). A mouse model of the disease showing the lack of HGD has been detailed in the literature, but only recently was it shown to display the cartilaginous pigmentation synonymous with the human condition. Recent work has also shown that Nitisinone, a tri-ketone herbicide, is a potential therapy for AKU, work in a mouse model has shown a significant reduction of HGA (9) and early data from the latest human studies show that a similar effect is seen (10). Whilst there is an increase in the recent knowledge on the commencement of pigmentation in joints and the end stage of the disease, there is a gap in the knowledge on the intermediate steps that see the conversion of the monomer HGA to its destructive pigment polymer.

Our hypothesis is that an enzyme that is present in the body, tyrosinase, may be acting non-specifically using HGA as a substituted substrate following an alteration in physiological conditions due to the long-term elevation of HGA in the body.

HGA and TYR; their structure and function.

HGA, chemically known as 2,5-dihydroxyphenylacetic acid, is a *p*-diphenol and an intermediary in the breakdown of TYR. It is metabolised intracellularly by hepatocytes which produce the enzyme HGD that cleaves the benzene ring of the HGA molecule to give a linear structure; maleylacetoacetic acid which is then further metabolised. HGA is usually present in a non-alkaptonuric liver for fractions of a second and is usually undetectable in non-alkaptonurics (11). In AKU, HGA is rapidly oxidised to the intermediate benzoquinone acetic acid (BQA) (structure currently unknown). This quinone intermediate is then polymerised into ochronotic pigment. The pigmentation process seen in AKU is remarkably similar to that observed in the conversion of TYR to melanin in the body, a monomer (also a diphenol) is

polymerised to a quinone intermediary and finally a pigmented polymer; L-DOPA and melanin respectively (12).

The conversion of TYR to its quinone intermediary and then to melanin is the result of a single enzyme; tyrosinase which is the rate limiting enzyme in melanin synthesis. Currently the conversion of HGA to its quinone intermediary and polymeric pigment by an enzyme, if present, is not clear. It has previously been postulated that HGA polyphenol oxidases (HGA PPO's) catalyse the formation of ochronotic pigment from HGA. Zannoni identified that BQA was produced via the oxidation of HGA and that BQA may polymerise to form ochronotic pigment by these oxidative enzymes (13,14). However, scientists are still unable to identify HGA PPO's. It could be argued that their existence is questionable since they incur no survival advantage. In a healthy individual, HGA is oxidised to maleylacetoacetic acid, under the action of HGD, rendering HGA PPO's needless.

Physiological changes in AKU.

The physiological changes seen in AKU are well documented in relation to the presence of pigment in collagenous tissues and gram quantities of HGA excreted daily in the urine (15). The kidney appears to be a key factor in controlling the amount of HGA in the body system and removing it from the body, as renal function declines the amount of plasma HGA in the system increases (16). Renal function deteriorates with age and it may well be linked to the onset of deposition of HGA polymer in tissues as the ability or quantity of HGA removed from the body declines (17). It could be this reason why the onset of ochronosis is not seen in the paediatric or adolescent group. Further support for the involvement of the kidney is observed in a number of cases where individuals with AKU have experienced renal failure associated with rapidly progressing ochronosis (18-22).

The role of the kidneys in efficiently removing HGA is demonstrated in one of the few cases in the literature where a patient with AKU had undergone kidney replacement and demonstrated an almost 3 fold reduction in plasma HGA concentration; from 126.3μmol/L to 43.7μmol/L (23). Admittedly, there is data lacking on the pH changes seen in urine and plasma of AKU patients, but what little there is describes decreased urinary pH in AKU. This is reflective of the plasma pH and is also an indicator of renal tubular function (24).

The pigmentation process.

As described before, the pigmentation process shows remarkable similarities to the formation of melanin, yet relatively little is known about the intermediary(ies) between HGA and the ochronotic pigment. HGA is a small water soluble molecule and therefore is seen in tissues with a large amount of water, such as the articular cartilage (25). The affinity for deposition in collagenous tissues is widely accepted but the reason for deposition being seen here is not fully understood. It has been demonstrated that the periodicity of collagen appears to have an association with the deposition of pigment, but the exact site of nucleation on the collagen molecule or molecule associated with the collagen, is as yet unclear (26). The evaluation of ochronotic vs control cartilage by NMR spectroscopy has shown that there is a broadening of peaks for most molecules indicative of disorder in the tissue affecting most regions (27). However, the biggest question still not answered around the pigmentation process is "where does the pigmentation commence?"

Tyrosinase.

Tyrosinase is found in many living organisms including plants and bacteria and has been extracted and purified from a variety of them, its primary function is conversion of TYR to DOPA and then melanin. Numerous studies have observed the enzymes specificity and distribution; demonstrating tyrosinase to be a non-specific enzyme. It shows action against

other phenols, observed in a range of studies, some functionality was dependent upon the species which the tyrosinase originated from (28-32). These studies have mainly used monophenols and *ortho*-substituted diphenols as substrates. One study had used tyrosinase for the hydroxylation of p-substituted phenols (30), but there are currently no studies which use HGA as a substrate for tyrosinase A further study showed that phenol could be converted by tyrosinase by as much as 93%, depending on the pH (pH 6.8) (33). Other studies have shown that different *ortho*-phenols can be used as alternative substrates for TYR in the production of melanin, with some of the phenols used being structurally similar to HGA (12), Mono-, di- and polyphenols have been tested to see if they are viable substrates, with results demonstrating that enzyme specificity was in part dependant on which organism tyrosinase originated from. However, the majority of enzymes displayed the ability to catalyse more than one substrate (34-36). Whilst studies in bacteria and plants are abundant, studies that use mammalian tyrosinase are limited. However there are studies emerging which demonstrate that mammalian tyrosinase also has low substrate specificity (37). These experiments offer potential that in the right physiological circumstances tyrosinase could act with HGA as its substrate, particularly in circumstances where HGA is in abundance, such as in AKU.

One question remains regarding the presence of tyrosinase in any of the tissues where ochronosis is seen. Ochronosis is observed in a variety of tissues including skin, sclera, heart valves and most commonly in cartilage and other connective tissues. Whilst these areas are specifically seen in AKU, other individuals not diagnosed with AKU can undergo exogenous ochronosis. Exogenous ochronosis occurs superficially in all skin types due to excessive use of hydroquinone cream for skin lightening therapy. In this form of therapy, the cream aims to inhibit tyrosinase activity in melanocytes, thus inhibiting melanin production (38-40). As well as skin, studies have also shown the presence of melanocytes in cardiac tissue (41). Considering the presence of tyrosinase within melanocytes, this further supports the theory

that tyrosinase is potentially the HGA PPO referred to by Zannoni that may be acting non-specifically (13, 42, 43). Recent work has shown that joint and connective tissues that are affected most commonly in AKU, including cartilage, demonstrate expression of enzymes on the TYR metabolic pathway (44, 45).

DISCUSSION

The ochronosis that occurs in AKU still presents a number of unanswered questions and each of these offers an alternative therapeutic opportunity. The fact that ochronosis takes a number of years to occur, well into adulthood, adds weight to the theory that there is a physiological change that promotes or precipitates the conversion of HGA to its ochronotic polymer. This is likely related to a reduction in kidney function with ageing but there are other factors that could play a role; evidence of the importance of cellular stress and polymerisation potentially marking damage to tissues appear to be associated with the pigmentation process (25, 46, 47, 48).

There is clearly a biochemical setting that allows for the natural conversion of HGA to ochronotic pigment, as seen in the urine of alkaptonuric patients (1). However this is unlikely to be the sole factor.

The potential for "natural" conversion of HGA to ochronotic pigment in the most severely affected tissue; cartilage at a rate similar to that seen in urine is unlikely. Given that HGA is elevated from birth and the effects of ochronotic pigment in cartilage is not seen for multiple decades shows that there is either a protective mechanism in cartilage or other pigment forming promoter mechanisms that are only activated in certain physiological circumstances. If the natural oxidation of HGA is the reason for the build-up of ochronotic pigment in cartilage, data seen in urine suggests that oxidation occurs straight away. If that is the case then why does arthropathy present in later life? Shouldn't ochronotic pigment be seen from

an early age? An explanation for this may be the fact that cartilage is avascular, so the tissue remains hypoxic so not much oxidation can occur. It is only when the patient ages, when there is an increase in inflammatory processes and an increase in free radicals in cartilage which may allow the oxidation of HGA leading to the formation of the ochronotic polymer. However, some studies have shown that the structure of ochronotic pigment is completely absent of inflammatory infiltrates (49).

A recent case report detailing AKU and acidosis had fatal complications, but a number of clinical details are of significance. The patient demonstrated marked skin darkening, indicative of ochronosis 7 days after admittance to hospital (50). This rapid progression of darkening of the skin is not usually seen in natural progression of AKU. The metabolic acidosis seen in this patient would likely not promote the natural oxidation of HGA as is seen in alkali environments such as urine. One could speculate that the rapid darkening in the skin and the acidic physiological setting in this patient may allow for the acidic environment to alter the specificity of tyrosinase to polymerise HGA. This has been described previously with tyrosinase catalysing other phenolic compounds displaying structural similarities to HGA at an acidic pH (12, 33).

The importance of the kidneys and the physiology *in vivo* can be seen with evidence of 4 reported cases of acidosis and 3 reported cases with acute kidney injury reported in separate individuals with AKU, from the literature, all resulting in death (51). Whilst the kidneys and other physiological mechanisms appear protective (to a point), there are other potential mechanisms that may become activated during ageing or pathology and these warrant further investigation to offer the potential for further development of therapeutic strategies to treat AKU. One link missing from the literature would be an individual who suffered from both AKU and albinism and any information on the pathogenesis of AKU in this individual may provide further clues for any potential action for tyrosinase in the disease process in AKU.

The physiological changes and circumstances that affect the specificity of tyrosinase are clearly documented in the literature. These changes offer the potential for the enzyme to act as a factor in the polymerisation of HGA in AKU patients and the action of tyrosinase and its potentially contributory factor in the disease process warrant further investigation in vitro, by means of assay and in vivo via means of tissue specific examination of cells for the production of tyrosinase.

Acknowledgements

Dr Adam Taylor would like to acknowledge the Rosetrees Trust for funding. The funders had no role in the production or decision to submit this manuscript for publication

Conflict of interest

The authors declare no conflicts of interest.

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