Isoform Specific Effect of Ischemia/Reperfusion on Cardiac Na,K-ATPase: Protection by Ouabain Preconditioning

Submitted by:
Cory Stebal

In partial fulfillment of the requirements for the degree of Master of Science in Biomedical Science

Examination Committee

Major Advisor: Sandrine Pierre, Ph.D.
Academic Advisory Committee: Zi-Jian Xie, Ph.D.
David Giovannucci, Ph.D.

Signature/Date

Senior Associate Dean
College of Graduate Studies
Michael S. Bisesi, Ph.D.

Date of Defense: March 26, 2009
Effect of Ischemia/Reperfusion on Cardiac Na,K-ATPase:
Protection by Ouabain Preconditioning

Cory Stebal

University of Toledo Health Science Campus

2009
DEDICATION

I dedicate this to my parents, Robert and Claudia Stebal, for their limitless love and support. I also wish to dedicate this to my grandpa, Joseph Frantz, for always telling me I was smart enough to accomplish anything I set my mind to.
ACKNOWLEDGEMENTS

I want to extend my greatest appreciation to Dr. Sandrine Pierre for serving as my major advisor. In addition, I want to thank the entire lab, particularly Dr. Eric Morgan and Yoann Sottejeau for their help on my project. I also want to thank the professors who sat on my committee, Dr. Zi-Jian Xie, Dr. David Giovannucci, Dr. Amir Askari, Dr. Andrew Beavis, and Dr. Jiang Tian. Lastly, I would like to thank the entire Department of Physiology/Pharmacology, especially Martha Heck and Marianne Miller-Jasper.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>TABLE OF CONTENT</td>
<td>iv</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td>3</td>
</tr>
<tr>
<td>METHODS</td>
<td>24</td>
</tr>
<tr>
<td>RESULTS</td>
<td>29</td>
</tr>
<tr>
<td>CONCLUSIONS</td>
<td>36</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>39</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>49</td>
</tr>
</tbody>
</table>
INTRODUCTION

Early after the description of acute myocardial infarction in the beginning of the 20th century, its treatment was limited to complete bed rest and dealing with complications when they arose. In 1974, Braunwald first proposed the concept that “just because myocardial tissue lies within the distribution of a recently occluded coronary artery does not mean that it is necessary condemned to death”. This statement was at the origin of the concept of “damage control”, which aims at limiting cardiac injury by mean of early reperfusion and adjunctive pharmacologic therapy. A second breakthrough was the discovery by Murry et al. in 1986, that brief intermittent periods of ischemia and reperfusion are protective against a longer ischemic episode. The protection afforded by this so-called “preconditioning” against cell death is dramatic and is observable in all species investigated, as well as in tissues other than the myocardium. Although a large number of studies have identified different triggers and mediators of preconditioning, their clinical applicability has been limited.

Our laboratory has recently shown the preconditioning effect of sub-inotropic doses of ouabain, a well-known member of the family of cardiac glycosides, like digoxin. Even though the latter has been used in the treatment of heart failure for over 200 years, this possible mechanism of action has not been considered until recently. Rather than the classical textbook explanation for the digitalis-induced positive inotropy via inhibition of Na,K-ATPase ion-pumping
function, evidence now suggest that ouabain preconditioning occurs via activation of Na,K-ATPase-mediated signaling to the mitochondria.

Because alteration of cardiac Na,K-ATPase enzyme activity plays a critical role in the pathogenesis of ischemia/reperfusion injury, it appears that the protein complex plays a key role in both ischemia-reperfusion injury and digitalis preconditioning. Because it might be key to the therapeutic management of heart failures precipitated by myocardial ischemia, we conducted a study of ischemia-reperfusion-induced remodeling of Na,K-ATPase at key time-points, and examined the effect of ouabain pretreatment on these alterations.
1. Structure and functions of the cardiac Na,K-ATPase.

The Na,K-ATPase is the ubiquitous transmembrane protein complex that transports 3 Na\(^+\) out of and 2 K\(^+\) into the cells against their electrochemical gradients across the plasma membrane by hydrolysis of ATP. The definitive characterization of the sodium pump as an ATPase was reported in 1957 by J.C. Skou (1957), for which he was awarded the Nobel Prize in 1997 (Clausen and Persson, 1998; Skou, 1957). Na,K-ATPase activity plays a critical role in generating and maintaining the Na\(^+\) and K\(^+\) gradients that contribute to membrane potential, cell volume, secondary active transport, and excitability (Kaplan, 2002). In cardiac cells, approximately 40% of the resting ATP consumption has been attributed to the Na,K-ATPase (Ismail-Beigi and Edelman, 1971).

1.1 Structure.

The Na,K-ATPase is a member of the P-type family of ATPases and is closely related to the Ca\(^{2+}\) ATPase (Horisberger, 2004; Kaplan, 2002). It is composed of two major subunits. The catalytic $\alpha$ subunit hydrolyzes ATP and transports the cations (Nesher et al., 2007). The glycosylated $\beta$ subunit has a crucial role in the structural and functional maturation of Na,K-ATPase and modulates its transport properties. Other proteins, such as members of the FXYD family, associate with the enzyme and modulate Na,K-ATPase activity in a
tissue-specific manner. FXYD1 (phospholemman) regulates Na,K-ATPase activity in the heart (Geering, 2008; Sweadner, 2005). A schematic representation of the protein complex and key sites that have been identified thus far is shown in Figure 1.

Figure 1. Schematic illustration of the Na,K-ATPase subunits. Major ligand binding and active sites on the α1 (red), β1 (blue) and FXYD1 (green) subunits are depicted. Ellipses in the transmembrane domains indicate regions involved in Na⁺ and/or K⁺ binding. Major binding site for digitalis (1st extracellular loop) and other sites involved are indicated by yellow rectangles. Phosphorylation sites by PTK: protein tyrosine kinase, PKC: protein kinase C, and PKA: protein kinase A are shown, as well as binding sites for PI3K: phospho-inositol-3 kinase, IP3: inositol 1,4,5-trisphosphate binding site, 14-3-3: 14-3-3 protein, CMB: caveolin 1 and 2, Ank: ankyrin, AP2: clathrin adaptor protein 2, Mg⁺⁺: magnesium. P-ATPase: conserved motif of the P-ATPases. FXYD: conserved motif of the FXYD protein family. (Slightly modified from Nesher et al., Life Sci. 2007: 80: 2093-2107).

Isozyme diversity for the Na,K-ATPase results from the association of different molecular forms (each derived from a different gene) of the α (α1-4) and β (β1-3) subunits. The pattern of expression of the various isozymes is highly specific, and varies with tissue, developmental stage, diseases and hormonal regulation (Blanco, 2005). While α1 is expressed ubiquitously, α2 is present in various tissues such as skeletal muscle, heart, brain, adipose tissue, or vascular smooth muscle. The α3-isoform is found almost exclusively in neurons and ovaries, but also occurs in the heart of some
species including human (McDonough et al., 2002; Shamraj et al., 1991). The expression of α4 is restricted to testis.

In rodents, the ubiquitous α1 has a low affinity for the digitalis drug ouabain, the specific inhibitors of the enzyme, whereas the other isoforms are highly sensitive. The adult rat heart, which is the model used in the present study, contains predominantly α1 and α2 isoforms, with the latter exhibiting lower protein levels (Sweadner, 1989; Zahler et al., 1996). In rat heart, it is estimated that the α2 isoform with high affinity for ouabain represents approximately 10 to 30% of the total enzyme activity (Lucchesi and Sweadner, 1991; Noel and Godfraind, 1984). The α3 isoform is well-expressed in fetal rat heart, but its expression decreases markedly after birth and the protein becomes hardly detectable (Lucchesi and Sweadner, 1991).

1.2. Ion-pumping function.

1.2.1 Mechanism.

The formation of a transient phosphorylated aspartate residue during the catalytic cycle is a hallmark of all P-type ATPase family members. There are two conformers of the enzyme, termed E1 and E2. Ion

**Figure 2.** P-type ATPase enzymatic cycle for Na,K-ATPase (simplified representation of the Albers-Post cycle, see text for details).
movement across the membrane is coupled to ATP hydrolysis via a cation-dependent E1 to E2 conformational change. A simplified representation of the scheme of Albers and Post for Na,K-ATPase is depicted in Figure 2 (Albers, 1967; Post et al., 1972). Briefly, intracellular Na\(^+\) binds to E1, allowing ATP to bind to a high affinity site and phosphorylate the \(\alpha\)-subunit on Asp-369. Na\(^+\) is occluded within the intramembrane helices in the so-called E1P(Na\(^+\)) state. E1P(Na\(^+\)) undergoes a conformational change to E2PNa\(^+\) and Na\(^+\) is released to the extracellular space. Extracellular K\(^+\) binds to E2P, releases Pi, and K\(^+\) is occluded in the E2(K\(^+\)) state. Spontaneous deocclusion of K\(^+\) is slow. However, when ATP concentration is high enough to allow the occupation of a low affinity ATP site on E2(K\(^+\)), deocclusion is accelerated, K\(^+\) is released to the inside, and the cycle is completed. Ouabain has the highest affinity for E2P. Upon ouabain binding and subsequent slow dephosphorylation, a dead-end complex is formed.

1.2.2 Cardiac contractility and positive inotropy by digitalis drugs.

In the heart, Na,K-ATPase activity not only generates and maintains the Na\(^+\) and K\(^+\) gradients across the membrane necessary for nutrient transport and membrane potential, it also regulates cardiac contractility by providing the driving force for Ca\(^{2+}\) extrusion via the Na\(^+\)/Ca\(^+\) exchanger (Bers et al., 1990). The importance of the ion-pumping function for the well-known positive inotropic action of digitalis drugs like ouabain or digoxin has long been established (Akera and Brody, 1977; Smith, 1988).

1.2.3 Isoforms specificity.

Although isoform-specific roles in the regulation of cardiac muscle
contractility have been suggested, both $\alpha_1$ and $\alpha_2$ containing Na,K-ATPases appear to co-localize with Na$^+$/Ca$^+$ exchanger, and either $\alpha_1$ or $\alpha_2$ containing enzymes can provide local Na$^+$ gradients that control the activity of the Na$^+$/Ca$^+$-exchanger, influence Ca$^{2+}$ stores, and digitalis-induced positive inotropy in the cardiac muscle (Dostanic-Larson et al., 2006; McDonough et al., 1996).

1.3. Signaling function.

1.3.1 Overview.

The Na,K-ATPase protein complex is also a signal transducer, which interacts with Src to form a functional digitalis receptor. Upon digitalis binding, the receptor complex initiates signaling cascades, resulting in increased protein tyrosine phosphorylation and recruitment of additional tyrosine kinases, lipid kinases and serine/threonine kinases to form a functional signaling complex that transmits the ouabain signal to different intracellular compartments and
organelles in a cell-specific manner, as summarized in Figure 3 (Pierre and Xie, 2006; Tian and Xie, 2008; Xie and Cai, 2003).

1.3.2 Isoforms specificity.

Very little is known about the signal transducing capabilities of Na,K-ATPase isoenzymes containing an \( \alpha \)-polypeptide other than \( \alpha_1 \). Indeed, studying the effect of ouabain on Na,K-ATPase isoforms has been a difficult task, as the various \( \alpha \) polypeptides are co-expressed in cells in different combinations and usually with the ubiquitous \( \alpha_1 \) polypeptide. To date, there has been no report of studies of isoform-specific signaling effects in cardiac tissue. In fact, the only study available was performed using a baculovirus expression system in Sf-9 insect cells, which contain negligible levels of endogenous Na,K-ATPase. This system allows for analysis in the absence of high background levels of the transporter. The results suggested that the \( \alpha_1, \alpha_3 \) and \( \alpha_4 \) isoforms, but not \( \alpha_2 \) can transmit ouabain induced phosphorylation of ERK in the insect cells (Pierre et al., 2007a).

2. Ischemia-Reperfusion (IR)-Induced Cardiac Injury and Preconditioning.

2.1 Incidence of IR.

IR-induced myocardial infarction is the leading cause of mortality in industrialized nations, accounting for nearly 1 in 3 deaths in the United States, and also resulting in significant disability among survivors. Among the various forms of cardiovascular disease, IR-induced myocardial injury accounts for the
majority of heart disease-related deaths in the industrialized world (Powers et al., 2007).

2.2 Ischemia/Reperfusion Injury.

2.2.1 Myocardial stunning.

Myocardial stunning is the best-established form of reperfusion injury (Ambrosio and Tritto, 2001; Kloner et al., 2001) and first described by Heyndrick et al. in 1975 (Heyndrickx et al., 1975). It is defined as prolonged post ischemic mechanical dysfunction, which persists after reperfusion (Braunwald and Kloner, 1982). The duration of this dysfunction greatly exceeds that of the antecedent ischemia (Bolli, 1988). After 15 min of ischemia in dogs, myocardial function remains depressed for 24 hours. Myocardial stunning can be found after reperfusion of a globally ischemic myocardium (cardiac arrest during cardiac surgery), or in the setting of regional ischemia and reperfusion (PCI, thrombolysis, unstable angina, and stressor exercise-induced angina) (Bernier et al., 1989; Kloner and Jennings, 2001; Piper et al., 2003). Stunning is an important causative factor in the development of ischemic cardiomyopathy, wherein repeated episodes of myocardial ischemia and reperfusion may lead to the development of heart failure (Ambrosio and Tritto, 2001; Kloner et al., 2001).

2.2.2 Reperfusion arrhythmias.

Reperfusion of the heart after an ischemic period may lead to potentially lethal arrhythmias (Manning and Hearse, 1984). In humans, the most common reperfusion arrhythmia is an accelerated idioventricular rhythm (Goldberg et al.,
However, ventricular tachycardia and ventricular fibrillation remain the most important causes of sudden death following spontaneous restoration of antegrade flow (Manning and Hearse, 1984; Tzivoni et al., 1983). It was suggested that oxygen derived free radicals might play a key role in the genesis of ventricular arrhythmias (Meerson et al., 1987; Shen and Jennings, 1972b).

### 2.2.3 Myocyte death and necrosis.

Cardiomyocyte cell death during ischemia is named “oncosis”, during reperfusion it is part of the myocardial reperfusion injury. Development of cardiomyocyte contracture (contraction band necrosis) seems to be the primary cause for necrotic cardiomyocyte injury during the earliest phase (i.e. minutes) of reperfusion. Thereafter (i.e. minutes to hours), various additional causes can lead to a further increment of cell death either by necrosis or apoptosis (Piper et al., 2003). The myocardium can tolerate brief periods (up to 15 min) of severe and even total myocardial ischemia without resultant cardiomyocyte death. This is observed in clinical settings like coronary vasospasm, angina and balloon angioplasty, and is therefore not associated with concomitant myocyte death (Kloner and Jennings, 2001).

### 2.3 Mechanisms of I/R Damage.

#### 2.3.1 Oxygen derived free radicals.

A free radical may be defined as any atom or molecule that can exist independently with one or more unpaired electrons in its outer orbital. Because of the existence of an unpaired electron, the atom or molecule is relatively
unstable and, in general, it is highly reactive. They are generated in small
amounts during the normal metabolism of cells and are normally inactivated by
endogenous scavenging systems. Reintroduction of abundant oxygen at the
onset of reperfusion evokes within the first few minutes of reflow a burst of potent
free radicals, such as superoxide anion, hydroxyl radical and peroxynitrite as
demonstrated in experimental settings as well as in humans with acute
myocardial infarction undergoing thrombolysis or percutaneous coronary
intervention (Roberts et al., 1990), as in patients undergoing open heart surgery
(Kim et al., 1994). In the heart, oxygen radicals may be generated by several
mechanisms, such as mitochondrial respiration, activated neutrophils and, in
some species, by xanthine oxidase activity. The release of free radicals in the
early phase of reperfusion, in combination with the IR-induced decrease in
antioxidant activity, renders the myocardium extremely vulnerable. Membranes
are composed mostly of phospholipids and proteins. Alterations in membrane
proteins by free radicals are among the important factors in the evolution of
myocardial IR damage. Large quantities of ODFR cause an overwhelming of
body’s endogenous antioxidant defenses. This leads to peroxidation of lipid
membranes and loss of membrane integrity and results in necrosis and cell death
(Lazzarino et al., 1994). Lipid peroxidation is not observed in those who
underwent ischemia without reperfusion, nor when oxygen radicals were
inactivated by specific scavengers (Becker and Ambrosio, 1987). Administration
of antioxidants prevents lipid peroxidation. ODFR can also be generated from
sources other than reduction of molecular oxygen. These sources include (i)
enzymes such as xanthine oxidase, cytochrome oxidase, and cyclooxygenase, and (ii) the oxidation of catecholamines. ODFR stimulate also the endothelial release of platelet activating factor, which attracts more neutrophils (Jordan et al., 1999) and amplifies the production of oxidant radicals and the degree of reperfusion injury.

2.3.2 Calcium overload and hypercontracture development.

Changes in intracellular calcium homeostasis play an important role in the development of reperfusion injury (Gross et al., 1999). Shen and Jennings (Shen and Jennings, 1972a) first documented that myocardial reperfusion after temporary coronary artery occlusion accelerated the accumulation of tissue Ca2+ in the canine heart. The calcium hypothesis suggests that ischemia induces defect in the cell’s viability to regulate calcium such that upon reperfusion the cell accumulates toxic levels of calcium. Ischemia and reperfusion are both associated with an increase in intracellular calcium. This effect may be related to increased sarcolemmal calcium entry through L-type calcium channels as a consequence of the sodium overload, or it may be secondary to alterations in sarcoplasmic reticulum calcium cycling. The glycocalyx layer of the sarcolemmal membranes also plays an important role in calcium binding and entry into normal myocardial cells. After significant ATP depletion, calcium-binding sites on the glycocalyx layer are disrupted, thus facilitating calcium entry in the myocyte. Additionally, to intracellular calcium overload, alterations in myofilament sensitivity to calcium have been implicated in reperfusion injury. Reperfusion injury results in significant desensitization of the myofibrils to calcium. In the first
minutes of reperfusion, the myocardium can be damaged by contracture (i.e., a sustained shortening and stiffening of myocardium) development, causing mechanical stiffness, tissue necrosis, and the “stone heart” phenomenon. This term is used to indicate that the contracture affects the entire heart, as may occur after global ischemia, e.g. during prolonged cardiac surgery. Reperfusion-induced contracture can have two different causes, namely, Ca2+ overload-induced contracture or rigor-type contracture. Ca2+-overload-induced contracture occurs when high levels of cytosolic Ca2+ together with high energy leads to uncontrolled activation of the contractile machinery. Rigor-contracture may be activated during reoxygenation, if reenergization proceeds very slowly. The low concentrations of ATP provoke rigor contracture. It may, therefore, be observed after prolonged or severe ischemia. Rigor-contracture is not essentially dependent on Ca2+-overload. A first approach to prevent rigor-contracture could possibly be the application of mitochondrial energy substrates, e.g., succinate (Linz et al., 1988). Cells undergoing contracture exchange forces with their neighbors and may disrupt these. This way, the development of contracture contributes to the spreading of necrosis.

2.4 Preconditioning.

2.4.1 Physiology.

Myocardial preconditioning is the protective effect either produced by short intervals of coronary occlusion and reperfusion or a pharmacological agent added prior to a sustained coronary occlusion. Preconditioning results in a major
reduction of infarct size compared with the non-preconditioned state when hearts are subjected to approximately 30 to 90 min of coronary occlusion, with more extensive necrosis eventually developing if the coronary occlusion is maintained for two or more hours (Murry et al., 1986). The preconditioning effect is operative for 1 to 2 h prior to sustained coronary occlusion, then is lost for several hours (refractory period), but redevelops when sustained coronary occlusion is induced approximately 24 h after the preconditioning. The first phase is known as early or classical preconditioning and the delayed phase as the second window of protection (Yellon and Downey, 2003).

2.4.2 Molecular mechanism.

Ischemic preconditioning is thought to operate via agonist binding to Gi protein-coupled receptors prior to ischemia, which triggers a signaling cascade that protects the heart from mitochondrial permeability transition (MPT). In our current understanding, these triggers induce the formation of a vesicular caveolar signaling platform (signalosome) that contain the enzymes of the pathway and terminates in activation of guanylyl cyclase resulting in increased production of cGMP and subsequent activation of protein kinase G (PKG). cGMP-activated PKG phosphorylates a protein on the mitochondrial outer membrane (MOM), which then causes the mitochondrial KATP channel (mitoKATP) on the mitochondrial inner membrane to open, leading to increased production of reactive oxygen species (ROS) by the mitochondria. The protective signal is the transmitted from the MOM to the mitochondrial inner membrane by a series of intermembrane signaling steps that includes PKC activation. The resulting ROS
then activate a second PKC pool which, through another signal transduction pathway termed the mediator pathway, causes inhibition of MPT and reduction in cell death (Figure 4) (Costa et al., 2007; Garlid et al., 2008; Quinlan et al., 2008).

2.4.3 Digitalis Preconditioning.

Both ouabain and digoxin have been shown to protect the heart against ischemia-reperfusion injury at concentration that do not induce significant changes in intracellular ion concentration or inotropy (D'Urso et al., 2008; Insete, 2007; Pasdois et al., 2007; Pierre et al., 2007b). Ouabain cardioprotection is blocked by 5-hydroxydecanoate (5-HD, a mitoKATP blocker) and the ROS scavenger N-2-mercaptopropionylglycine (Pasdois et al., 2007). Ouabain cardioprotection exhibits several interesting features. First, it occurs in rat heart at concentrations much lower than those required to induce an inotropic response. Pasdois et al (Pasdois et al., 2007) showed that low micromolar concentrations of ouabain induce cardioprotection whereas maximum inotropic
response is achieved with higher micromolar concentrations (10 vs 80 µM).
Second, it does not depend on guanylyl cyclase or PKG activities, showing that
its signaling pathway differs from that triggered by GPCR agonists (Figure 4).
Ouabain-induced inotropy also signals mitochondria to open mitoKATP, and this
pathway involves not only mitoKATP opening and ROS production, but also Src
tyrosine kinase, guanylyl cyclase and PKG (Pasdois et al., 2007).

3. Cardiac Na,K-ATPase enzyme function during Ischemia/Reperfusion
Injury (IRI).  Effect of ischemic Preconditioning.

Beller et al. first correlated post-ischemic alterations in cardiac glycoside
binding to decreased in vitro Na,K-ATPase activity in dog myocardium in 1976
(Beller et al., 1976).  This study and many others that are reviewed in this section
were undertaken with the hope to understand the increased toxicity of digitalis
when used to treat heart failures precipitated by myocardial ischemia.  As can be
inferred from the brief review to follow, even though it is clear that ischemia-
reperfusion-induced alteration of the cardiac Na,K-ATPase isoforms occurs over
time and plays an important role in the recovery or failure to recover of the
cardiac myocyte, the mechanisms involved remain unclear.

3.1 Ischemia without reperfusion.

In purified sarcolemmal vesicles from rabbit heart submitted to 1h
ischemia without reperfusion, Bersohn reported a large reduction of ATP-
dependant Na\(^+\) pumping with unchanged Na, K-ATPase activity and increased
$^3$H ouabain binding, suggesting an uncoupling of the Na+/K+ pumping function by ischemia (Bersohn, 1995). Fuller and co-workers exposed rat hearts to 15 or 30 min of ischemia without reperfusion and observed a profound decrease in activity in crude homogenate fractions. Interestingly, when they purified out the sarcolemmal membranes from the cytosolic fraction, they observed a substantial increase, and concluded that ischemia caused Na,K-ATPase inhibition via a labile cytosolic compound (Fuller et al., 2003). The same authors suggested that the observed activation in sarcolemmal membranes may occur indirectly, via the phosphorylation of phospholamban, and be $\alpha_1$-specific (Fuller et al., 2004). In ventricular slices from guinea-pig hearts exposed to 2h of ischemia without reperfusion, Kim et al found no change of the ouabain-sensitive Rb+ uptake (Kim et al., 1983).

### 3.2 Ischemia and short reperfusion (5 min).

In rat hearts exposed to 1h ischemia and 5 min reperfusion, Inserte et al. have reported a reduction of 80% of Na,K-ATPase activity in homogenate, associated with detachment of $\alpha_1$ and $\alpha_2$ from the membrane-cytoskeleton complex (Inserte et al., 2005). Other studies have reported decreased Na,K-ATPase activity in sarcolemmal vesicles of hearts exposed to 30 min of ischemia and 5 min of reperfusion, with a decrease in expression of all 3 $\alpha$-isoforms (Ostadal et al., 2004).
3.3 Ischemia and Long reperfusion (20 min -1h).

Thirty min. of ischemia followed by 30 min of reperfusion significantly decreases Na,K-ATPase at the mRNA-, protein-, and ATPase activity level. The $\alpha_1$ isoform was found less sensitive to this IR-induced remodeling than the other $\alpha$-isoforms (Elmoselhi et al., 2003; Ostadal et al., 2003). On the hand, Ostadal et al. reported a decrease of Na,K-ATPase is sarcolemmal vesicles of hearts exposed to 30 min of ischemia, with a decrease in expression of all $\alpha$-isoforms. Isoforms were not protected equally by antioxidant, with $\alpha_1$ being the least protected (Ostadal et al., 2004). In ventricular slices from guinea-pig hearts exposed to 2h of ischemia without reperfusion, Kim et al found no change of the ouabain-sensitive Rb+ uptake, but a significant reduction after 20 min of repfifuion (Kim et al., 1983).

3.4 Ischemic Preconditioning (IP).

Ischemic preconditioning has been shown to protect Na,K-ATPase activity and isoform dissociation from the membrane-cytoskeleton during early reperfusion (Inserte et al., 2006) and remodeling in hearts subjected to 30 min of ischemia followed by 30 min reperfusion (Elmoselhi et al., 2003). Although the precise mechanism by which IP protects Na,K-ATPase remains to be established, studies have suggested that Na,K-ATPase itself may be one key mediator of the IP-induced protection (Imahashi et al., 2001; Yorozuya et al., 2004).
Hence, it appears that the cardiac Na,K-ATPase plays a key role in both ischemia-reperfusion injury and preconditioning. Because it might be key to the therapeutic management of heart failures precipitated by myocardial ischemia, we conducted a study of ischemia-reperfusion-induced remodeling of Na,K-ATPase at key time-points, and examined the effect of ouabain pretreatment on these alterations.

**AIM OF THE STUDY**

The aim of this study was to investigate the fate of cardiac Na,K-ATPase activity, \( \alpha_1 \) and \( \alpha_2 \) isoforms during ischemia and reperfusion and to test the hypothesis that the digitalis ouabain can protect Na,K-ATPase against ischemia-reperfusion injury.
METHODS

Isolated Perfused Rat Heart Model

**Langendorff-perfused rat hearts.** Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health NIH (Publication No. 85-23, revised 1996). Male Sprague-Dawley rats (350-400 g) were anesthetized with 50 mg phenobarbital sodium injected intraperitoneally. Hearts were excised and placed into ice cold Krebs-Henseleit buffer. Within 40 s, hearts were perfused in the Langendorff mode with oxygenated Krebs-Henseleit buffer containing (in mM) NaCl (118.0), KCl (4.0), CaCl2 (1.8), KH2PO4 (1.3), MgSO4 (1.2), Ethylene glycol-bis (2-aminoethylether)-N,N′,N′-tetraacetic acid (0.3), NaHCO3 (25.0), D-glucose (11.0). The gas used for oxygenation was a mixture of O2 (95%) and CO2 (5%), resulting in a pO2 of 618 mmHg, a pCO2 of 30 mmHg and a pH of 7.5. The coronary flow was kept constant at a rate set to obtain a perfusion pressure of approximately 100 mmHg at the end of a 10 min equilibration period. A drainage cannula was inserted into the apex of the left ventricular cavity to vent the Thebesian flow. Isovolumic left ventricular developed pressure (LVDP) was measured by inserting a water-filled latex balloon into the left ventricle connected to a P23XL Becton Dickinson pressure transducer, a CP122 AC/DC strain gage amplifier and a Grass Telefactor recording system. The end diastolic pressure (EDP) was adjusted initially at 10 mmHg. Hearts were paced at 4.5 Hz and the
latex balloon was kept inflated throughout the experiment, including during ischemia, as described (Pierre et al., 2007b).

**Experimental Protocols**

Three groups of rat hearts were studied (Figure 5). The control group was stabilized and perfused without any period of ischemia. The ischemia-reperfusion group (IR) was stabilized and perfused for 30 min before 30 min of global ischemia and reperfusion. The ouabain-preconditioned group (OIR) was stabilized and perfused for 8 min with normal Krebs-Henseleit solution followed by 15 min of perfusion with ouabain (50 µM) before 4 min global ischemia and reperfusion for 30 min. For each of this groups, tissue were collected at 0, 5 or 30 min reperfusion, as indicated by the arrows (Figure 5).
Preparation of Homogenates

Hearts were perfused as described above. At the end of the experiment, hearts were flash frozen in liquid nitrogen and stored at -80 °C. Sample preparations were made following a modified protocol for tissue homogenization used by Huang et al. (1994). 0.5-1.0 g of ventricular tissue was cut into pieces in 10 ml of 1M KCl and homogenized with a Polytron PT-10/ST for 30 sec. The homogenate was vacuum-filtered and suspended in 40 ml of 50 mM Tris-HCl and 10 ml of 50 mM KCl and centrifuged at 1000 g (4190 rpm) for 10 min. The pellet was centrifuged twice with 50 ml of 50 mM Tris-HCl (pH 7.4) for 10 min. The final pellet was suspended in 1.25-3 ml of 1 mM Tris-EDTA (pH 7.4) plus 10 µl of a protease inhibitor using a hand homogenizer. This procedure was conducted on ice and with the use of a refrigerated centrifuge at 4°C.

Differential Fractionation

Ventricular tissue from one heart was diced and incubated for 30 min in 1M NaCl, 20mM Hepes, then centrifuged at 1000g for 5 min, rinsed in Buffer A and centrifuged again. (Buffer A 20mM hepes pH 7.4, 2mM EDTA pH 8.0, 1mM MgCl2, 250mM sucrose.) Tissue was then homogenized by hand as described (Fuller et al., 2001). Homogenates were then centrifuged according to Table 2.

TABLE 2

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Speed (g)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>5,000</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>20,000</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>50,000</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>100,000</td>
<td>60</td>
</tr>
</tbody>
</table>
Determinaction of Na,K-ATPase activity

Na,K-ATPase activity was measured in crude homogenate by colorimetric determination of inorganic phosphate released after incubation for 10 min at 37°C in a buffer containing 20 mM Tris-HCl, 1mM MgCl2, 100 mM NaCl, 20mM KCl, 1mM EGTA-Tris, and NaAzide. After addition of MgATP, the reaction was allowed to run for 10 min before the addition of 1 ml cold 8% tricholoroacetic acid and rapid placement of each test tube into ice to terminate the reaction. Using an inorganic phosphate detection kit (BIOMOL GREEN, AK-111), released phosphate in each test tube was quantified using a spectrophotometer at 620 nm. Ouabain insensitive activity was measured in a separate reaction in the presence of 1mM ouabain in the same buffer. Ouabain sensitive Na,K-ATPase activity was then determined by subtracation of ouabain insensitive from total ATPase activity.

Alamethicin Treatment

The ionophore alamethicin was used to insure access of substrates and inhibitor to both the ATP and ouabain binding sites of the closed membrane vesicles formed in crude homogenates. Reduced access of ATP and ouabain to their respective binding sites on Na,K-ATPase would result in an underestimation of Na,K-ATPase activity. Accordingly, crude homogenates were exposed to increasing concentrations of alamethicin for 10 min prior to ATPase measurement.
Immunodetection of Na⁺,K⁺-ATPase

Samples were dissolved in 1.5x diluted Laemmli buffer and separated on 10% SDS-PAGE before being transferred to a nitrocellulose membrane via electroblotting. Na⁺,K⁺-ATPase catalytic subunit α1 was probed using α6F primary-antibody and goat anti-mouse-IgG-HRP secondary-antibody. Na⁺,K⁺-ATPase catalytic subunit α2 was probed using HERED primary-antibody (a gift from Dr TA Pressley, Lubbock, TX) and donkey anti-rabbit-IgG-HRP secondary-antibody. Actin was probed as a loading control using actin C-11 primary-antibody (Santa Cruz, CA) and donkey anti-goat-IgG-HRP secondary-antibody. Western blot films were electronically scanned and analyzed with the use of ImageJ software allowing for the quantification for each of the probed proteins.

Statistical Analysis

Statistical analysis between control and pharmacologically treated groups were conducted using a one-way ANOVA followed by Tukey’s multiple comparison post hoc test. P<0.05 was considered statistically significant.
RESULTS

1. Functional data confirmed that ouabain 10µM protects the rat heart against ischemia/reperfusion (IR) injury.

Our laboratory has reported that ouabain exposure activates a cardioprotective signaling cascade that results in protection against functional and structural damage induced by ischemia-reperfusion (Pierre et al., 2007b). Although the same settings were used to prepare the tissues needed for the present study according to the protocols presented in Figure 5, user-to-user variations needed to be controlled for. Thus, we first collected a series of functional and structural data to verify that the extent of injury and protection obtained were comparable to the previously published study. As shown in Figure 6, ouabain treatment prevented ischemia-induced increase in end diastolic pressure (EDP) and decrease in left ventricular developing pressure (LVDP). Further, lactate dehydrogenase release and infarct size were significantly reduced, indicative of protection against cell death. These results were qualitatively and quantitatively comparable to the previously reported study of our laboratory (Pierre et al., 2007b).

Na+/K+-ATPase activity was measured by the ouabain-sensitive ATPase activity following alamethicin treatment as described in the methods section. In crude homogenates prepared from hearts reperfused for 5 min following ischemia, we did not detect any change in ouabain-sensitive activity, whether ischemia was preceded by a treatment with ouabain or not. After 30 min of reperfusion, ouabain sensitive ATPase activity was significantly decreased (P≤0.05). This decrease was prevented by ouabain treatment prior to ischemia (Figure 7).
Figure 7. Na,K-ATPase activity alamethacin-treated crude homogenates from continuously perfused hearts (control) or hearts exposed to ischemia and 5 or 30 min of reperfusion following ischemia. * P<0.05 vs control

3. Total cardiac Na,K-ATPase α1 and α2 protein content during ischemia and reperfusion. Protective effect of ouabain preconditioning.

To determine the effect ischemia reperfusion has on Na,K-ATPase α1 and α2 protein expression a time dependent study was performed using western blot analysis. 5 minutes of reperfusion revealed that there was no change in protein expression between control, IR, and OIR in either α1 or α2 (Figure 8). The experiment was repeated with hearts that have been reperfused for 30 min and a 53.5 ± 3.7% decrease in α1 and 43.7 ± 2.6% in α2 was observed in IR group vs. Control (P≤0.05). This decrease in protein expression was prevented with ouabain treatment (Figure 8).
Figure 8. Total protein expression of Na,K-ATPase α1 and α2 after in crude heart homogenates after 0 (A), 5 min (B) and 30 min (C) of reperfusion following 30 min of ischemia. Top panels: representative western blots. Bottom panels: means ± SEM of 3 independent experiments. * P<0.05 vs control.

We hypothesized that ischemia/reperfusion caused time-dependant endocytosis, leading to degradation of cardiac Na,K-ATPase. To further test this hypothesis, a differential centrifugation was performed on samples reperfused for 5 min, after ischemia. This resulted in 5 separate fractions.

Figure 9. Effect of ischemia and 5 min of reperfusion on the location of Na⁺/K⁺-ATPase α1 in comparison with EEA1 (Early Endosome Antigen 1).
Top Panel: representative western blots of proteins collected by differential fractionation.
Bottom panel: quantification of the means of each fraction (n=3) over the total protein.
Western blot analysis revealed that fractions 4 and 5 were enriched with the early endosomes marker EEA1. We then proceeded to the analysis of the amount of Na+/K+-ATPase α1 in each fraction. As shown in Figure 9, we found that there is significantly more α1 in fractions 4 and 5 in the IR group vs. control and OIR (P≤0.05) (Figure 9).
CONCLUSION AND PERSPECTIVES

Since Beller et al. first correlated post-ischemic alterations in cardiac glycoside binding to decreased in vitro Na,K-ATPase activity in dog myocardium in 1976, many studies have aimed at understanding the increased toxicity of digitalis when used to treat heart failures precipitated by myocardial ischemia. Although the idea of a time dependent alteration of Na,K-ATPase expression throughout ischemia and reperfusion has been proposed, this study is the first undertaken to report a sequential evaluation of total Na,K-ATPase activity and protein expression at 0, 5 and 30 min of reperfusion in a same model. Further, we evaluated the protective role of the digitalis ouabain on ischemia-reperfusion-induced changes in cardiac Na,k-ATPase.

Hence, the aim of this study was to investigate the fate of cardiac Na,K-ATPase activity, α1 and α2 isoforms during ischemia and reperfusion and to test the hypothesis that the digitalis ouabain can protect Na,K-ATPase activity and isoforms expression against ischemia-reperfusion injury. Using a model of Langendorff-perfused rat heart preparation, the data presented here confirm that ouabain preconditioning protects the heart against structural and functional damage induced by ischemia/reperfusion (Figure 6), as previously reported (Pierre et al., 2007b). In addition, Na⁺,K⁺-ATPase activity was significantly decreased in the IR groups after 30 min of reperfusion, but not after 5 min of reperfusion. This decrease was prevented by ouabain preconditioning. This was correlated with total Na⁺,K⁺-ATPase α1 and α2 isoforms protein contents, which
were not affected after 0 or 5 min, but decreased after 30 min of reperfusion and protected by ouabain. Finally, immunodetection following differential cell fractionation showed that Na\(^+\),K\(^+\)-ATPase \(\alpha_1\) after IR was enriched in fractions enriched with the Early Endosome Antigen 1 (EEA1) marker in the IR group, and this was prevented by ouabain pre-treatment.

Taken together, these data suggest that, during the first 5 min of reperfusion that follow 30 min of ischemia, the Na\(^+\),K\(^+\)-ATPase complex is redistributed into intracellular compartments. After 30 min, Na\(^+\),K\(^+\)-ATPase is degraded. Such redistribution may explain previous reports of decreased sarcolemmal activity without changes in total protein contents and contribute to the increase in intracellular Na\(^+\) observed during ischemia/reperfusion injury. Ouabain preconditioning prevented the redistribution of \(\alpha_1\) at 5 min reperfusion and protected against \(\alpha_1\) and \(\alpha_2\) protein degradation and loss of enzyme activity. Future studies shall determine whether the redistribution of \(\alpha_1\) observed at 5 min of reperfusion occurs at 0 min (i.e., at the end of the 30 min of ischemia and before reperfusion), and establish the fate of \(\alpha_2\). Indeed, studies have suggested that the various \(\alpha\) isoforms may have different sensitivities to oxidative stress and be altered differentially during ischemia-reperfusion (Huang et al., 1994; Ostadal et al., 2004; Xie et al., 1990; Zolotarjova et al., 1994). The presence or absence of the signaling form of the digitalis receptor at the cell surface following ischemia and reperfusion may explain the increased toxicity of digitalis in ischemic heart disease. Specifically, in those patients with ischemic heart disease, it may be critical that digitalis treatment be given in very low doses
(to trigger protection of the receptor) before reperfusion (to prevent internalization), before increasing the dose to trigger positive inotropy.


dysfunction ("stunned myocardium"). J Am Coll Cardiol 12, 239-249.


prize in chemistry for the identification of the Na+, K(+)-pump. Acta

(2007). cGMP signalling in pre- and post-conditioning: the role of

Cardioprotection by ouabain and digoxin in perfused rat hearts. J
Cardiovasc Pharmacol 52, 333-337.

Dostanic-Larson, I., Lorenz, J. N., Van Huysse, J. W., Neumann, J. C., Moseley,
alpha2-isoforms of the Na+-K+-ATPase and biological significance of their
cardiac glycoside binding site. Am J Physiol Regul Integr Comp Physiol
290, R524-528.

Preconditioning attenuates ischemia-reperfusion-induced remodeling of
1063.


ABSTRACT

Ouabain and other digitalis drugs are specific inhibitors of the purified Na\(^+\),K\(^+\)-ATPase, the ubiquitous enzyme that transports Na\(^+\) and K\(^+\) across the plasma membrane by hydrolysis of ATP. Independent of changes in ion pumping activity, ouabain binding to the cardiac Na\(^+\), K\(^+\)-ATPase also triggers the assembly and activation of a cardioprotective signaling cascade initiated by the formation of the Na\(^+\),K\(^+\)-ATPase/c-Src binary receptor. Activation of this complex by low doses of ouabain before ischemia/reperfusion protects the heart against infarction, a phenomenon called ouabain preconditioning. The aim of this study was to investigate whether ouabain preconditioning affects the fate of the cardiac membrane Na\(^+\),K\(^+\)-ATPase enzyme complex following ischemia/reperfusion (IR) injury. In our model of Langendorff-perfused rat heart preparation exposed to 30 min of zero flow ischemia, Na\(^+\),K\(^+\)-ATPase activity was significantly decreased in the IR group after 30 min of reperfusion, but not after 5 min of reperfusion. This decrease was prevented by ouabain preconditioning. This was correlated with total Na\(^+\),K\(^+\)-ATPase \(\alpha\)1 and \(\alpha\)2 isoforms protein contents, which were not affected after 0 or 5 min, but decreased after 30 min of reperfusion and protected by ouabain. Finally, immunodetection following differential cell fractionation showed that, after IR, Na\(^+\), K\(^+\)-ATPase \(\alpha\)1 was enriched in fractions enriched with the Early Endosome Antigen 1 (EEA1) marker in the IR group, and this was prevented by ouabain pre-treatment. Taken together, these data suggest that, during the first 5 min of reperfusion that follow 30 min of ischemia, the Na\(^+\),K\(^+\)-
ATPase complex is redistributed into intracellular compartments. After 30 min, Na⁺,K⁺-ATPase is degraded. Such redistribution may explain previous reports of decreased sarcolemmal activity without changes in total protein contents and contribute to the increase in intracellular Na⁺ observed during ischemia/reperfusion injury. Ouabain preconditioning prevented the redistribution of α1 at 5 min reperfusion and protected against α1 and α2 protein degradation and loss of enzyme activity.