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The Potential Role of ABCC6 in Purinergic Signaling and Regulation of Cardiac Functions

Abstract

Ischemic cardiac diseases, including acute myocardial infarction (MI), represent a significant health problem that can lead to heart failure. Understanding innate mechanisms of cardioprotection is essential to develop novel therapy for patients with acute MI. In the heart, purinergic signaling, including adenosine and ATP, has a well-established role in mitigating myocardial damage from ischemia. ABCC6, an ATP-binding cassette membrane transporter, is primarily expressed in the liver and kidney and contributes to calcification inhibition by facilitating the efflux of cellular ATP into the extracellular space. The released ATP is rapidly converted into pyrophosphate (PPi) and adenosine by ectonucleotide pyrophosphatase phosphodiesterase 1 (ENPP1) and ecto-5'-nucleotidase (NT5E). PPi is a major inhibitor of calcification and adenosine indirectly prevents calcification by inhibiting the expression of the tissue nonspecific alkaline phosphatase (TNAP). Mutations in ABCC6 result in two currently incurable human disorders, pseudoxanthoma elasticum (PXE) and some cases of generalized arterial calcification of infancy (GACI). Both disorders lead to extensive ectopic calcification, notably affecting cardiovascular tissue. ABCC6 also influences genes involved in nucleotide and purinergic signaling. As a note, ABCC6 unitalicized is in reference to the protein and Abcc6 italicized is in reference to the gene.
Hypothesis

We hypothesize that ABCC6 acts as an upstream modulator of extracellular purinergic signaling in distal tissues, and that it contributes to the regulation of cardiac functions.

Study Objectives

This study aims to understand and evaluate the cardioprotective effects of ABCC6 deficiency during ischemia in in vivo and ex vivo models and analyze how the expression of genes involved in extracellular purine and phosphate metabolism, Hmgcs2, Angpt14, Cpt1a, and Ptdgs, are regulated by ABCC6.

Significance

This study will be one of the first studies to explore the roles and functions of ABCC6 in altering purinergic metabolism and its effect on regulating cardiac functions distally to potentially provide insights in new therapeutic targets in cardiovascular diseases.

Background Information and Literature Review

Soft tissue calcification is a condition that is commonly associated with aging and can be seen in some common conditions such as diabetes, hypercholesterolemia, and certain genetic disorders. Soft tissue calcification can be defined as either ectopic or dystrophic calcification.\(^1\) Ectopic, or abnormal calcification occurs when calcium and/or phosphate salts deposit in soft tissue, most commonly in vascular tissues.\(^4\) Dystrophic calcification is the calcification of damaged, injured, and/or necrotic (dead) tissues. Extensive dystrophic calcification can be seen in long-term survivors after substantial myocardial infarctions (MI).\(^5\) Calcification is particularly problematic in that it can result in further chronic damage of tissues regardless of the cause of the
calcification. Calcification of soft tissues can also be seen in rare genetic disorders such as pseudoxanthoma elasticum (PXE), generalized arterial calcification of infancy (GACI), and arterial calcification due to deficiency of CD73 (ACDC) in humans and dystrophic cardiac calcification (DCC) in mice. These three conditions are all linked to ABCC6, an ATP-binding cassette (ABC) membrane transporter that appears to regulate calcification in humans and mice.

ABCC6 is part of a large ABC gene subfamily C and is expressed primarily in the liver and kidneys. A model of the membrane transporter with positions of known missense mutations is shown in Figure 1. This membrane transporter uses adenosine triphosphate (ATP) as an energy source to efflux or transport out of the cell an unknown substrate(s), the identification of which has been difficult despite many attempts. However, ABCC6 function has been shown to be related to calcification inhibition. Previous research has shown that ABCC6 significantly increases extracellular nucleosides, nucleoside monophosphates, and nucleotide sugars, especially adenosine monophosphate (AMP), and pyrophosphate (PPi). The increase of extracellular nucleotide triphosphates, specifically ATP, is rapidly broken down into AMP and PPi by ectonucleotide pyrophosphate phosphodiesterase 1 (ENPP1). AMP is further broken down into adenosine and phosphate by ecto-5'-nucleotidase (NT5E). Mutations in any of these proteins result in calcification, suggesting that they all participate in the same pathway leading to soft tissue calcification.

Figure 1. Model of ABCC6 membrane transporter and position of missense mutants.
Dysfunction in ABCC6 results in a decreased cellular efflux of ATP and significantly decreased production of extracellular PPI. PPI is the main inhibitor of hydroxyapatite crystallization and hydroxyapatite crystallization results in soft tissue calcification. As a result, mutations in ABCC6 are associated with increased calcification as seen in the rare genetic disorders PXE and GACI. Supplementation with PPI by single daily injections and by providing PPI in the drinking water has been shown to prevent the development of acute and chronic calcification associated with ABCC6 deficiency, DCC, and calcification of vibrissae in mice. Mice lacking expression of ABCC6 (Abcc6−/−) have also been shown to have decreased expression of ENPP1 and NT5E in the liver, suggesting that ABCC6 is acting upstream of both these enzymes. Figure 2 suggests a model of the ABCC6 molecular pathway involved in the generation of PPI and inhibition of ectopic calcification.
generation of PPI to inhibit ectopic calcification.\textsuperscript{2} The adenosine produced by NT5E is an inhibitor of tissue-nonspecific alkaline phosphatase (TNAP) synthesis, which, when activated, hydrolyzes PPI into phosphate and results in ectopic calcification.

Heritable mutations in \textit{Abcc6} result in the currently incurable calcification disorder, PXE. PXE is an autosomal-recessive disease that occurs in approximately 1 in 25,000 individuals according to the Online Mendelian Inheritance in Man \textsuperscript{264800}. This disease results in late-onset of extensive mineralization of soft tissue, specifically through calcium deposits of skin, the Bruch’s membrane of the eyes, and cardiovascular system.\textsuperscript{8} PXE also results in skin sagging and the progressive loss of vision and peripheral arterial disease (PAD) associated with gastrointestinal bleeding and intermittent claudication (pain caused by lack of blood flow, typically in legs).\textsuperscript{1} Progressive loss of vision in PXE patients results from calcification in the Bruch’s membrane in the eye, which is a thin membrane under the retina. Calcification in Bruch’s membrane leads to cracks in the retina and development of angioid streaks.\textsuperscript{13} Angioid streaks are associated with breaks in Bruch’s membrane and grow longer and wider over time and can lead to visual impairment. An example of the angioid streaks can be seen in Figure 3.\textsuperscript{14}

\textbf{Figure 3.} Angioid streaks found in the breaks of Bruch’s membrane indicated by the arrows.\textsuperscript{14}
GACI is also a rare autosomal-recessive disease that occurs in approximately 1 in 391,000 individuals according to the U.S. National Library of Medicine (NLM). This disease was initially found to result due to mutations in ENPP1; however, some patients diagnosed with GACI were found to have mutations in Abcc6 and no mutations in ENPP1. The overlapping diagnosis of GACI found due to mutations in either Abcc6 or ENPP1 provide support to their converging molecular pathways seen in Figure 2. This disease results in severe calcification of the cardiovascular system prior to or within the first few months of life and is often fatal within the first six months of life. Symptoms include thickening of the arteries from calcification and hypertension (high blood pressure) that eventually lead to severe MI and congestive heart failure. The similar soft tissue calcification symptoms seen in PXE and GACI patients and the fact that Abcc6 mutations can also lead to GACI led to a study that showed that there were overlapping characteristics between the two diseases and that mutations in ENPP1 and/or Abcc6 result in a spectrum of severity in ectopic calcification.

DCC is an autosomal-recessive trait that occurs from ABCC6 deficiency in several inbred mouse strains. Calcification of cardiac tissue can occur either spontaneously over a long period of time or by initiating the symptoms through a specific diet. DCC can also be induced in an acute or localized area by a severe injury, such as surface freeze-thaw injuries or ischemia, and develop the calcification symptom very quickly. The DCC trait induced in mice by injury serves as a controlled model of the human PXE disease as compared to mice that have no expression of ABCC6 alone.

All these diseases have similar symptoms such as vascular calcification, which plays a significant role in a broad range of cardiovascular disorders. Heart disease is the leading cause of death in the United States according to the Centers for Disease Control and Prevention, so studying
and understanding the mechanisms behind related symptoms in various heart diseases is particularly important in finding new novel treatments. Purine and pyrimidine nucleotides seem to contribute to injury, but adenosine is generally protective.\textsuperscript{16} Patients with chronic heart failure (CHF) have been shown to have an increased accumulation of adenosine within cardiac tissues.\textsuperscript{17} As a result, long-term oral administration of dipyridamole has been used to improve the cardiac status in patients with mild to moderate heart failure by increasing the levels of extracellular adenosine.\textsuperscript{16} Additionally, since NT5E is associated with the breakdown of AMP into adenosine and phosphate, increasing NT5E could potentially contribute to increased levels of adenosine.\textsuperscript{18} According to the proposed molecular pathway, ABCC6 is upstream from NT5E and ENPP1, an increase of ABCC6 should potentially result in increased adenosine.\textsuperscript{2}

ABCC6 deficiency in mice was found to be associated with increased cardiac infarct size (dead tissue) after ischemia-reperfusion, suggesting the potential role of ABCC6 in cardioprotection.\textsuperscript{19} However, our preliminary data surprisingly suggests that ABCC6 deficient mice presented improved heart functions when compared to WT mice following MI in \textit{in vivo} experiments (taking place within a living organism). For the \textit{in vivo} experiments, mice were given permanent coronary ligation to imitate MI and heart functions were measured for 30 days after MI and another set of WT and Abcc6\textsuperscript{-/-} mice were terminated 24 hours after MI and size of infarct was determined. Figure 4 shows the \textit{in vivo} heart function measurements after MI. Abcc6\textsuperscript{-/-} mice showed significantly higher heart rate (Figure 4A), ejection fraction (Figure 4B), which is the percentage of blood flow exiting the heart with every contraction, and fractional shortening (Figure 4C), which is the percentage of length shortening that occurs in the heart during contraction, than WT mice following coronary ligation. Figure 5A presents representative images of hearts in both
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**Figure 4.** Heart rate (A.), ejection fraction (B.), and fractional shortening (C.) at 0 days (basal level) to 30 days after coronary ligation in WT (n=5) and *Abcc6*–/– mice (n=6). n.s. not significant, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.001.

Wild-type (WT) and *Abcc6*–/– mice 24 hours after coronary ligation that are stained with Triphenyl tetrazolium chloride (TTC), which stains healthy tissue red and leaves areas of infarct white. Figure 5B presents a ratio of infarcted area (IA) over the area at risk (AAR) for both WT and *Abcc6*–/– mice and shows that the *Abcc6*–/– mice had significantly less IA/AAR than WT mice following coronary ligation. These findings suggest that the absence of ABCC6 is beneficial during the ischemic phase. A volcano plot is shown in Figure 6 that illustrates the global impact of ABCC6 deficiency on gene expression in the liver and aorta of mice. This figure illustrates the genes that are up or downregulated as a result of ABCC6 deficiency and suggests the impact that ABCC6 has on vascular purine metabolism. Interestingly, there is little to no expression of
ABCC6 in cardiac tissue, however ABCC6 deficiency seems to affect the expression of genes associated with purinergic signaling in distal tissues. Understanding ABCC6’s involvement in regulating these genes will provide insight on how ABCC6 influences the homeostasis of cardiac tissues and advance fundamental knowledge of molecular determinants of cardiac functions and may help identify new therapeutic targets.

**Methodology**

C57BL/6J mice, designated as wild-type, were derived from mice purchased from Jackson Laboratories (Bar Harbor, ME). Purchased mice were given two weeks to acclimate before being included in experiments. Abcc6<sup>tm1Aabb</sup> mice were generated on 129/Ola background and backcrossed into a C57BL/6J greater than 10 times and are designated as Abcc6<sup>/−</sup>. All animals

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**Figure 5.**

A. Representative images of hearts after Triphenyl tetrazolium chloride (TTC) staining of wild-type (WT) and Abcc6<sup>/−</sup> hearts 24 hours after coronary ligation. Size of infarct outlined with dashed lines (white area).

B. Ratio of infarcted area (IA) over area at risk (AAR) for wild-type and Abcc6<sup>/−</sup> mice. **p = 0.065.**

ABCC6 in cardiac tissue, however ABCC6 deficiency seems to affect the expression of genes associated with purinergic signaling in distal tissues. Understanding ABCC6’s involvement in regulating these genes will provide insight on how ABCC6 influences the homeostasis of cardiac tissues and advance fundamental knowledge of molecular determinants of cardiac functions and may help identify new therapeutic targets.
were housed in approved animal facilities at John A. Burns School of Medicine and were kept under routine laboratory conditions with 12-hour light-dark cycles with ad libitum access to water and chow. All mice will be mated, weened at two weeks of age, and genotyped by clipping the tails of the mice, extracting the ribonucleic acid (RNA) from the tails, producing complementary deoxyribonucleic acid (cDNA) from the RNA, and running the cDNA samples on an agarose gel to determine the exact genotype of the mice.

Conditional Abcc6-knockout (KO) mice are also being developed using a Cre-Lox recombination system. We have generated floxed Abcc6 mice on a C57BL/6N background with the assistance of the transgenic core facility at the Institute for Biogenesis Research (UH). The floxed Abcc6 mice will be crossed with Alb-Cre (liver-specific) and aMyHC-Cre (cardiomyocyte-specific) mutant mice purchased from Jackson Laboratory. Conditional Abcc6-KO mice will have

**Figure 6.** Volcano plot showing distribution of relative gene expression in Abcc6<sup>−/−</sup> mice in (a) liver and (b) aorta.²⁰
no expression in only the liver or cardiac muscle tissues to examine the role of *Abcc6* in tissue specific KO on cardiac function. The Cre-Lox recombination system selectively deletes the *Abcc6* sequence in specific tissues by flanking the sequence of interest with loxP sites. This is done by injecting a deoxyribonucleic acid (DNA) construct into the genome. These mice are then crossed with a mice strain that contains the Cre sequence, which will snip the genome at the loxP site, essentially deleting the *Abcc6* sequence contained within the loxP sites in the tissues of interest.

To measure the effect of tissue-specific *Abcc6*-KO on cardiac function and purinergic signaling, mice will be given a permanent coronary ligation to introduce ischemic stress *in vivo*. The heart rate, fractional shortening, and ejection fraction will be monitored before coronary ligation and for 30 days afterwards. Heart, liver, and blood serum will be harvested for analysis. An ELISA kit will be used on the blood serum for plasma analysis, to measure enzyme levels, and molecules of interest.

**Materials and Resources**

All materials and resources are available and provided by Le Saux’s Laboratory.

**Role of the Researcher**

The student will be working under a larger research project conducted by Bianca Calio, a graduate student at Le Saux’s laboratory. The student will work together with Ms. Calio to maintain, breed, wean, and genotype the mouse strains and harvest tissues of interest. The student will also perform and analyze the protocols stated in the methodology.

**Research Ethics Statement**
The student has completed all necessary trainings and received all certificates, which are all up to date. Trainings include General Lab Safety, Hazardous Waste Generator, General Biosafety Principles and Practices, Bloodborne Pathogens and Safe Sharps Use, CITI Program Training – Reducing Pain and Distress in Laboratory Mice and Rats, and Animal and Veterinary Services Vivarium Orientation.

**Timetable**

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