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Characterization of Prolylcarboxypeptidase Expression Pattern in Rat Cardiomyocytes in Nutrition Overload Conditions

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CHARACTERIZATION OF PROLYLCARBOXYPEPTIDASE EXPRESSION PATTERN IN RAT CARDIOMYOCYTES IN NUTRITION OVERLOAD CONDITIONS

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ABSTRACT

Prolylcarboxypeptidase (PRCP) is a serine protease that cleaves the last amino acid at the carboxy-terminus of peptides with a penultimate proline such as angiotensin II (Ang II), angiotensin III (Ang III), prekallikrein (PK) and α-melanocyte-stimulating-hormone (1-13) (α-$MSH_{1\text{-}13}$). By inactivating Ang II and Ang III, PRCP promotes vasodilation and insulin sensitivity. As well by activating PK, PRCP increases nitric oxide (NO) generation and vasorelaxation through bradykinin (BK) liberation from high molecular weight kininogen (HK). PK is activated to kallikrein by PREP, implicating a role for PRCP in the coagulation pathway. The resultant kallikrein will activate FXII which in a reciprocal manner leads to the activation of FXI. Lastly, by metabolizing α-$MSH_{1\text{-}13}$ to an inactive metabolite (α-$MSH_{1\text{-}12}$), PRCP inhibits the anorexigenic response to the endogenous α-$MSH_{1\text{-}13}$, leading to an increase in appetite. Thus, by means of vasodilation, insulin sensitization (via direct stabilization of IRS-1 and indirect reduction of Ang II), and suppressing reactive oxygen species (ROS) generation (via direct increase in NO generation and indirect reduction of Ang II) PRCP functions to protect cardiovascular system. Moreover, PRCP mutation is related to acute coronary syndrome in men, and individuals with obesity, diabetes and arteriosclerosis have elevated plasma PRCP concentration and activity. Since cardiovascular disease is the leading cause of mortality in diabetes and obesity, PRCP overexpression in nutrition excess conditions represents an important target for studying metabolic syndrome-related cardiac dysfunction. The overall goal of this study was to elucidate the hormetic effects of major nutrients on PRCP-dependent pathways. Therefore, the following methods

were exploited to address our goals: cell culture of rat H9c2 cardiomyocytes, enzymatic assays for detecting plasma and cardiomyocyte PRCP activity, PRCP RNA and protein studies in high glucose and high fatty acid (saturated and unsaturated) conditions, enzymatic assays to detect PRCP-stimulated activation of PK and plasma protein studies of PK and its downstream target in the coagulation cascade, FXI. Using *in vitro* biochemical assays, plasma PRCP and kallikrein activity were significantly increased in uncontrolled diabetic patients; however metformin and insulin treated diabetic patients had reduced plasma PRCP and kallikrein activity. As well, uncontrolled diabetic patients had markedly elevated plasma prekallikrein (PK), PRCP and FXI protein which was reduced in insulin and metformin treated groups. In a rat cardiomyocyte model of nutrition overload, saturated fatty acid palmitate, unlike glucose, suppressed PRCP levels by 60% in a dose-dependent and time-dependent manner without affecting cell viability, while other tested saturated and unsaturated fatty acids did not alter the basal cardiomyocyte PRCP expression. Thyroxine and insulin, but not metformin, restored palmitate-induced cardiac PRCP depletion. Lastly, although fatty acid uptake inhibition by the CD36 antagonist increased palmitate-induced PRCP depletion by 2-fold, NFκB inhibition did not restore palmitate-stimulated PRCP suppression. Our results indicate that fatty acid-accelerated cardiomyocyte PRCP depletion along with diabetes-stimulated increase in plasma PK and FXI concentration, may contribute to nutrition overload-stimulated cardiovascular dysfunction. The details of fatty acid-induced PRCP down regulation in the heart could open new therapeutic avenues to protect against metabolic syndrome-related cardiovascular complications.

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CHAPTER 1

INTRODUCTION

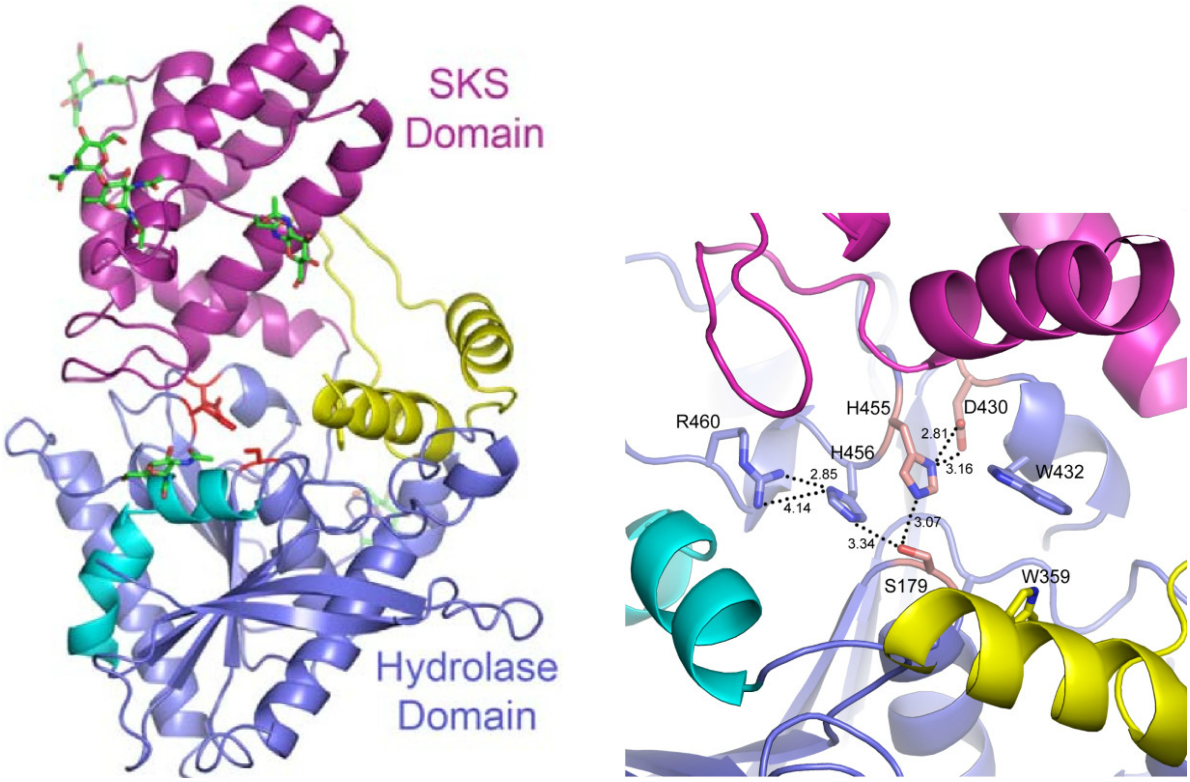
Prolylcarboxypeptidase (PRCP) was first isolated from lysosomal kidney fractions (Odya et al. [2]) and later in the endothelial cells (Shariat-Madar et al. [3]), plasma, urine, liver, pancreas, brain, lung, heart, gut and adipose tissue (Yang et al. [4], Kakimoto et al. [5], Jackman et al. [6] and Miller et al. [7]). The wide distribution of PRCP suggests the importance of this enzyme in the maintenance of normal and pathological body functions.

1.1 PRCP Structure

PRCP belongs to the S28 family of serine proteases. Cloned in 1993 (Tan et al. [8]), human PRCP gene is mapped to chromosome 11q14 . Two splice variants of PRCP have been identified: PRCP1 (NCBI: NM-005040) and PRCP2 (NCBI: NM 199418). PRCP2 has a longer transcript compared to PRCP1, however so far no functionally active protein has been coded by PRCP2 (Odya et al. [2]). PRCP structure consists of a prototypical α/β hydrolase domain and a SKS domain. The SKS domain caps the active site (catalytic domain) that consists of Asp430-His455-Ser179 triad (Soisson et al. [9]) (Fig. 1.1).

1.2 Physiological Roles of PRCP

PRCP cleaves the last amino acid at the carboxy terminus of peptides, if proline is the penultimate amino acid. The described endogenous substrates of PRCP consist of angiotensin II (AngII), angiotensin III (Ang III) and prekallikrein (PK). The enzyme is also responsible for two other functions, which include metabolism of des-arg^9-bradykinin and α-melanocyte stimulating hormone$_{1\text{-}13}$, also known as α-MSH$_{1\text{-}13}$ (Fig. 1.2). The ubiquitous



(a) (b)

Figure 1.1. The Structure of PRCP.
(a) Ribbon diagram of tertiary structure of PRCP. The Asp-His-Ser catalytic triad is shown in red. The α/β hydrolase fold is shown in blue and cyan and the SKS domain in magenta. (b) The active site of PRCP. The Asp-His-Ser catalytic triad is shown in salmon. Reprinted with copyright permission from Soisson et al. [9].

distribution and various substrate range of PRCP indicate the possible regulatory roles of this enzyme in different physiological systems, i.e., the renin-angiotensin system (RAS), the kallikrein-kinin system (KKS) and the proopiomelanocortin (POMC) system.

1.2.1 PRCP in Renin-Angiotensin-System (RAS)

In 1978, Odya et al. [2] purified PRCP from human kidney and observed that it could cleave Ang II and Ang III to Ang 1-7 and Ang 2-7, respectively. Thus, they named it Angiotensinase C. Since RAS is an important regulator of blood pressure and peripheral vascular resistance, by metabolizing two of its members (Ang II and Ang III), PRCP could also be considered a modulator of RAS function in the body.

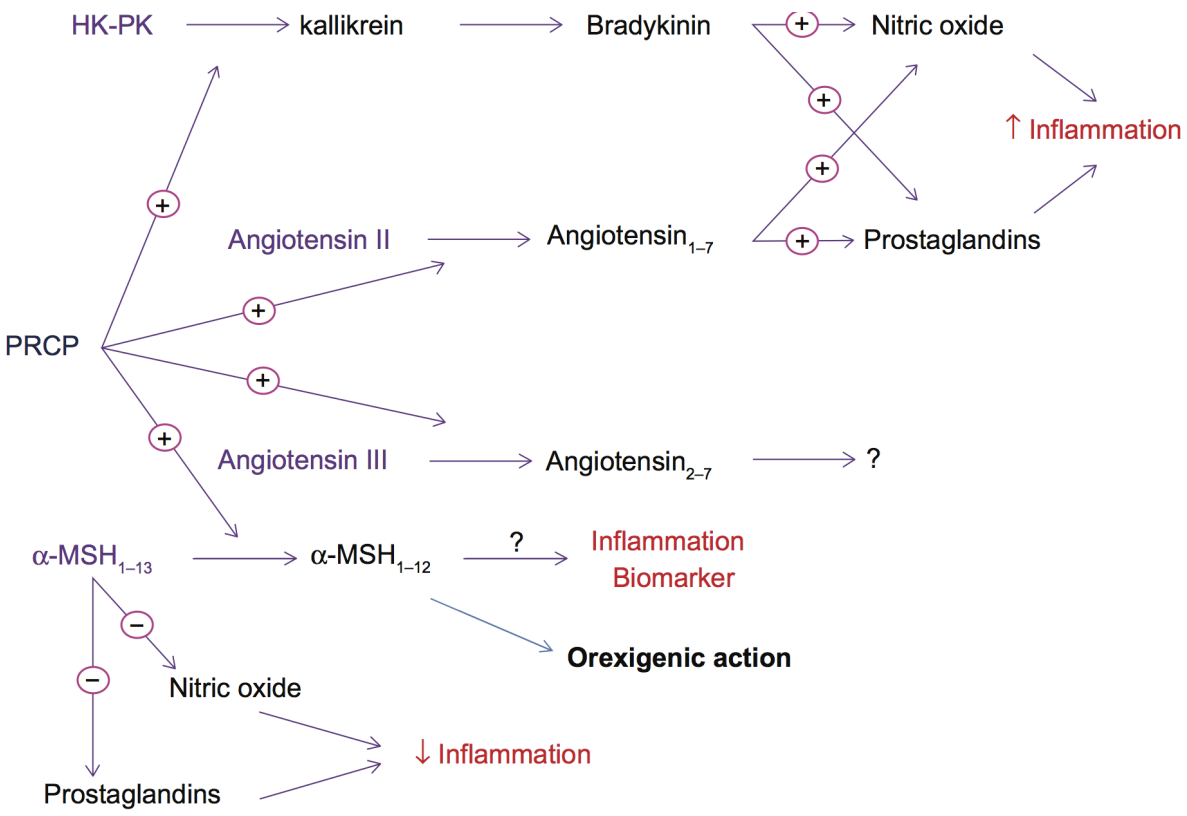


Figure 1.2. The Physiologic Role of PRCP.
By metabolizing Ang II and Ang III, PRCP tips the balance in favor of vasodilation in the renin angiotensin system. PRCP also activates PK to kallikrein which further liberates BK from HK. Lastly, α-$MSH_{1\text{-}13}$ is metabolized to α-$MSH_{1\text{-}12}$ by PRCP. Shariat-Madar et al. [10]

1.2.2 PRCP in Kallikrein-Kinin-System (KKS)

At the endothelial cell surface, PRCP is colocalized with High molecular weight Kininogen (HK) membrane-binding proteins such as complement C1q receptor (gC1qR), urokinase plasminogen activator receptor (u-PAR), and cytokeratin 1 (CK1) (Joseph et al. [11], Hasan et al. [12]). Therefore, upon formation of HK-PK complex at the cell surface, PRCP activates PK to kallikrein which then liberates Bradykinin (BK) from HK (Shariat-Madar et al. [3]). BK exerts its effects via its G-protein-coupled receptor (B2) to increase nitric oxide and prostacyclin generation (Palmer et al. [13] and Hong [14]) resulting in vasodilation, increased vascular permeability, pain and inflammation.

1.2.3 PRCP in Proopiomelanocortin-System

Proopiomelanocortin (POMC) is a prohormone expressed especially in the hypothalamus and plays an important role in appetite regulation and energy homeostasis. By the action of prohormone convertases, POMC is processed to several active peptides such as endorphins, adrenocorticotropin hormone (ACTH) and α-MSH. α-MSH is a 13 amino acid peptide which acts via melanocortin receptors (MC3 and MC4) to promote energy expenditure and anorexia and also to suppress inflammation. In the hypothalamus, PRCP has been shown to metabolize α-$MSH_{1\text{-}13}$ to an inactive metabolite, α-$MSH_{1\text{-}12}$. Thus, by decreasing hypothalamic α-$MSH_{1\text{-}13}$ concentration, PRCP promotes food intake and weight gain (Wallingford et al. [15]).

1.3 Pathophysiological Roles of PRCP

PRCP is also linked to multiple pathophysiological conditions such as cancer, inflammation, cardiovascular disease and metabolic syndrome.

1.3.1 PRCP in Cancer

Overexpression of PRCP was seen during active growth of vasculature and in glioblastoma in 2009 (Javerzat et al. [16]). Later, PRCP was linked to cell proliferation and autophagy as knocking down PRCP decreased cell proliferation and increased sensitivity to tamoxifen in a breast cancer cell line (Duan et al. [17]). To elaborate the role of PRCP in angiogenesis, Adams and colleagues ([18]) observed that PRCP depletion both *in vitro* and *in vivo* resulted in a decreased cell migration, defective angiogenesis and impaired wound healing. Phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/the mammalian target of rapamycin (mTOR) signaling pathway, important in cell proliferation and metabolism, is over-activated in most cancer cells. Recently, PRCP has been shown to activate the latter pathway by stabilizing the adaptor protein, insulin receptor substrate-1 (IRS-1), further increasing cell proliferation and survival in a pancreatic cancer cell line. As suggested by Duan et al. [19], PRCP inhibitors could be useful in treating cancer in the future.

1.3.2 PRCP in Inflammation

Implication of PRCP in inflammation has been described through different mechanisms. PRCP-dependent plasma PK activation, promotes BK generation (Kerbiriou and Griffin [20]) which upon binding to its constitutive BK B2 receptors (B2) on endothelial cells induces inflammation and vascular permeability through nitric oxide and prostacyclin generation (Hong [21], Palmer et al. [22] and Zhao et al. [23]). Activated plasma kallikrein can also stimulate complement system activation (Ghebrehiwet et al. [24] and DiScipio [25]), helping recruitment of the proinflammatory cells and supporting inflammation via the release of potent chemotactic mediators (Muller-Eberhard [26]). Furthermore, by metabolizing Ang II to Ang 1-7, PRCP mediates Ang 1-7 receptor, Mas, activation and the release of prostaglandins as well as nitric oxide (de Castro et al. [27]). Lastly, PRCP truncates α-$MSH_{1\text{-}13}$ to α-$MSH_{1\text{-}12}$ (Wallingford et al. [15]). α-$MSH_{1\text{-}13}$ acts as a potent anti-inflammatory hormone (Chiao et al. [28]), via activation of melanocortin receptors, mainly MC1R. Therefore, it decreases proinflammatory cytokine expression and release as well as nitric oxide generation (Luger et al. [29], Lipton and Catania [30] and Manna and Aggarwal [31]). In support of the role for PRCP as a modulator of inflammation, studies have found PRCP activity in inflammatory synovial exudates of arthritic patients (Kumamoto et al. [32]) as well as on the tonsils of a tonsillitis patient (Palmeri et al. [33]). Also in an *in vitro* model of inflammation, lipopolysaccharide (LPS)-treated endothelial cells had a significant upregulation of PRCP mRNA expression along with an increase in kallikrein generation (Ngo et al. [34]). Thus, a strong body of evidence highlights the role of PRCP in inflammation.

1.3.3 PRCP in Cardiovascular Diseases

Cardiovascular disease (CVD) is the number one cause of mortality and morbidity in the United States. To date, several features of CVD have been separately linked to PRCP such as inflammation, thrombosis, hypertension, oxidative stress, obesity and insulin resistance. In 2003, McCarthy et al carried out a candidate gene survey to elucidate the major genetic determinants of the metabolic syndrome and showed that PRCP is significantly associated

with coronary heart disease in men (McCarthy et al. [35]). Later in 2012 Xu et al ([36]) proved that increased plasma concentration of PRCP is a strong indicator of obesity, diabetes mellitus, and arteriosclerosis. In a rat model of hypertension, rutacarpine treatment enhanced PRCP mRNA but not protein expression, and inhibited cardiac hypertrophic remodeling via increasing Ang II degradation (Qin et al. [37]). It was inferred form this study that the observed increased Ang II inactivation might be caused by increased PRCP activity. In 2009, Mallela et al ([38]) suggested that PRCP is a cardioprotective enzyme because firstly it metabolizes angiotensin II. Elevated Ang II triggers cardiovascular dysfunction by promoting vasoconstriction, platelet aggregation, collagen synthesis, nitric oxide destruction and insulin resistance (Olivares-Reyes et al. [39], Regoli et al. [40], Carey and Padia [41] Echeverria-Rodriguez et al. [42]) through angiotensin II receptor 1 (AT1R) . However by converting angiotensin II to angiotensin 1-7, a vasodilator (Ren et al. [43]) and an insulin sensitizer (Giani et al. [44]), PRCP can protect the cardiovascular system from atherosclerosis, thrombosis, hypertension, and insulin resistance. Secondly, PRCP metabolizes angiotensin III to angiotensin 2-7. Angiotensin III promotes vasoconstriction, inflammation and increases aldosterone secretion thus triggering arterial hypertension (Yugandhar and Clark [45]). By inactivating angiotensin III, PRCP reduces the risk of cardiovascular disease. The last mechanism by which PRCP acts as a cardioprotective enzyme is through increasing bradykinin (BK) production (Shariat-Madar et al. [3]). BK increases nitric oxide generation and vasodilation, decreases platelet aggregation and thrombus formation, and improves insulin sensitivity (Sowers et al. [46], McFarlane et al. [47], Couture and Girolami [48] and Manolis et al. [49]).

PRCP in Hypertension

Several studies have linked PRCP to hypertension. PRCP polymorphism is associated with increased risk of preeclampsia (Wang et al. [50]), hypertension (Wu et al. [51]) and a reduced response to antihypertensive drug, benazepril (Zhang et al. [52]). In 2005, Campbell et al reported an increase in bradykinin levels in hypertensive humans upon losartan treat-

ment (Campbell et al. [53]), pointing to a possible role for angiotensin receptor blockers in increasing PRCP levels and subsequently BK levels. Later Quin et al observed that PRCP expression is significantly decreased in kidneys and mesentric arteries of hypertensive rats (Qin et al. [37]). As well, murine PRCP hypomorph had significantly higher mean arterial pressure coupled with increased reactive oxygen species (ROS) generation, which subsided when the animals were treated with anti-oxidants (Adams et al. [54]).

PRCP in Thrombosis

PRCP can act both as a pro-thrombotic and an anti-thrombotic enzyme. As a pro-thrombotic enzyme, PRCP contributes to clot formation by converting PK to kallikrein at the endothelial cell surface. The resultant kallikrein, a key activator of the intrinsic (contact) pathway of blood coagulation, will then activate FXII to FXIIa. FXIIa can directly consolidate the clot by stabilizing fibrin structure (Konings et al. [55]), and it can indirectly promote thrombin generation through the intrinsic pathway.

On the other hand, PRCP stimulates BK generation via kallikrein activation. BK can exert anti-thrombotic effects via three mechanisms. Firstly, BK and its stable metabolite, BK 1-5, inhibit thrombin-induced platelet aggregation (Hasan et al. [56] and Murphey et al. [57]). Secondly, via B2 receptor, BK promotes nitric oxide and prostacyclin generation which both are potent inhibitors of platelet adhesion and aggregation (Radomski et al. [58]). Thirdly, BK increases tissue plasminogen activator (tPA) release which acts as a fibrinolytic agent (Minai et al. [59] and Brown et al. [60]). Studies are also controversial on the role of PRCP in coagulopathies. Adams et al ([54]) observed that $PRCP^{gt/gt}$ mice has a pro-thrombotic phenotype, but in the study of Rabey and coworkers ([61]), pharmacological inhibition of PRCP did not promote thrombus formation in the wild type mice. However, in the same study, treating FXII deficient mice with UM8190, a potent selective inhibitor of PRCP, resulted in an anti-thrombotic effect (Rabey et al. [61]) suggesting a non-PK-mediated role for PRCP in coagulation. Clearly, additional studies are needed to better understand the role of PRCP in blood clot formation.

1.3.4 PRCP in Metabolic Syndrome

Metabolic syndrome is a group of symptoms that raise the risk of cardiovascular disease (CVD) and type 2 diabetes (T2DM) (Grundy et al. [62]). Six components of the metabolic syndrome are related to CVD: central or abdominal obesity (waist circumference $>$ 102 cm (40 inch) in men or $>$ 88 cm (35 inch) in women), atherogenic dyslipidemia (plasma triglyceride $>$ 150 and HDL cholesterol $<$ 40 in men or $<$ 50 in women), high blood pressure (blood pressure $>$ 130/85 mm Hg or receiving anti-hypertensive drug), insulin resistance (fasting blood glucose $>$ 100 mg/dL or receiving hypoglycemic agent) and pro-thrombotic state (increased plasminogen activator inhibitor-1 (PAI-1) and fibrinogen) (The National Cholesterol Education Programs Adult Treatment Panel III report (ATPIII), 2002 and Grundy et al. [62]) (Table 1.1). An estimated 29 million children and adults in the United States, 8.3% of the population, are affected with diabetes while in 2010 more than one third of the US population (35.7%) was obese (Center for Disease Control and Prevention, 2013). With the growing epidemic of obesity and T2DM as the leading underlying causes of CVD and the limitations in the treatment of resultant cardiovascular disorders, better understanding of the molecular mechanisms underlying cardiometabolic pathologies could open new avenues toward potential therapeutic targets. PRCP has recently gained increasing attention due to its implication in obesity and insulin resistance, both components of metabolic syndrome.

PRCP in Obesity

In 2009, Wallingford et al introduced PRCP as an enzyme that could regulate feeding behavior by metabolizing hypothalamic α-$MSH_{1\text{-}13}$ (Wallingford et al. [15]). They observed that on a high fat diet, PRCP deficient mice have a lower body weight, significantly less food intake and fat mass as compared to their wild type littermates and revealed that this phenomenon was mainly due to a decreased α-$MSH_{1\text{-}13}$ metabolization by PRCP and a resultant increase in hypothalamic α-$MSH_{1\text{-}13}$ concentration. Later, Jeong and Diano [63] exploited radioactive *in situ* hybridization to localize PRCP expression in the brain. They noticed that PRCP is extensively expressed throughout the brain and its expression in the hypothalamus

Table 1.1. WHO and ATPIII Definitions of Metabolic Syndrome. Adapted and modified from Ritchie and Connell [1]

WHO (1999)	ATPIII (2001)
Diabetes, impaired fasting glucose, glucose intolerance or insulin resistance (defined by hyperinsulinaemic, euglycaemic clamp mechanism), plus two or more of the following:	Three or more of the following:
BMI > 30 kg/m^2, or waist to hip ratio > 0.9 (M) or > 0.85 (F)	WC > 102 cm (M),
TG > 1.7 mmol/L, or HDL-C < 0.9 (M) or < 1.0 mmol/L (F)	TG > 1.7 mmol/L
BP $> 130/90$ mmHg	BP $> 135/85$ mmHg or antihypertensive medication
Albumin excretion > 20 mg/min	FPG > 6.1 mmol/L

BMI: body mass index; BP: blood pressure; F: female; M: male; FPG: fasting plasma glucose; HDL-C: high-density lipoprotein cholesterol; TG: triglycerides; WC: waist circumference.

is in close proximity to areas with notable melanocortin receptors (MC3R and MC4R) expression, confirming the association of PRCP with melanocortin signaling (Jeong and Diano [63]). Furthermore, administration of UM8190 (a novel synthetic inhibitor of PRCP) attenuated food intake in mice in a time-dependent and dose-dependent manner (Rabey et al. [61]). Not only is PRCP implicated in appetite regulation, but also it affects energy homeostasis. PRCP null mice have higher energy expenditure and significantly elevated circulating free thyroxine (T4) level compared to their wild type control (Jeong et al. [64]), probably due to an increased α-MSH$_{1\text{-}13}$-mediated stimulation of hypothalamus-pituitary-thyroid (HPT) axis (Jeong et al. [65]). Moreover, PRCP deficient mice were resistant to high fat diet-induced hepatic steatosis and had an attenuated plasma circulating nonesterified fatty acid (NEFA) as well as lower plasma triglycerides level (Jeong et al. [64]). Implication of PRCP in metabolism regulation, increased plasma PRCP in obesity (Xu et al. [36]) and the fact that hyperlipidemia and obesity are major risk factors for CVD, highlight the importance of cardiac PRCP expression regulation in metabolic syndrome.

PRCP in Insulin Resistance

Like PRCP, insulin is also considered a cardioprotective hormone (Fig. 1.3), since several features of insulin signaling protects the cardiovascular system. Insulin resistance is the core pathophysiology underlying the metabolic syndrome-stimulated cardiac dysfunction. Not much is known about the role of PRCP in insulin signaling pathway. However, PRCP depletion in mice was associated with a better insulin sensitivity, decreased fasting glucose and attenuated hepatic gluconeogenesis (Jeong et al. [64]) suggesting that PRCP might exacerbate insulin resistance. The latter finding is in agreement with previous studies that pointed to PRCP as an orexigenic enzyme that its depletion improved lipidemic control (Jeong et al. [64]). One explanation behind these findings is that a major mechanism to develop insulin resistance is through high fatty acid-induced protein kinase C (PKC) activation and impairment of insulin signaling by serine phosphorylation of insulin receptor substrates (Abel et al. [66]). In contrast to these findings, Duan et al ([19]) showed that PRCP depletion or pharmacologic inhibition promotes insulin receptor substrate 1 (IRS-1) degradation and that PRCP regulates PI3K/Akt pathway by stabilizing IRS-1. Since IRS-1 is a downstream of insulin signaling (Yu et al. [67]) and its serine phosphorylation causes insulin resistance, one could suggest that by stabilizing IRS-1, PRCP could have an insulin-sensitizing role. Moreover, Ang II, a substrate of PRCP, could also deteriorate insulin sensitivity by several mechanisms such as blocking insulin-stimulated PI3K activity (Velloso et al. [68]), inhibiting adipocyte proliferation and differentiation (Janke et al. [69]), fibrosis of pancreatic islets (Jandeleit-Dahm et al. [70]) and increasing ROS generation (Chabrashvili et al. [71] and Barton et al. [72]). Thus by metabolizing Ang II, PRCP could further improve insulin sensitivity. Clearly, more research in this area is needed to clarify the involvement of PRCP in insulin signaling and its role in developing T2DM.

1.4 Objectives

Not much is known about how PRCP expression is regulated in different tissues. However, decreased renal and vascular PRCP expression in the hypertensive rats (Qin et al. [37])

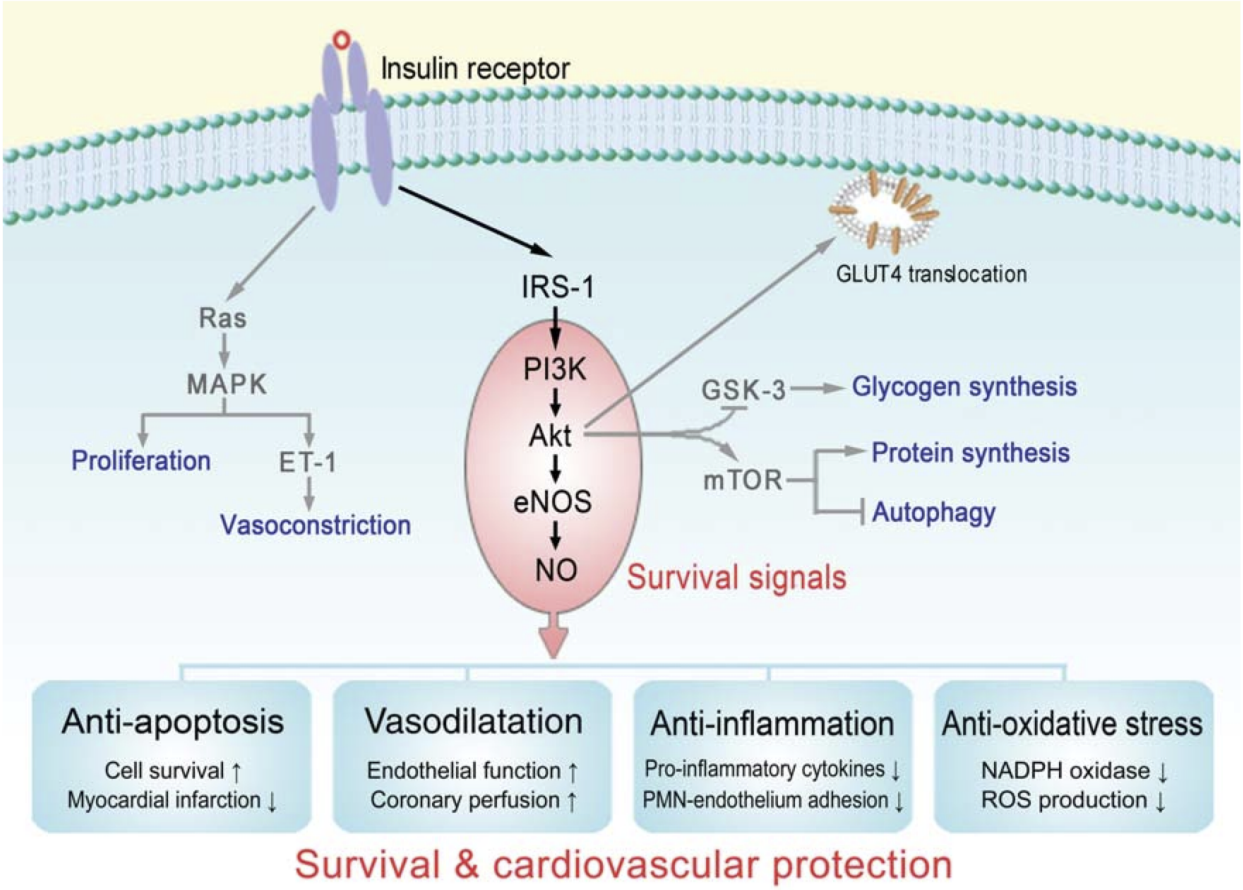


Figure 1.3. Insulin Signaling and Cardiovascular Protection.
Binding of the insulin to its membrane bound insulin receptor (IR) results in autophosphorylation of the IR tyrosine residues, followed by activation of 2 signaling cascades: the proliferative Ras-MAPK signaling cascade and the IR substrates-PI3K-Akt-eNOS-NO cascade. The latter would result in translocation of the major cardiac glucose transporter GLUT4 to the cell membrane increasing glucose uptake. Besides, the PI3K-Akt-eNOS-NO cascade elicits cardioprotective effects via promoting vasodilation and attenuating apoptosis, inflammation and oxidative stress. Akt: protein kinase B; eNOS: endothelial nitric oxide synthase; ET-1: endothelin-1; GLUT4: glucose transporter 4; GSK: glycogen synthase kinase; IRS: insulin receptor substrate; MAPK: mitogen-activated protein kinase; mTOR: the mammalian target of rapamycin; PI3K: phosphatidylinositol 3-kinase; PMN: polymorphonuclear neutrophil; and ROS: reactive oxygen species.Reprinted with copyright permission from Yu et al. [67].

and increased plasma PRCP concentration and activity in obesity, diabetes and CVD (Xu et al. [36]) have been reported. PRCP is implicated in metabolic syndrome by inactivating the anorexic peptide α-$MSH_{1\text{-}13}$ and also by improving insulin sensitivity (via stabilizing IRS-1 and metabolizing Ang II). As well, PRCP protects several features of cardiovascular health. Based on the fact that CVD is the most common cause of mortality in metabolic syndrome, PRCP appears to be a plausible enzyme to study, which plays an important role in both conditions (metabolic syndrome and its resultant CVD). However until now, no studies have been conducted on the cardiac PCRP expression pattern in metabolic syndrome. Considering PRCP as a cardioprotective enzyme, metabolic syndrome-accelerated alteration of PRCP expression in the heart might be a mechanism by which nutrition overload could exacerbate cardiovascular dysfunction. Furthermore, PRCP promotes inflammation and both diabetes and obesity are also proinflammatory conditions (Fig. 1.4). Based on these findings, increased plasma PRCP levels in diabetes and obesity may deteriorate cardiac inflammation. On the other hand, increased plasma PRCP in metabolic syndrome might be a defense mechanism to protect cardiovascular system from the detrimental effects of Ang II.

To address these questions, we aimed to study the cardiac PRCP expression pattern in an *in vitro* model of cardiac nutrition overload (high glucose/fatty acid concentration), resembling metabolic syndrome. Our results could highlight the importance of glycemic and lipid control for cardiac health through the regulation of PRCP expression. It will also point to possible strategies to target PRCP for improving cardiac outcomes in patients with metabolic syndrome.

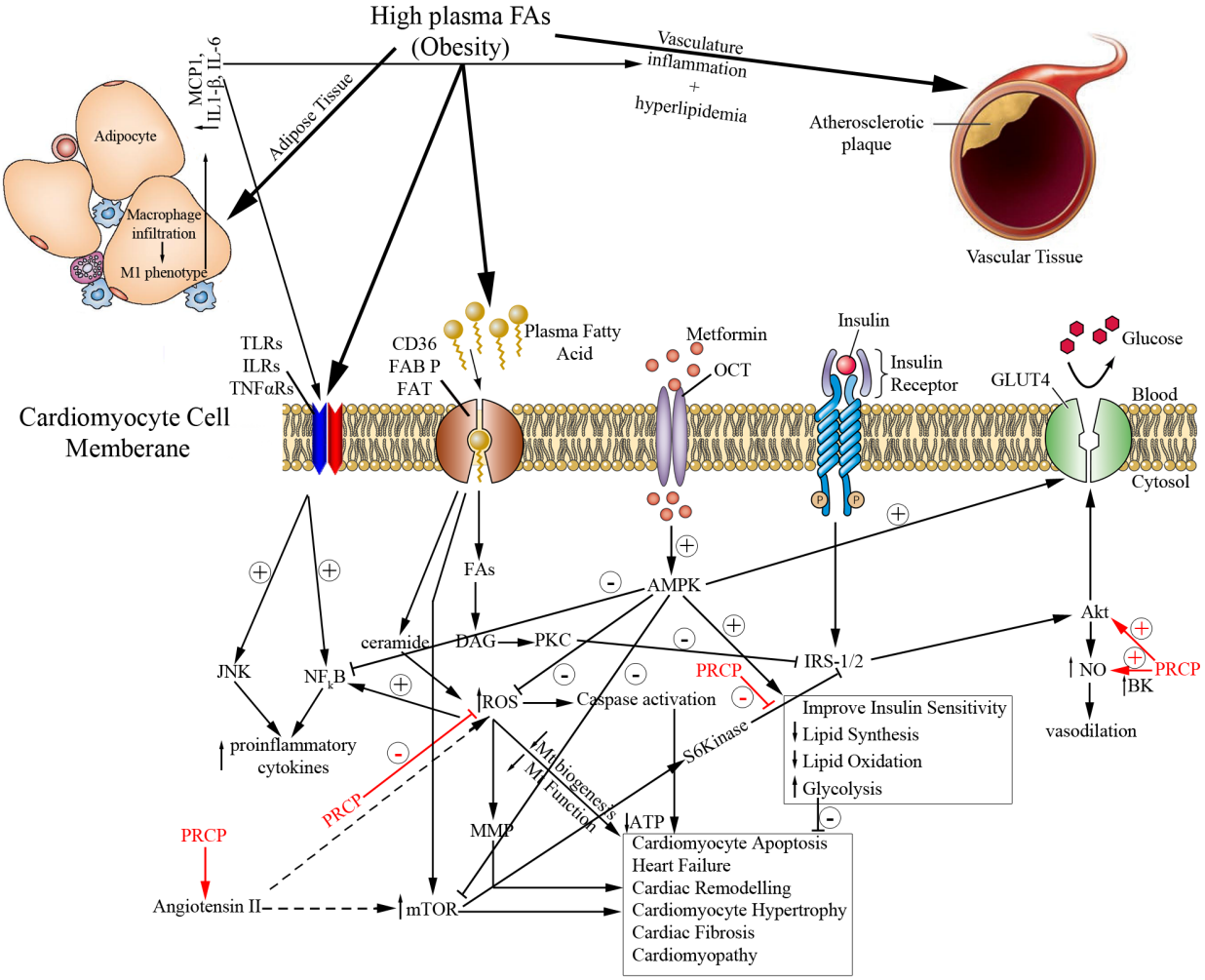


Figure 1.4. The Molecular Pathways Linking Obesity to Cardiovascular Disease and the Role of PRCP in the Cardiovascular System.

High fatty acid concentration can impair insulin signaling by increasing ROS generation, and DAG-induced PKC activation. Moreover, hyperlipidemia is an inflammatory condition, since intracellular fatty acids can activate NF-kB and JNK pathways, and polarize adipocytes macrophages to a inflammatory phenotype (M1). PRCP, however can improve insulin sensitivity by stabilizing IRS-1, attenuating ROS generation, increasing NO generation and metabolizing Ang II. MCP1: Monocyte Chemoattractant Protein 1, FA: Fatty Acid, TLR: Toll Like Receptor, OCT: Organic cation transport proteins, DAG: Diacylglycerol, AMPK: 5' adenosine monophosphate-activated protein kinase, PKC: Protein Kinase C, ROS: Reactive Oxygen Species, JNK: c-Jun N-terminal kinase, NFκB: Nuclear Factor kappa-light-chain-enhancer of activated B cells, MMP: Matrix MetalloProteinase, mTOR: Mechanistic Target of Rapamycin (serine/threonine kinase), Mt: Mitochondria, IRS: Insulin Receptor Substrate, NO: Nitric Oxide.

CHAPTER 2

EXPERIMENTAL METHODS

2.1 Materials Used

Essential fatty acid free bovine serum albumin (BSA), caprylic acid, decanoic acid, dodecanoic acid, palmitic acid, linoleic acid, arachidonic acid, docosahexanoic acid, thyroxine, insulin and metformin were purchased from Sigma Aldrich (St. Louis, MO). CD-36 inhibitor, Sulfosuccinimidyl Oleate (SSO), and NFκB inhibitor (BAY 11-7082) were from Santa Cruz Biotechnology, Inc (Dallas, TX). Human plasma from 4 groups of patients (control non diabetic, diabetic on metformin, diabetic on insulin, and uncontrolled diabetic patients) were purchased from Physicians Plasma Alliance (Johnson City, TN).

2.2 Fatty Acid-BSA Preparation

Since fatty acids have low solubility in aqueous phase, ultra fatty acid free bovine serum albumin (BSA) was conjugated to them as a carrier. The resultant aqueous-soluble fatty acid-BSA is easily absorbed and utilized by the cells. Fatty acid-BSA conjugate (6:1 molar ratio fatty acid:BSA) was prepared as described previously by Cousin et al. [73]. Briefly, a 6 mM fatty acid solution in 150 mM NaCl was prepared while heating at 70 °C on a heated stirring plate. Meanwhile, on an adjacent heated stirring plate a 1 mM ultra free fatty acid BSA solution was prepared at approximately 37 °C but not higher than 40 °C. After both solutions became clear, 40 ml of the 70 °C fatty acid solution was transferred to the BSA solution while stirring at 37 °C for 1 hour. Finally, pH was adjusted to 7.4 with 1 N NaOH and aliquots were stored at −20 °C. Prior to use, fatty acid-BSA and BSA vehicle control were thawed at 37 °C for 7-10 minutes.

2.3 Cell Culture

Rat H9c2 cardiomyoblasts, originally derived from BDIX rat heart tissue, were purchased from the American Type Culture Collection (Rockville, MD) and cultured in 8 mL of Dulbeccos modified Eagles medium (DMEM)(Sigma Aldrich, MO) containing 10% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, 100 µg/mL streptomycin (Life Technologies,NY) and 1.5 g/liter NaHCO3 under 5% CO2 at 37 °C. While maintained in 10% FBS, each flask of cardiomyoblasts were subcultured to 3 flasks when cells reached approximately 80% confluency. For studying cardiac features, the H9c2 cells were differentiated into cardiomyocytes upon FBS starvation (2% FBS) for 24 hours.

2.4 Treatment and Exposure

Flasks of differentiated H9c2 cell line were incubated overnight to reach confluency. The next day cells were treated with appropriate concentrations of BSA or fatty acid-BSA in the presence or absence of increasing concentrations of insulin, thyroxine or metformin for the time periods mentioned. For the SSO study, flasks of differentiated H9c2 cell line were incubated with increasing concentrations of SSO 4 hours prior to adding BSA vehicle or palmitate-BSA. Upon completion of the incubation period, the condition media was collected from each flask and the cells were either resuspended with TRIzol Reagent or RIPA buffer for further investigations. The media was then dialyzed in 1x Tris Buffer (10 mM Tris, 25 mM NaCl, 0.5 mM EDTA, 0.5 mM β- mercaptoethanol; pH 7.1) for 18 hours at 4 °C with one change of buffer every 6 hours, freeze dried and concentrated for future studies. As for the 16 days study, flasks of cells were treated with BSA, 0.08 mM palmitate, 100 nM insulin plus 0.08 mM palmitate or 500 nM thyroxine plus 0.08 mM palmitate. After 2 days, one flask of each treatment condition was collected in TRIzol for RNA extraction and the other flask was subcultured to two flasks while maintained in the same treatment condition. This approach was performed every two days all the way to 16 days.

2.5 Cell Viability

To figure the sub-lethal concentration of palmitate suitable for chronic treatment, MTT assay was performed according to the method developed by Mossman (Mosmann [74]). Briefly 100 µL of cell suspension (3×10^5 cells/mL) were seeded overnight in 96 well plate. The next day cells were treated with BSA or increasing concentrations of palmitate-BSA for 24, 48 or 72 hours. At the end of the incubation period, 200 µL of 0.5 mg/mL filtered MTT (Calbiochem, MA) was added to each well, covered in aluminum foil and incubated at 37 °C in culture hood for 4 hours. To solublize formazan crystals, media was then carefully replaced by 150 µL of MTT solvent (4 mM HCl, 0.1% Nondet P-40 (NP40) in isopropanol) and the plate was shaken gently for 15 minutes. Finally, the absorbance was read at 570 nm with subtraction of the background at 690 nm.

2.6 Protein Extraction

Confluent monolayers of H9c2 cell line were treated with BSA or increasing concentrations of palmitate-BSA for 24, 48, 72 and 96 hours. The next day, cell lysate was collected in 300 µL of Protease Inhibitor Cocktail (BD Biosciences,CA) in ice-cold RIPA buffer (10 mM TrisHCl pH=7.5, 150 mM NaCl, 2 mM EDTA, 1% NP40, 1% sodium deoxycholate, 0.1% SDS) (Thermo scientific, MA) and cell protein was extracted by transferring the supernatant to new tubes after centrifugation at 13000×g for 15 minutes. Aliquots of the extracted protein were stored at −20 °C and western blot analysis of the samples were performed later, after measuring the protein concentration by BCA assay.

2.7 BCA Protein Assay

Protein concentration in H9c2 cell lysate or human plasma samples were measured using Pierce BCA Protein Assay Kit (ThermoFisher Scientific, MA). Briefly, serial dilutions of bovine serum albumin were created as protein standards using albumin standard ampules (2 mg/ml) provided in the kit. Next, each sample was diluted 50 and 100 fold, in deionized water. Then, the working reagent was prepared by adding BCA Reagent A (containing

sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide) and BCA Reagent B (containing 4% cupric sulfate) to a reservoir, mixing well. Finally 150 µL of protein standards (in duplicate) as well as 50 and 100 fold diluted samples were added to a 96 well plate. On top of that, 150 µL of working reagent was added, the plate was covered, shaken thoroughly and incubated at 37 °C for 30 minutes. At the completion of incubation, the absorbance was measured at 562 nm on a plate reader. The standard curve was then plotted via Prism (Graph Pad Software, Inc., USA) and samples protein concentration was interpolated based on the standard curve.

2.8 Gel Electrophoresis and Western Blot Studies

Dialyzed plasma proteins (155 µg of protein) or cell protein extracts (200 µg of protein) were separated at 25 mA by SDS/PAGE using a 10% acrylamide gel (BioRad, CA). Following electrophoresis, proteins were transferred onto a nitrocellulose membrane (BioRad, CA) at 100 V for 1 hour. Blots were then blocked in 3% nonfat dry milk (BioRad, CA) in PBS-0.1% Tween-20 for 1 hour at room temperature and incubated with primary antibody against human Angiotensinase C in goat 1:100, primary antibody against α-tubulin in rabbit 1:250 (Santa Cruz Biotechnology INC, TX), primary antibody against prekallikrein in sheep 1:200 or primary antibody against human serum albumin in rabbit 1:500 (ThermoFisher Scientific, MA) in PBS-0.1% Tween-20 containing 1% nonfat dry milk overnight at 4 °C. The next day, the membrane was washed 4 time (80 minutes) with PBS-0.1% Tween-20 and incubated with horseradish peroxidase (HRP)-conjugated anti-goat, anti-rabbit or anti-sheep IgG secondary antibodies (ThermoFisher Scientific, MA) at room temperature for 60 minutes. Finally, the membranes were washed 3 time (60 minutes) with PBS-0.1% Tween-20 and the PRCP, α-tubulin, prekallikrein or serum albumin-bound antibodies were detected using SuperSignal West Femto Maximum Sensitivity Substrate (ThermoFisher Scientific, MA). Then, PRCP or prekallikrein abundance were normalized to serum albumin (for plasma samples) or to α-tubulin (for H9c2 cell lysate) and quantified by NIH ImageJ software.

2.9 RNA Isolation and Reverse-Transcriptase PCR

Total RNA was isolated from cultured cells by using RNase-Free DNase Set (Qiagen, Netherlands) as per manufacturer's standard instructions. Briefly, cells were collected in 500 µL (per flask of cells) of TRIzol Reagent (Life Technologies, NY). DNase treated RNA was then separated into an aqueous phase by adding 100 µL chloroform and precipitated from the aqueous phase using 200 µL isopropanol per 500 µL of TRIzol. The pellet was then washed three times with 600 µL of 75% ethanol, and suspended in 35 µL of RNase-free water. RNA concentration was finally determined using the NANODROP 2000c Spectrophotometer. Next, a total of 1 µg of RNA was reverse transcribed and amplified into cDNA using the SuperScript III One-Step-PCR system with Platinum Taq DNA Polymerase (Life Technologies, NY) according to the manufacturer's protocol. The detection of rat PRCP was performed using MPCP-S1 and MPCP-AS1 (annealing temperature 58 °C) (sense: CGCTGATAAACACTGGCAGA and anti-sense: TATCGATGCTCAGCGAACAC) primers. Abundance of PRCP genes were normalized to β-actin genes using rat β-actin primers (annealing temperature 60 °C) (sense: TTGTAACCAACTGGGACGATATGG and anti-sense: GATCTTGATCTTCATGGTGCTAGG) followed by running on a 1.5% agarose gel containing ethidium bromide. Finally, the band density was analyzed with the NIH ImageJ software.

2.10 Kallikrein Activity in H9c2 Cell Line

Blast and differentiated H9c2 cells (3×10^5 cells/mL) were seeded overnight in a 96 well microtiter plate (Greiner Bio-One, Monroe, NC). Confluent monolayers (semi-confluent for blast H9c2) of H9c2 cells were then washed three times with HEPES-NaHCO3 buffer (137 mM NaCl, 3 mM KCl, 12 mM NaHCO3, 14.7 mM HEPES, 5.5 mM glucose, 0.1% gelatin, 2 mM CaCl2, 1 mM MgCl2, 7.1 pH, filtered) and blocked with 1% gelatin for 1 hour at 37 °C to prevent non-specific binding. After washing three times with HEPES-NaHCO3 buffer, cells were then incubated with increasing concentrations of HK (DiaPharma Group, Inc., West Chester, Ohio) for 1 hour at 37 °C. At the end of the incubation, cells were washed

three times with HEPES-NaHCO3 buffer to remove the unbound HK and then treated with increasing concentrations of PK (DiaPharma Group, Inc., West Chester, Ohio). After 1 hour incubation at 37 °C, cells were washed three times and 0.5 mM S2302 (H-D-Pro-Phe-Arg-pNA-2HCl, DiaPharma Group, Inc., West Chester, OH) in 100 µL HEPES-NaHCO3 buffer was added to determine the activity of kallikrein produced. Substrate hydrolysis was allowed to proceed for 1 hour at 37 °C. Kallikrein activity was measured as the change in absorbance at 405 nm, using BioTek ELx800 Absorbance Microplate Reader.

2.11 Kallikrein Inhibition In Fluid Phase

4 µL (155 µg) of dialyzed human plasma samples were incubated with 0.5 mM S2302 in the absence or presence of increasing concentrations of soy bean tripsin inhibitor (SBTI) (Sigma Aldrich, MO) in a final volume of 100 µL HEPES-NaHCO3 buffer. After 1 hour of incubation at 37 °C, kallikrein activity was measured as the change in absorbance at 405 nm, using BioTek ELx800 Absorbance Microplate Reader.

2.12 Assay of Proteolytic Activity

In an enzymatic assay, PRCP activity was measured using APpNA (Bachem, PA) as described previously (Chajkowski et al. [75]). For this purpose the plasma from diabetic patients along with the proper control plasma as well as the treated H9c2 collected growth media, underwent dialysis in 1x Tris Buffer (10 mM Tris, 25 mM NaCl, 0.5 mM EDTA, 0.5 mM β- mercaptoethanol; pH 7.1) for 18 hours at 4 °C with one change of buffer every 6 hours. Then, 155 µg total protein from human plasma or H9c2 condition media was incubated in 1x CH3COONa buffer (10 mM CH3COONa, 0.5 mM EDTA, 0.5 mM β-Mercaptoethanol; pH 4.8) containing 1.0 mM APpNA, at 37 °C for 1 hour. Finally, the rate of paranitroanilide liberation from APpNA by plasma PRCP or PRCP secreted into the media by H9c2 was determined by measuring the absorbance at 405 nm using BioTek ELx800 Absorbance Microplate Reader.

2.13 Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM) of n determinations. Experiments were performed at least three times in duplicates or triplicates. Data was analyzed using one-way analysis of variance (ANOVA) with Newman-Keuls or Tukeys post-hoc test (Prism Graph Pad 5.0, Graph Pad Software, Inc., USA) to assess statistical significance of the observed differences between drug-treated and corresponding control groups. For all statistical comparisons, significance was determined by $P < 0.05$.

CHAPTER 3

RESULTS

3.1 Human Plasma Studies

Xu et al. [36] developed a sensitive enzyme-linked-immunosorbent-assay (ELISA) using rabbit anti-PRCP polyclonal antibody to measure PRCP concentration in plasma. They showed that plasma PRCP levels were significantly correlated with signs and symptoms of obesity and T2DM. Moreover, they observed that plasma PRCP levels were positively associated with plaque formation, plaque size, vessel wall stiffness and cardiac posterior wall thickness. Therefore, PRCP appears to be a promising screening biomarker for obesity, diabetes mellitus and arteriosclerosis.

3.1.1 Plasma PRCP Detection

To characterize PRCP levels in diabetes, we used an enzymatic assay to measure PRCP activity in the plasma of diabetic patients (either poorly controlled or on hypoglycemic agents). As shown in Fig. 3.1, having T2DM was consistent with significantly increased plasma PRCP activity, and although metformin and insulin-treated patients had a lower plasma PRCP activity compared to the uncontrolled patients, these findings were not significant.

Metabolic acidosis, characterized as an increased plasma acidity, is seen in diabetic patients. Diabetic metabolic acidosis could be due to an impaired renal function which is a major complication of T2DM. Ketoacidosis is another possible cause of increased plasma acidity in T2DM, because insulin resistance favors delivery of fatty acids to the mitochondria

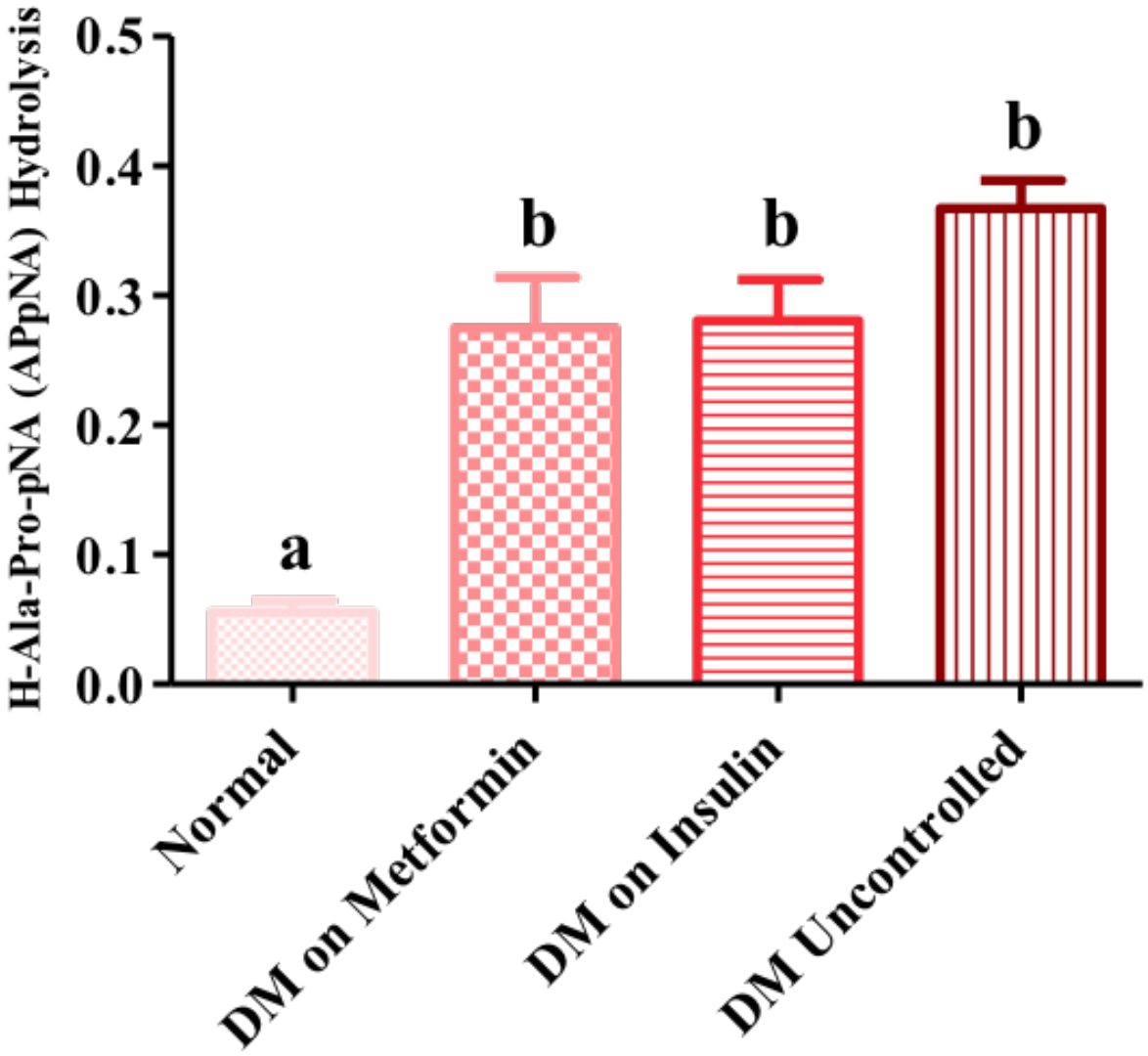


Figure 3.1. PRCP Activity in the Plasma of Diabetic Patients.
EDTA treated plasma from uncontrolled diabetic patients, diabetic patients on insulin and diabetic patients on metformin were assessed for PRCP activity and compared to the control plasma from non-diabetic patients. Dialyzed in 1x Tris Buffer for 18 hours at 4 °C with one change of buffer every 6 hours, 155 µg total plasma protein from each group was incubated in 1x CH3COONa buffer containing 1.0 mM APpNA, at 37 °C for 1 hour. Finally, the rate of paranitroanilide liberation from APpNA by plasma PRCP was determined by measuring the absorbance at 405 nm. Data are expressed as mean ± SEM (n = 3-5 patients/group/experiment, done in triplicate). Bars with the same letter are not statistically different (ANOVA, $P < 0.05$).

and their metabolization to ketoacids. Furthermore, a diabetic diet restricted in carbohydrate and enriched with protein also alters plasma pH toward a more acidic condition (Souto et al. [76] and Avogaro et al. [77]). Considering the fact that PRCP metabolizes its substrates at acidic pH (Grobe et al. [78]), a decreased plasma pH in T2DM could enhance plasma PRCP activity. Therefore, the increased plasma PRCP activity in diabetes seen in Fig. 3.1 could be attributable to increased plasma acidity and/or increased plamsa PRCP expression in diabetes. Thus, we evaluated PRCP protein expression in the human plasma samples of diabetic patients using anti-human angiotensinase C antibody and observed that PRCP protein expression is accordingly increased in diabetes (Fig. 3.2). To our surprise, metformin and insulin treated patients, had significantly lower plasma PRCP protein expression compared to the uncontrolled patients however plasma PRCP activity was not markedly impacted in the latter groups compared to the uncontrolled diabetic samples.

3.1.2 Plasma Prekallikrein Detection

T2DM is a prothrombotic states since the coagulation factors (including fibrinogen, factor VII, factor VIII, factor XI, factor XII, kallikrein, and vWF) are markedly elevated in the plasma of diabetic patients (Madan et al. [79]). As well, the anticoagulant protein C is decreased in diabetic subjects (Ceriello et al. [80]). Moreover, hyperglycemia and insulin resistance enhance platelet adhesion and aggregation as well as deteriorating endothelial function (Taylor [81]). Dysfunction of kallikrein-kinin-system (KKS) has been largely linked to diabetes hypercoagulable states (Uehara et al. [82]). Studies have shown that plasma prekallikrein (PK) is increased in diabetes (Kedzierska et al. [83]) due to an increased liver PK synthesis (Federspil et al. [84]) or a proteinuria-associated decreased plasma kallikrein inhibitor (C1 inhibitor) (Jaffa et al. [85]). Clermont et al. [86] observed a 74% increased plasma PK protein in diabetic rats. With the possible role of increased PK in pathogenesis of diabetes-related micro- and macrovascular complications and the fact that PRCP activates plasma PK to kallikrein, we decided to examine how the increased plasma PRCP activity and expression in diabetes, would influence plasma PK protein and the resultant

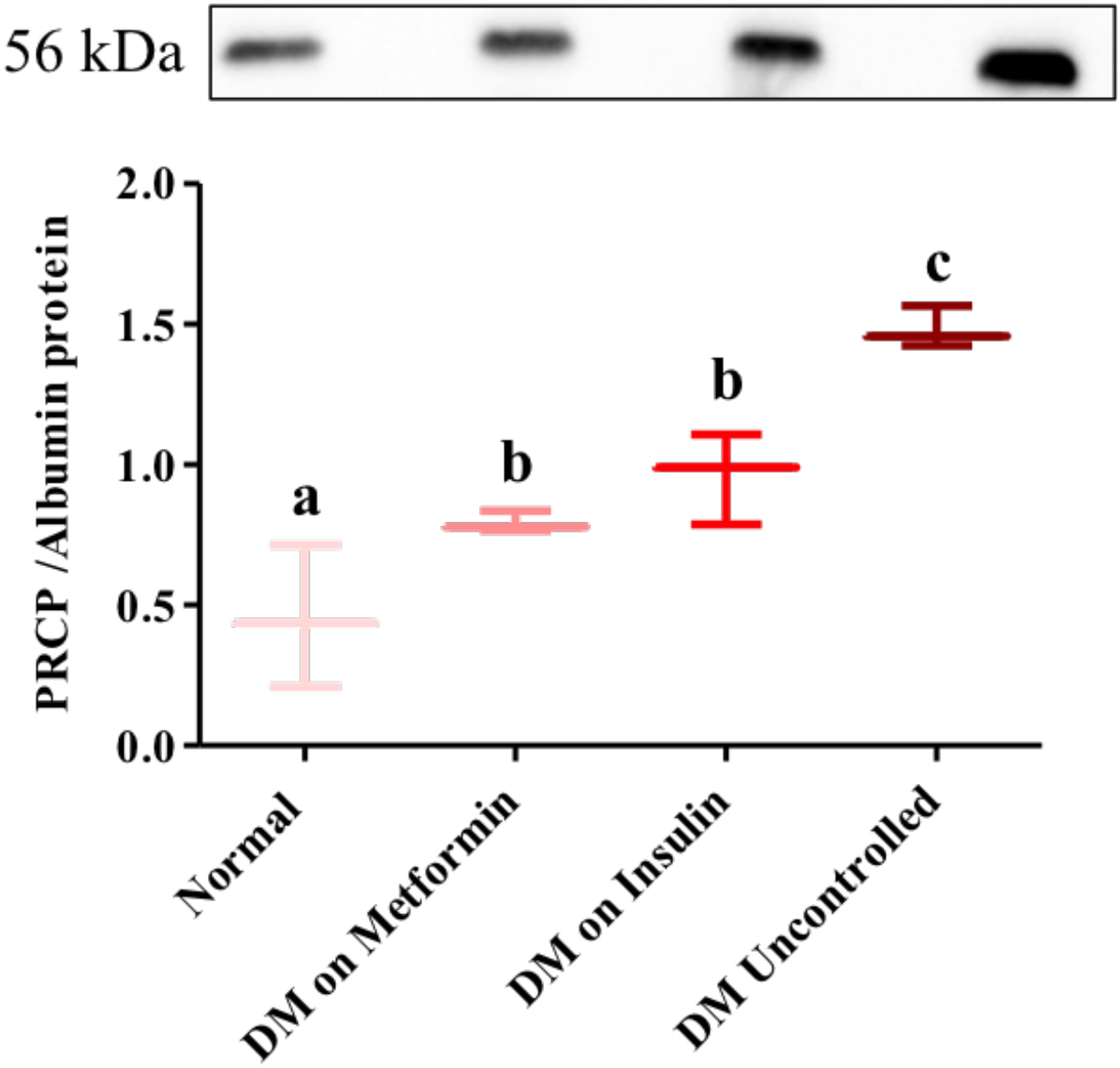


Figure 3.2. PRCP Protein Expression in the Plasma of Diabetic Patients.
EDTA treated plasma from uncontrolled diabetic patients, diabetic patients on insulin and diabetic patients on metformin were assessed for PRCP protein expression and compared to control plasma from non-diabetic patients. Dialyzed plasma protein (10 µg) was loaded to a 10% acrylamide gel and the western blot analysis was performed using anti-human angiotensinase C antibody. Plasma PRCP protein was normalized to serum albumin protein. Data are expressed as mean ± SEM (n = 3-5 patients/group/experiment, done in triplicate). Bars with the same letter are not statistically different (ANOVA, $P < 0.05$).

kallikrein activity. We observed that diabetes significantly increases plasma PK concentration and metformin or insulin treated patients had lower plasma PK level compared to the uncontrolled diabetic patients.

Next we determined the effect of soy bean trypsin inhibitor (SBTI), a known inhibitor of kallikrein, on plasma kallikrein activity. Plasma kallikrein activity was significantly higher in uncontrolled diabetic patients compared to the control plasma and this activity was inhibited by SBTI. Interestingly, kallikrein activity was significantly decreased in metformin or insulin treated diabetic patients compared to the uncontrolled diabetic patients.

3.1.3 Plasma FXI in Diabetic Patients

Plasma PK is a key component of intrinsic (contact activation) pathway of coagulation. At the endothelial cell surface, PK is activated to kallikrein by PRCP. However, at the negatively charged surfaces PK and FXII can reciprocally activate each other to kallikrein and FXIIa, respectively (Cochrane and Griffin [87]). FXIIa in turn activates factor XI (FXI) to FXIa, thereby initiating blood clot formation cascade (Macfarlane [88] and Davie and Ratnoff [89]). Available reports regarding the level of FXI in the plasma of diabetic patients are conflicting. Some studies demonstrate a significant increase in plasma FXI in diabetic patients contributing to diabetes vascular complications and hypercoagulation (Patrassi et al. [90] and Mard-Soltani et al. [91]), however in other studies the alteration in plasma FXI in diabetes was negligible (Kim et al. [92]). Therefore, to clarify how diabetes could alter plasma FXI concentration, we explored the plasma FXI protein in our diabetic plasma samples and observed that in uncontrolled diabetic patients there is a significant increased plasma FXI levels as compared to the control and insulin or metformin treated patients.

3.2 Designing an *In Vitro* Model of Cardiac Nutrition Overload

H9c2 cell line, derived from embryonic rat heart myoblasts, can present myoblastic proliferative phenotype while maintained in 10% fetal bovine serum (FBS) containing culture media (DMEM) and can differentiate into cardiac muscle cells upon FBS starvation (2%

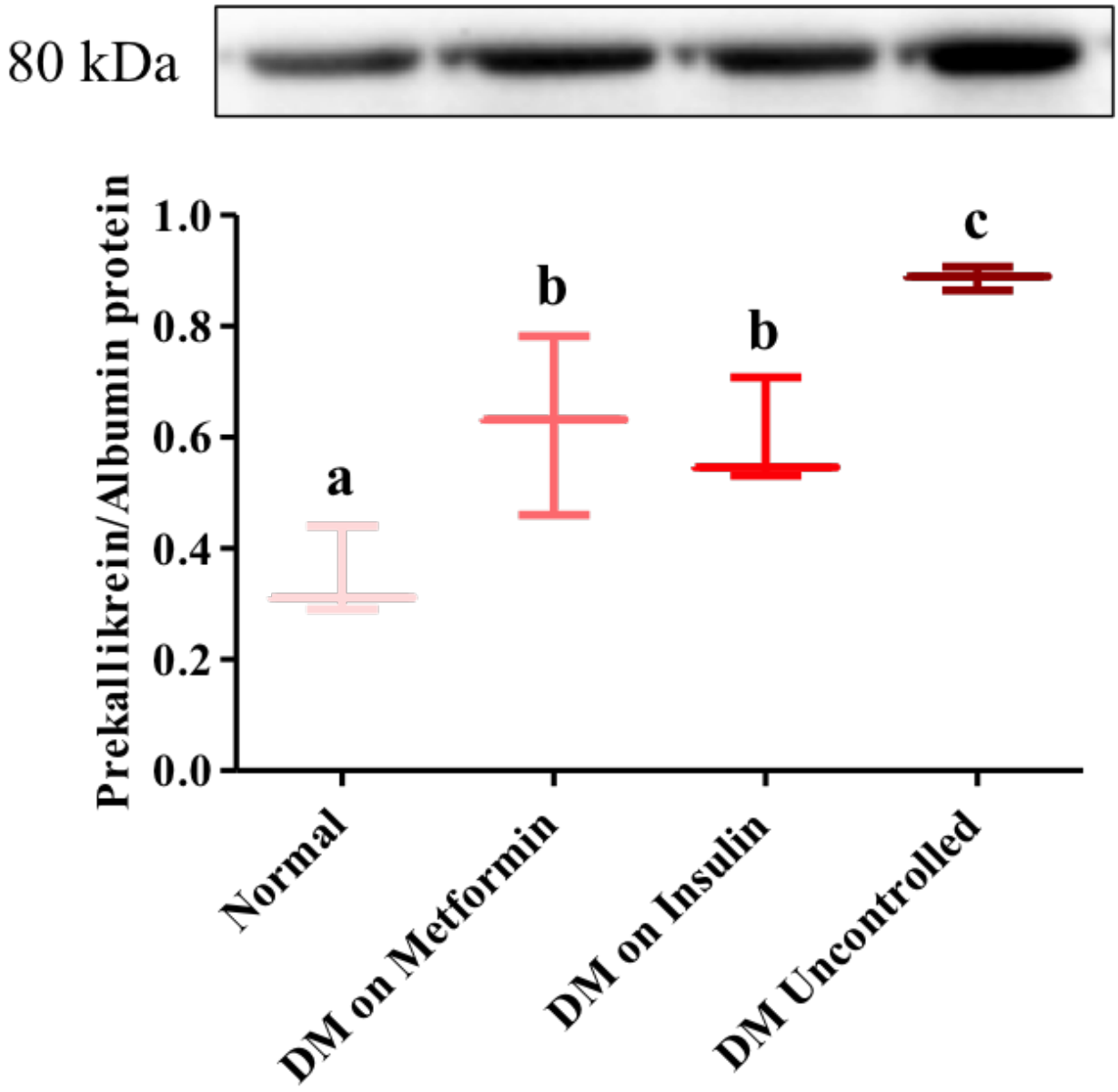


Figure 3.3. Prekallikrein Protein Expression in the Plasma of Diabetic Patients. EDTA treated plasma from uncontrolled diabetic patients, diabetic patients on insulin and diabetic patients on metformin were assessed for PK protein expression and compared to control plasma from non-diabetic patients. Dialyzed plasma protein (10 µg) was loaded to a 10% acrylamide gel and the western blot analysis was performed using anti-human prekallikrein antibody. Plasma PK protein was normalized to serum albumin protein. Data are expressed as mean ± SEM (n = 3-5 patients/group/experiment, done in triplicate). Bars with the same letter are not statistically different (ANOVA, $P < 0.05$).

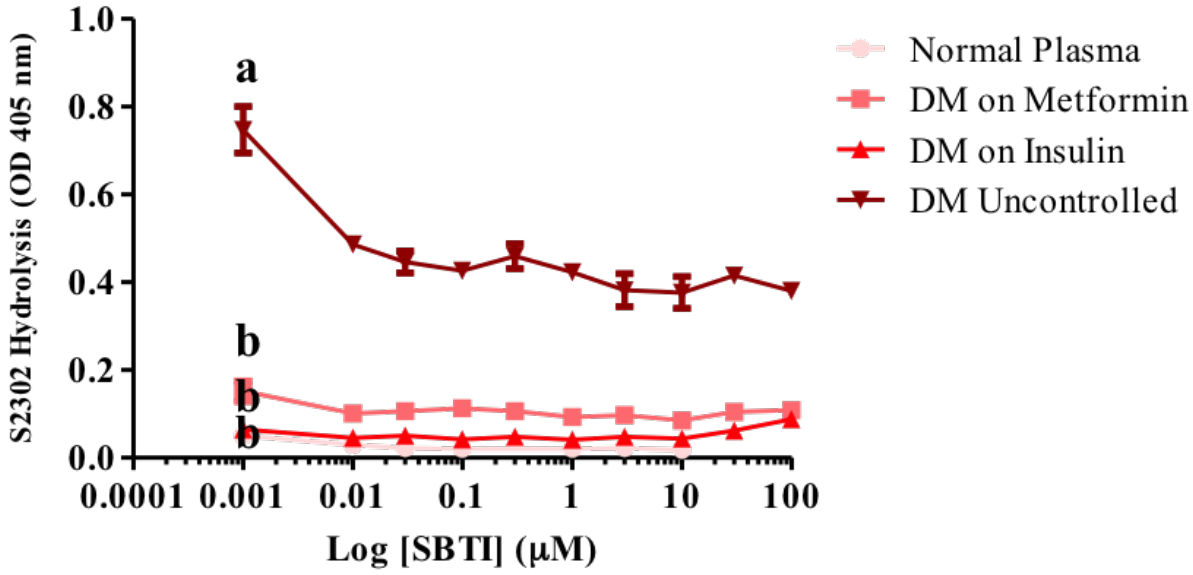


Figure 3.4. SBTI Inhibits Kallikrein Activity in the Plasma.
The liberation of paranitroaniline from S2302 (0.5 mM) by plasma kallikrein (155 µg total plasma protein) in the absence or presence of increasing concentrations of SBTI was measured as the change in absorbance at 405 nm after 1 hour incubation at 37 °C. Data are expressed as mean ± SEM (n = 3-5 patients/group/experiment, done in triplicate). The absence of standard error bars indicates that the variation was too small to be visualized. Bars with the same letter are not statistically different (ANOVA, $P < 0.05$).

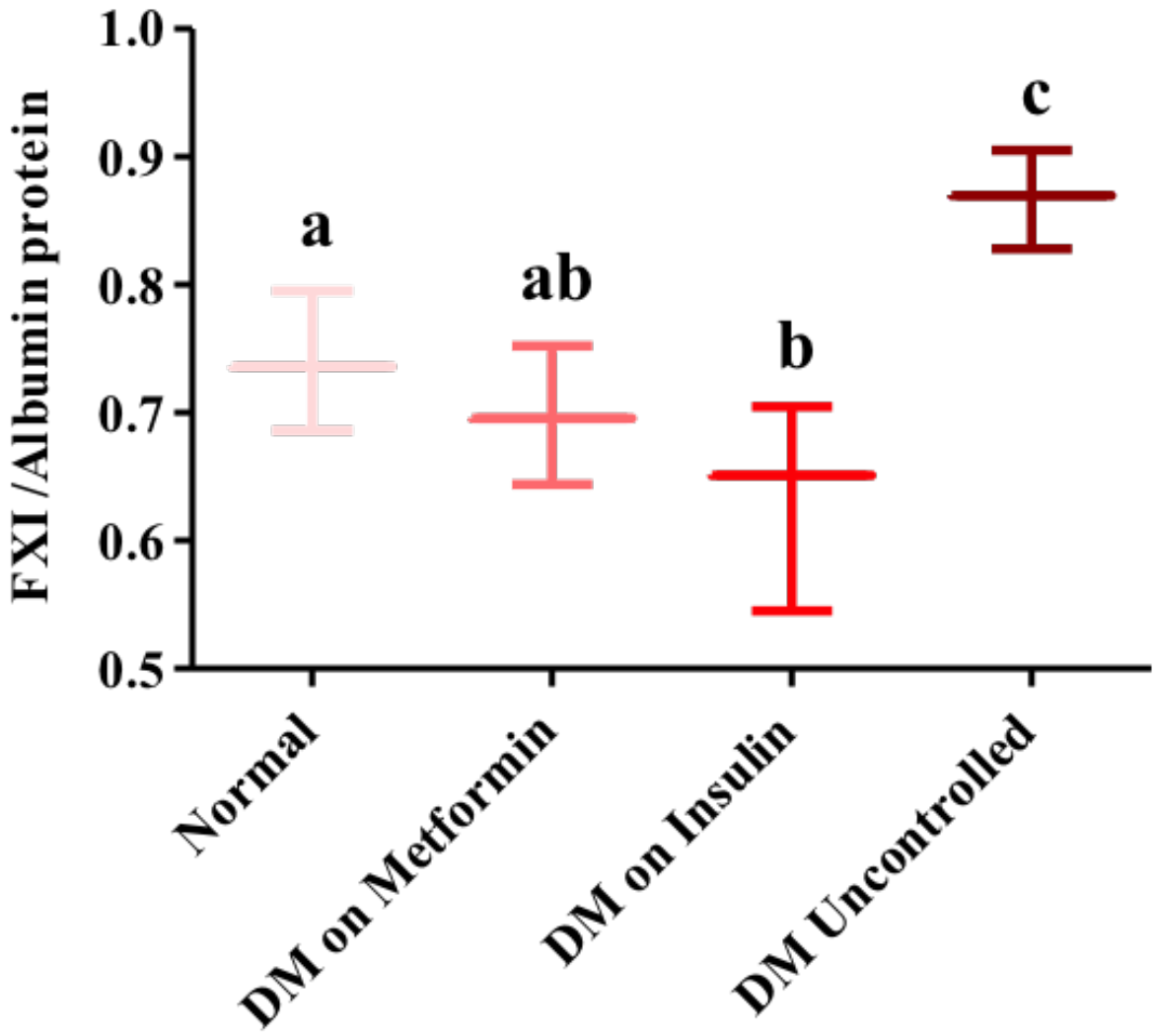
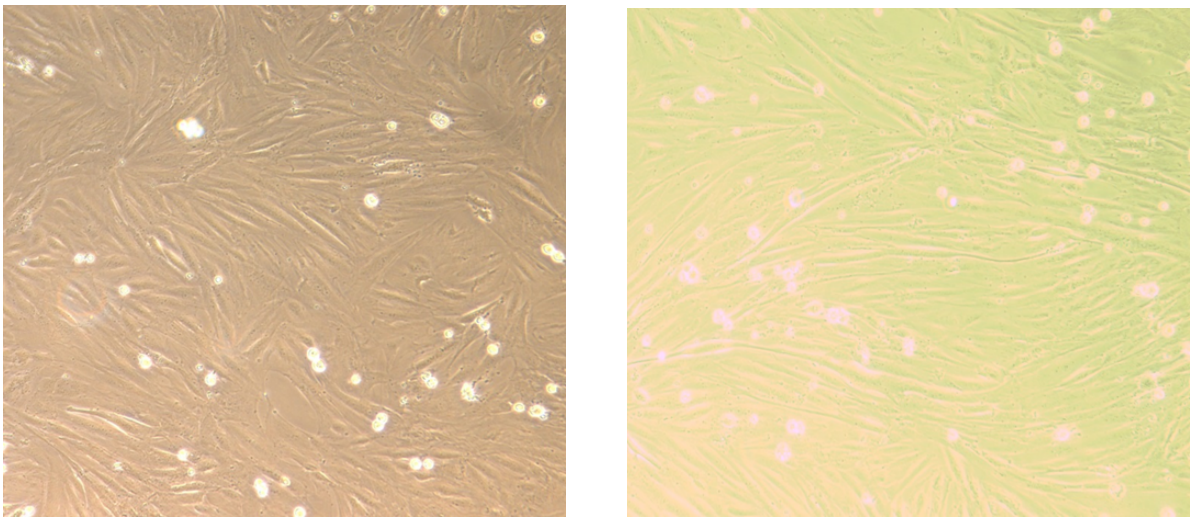


Figure 3.5. FXI Protein Expression in the Plasma of Diabetic Patients.
EDTA treated plasma from uncontrolled diabetic patients, diabetic patients on insulin and diabetic patients on metformin were assessed for FXI protein expression and compared to the control plasma from non-diabetic patients. Dialyzed plasma protein (10 µg) was loaded to a 10% acrylamide gel and the western blot analysis was performed using anti-human FXI antibody. Plasma FXI protein was normalized to serum albumin protein. Data are expressed as mean ± SEM (n = 3-5 patients/group/experiment, done in triplicate). Bars with the same letter are not statistically different (ANOVA, $P < 0.05$).



(a) (b)

Figure 3.6. The Morphological Features of H9c2 Cell Line.
Upon differentiation, the stellate/spindle shaped H9c2 myoblasts fuse together to form thin, elongated and multi-nucleated H9c2 myocytes (Sardao et al. [96] and Zara et al. [97]). (a) H9c2 cardiomyoblast. (b) H9c2 cardiomyocyte.

FBS) (Pereira et al. [93] and Menard et al. [94]). While in 10% FBS-DMEM, myoblastic features were restored by subculturing the cells before reaching confluency, usually every other day. However, for differentiation, cells were cultured in 2% FBS-DMEM and the growth media was replaced every two days. Upon differentiation, H9c2 cells fuse to form multi-nucleated myotubes (Fig. 3.6) and increase biogenesis of mitochondria (Comelli et al. [95]), therefore while the myoblastic form maintains its energy by glycolysis, the differentiated phenotype shows an increased rate of fatty acid β-oxidation and a more efficient ATP production (Pereira et al. [93]). H9c2 cell line has been widely used for the study of cardiac hypertrophy, cardiotoxicity, ischemia/reperfusion injury and oxidative stress, since its derivation and it is one of the closest cell line to human cardiac muscle, therefore it appears to be a proper model for our studies.

3.2.1 PRCP is Expressed in H9c2 Cell Line

To determine PRCP mRNA and protein expression in H9c2 cell line, flasks of H9c2 cell line underwent RNA and protein extraction. Then the samples were run through 1.5%

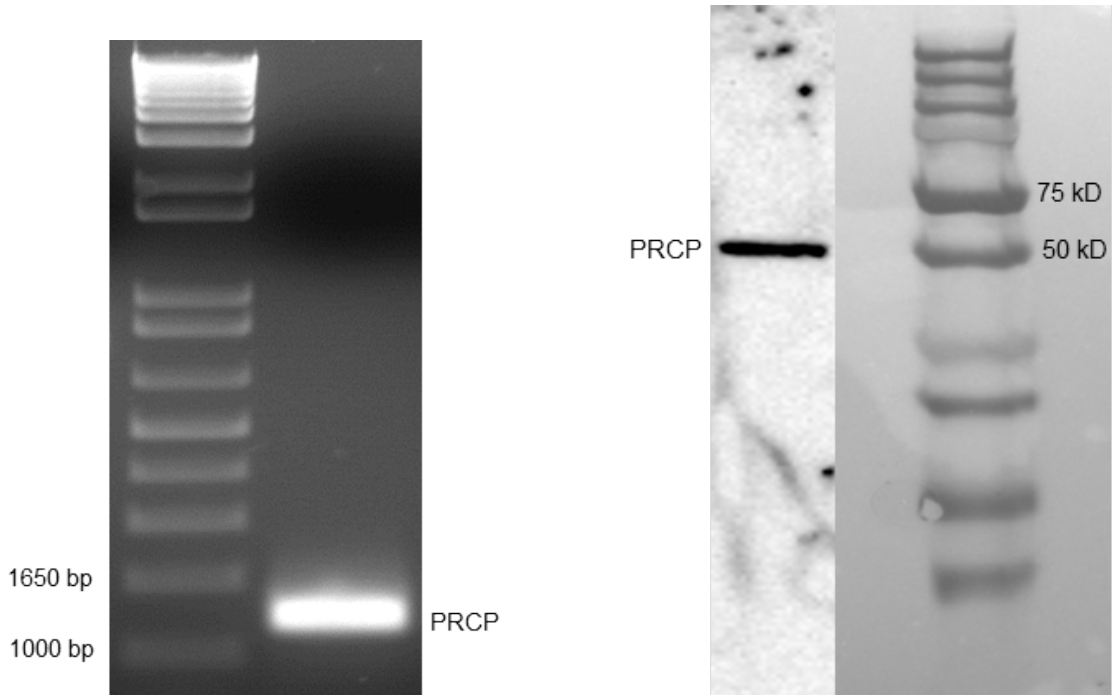


Figure 3.7. PRCP Expression in H9c2 Cell Line.
(a) PRCP mRNA expression in differentiated H9c2 cell line. (b) PRCP protein expression in differentiated H9c2 cell line. The ladder was visualized via colorimetric exposure and the sample was visualized using the chemiluminescence exposure.

agarose gel and 10% acrylamide gel for detection of PRCP mRNA and protein, respectively. As shown in Fig. 3.7, PRCP is expressed in the H9c2 cell line, thus H9c2 is an appropriate cardiac cell line for the study of PRCP regulation of expression.

3.2.2 Characterizing PRCP Activity in H9c2 Cell Line

To elucidate PRCP activity in H9c2 model, we next carried out an enzymatic assay to assess PRCP activity in both H9c2 cardiomyoblast and cardiomyocyte. In the presence of PRCP and HK, PK is activated to kallikrein and the resulted kallikrein activity can be measured using an available chromogenic substrate (S2302). We observed that although both myoblastic and myocytic H9c2 demonstrate PRCP activity, differentiated cells have a 3 fold higher PRCP activity compared to the blast H9c2 cells (k_m values of 34.8 nM and 62.93 M, respectively) (Fig. 3.8). Thus, we decided to continue our studies with the differentiated

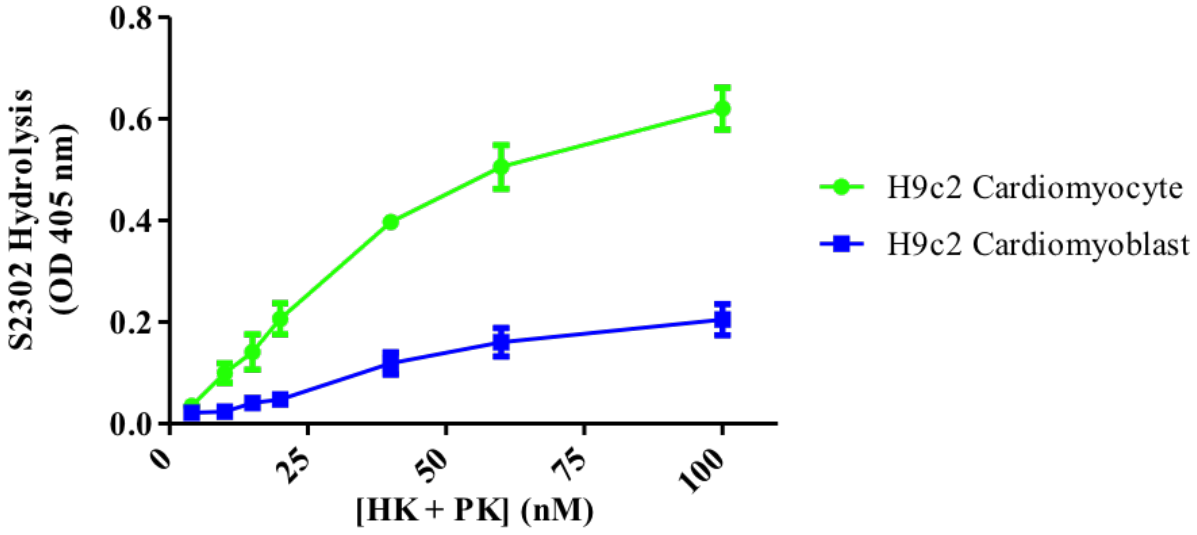


Figure 3.8. PRCP Activity in Myoblast versus Differentiated H9c2 Cell Line.
Rat H9c2 cells were incubated with increasing concentrations of HK (0, 4 nM, 10 nM, 15 nM, 20 nM, 40 nM, 60 nM and 100 nM) for 1 hour, followed by incubation with increasing concentrations of PK (same concentrations as HK) for an additional 1 hour. The activity of kallikrein was then determined by addition of 0.5 mM S2302 (kallikrein chromogenic substrate). Substrate hydrolysis was allowed to proceed for 1 hour at 37 °C. Kallikrein activity was measured as the change in absorbance of the reaction mixture at 405 nm. Data are expressed as mean ± SEM (n = 9).

H9c2 cells because not only they have higher rate of fatty acid oxidation (a feature of adult cardiac muscles) but also they possess higher PRCP activity.

3.3 The Effect of Nutrition Overload on PRCP Alteration of Expression

Since plasma PRCP concentration is increased in obesity, diabetes and cardiovascular pathologies (Xu et al. [36]), we hypothesized that chronic nutrition overload (hyperglycemia and/or hyperlipidemia) as seen in obesity and diabetes might account for changes in PRCP levels. Therefore, we evaluated the influence of high glucose or fatty acid concentration on cardiomyocytes PRCP expression after a minimum of 48 hours incubation.

3.3.1 High Glucose Concentration Increases PRCP Expression in H9c2 Cell Line

The effect of high glucose concentration on PRCP expression was investigated. The cut off point for diagnosis of diabetes based on the American Diabetes Association (ADA) guidelines (Sacks et al. [98]) is considered fasting plasma glucose (FPG) $\geq$ 126 mg/dL (7 mM) or 2 hours plasma glucose $\geq$ 200 mg/dL (11.1 mM). However, the manufacturer's (ATCC) required condition for culture of H9c2 cell line is the presence of 25 mM (450 mg/dL) glucose in the growth media (DMEM). Therefore, we treated H9c2 cells with 40 mM (720 mg/dL) and 80 mM (1440 mg/dL) glucose in condition media to evaluate alterations in PRCP expression. Exposure to 80 mM glucose for 48 hours significantly increased PRCP mRNA expression in the H9c2 cells, however lower glucose concentrations did not alter the basal PRCP expression (Fig. 3.9).

3.3.2 High Fatty Acid Concentration Suppresses PRCP mRNA Expression

Next, H9c2 cells were incubated with increasing concentrations of palmitate-BSA for 48 hours. In a murine macrophage cell line (RAW 264.7), palmitate concentrations of more than 0.2 mM (for 16 hours) were shown to be toxic (Shrestha et al. [99]). However, 24 hours palmitate treatment of 0.2 mM-0.6 mM in the mouse C2C12 myoblast was shown to induce insulin resistance without affecting cell viability (Yang et al. [100]). A recent study has shown that the number of apoptotic H9c2 cells significantly increases at palmitate concentrations of higher than 0.15 mM upon 12 hours treatment (Wei et al. [101]). Since we were planning to perform longer incubation periods (48 hours minimum), we decided to use a range of 0.02 mM-0.08 mM palmitate-BSA concentrations. Therefore, H9c2 cardiomyocytes were treated with 0.02, 0.04 and 0.08 mM of palmitate-BSA for 48 hours and the RNA extraction was performed as described in the previous section. Interestingly, we observed that palmitate-BSA decreases PRCP mRNA expression in a dose-dependent manner after 48 hours incubation period (Fig. 3.10).

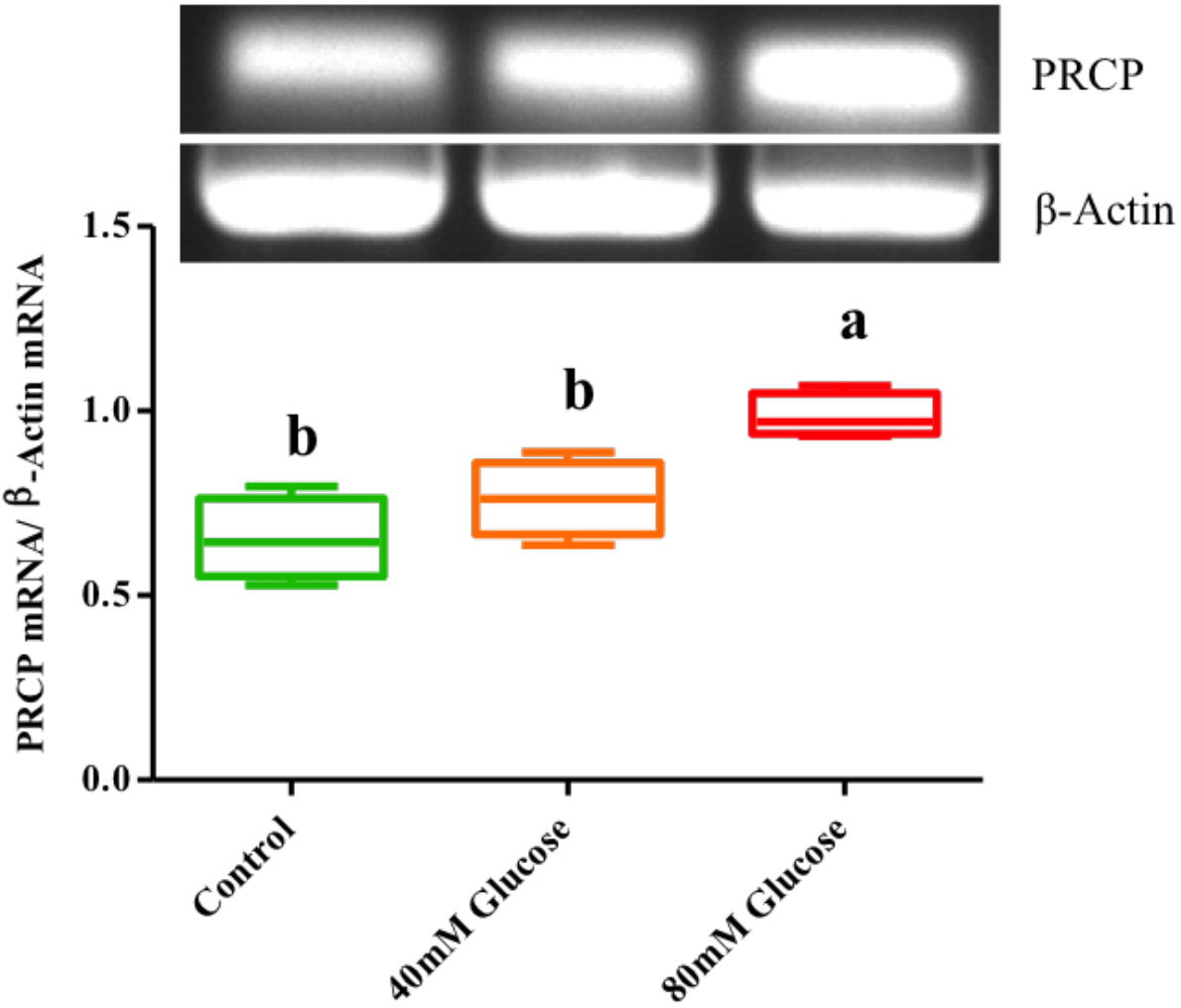


Figure 3.9. High Glucose Concentration Increases PRCP mRNA Expression in H9c2 Cell Line.

Flasks of differentiated H9c2 cells were incubated with 40 mM or 80 mM glucose in the growth media for 48 hours. Glucose (25 mM) containing DMEM was used as a control. At the completion of incubation, cells were collected in TRIzol reagent and RNA was extracted, reverse transcribed and run through a 1.5% agarose gel. For each study group, four different flasks of cells were analyzed and PRCP mRNA was finally normalized to each flasks corresponding house keeping gene (β-actin) expression. Data are expressed as mean ± SEM (n = 4). Bars with the same letter are not statistically different (ANOVA, $P < 0.05$).

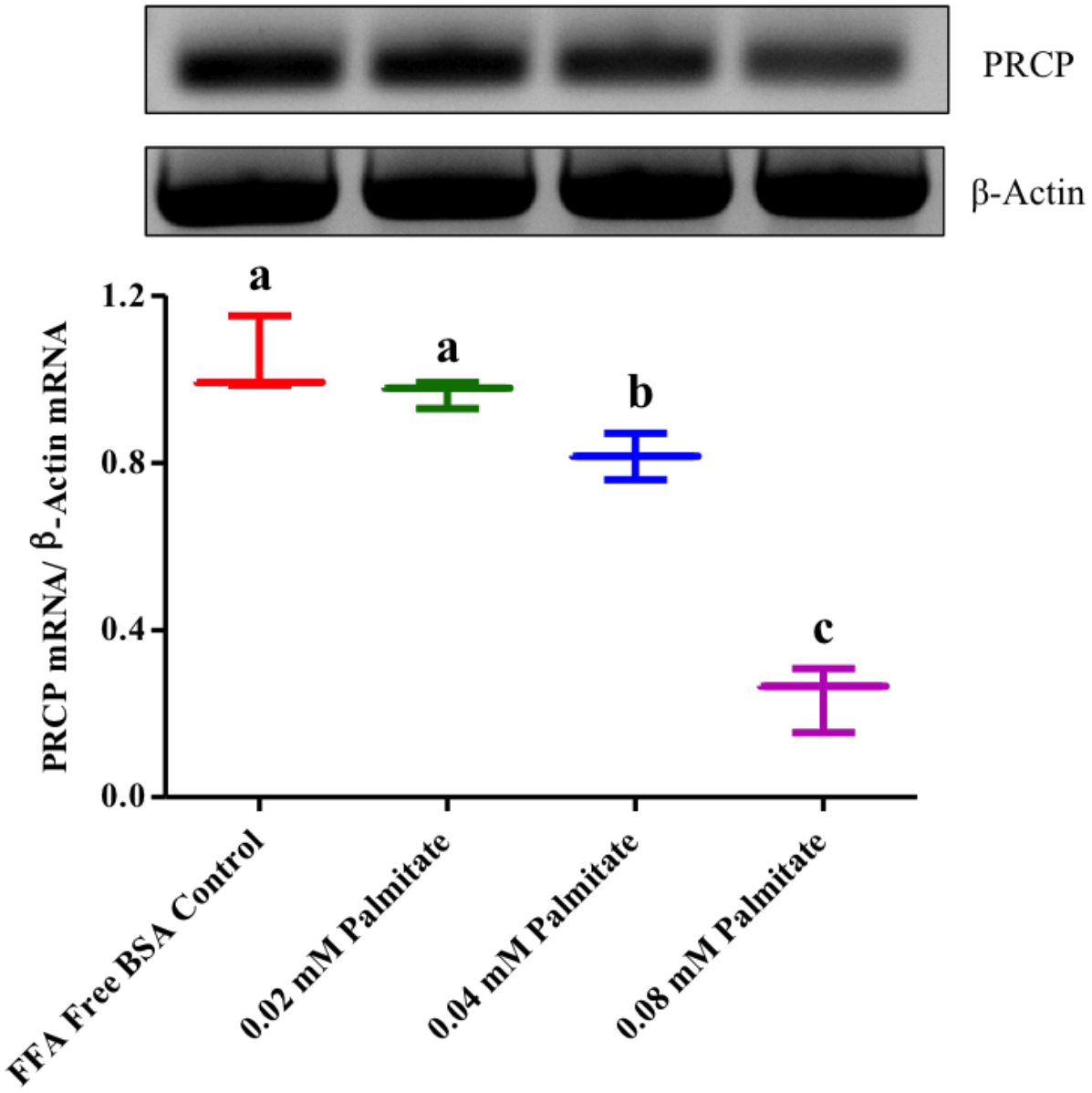


Figure 3.10. High fatty Acid Palmitate Concentration Decreases PRCP mRNA Expression in H9c2 Cell Line.

The differentiated H9c2 cells were treated with BSA vehicle, 0.02 mM, 0.04 mM or 0.08 mM palmitate-BSA for 48 hours. At the completion of incubation total RNA extraction, RT-PCR and agarose gel studies were performed. PRCP mRNA expression was then normalized to β-actin mRNA expression. For each study group, three different flasks of cells were analyzed and the change in PRCP expression upon palmitate treatment was compared to the BSA-vehicle. Data are expressed as mean $\pm$ SEM (n = 3). Bars with the same letter are not statistically different (ANOVA, $P < 0.05$).

Then we asked the question if the decrease in PRCP expression might be due to a palmitate-induced cytotoxicity, since palmitate could be directly toxic to cardiac cells. Therefore, the cell viability assay was performed after incubation of H9c2 cells with increasing concentrations of palmitate-BSA for 24, 48 and 72 hours. As demonstrated in Fig. 3.11, 0.08 mM palmitate did not influence cell viability in the mentioned incubation periods. However, higher concentrations of palmitate decreased H9c2 cell viability. Thus, we concluded that the sub-lethal concentration of 0.08 mM palmitate significantly inhibits PRCP mRNA expression in cardiomyocytes after 48 hours incubation .

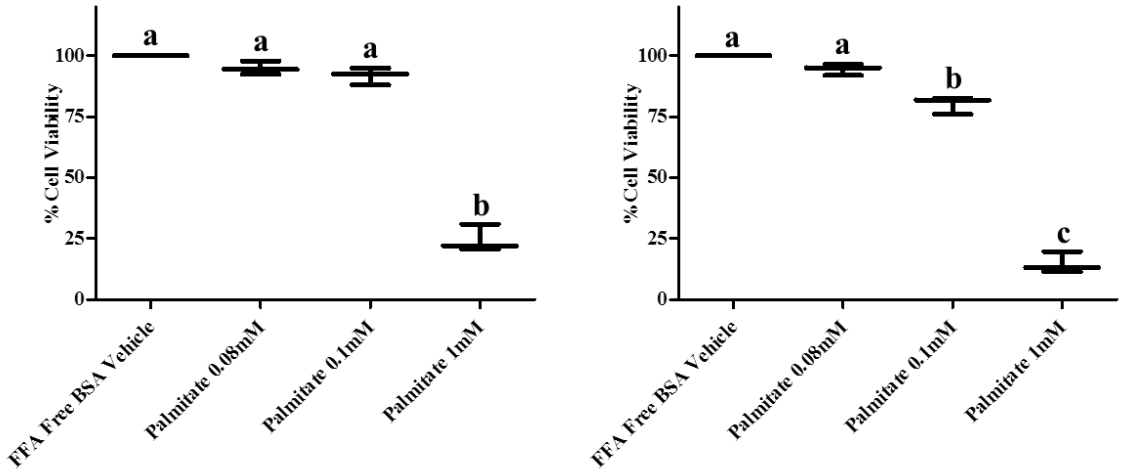
3.3.3 High Palmitate Concentration Decreases PRCP Protein Expression

To confirm our previous finding of palmitate-induced attenuation of PRCP expression in the cardiomyocytes and to elucidate if this alteration is translated to PRCP protein, we explored the effect of palamitate treatment on PRCP protein expression. In support of our mRNA results, we observed that both 0.08 mM and 0.1 mM concentration of palmitate-BSA decreased PRCP protein expression in the H9c2 cardiomyocytes (Fig. 3.12). Our results, suggest that fatty acid palmitate could regulate cardiomyocyte PRCP expression.

In order to further characterize the palmitate alterations of PRCP expression, PRCP protein expression was studied after 24, 48, 72 and 96 hours of palmitate treatment. As shown in Fig. 3.13, palmitate decreased PRCP protein expression in a time-dependent manner. The palmitate stimulated decreased PRCP expression in a dose- and time-dependent manner further implicating the 16 carbon fatty acid, palmitate, in the PRCP regulation in H9c2 cells.

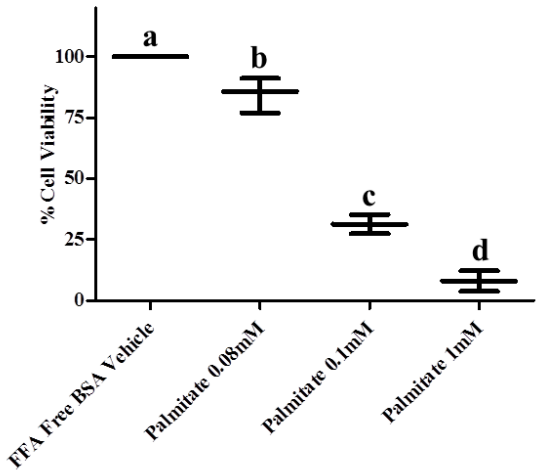
3.3.4 PRCP Activity is Decreased in Palmitate Treated H9c2 Cardiomyocytes

PRCP was first localized in lysosomal fractions (Kumamoto et al. [32]) and later on the endothelial cell membrane (Shariat-Madar et al. [3]). In addition, Skidgel and Erds [102] suggested that upon stimulation, the lysosomal PRCP could be released into the extracellular



(a)

(b)



(c)

Figure 3.11. The Effect of Palmitate on H9c2 Cell Viability.
Cell viability was measured by MTT assay after (a) 24 hours (b) 48 hours and (c) 72 hours of palmitate-BSA incubation. At the completion of incubation, the growth media was replaced with MTT solution (final concentration of 0.5 mg/L) and the cells were incubated for four hours at 37 °C covered from light. Then, formazan crystals were solubilized by replacing MTT solution with 150 µL of MTT solvent (4 mM HCl, 0.1% Nondet P-40 (NP40) in isopropanol) while shaking gently for 15-30 minutes. The absorbance was measured at 570 nm and subtracted from the background (read at 690 nm). Percent cell viability after palmitate treatment was compared to the BSA vehicle treated cells. Data are expressed as mean ± SEM (n = 3). Bars with the same letter are not statistically different (ANOVA, $P < 0.05$).

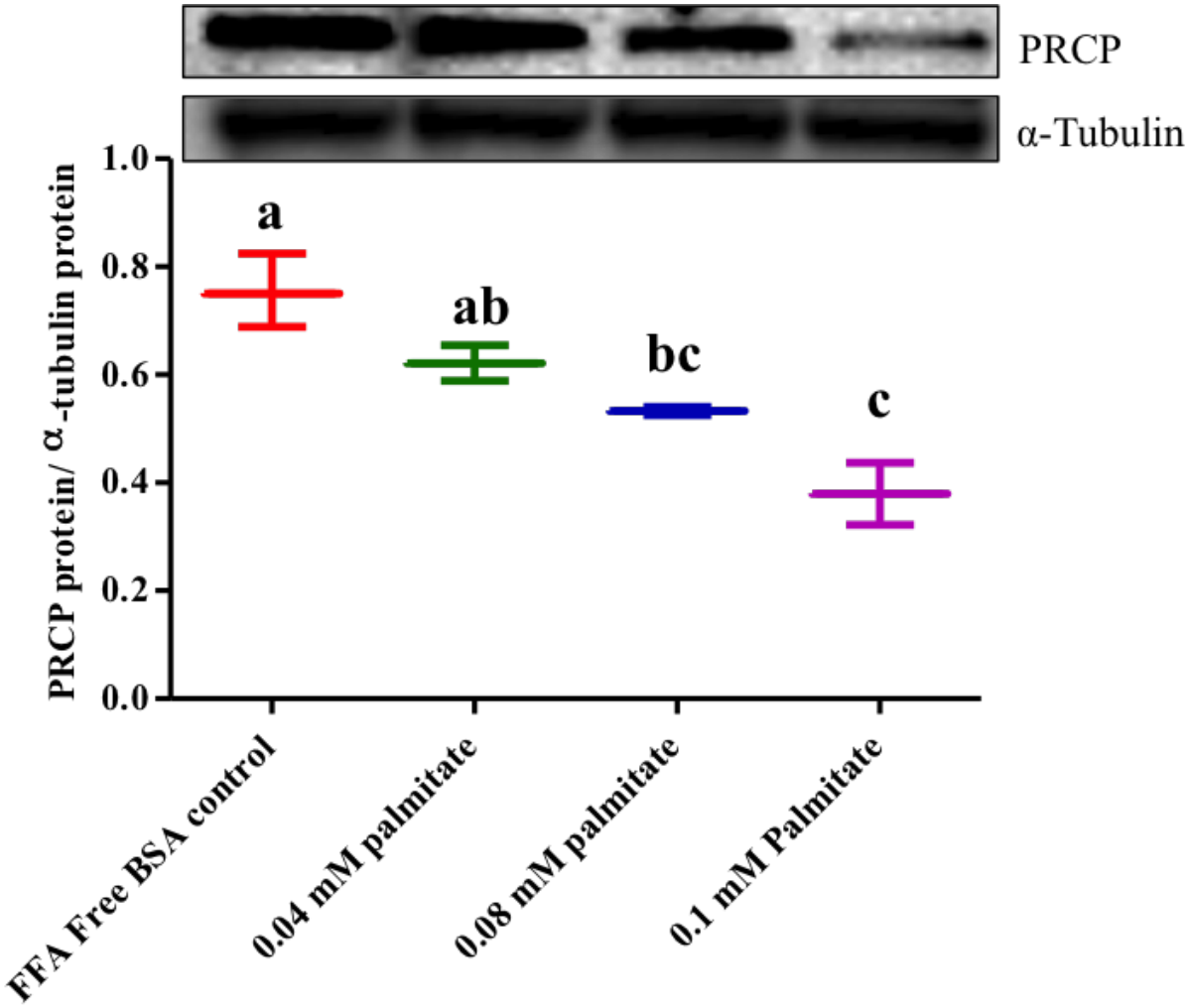


Figure 3.12. High fatty Acid Palmitate Concentration Decreases PRCP protein Expression in H9c2 Cell Line.

The differentiated H9c2 cells were treated with BSA vehicle, 0.04 mM, 0.08 mM or 0.1 mM palmitate-BSA for 48 hours. At the completion of incubation, cell lysate was collected in RIPA buffer and total protein was extracted, followed by measuring protein concentration with BCA assay. Western blot analysis was performed using anti-human angiotensinase C in goat (1:100). H9c2 PRCP protein was then normalized to α-tubulin protein. For each study group, three different flasks of cells were tested and the change in PRCP protein expression after palmitate treatment was compared to the BSA-vehicle. Data are expressed as mean $\pm$ SEM (n = 3). Bars with the same letter are not statistically different (ANOVA, $P < 0.05$).

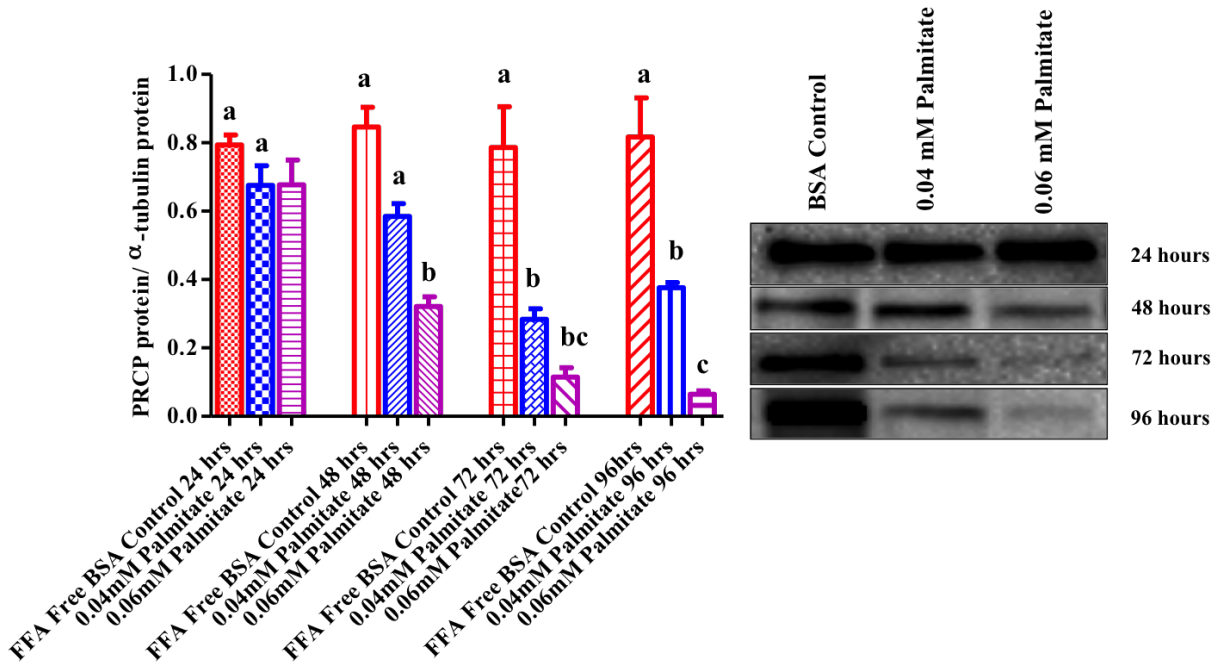


Figure 3.13. Palmitate Decreases PRCP Protein Expression in a Time-Dependent Manner. The differentiated H9c2 cells were treated with BSA vehicle, 0.04 mM or 0.06 mM palmitate-BSA for 24, 48, 72 and 96 hours. Western blot analysis of H9c2 cell lysate was performed and H9c2 PRCP protein was normalized to α-tubulin protein. For each study group, three different flasks of cells were tested and the change in PRCP protein expression after palmitate treatment was compared to the BSA-vehicle. Data are expressed as mean $\pm$ SEM (n = 3). Bars with the same letter are not statistically different (ANOVA, $P < 0.05$).

or body fluid such as synovial fluid (Kumamoto et al. [32]) and urine (Miller et al. [7] and Yang et al. [103]). Therefore, we hypothesized that a decrease in PRCP protein expression upon palmitate treatment will result in a decrease in PRCP activity. To address this question, we used an enzymatic assay to characterize PRCP activity after palmitate treatment in H9c2 cells. We observed that after 48 hours, 0.08 mM palmitate-BSA significantly decreases PRCP activity in the H9c2 cells compared to the vehicle treated cells (Fig. 3.14). This finding is in agreement with our previous observations regarding palmitate-induced cardiac PRCP inhibition.

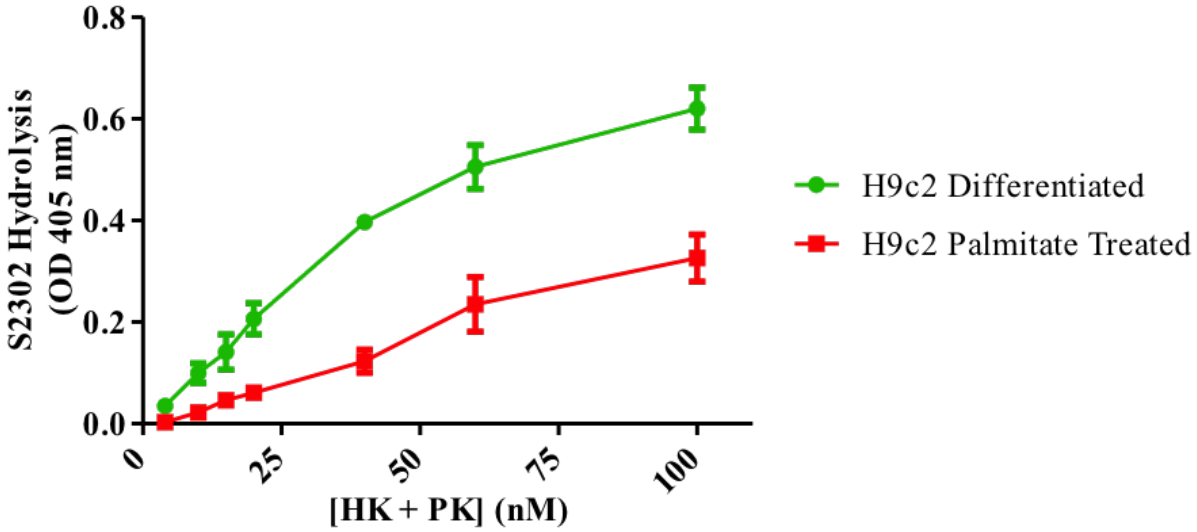


Figure 3.14. Palmitate Decreases Cardiomyocytes PRCP Activity.
PRCP activity after palmitate treatment of differentiated H9c2 cells was compared to PRCP activity in non-treated differentiated H9c2 cells. The differentiated H9c2 cells were seeded overnight in 96 well microtiter plates and the next day they were incubated with 0.08 mM palmitate-BSA or BSA-vehicle for 48 hours. At the completion of incubation, H9c2 cells were incubated with increasing concentrations of HK (0, 4 nM, 10 nM, 15 nM, 20 nM, 40 nM, 60 nM and 100 nM) for 1 hour, followed by incubation with increasing concentrations of PK (same concentrations as HK) for another 1 hour. The activity of kallikrein was then determined by addition of 0.5 mM S2302 (kallikrein chromogenic substrate). Substrate hydrolysis was allowed to proceed for 1 hour at 37 °C. Kallikrein activity was measured as the change in absorbance of the reaction mixture at 405 nm. For each HK/PK concentration 9 replicates were examined and the change in PRCP activity after palmitate treatment was compared to the vehicle treated cells. Data are expressed as mean ± SEM (n = 9).

3.4 Restoring PRCP Expression in Cardiomyocytes

3.4.1 The Effect of Insulin on Palmitate-Induced PRCP Inhibition in H9c2 Cardiomyocytes

Unlike our model, mammalian heart is a complicated system influenced by hormones and other surrounding systems. Under physiological conditions, insulin regulates cardiac substrate utilization by stimulating glucose and fatty acid uptake and metabolism in the heart. Nearly 70% to 90% of the ATP in the heart is produced by fatty acids (FAs) β-oxidation. Oxidation of glucose, lactate, ketone bodies and certain amino acids accounts for the remaining 10% to 30% (Doenst et al. [104], Abel et al. [66], Mandavia et al. [105] and Bayeva et al. [106]). Although FAs are the main and most efficient source of energy for the heart, yet a balance between FA and glucose usage is needed for the maintenance of heart performance. High plasma free fatty acids concentration and insulin resistance as seen in obesity and diabetes, result in a change in substrate utilization by the heart with 100% reliance on FAs oxidation for ATP synthesis. In addition to energy balance, insulin also promotes cardioprotection as discussed earlier in Fig. 1.3. Therefore, insulin resistance will deteriorate cardiac function promoting atherosclerosis, cardiac hypertrophy, cardiomyopathy and hypertension. PRCP on the other and, could have insulin sensitizing effects by stabilizing IRS-1 (Duan et al. [19]) and metabolizing Ang II. Furthermore, both PRCP and insulin improve cardiac health. Therefore, finding a possible link between insulin and PRCP pathways might tremendously benefit us in targeting cardiovascular diseases in metabolic syndrome. We hypothesized that insulin treatment may subside palmitate-induced cardiac PRCP depletion. To address this hypothesis, we first tested PRCP expression alteration after 48 hours insulin treatment (Fig. 3.15(a)). We observed that increasing concentrations of insulin do not alter the basal PRCP expression in H9c2 cells.

Insulin resistance is a reduced glucose uptake in the peripheral tissues that would result initially in a hyperinsulinemic state. High fatty acid concentration could exacerbate insulin resistance by impairing insulin signaling in the heart muscle. Several mechanisms have linked

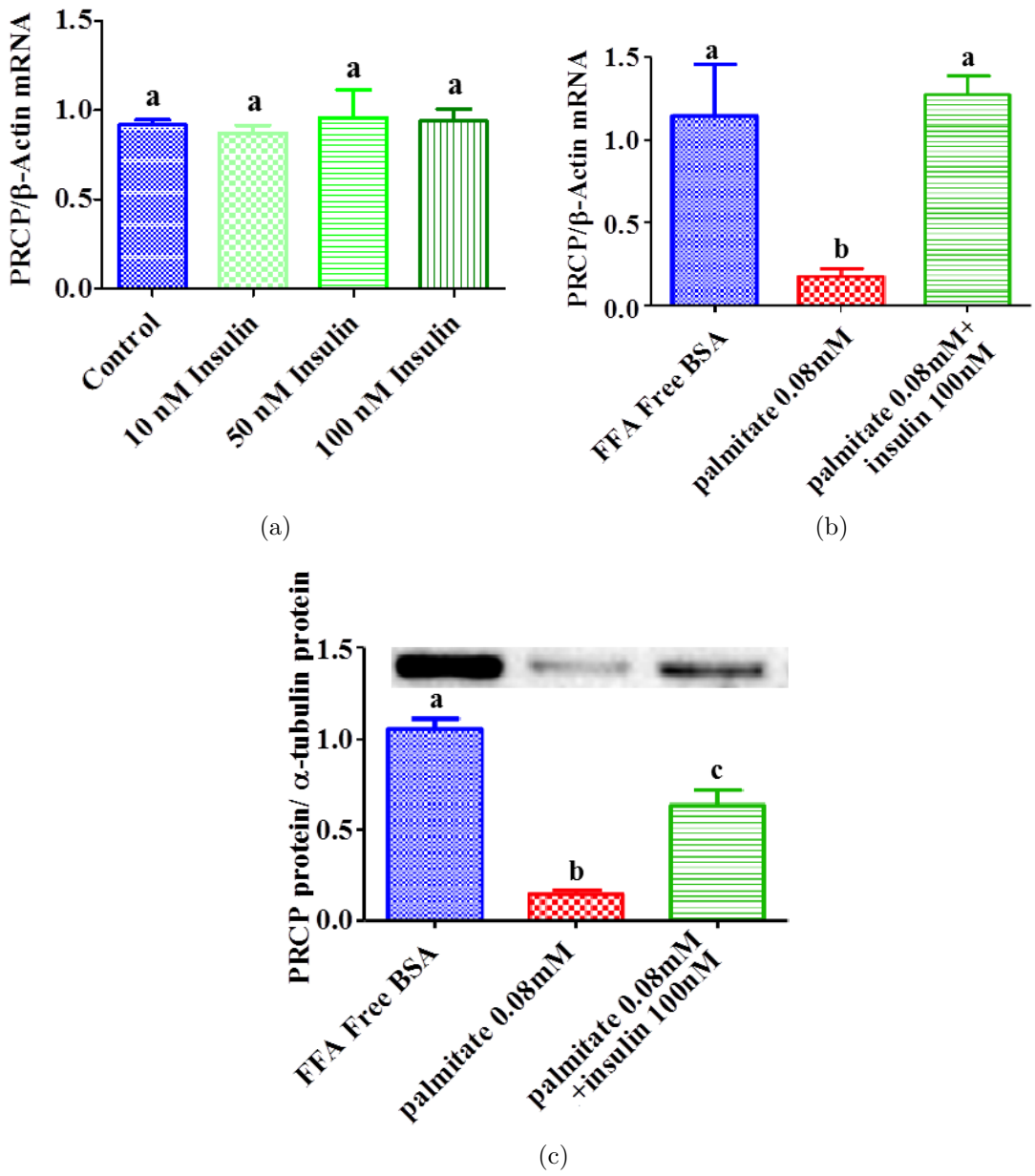


Figure 3.15. The Effect of Insulin on PRCP mRNA Expression.
(a) Increasing concentrations of insulin do not alter H9c2 PRCP expression. Cardiomyocytes were incubated with insulin at 10 nM, 50 nM and 100 nM concentrations for 48 hours. Total RNA was extracted and the fold change in PRCP mRNA was normalized to β-actin mRNA. (b) Insulin restored palmitate-induced PRCP mRNA expression inhibition. H9c2 cells treated for 48 hours with 0.08 mM palmitate in the absence or presence of 100 nM insulin, underwent RNA extraction and PRCP mRNA was normalized to β-actin mRNA. (c) Insulin restored palmitate-induced PRCP protein expression inhibition. Western blot analysis of H9c2 cells treated for 48 hours with 0.08 mM palmitate in the absence or presence of 100 nM insulin were performed and PRCP protein was normalized to α-tubulin protein. Data are expressed as mean $\pm$ SEM (n = 3). Bars with the same letter are not statistically different (ANOVA, $P < 0.05$).

excess lipid levels to insulin resistance such as increased ROS and diacylglycerol (DAG)-induced protein kinase C (PKC) activation (Abel et al. [66]). Therefore, we explored the effect of insulin on palmitate-induced altered PRCP expression. Interestingly, we found that 100 nM insulin treatment for 48 hours could subside palmitate-induced PRCP inhibition, at both messenger RNA (Fig. 3.15(b)) and protein (Fig. 3.15(c)) level, suggesting a role for insulin downstream signaling in PRCP regulation in the cardiomyocytes.

3.4.2 The Effect of Thyroxine on Palmitate-Induced PRCP Inhibition in H9c2 Cardiomyocytes

Aside from insulin, thyroxine is also an important regulator of energy balance and lipid metabolism in the body. Thyroid hormone acts through its ligand activated nuclear receptor to promote energy expenditure (Cheng et al. [107]) and it also cross talks with other nuclear hormone receptors including peroxisome proliferator-activated receptor (PPAR) and liver X receptor (LXR) (Mullur et al. [108]). More importantly, by activating PI3K-MAPK pathway, thyroid hormone is believed to synergize insulin signaling and increase glucose uptake (Cao et al. [109]). On the other hand, PRCP has been implicated in the regulation of hypothalamus-pituitary-thyroid (HPT) axis. α-$MSH_{1\text{-}13}$, a known substrate of PRCP, is believed to activate thyrotropin-releasing hormone (TRH) neurons in the hypothalamus, enhancing thyroid hormone release and energy expenditure. Jeong et al. [65] observed that PRCP ablation resulted in an increased circulating level of free T4 and not T3 in the mice, suggesting an interplay between thyroxine (T4) and PRCP. Therefore, we characterized the effect of T4 on palmitate-induced H9c2 PRCP inhibition. Although increasing concentrations of thyroxine did not alter PRCP mRNA expression (Fig. 3.16(a)), co-treatment of T4 and palmitate, significantly increased PRCP mRNA and protein expression (Fig. 3.16(b) and Fig. 3.16(c)). Based on our results, it appears that multiple molecules have the ability to regulate PRCP expression in cardiomyocytes in nutrition overload states.

To summarize and further support our findings in previous sections, we tested the long term effect of palmitate, insulin and T4 on PRCP expression in cardiomyocytes, since

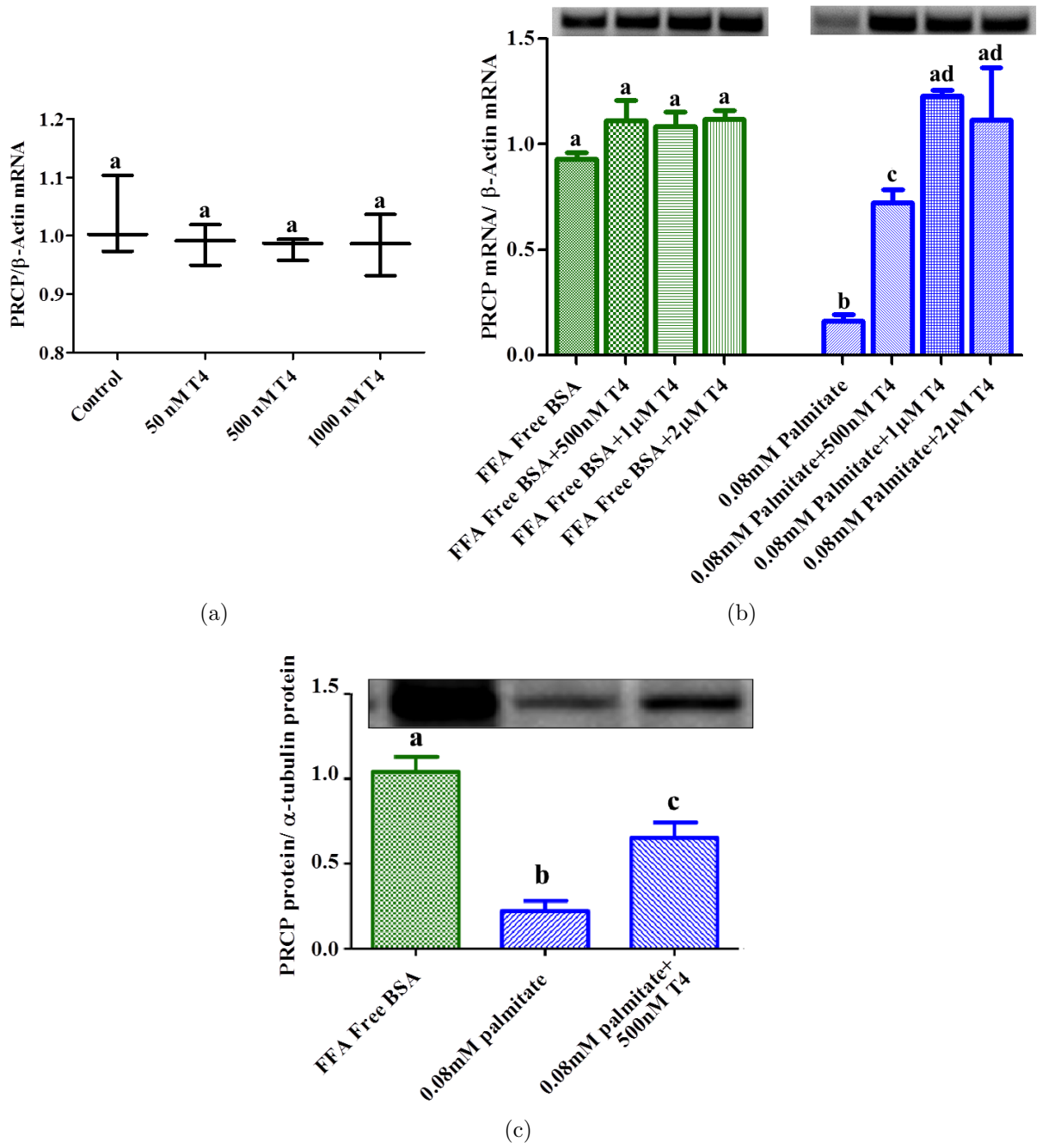


Figure 3.16. The Effect of Thyroxine on PRCP mRNA Expression.

(a) Increasing concentrations of thyroxine do not alter H9c2 PRCP expression. Cardiomyocytes were incubated with thyroxine at 50 nM, 500 nM and 1 µM for 48 hours. Total RNA was extracted and the fold change in PRCP mRNA was normalized to β-actin mRNA. (b) Thyroxine restored palmitate-induced PRCP inhibition. H9c2 cells treated for 48 hours with 0.08 mM palmitate in the absence or presence of 500 nM, 1 µM and 2 µM T4 underwent RNA extraction and PRCP mRNA was normalized to β-actin mRNA. (c) Thyroxine restored palmitate-induced PRCP protein expression inhibition. Western blot analysis of H9c2 cells treated for 48 hours with 0.08 mM palmitate in the absence or presence of 500 nM thyroxine were performed and PRCP protein was normalized to α-tubulin protein. Data are expressed as mean ± SEM (n = 3). Bars with the same letter are not statistically different (ANOVA, $P < 0.05$).

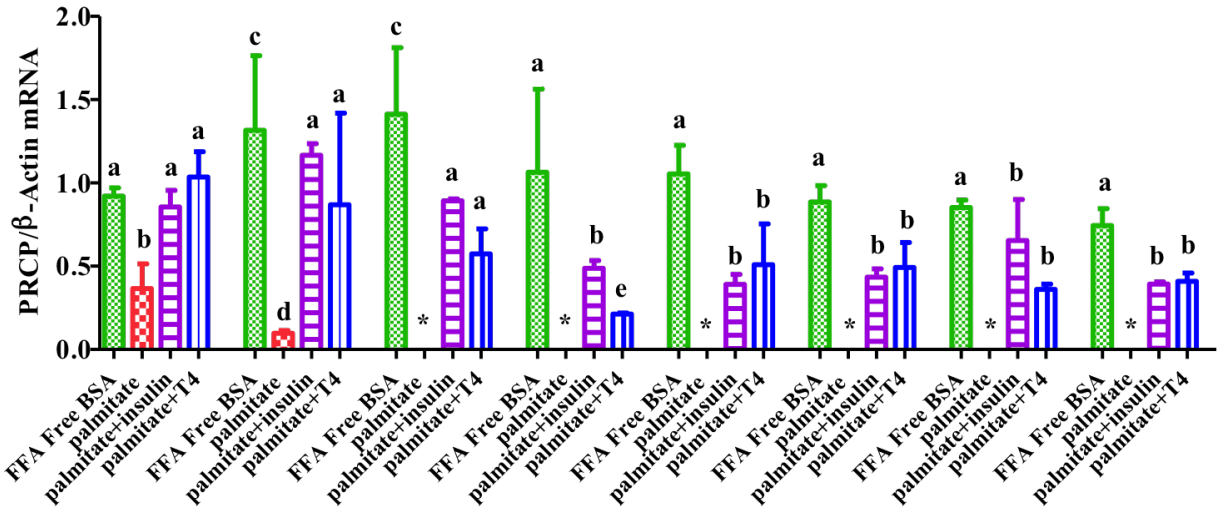
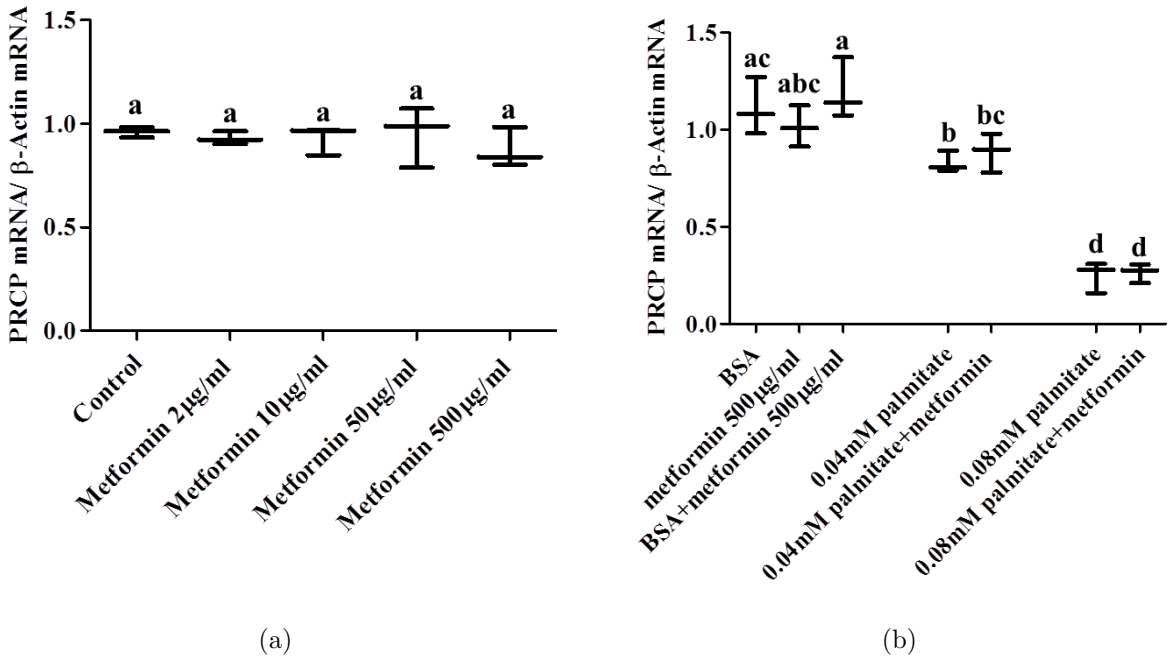


Figure 3.17. Thyroxine and Insulin Restore PRCP Expression in Cardiomyocytes. The differentiated H9c2 cells were treated with 0.08 mM palmitate-BSA in the presence or absence of 500 nM T4 or 100 nM insulin. For 16 days, every 2 days, each flask of cells were subcultured into two flasks and one flask of cells were collected in TRIzol reagent for further RNA analysis. H9c2 PRCP mRNA was normalized to β-actin mRNA for each sample. Data are expressed as mean $\pm$ SEM (n = 3). Bars with the same letter are not statistically different (ANOVA, $P < 0.05$). $*$ denotes that PRCP/β-actin mRNA expression was undetectable.

metabolic syndrome is also a chronic condition. We observed that upon 0.08 mM palmitate treatment, PRCP expression is decreased all the way to undetectable levels and both T4 (500 nM) and insulin (100 nM) restored PRCP expression in H9c2 cells (Fig. 3.17). So far we showed that palmitate, insulin and T4 can regulate the cardiomyocyte PRCP levels.

3.4.3 The Effect of Metformin on Palmitate-Induced PRCP Inhibition in H9c2 Cardiomyocytes

In our diabetic plasma studies, we observed that metformin and insulin-treated diabetic patients had decreased plasma PRCP protein and activity, therefore we tested the effect of metformin on palmitate-induced PRCP depletion in H9c2 cardiomyocytes and observed that metformin-palmitate co-treatment do not alter palmitate-induced suppression of H9c2 PRCP expression (Fig. 3.18).



(a) (b)

Figure 3.18. Metformin Treatment Do Not Restore Cardiac PRCP Expression After Palmitate Treatment.

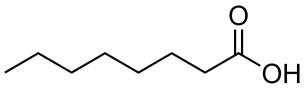
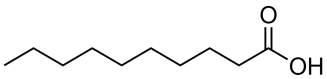
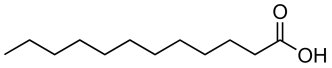
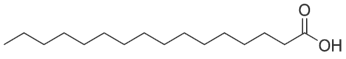
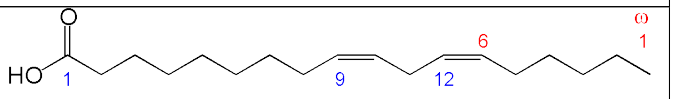
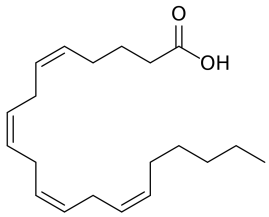
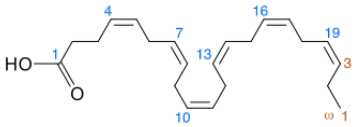
(a) Increasing concentrations of metformin do not alter H9c2 PRCP expression. Cardiomyocytes were incubated with metformin at 2 µg/mL, 10 µg/mL, 50 µg/mL and 500 µg/mL concentration for 48 hours. Total RNA was extracted and the fold change in PRCP mRNA was normalized to β-actin mRNA. (b) Metformin did not alter palmitate-induced PRCP inhibition. H9c2 cells treated for 48 hours with 0.04 mM or 0.08 mM palmitate in the absence or presence of 500 µg/mL metformin underwent RNA extraction and PRCP mRNA was normalized to β-actin mRNA. Data are expressed as mean ± SEM (n = 3). Bars with the same letter are not statistically different (ANOVA, $P < 0.05$).

3.5 Determine the Molecular Mechanism of PRCP Regulation by Palmitate Overload in Rat H9c2 Cardiomyocyte

Obesity is an inflammatory condition as several studies demonstrated an overexpression of the proinflammatory cytokine, tumor necrosis factor-alpha (TNF-α) and elevated plasma levels of other inflammatory mediators (IL-6, IL-1β, CRP, CCL-2, CCL-3 and CXCL-8) in obesity (Diamant et al. [110]). Several mechanisms have been proposed to link adiposity to inflammation such as adipose tissue macrophage infiltration (Xu et al. [111]), oxidative stress-induced activation of nuclear factor kappa-beta (NFκB) (Mohanty et al. [112] and Aljada et al. [113]) and activation of toll-like-receptors (TLR4) (Kim et al. [92] and Donath and Shoelson [114]). Moreover, lipid accumulation in the cardiomyocytes impairs insulin signaling further exacerbating cardiac lipotoxicity by decreasing glucose uptake into the heart muscle (Abel et al. [66]) (Fig. 1.3). Of interest, PRCP also promotes inflammation by releasing BK from HK and increasing nitric oxide and prostacyclin production (Zhao et al. [23], Shariat-Madar et al. [3] and Moreira et al. [115]). PRCP expression is upregulated during inflammation (Kumamoto et al. [32]). As well, Ngo et al. [34] showed that in an endothelial model of inflammation upon LPS treatment, there is a robust increase in PRCP mRNA expression along with increased ICAM-1 expression. In addition, we showed that palmitate can down regulate PRCP expression in the cardiomyocytes. Therefore, what remains elusive is the mechanism behind fatty acid induced PRCP regulation in the cardiomyocytes. To address this question, we studied fatty acid (FA) transport and its downstream signaling in the H9c2 cardiomyocytes. Several transporters are responsible for FA uptake and transport across the cells such as FA binding protein (FABP), FA transport protein (FATP) and CD36 (Angin et al. [116] and Steinbusch et al. [117]). Studies have shown that inhibiting CD36 decreases FA uptake into the cardiomyocyte (Luiken et al. [118]) and the CD36 null mice has 50%-80% lower cardiomyocyte FA uptake (Habets et al. [119] and Coburn et al. [120]). Thus, CD36-associated long chain fatty acid uptake appears to be a critical step in FA transport into the cardiomyoctes. We used a specific inhibitor of CD36, SSO (sulfo-N-succinimidyl

oleate), to block FA uptake into H9c2 cardiomyocytes (Harmon et al. [121]) and tested its impact on palmitate-induced PRCP inhibition. We observed that 100 µM (Luiken et al. [122]) SSO treatment, significantly restored PRCP expression in the cardiomyocytes (Fig. 3.19). We, therefore, concluded that CD36 mediated uptake of palmitate at the cardiomyocytes cell surface is one rate limiting step in regulating cellular PRCP expression.

Table 3.1: The Structure of Tested Saturated and Unsaturated Fatty Acids

Fatty Acid	Number of Carbons	Structure
Caprylic Acid	c:8	
Decanoic Acid	c:10	
Dodecanoic Acid	c:12	
Palmitic Acid	c:16	
Linoleic Acid	c:18	
Arachidonic Acid	c:20	
Docosahexanoic Acid	c:22	

Although controversies exist among investigators, evidence suggests that saturated (especially palmitic acid and lauric acid) but not unsaturated fatty acids can act as ligands for Toll-Like-receptor 4 (TLR4) and TLR2 (Shi et al. [123]), to enhance inflammatory response.

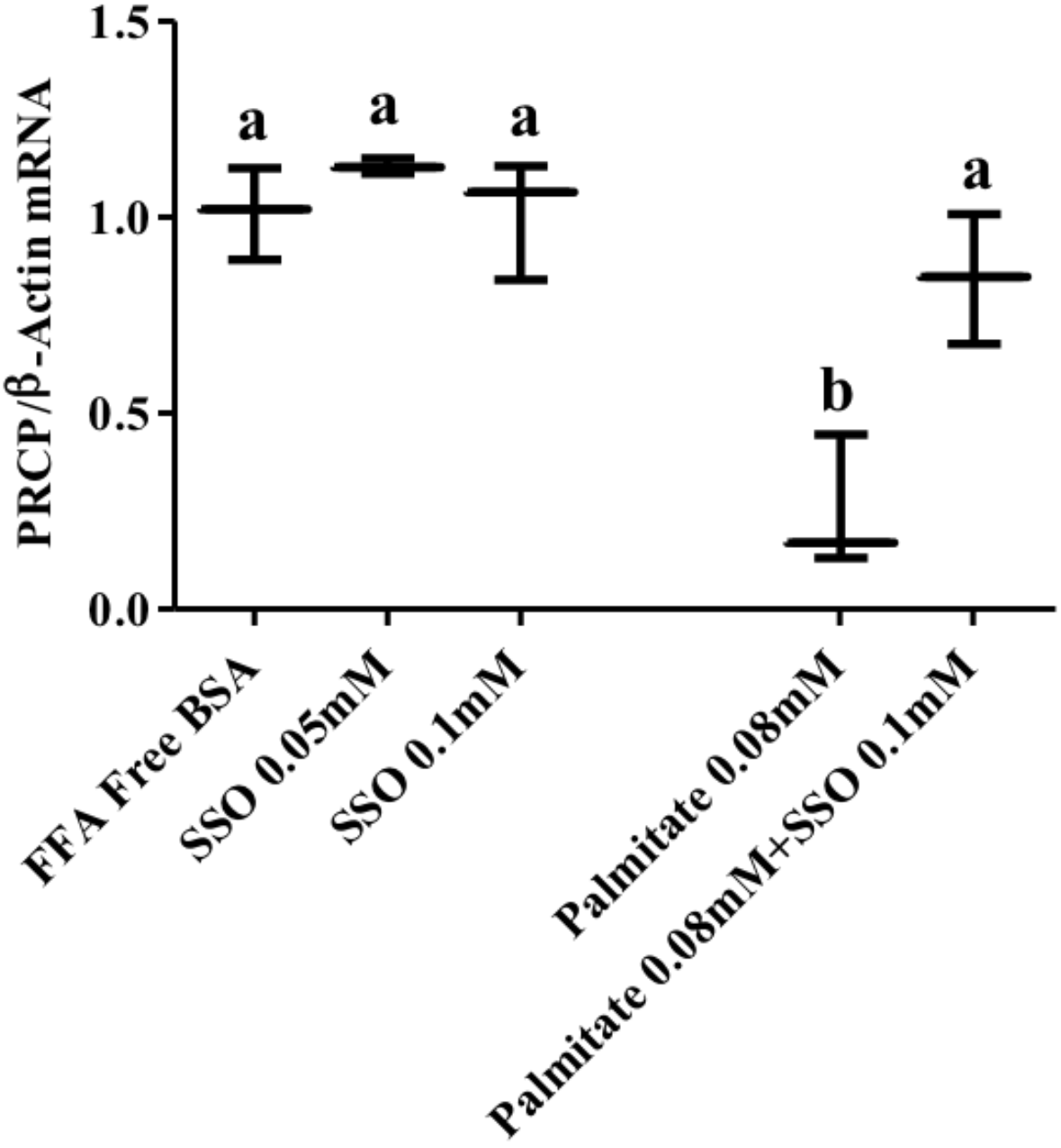


Figure 3.19. Sulfo-N-Succinimidyl Oleate (SSO) Blocks Palmitate-Induced Inhibition of PRCP Expression in Cardiomyocytes.

The differentiated H9c2 cells were treated with 50 and 100 µM SSO, 4 hours prior to 0.08 mM palmitate-BSA or BSA treatment for an additional 44 hours. Extracted PRCP RNA was normalized to β-actin mRNA for each sample. Data are expressed as mean ± SEM (n = 3). Bars with the same letter are not statistically different (ANOVA, $P < 0.05$).

Therefore, if palmitate-induced PRCP regulation is downstream to TLR-NFκB pathway, it is worth to explore the effect of other fatty acids on cardiomyocytes PRCP expression. To determine if the fatty acid-induced cardiac PRCP inhibition is specific to palmitate or other fatty acids can also suppress PRCP expression, we explored the effect of caprylic acid, decanoic acid, dodecanoic acid, linoleic acid, arachidonic acid and docosahexanoic acid (C8-C22) (Table 3.5) on PRCP expression in H9c2 cells. To our surprise, among all the tested saturated and unsaturated fatty acids only palmitate depleted cardiac PRCP expression in the differentiated H9c2 cells (Fig. 3.20). The fact that polyunsaturated fatty acids, like docosahexanoic acids (DHA), can have anti-inflammatory effects via inhibition of TLR-NFκB pathway (De Boer et al. [124]) lead us to explore the PRCP activity after co-exposure of H9c2 cells to DHA and palmitate for 48 hours. We observed that 0.08 mM, 0.12 mM or 0.16 mM DHA do not restore 0.04 mM palmitate-induced decrease in PRCP activity in H9c2 cells (Fig. 3.21).

Ligand-mediated activation of TLRs ultimately leads to the activation of NFκB and its translocation to the nucleus, regulating the expression of proinflammatory cytokines. Therefore, if the palmitate-induced alteration of PRCP expression is also regulated via TLR-NFκB pathway, inhibiting NFκB should restore PRCP levels in the cardiomyocytes. Thus, we tested the effect of BAY 11-7082, a known synthetic inhibitor of NFκB on palmitate-induced decrease in PRCP activity. We observed that 2 µM BAY 11-7082 do not restore palmitate-induced reduction of PRCP activity (Fig. 3.22). Thus based on our mechanistic studies, we can conclude that TLR-NFκB may not be involved in palmitate-induced regulation of cardiomyocyte PRCP expression. Indeed, more studies are needed in the future to clarify the molecular mechanism of PRCP regulation by fatty acids in the cardiomyocytes.

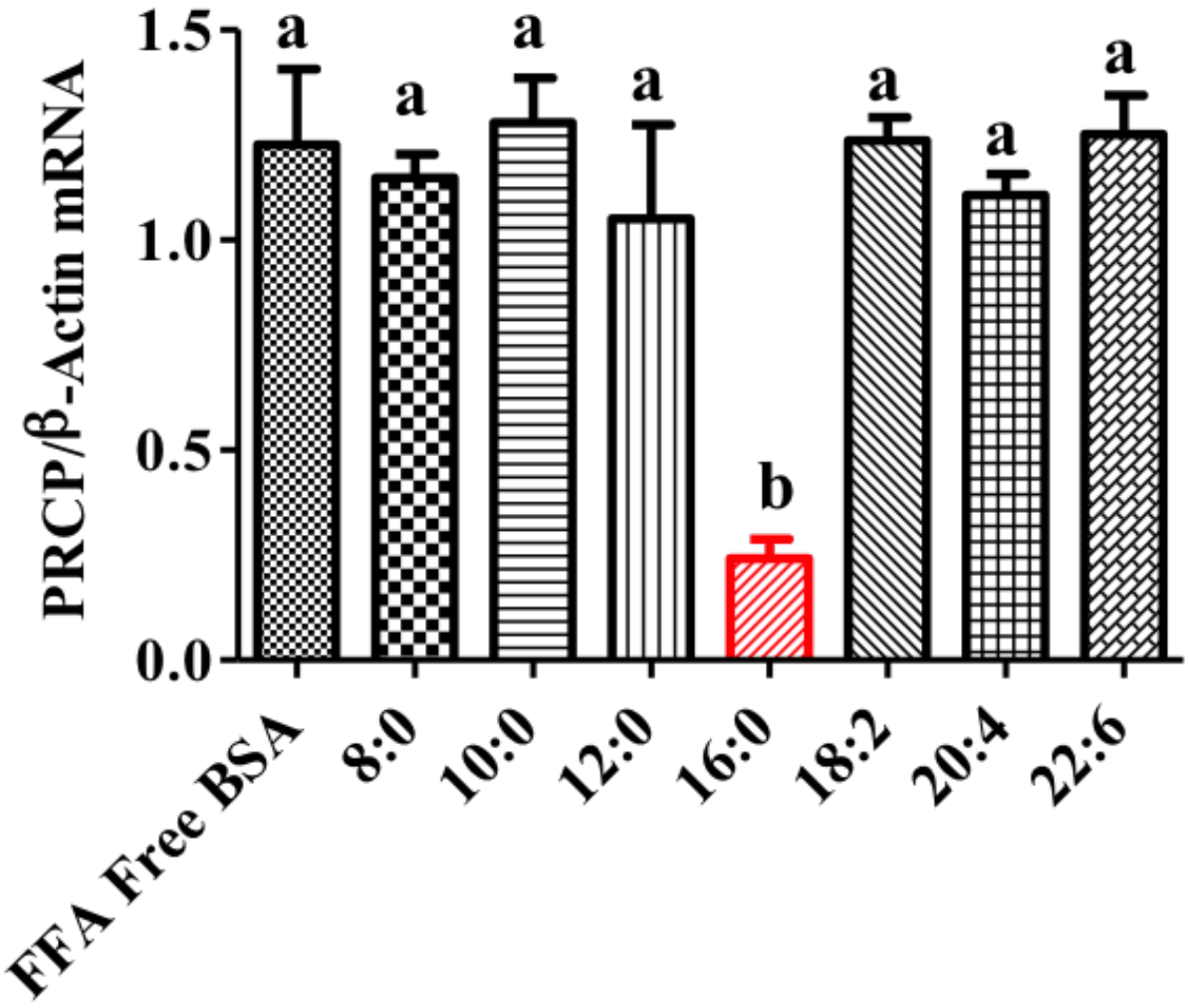


Figure 3.20. Fatty Acids, Except for Palmitate, Do Not Alter PRCP mRNA Expression in H9c2 Cell Line.

The differentiated H9c2 cells were treated with BSA vehicle, 0.08 mM caprylic-BSA, 0.08 mM decanoic acid-BSA, 0.08 mM dodecanoic acid-BSA, 0.08 mM palmitate-BSA, 0.08 mM linoleic acid-BSA, 0.08 mM arachidonic acid-BSA and 0.08 mM docosahexanoic acid-BSA for 48 hours. At the completion of the incubation period, total RNA extraction, RT-PCR and agarose gel studies were performed. PRCP mRNA expression was then normalized to β-actin mRNA expression. For each study group, three different flasks of cells were analyzed and the change in PRCP expression upon fatty acid treatment was compared to the BSA-vehicle. Data are expressed as mean $\pm$ SEM (n = 3). Bars with the same letter are not statistically different (ANOVA, $P < 0.05$).

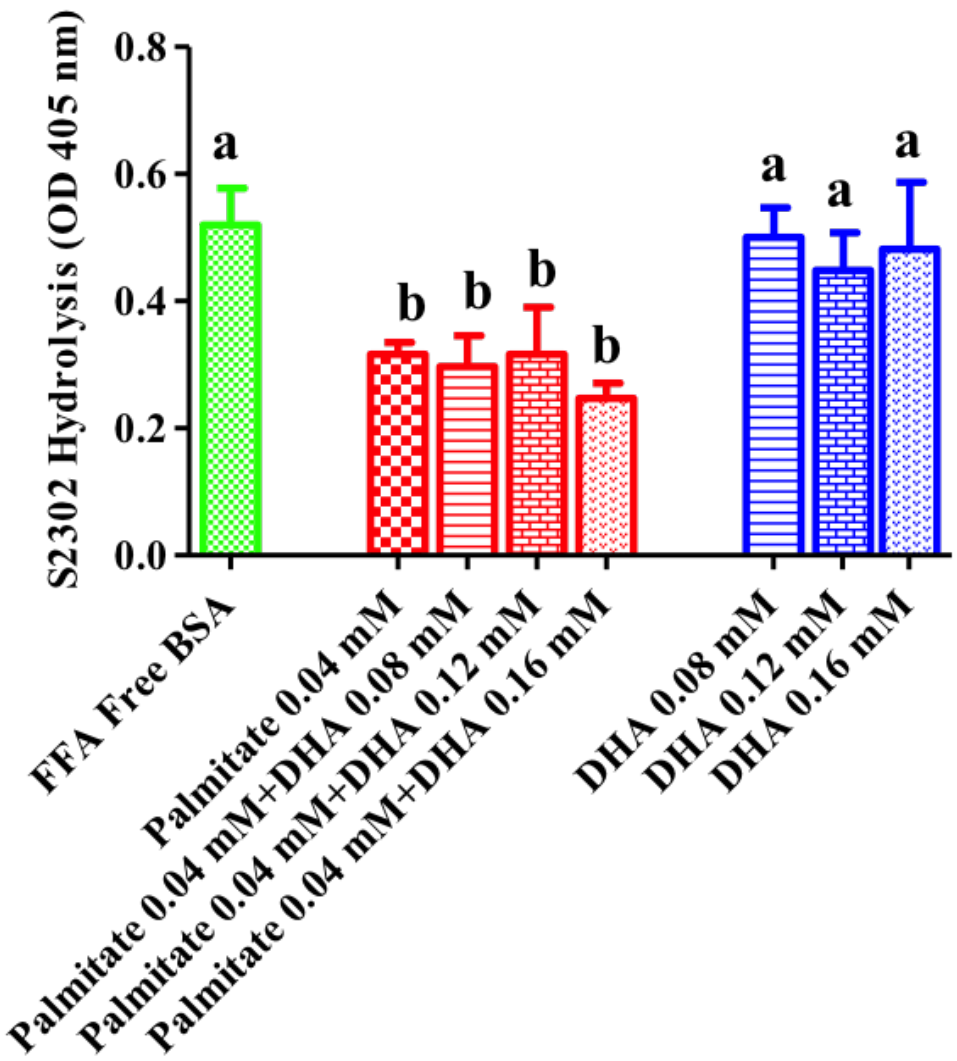


Figure 3.21. Increasing Concentrations of Docosahexanoic Acid (DHA) Do Not Restore Palmitate-Induced Decrease in PRCP Activity.

The differentiated H9c2 cells were seeded overnight in microtiter plates. The next day, cells were treated with BSA vehicle, 0.04 mM palmitate-BSA, 0.08 mM DHA-BSA, 0.12 mM DHA-BSA, 0.16 mM DHA-BSA, 0.04 mM palmitate-BSA plus 0.08 mM DHA-BSA, 0.04 mM palmitate-BSA plus 0.12 mM DHA-BSA or 0.04 mM palmitate-BSA plus 0.16 mM DHA-BSA for 48 hours. At the completion of the incubation period, cells were incubated with 40 nM HK for 1 hour followed by incubation with 40 nM PK for an additional hour. Lastly, the activity of the produced kallikrein was determined by addition of 0.5 mM S2302 (kallikrein chromogenic substrate). Substrate hydrolysis was allowed to proceed for 1 hour at 37 °C. Kallikrein activity was measured as the change in absorbance of the reaction mixture at 405 nm. Data are expressed as mean ± SEM (n = 3). Bars with the same letter are not statistically different (ANOVA, $P < 0.05$).

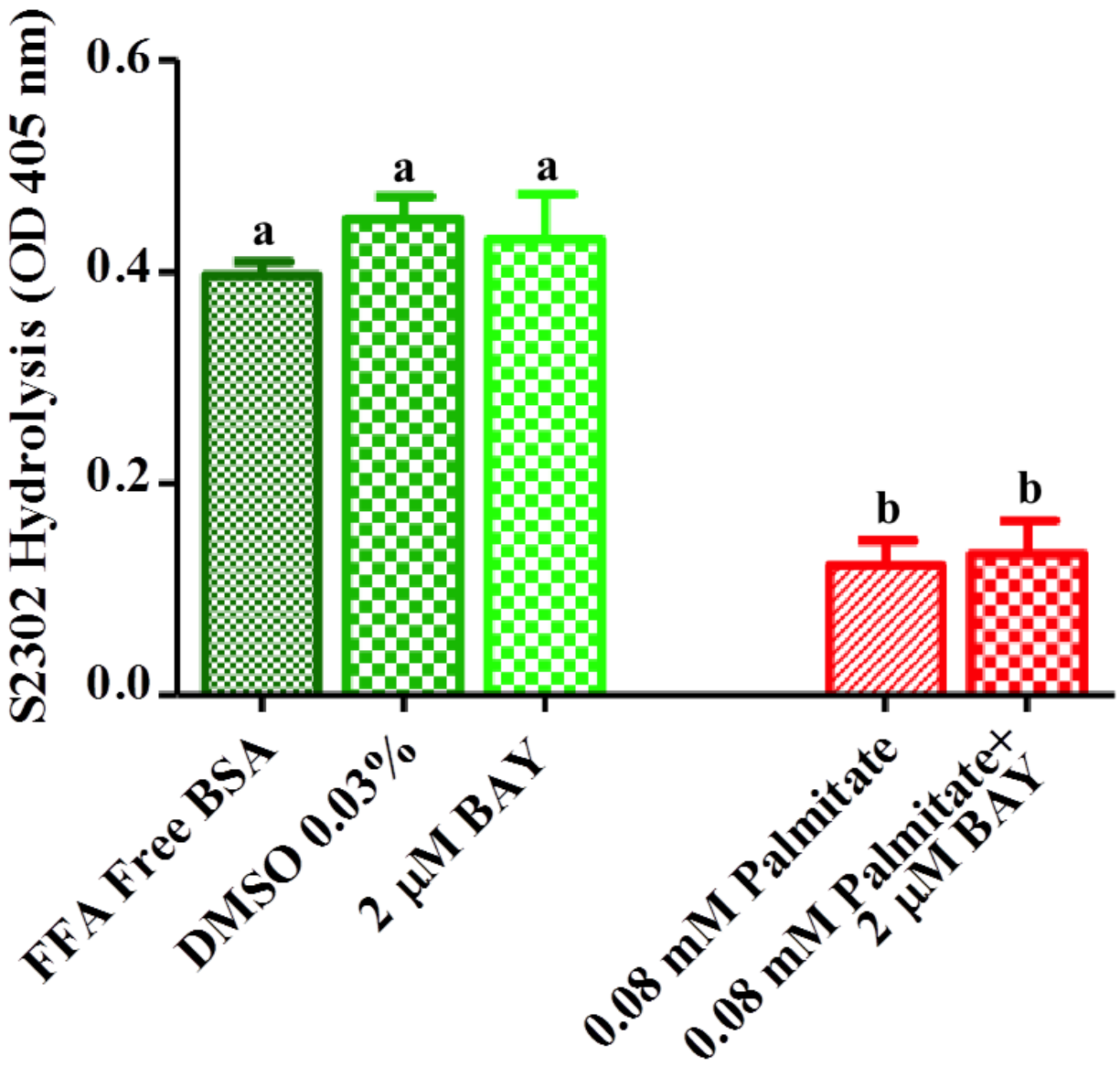


Figure 3.22. NFκB Inhibition via BAY 11-7082 Do Not Restore Palmitate-Induced Reduction of PRCP Activity.

The differentiated cells were seeded overnight in microtiter plates. The next day, cells were treated with BSA vehicle, DMSO 0.03% (BAY 11-7082 vehicle control), 2 µM BAY 11-7082 and 0.08 mM palmitate-BSA in the absence or presence of 2 µM BAY 11-7082 for 48 hours. At the completion of the incubation period, cells were incubated with 40 nM HK for 1 hour followed by incubation with 40 nM PK for an additional hour. Lastly, the activity of the produced kallikrein was determined by addition of 0.5 mM S2302 (kallikrein chromogenic substrate). Substrate hydrolysis was allowed to proceed for 1 hour at 37 °C. Kallikrein activity was measured as the change in absorbance of the reaction mixture at 405 nm. Data are expressed as mean ± SEM (n = 3). Bars with the same letter are not statistically different (ANOVA, $P < 0.05$).

CHAPTER 4

DISCUSSION

Our investigations have helped characterize the PRCP expression pattern in the cardiomyocytes and plasma in high fatty acid and glucose conditions. The major findings of this study are: (1) Plasma PRCP activity and protein level is increased in type 2 diabetic patients; (2) Plasma PK, FXI and kallikrein activity is increased in the uncontrolled diabetic patients and the kallikrein inhibitor, SBTI, blocked plasma kallikrein activity; (3) Metformin and insulin treated patients have lower plasma levels of prothrombotic factors, PK and FXI. (4) PRCP activity increases upon cell differentiation in H9c2 cardiomyocytes; (4) High glucose concentration increases cardiomyocyte PRCP expression in an *in vitro* model of nutrition overload; (5) Saturated fatty acid, palmitate, decreases PRCP expression and activity in the cardiomyocytes without affecting cell viability, and blocking fatty acid transport by the CD36 inhibitor (SSO) subsided PRCP suppression; (6) Unlike metformin, thyroxine and insulin restore palmitate-induced PRCP inhibition in H9c2 cell line; and (7) TLR-NFκB pathway may not be involved in palmitate-induced cardiomyocyte PRCP regulation.

Prolylcarboxypeptidase is a serine protease that was first discovered in 1968. Yang et al. [103] isolated an angiotensinase from lysosomal fractions of swine kidney that could cleave the last amino acid of peptides with a proline-X at the carboxy-terminus, such as Ang II. Since then, several groups have investigated other possible substrates, biological functions and significance of PRCP. Based on these findings PRCP was recognized to modulate inflammatory mediators, blood pressure, angiogenesis, coagulation and, most recently metabolism. In 2009, Wallingford et al. [15] introduced α-$MSH_{1\text{-}13}$, an anorexigenic and anti-inflammatory peptide, as a new substrate for PRCP. Co-expressed with α-$MSH_{1\text{-}13}$ in POMC neurons of the

hypothalamus, Wallingford and coworkers observed that by inactivating α-$MSH_{1\text{-}13}$, PRCP can promote obesity. The Wallingford et al. [15] PRCP deficient mice were leaner and had a significantly lower total body fat pad compared to their wild type control. Later, Rabey et al. [61] confirmed this finding by designing UM8190, a potent inhibitor of PRCP. Food intake was significantly reduced in UM8190-treated mice compared to vehicle treated wild type mice. Implication of PRCP in energy homeostasis made it a novel target in the investigations of metabolic syndrome. Xu et al. [36] purified PRCP from human granulocytes by chromatographic procedures and raised a polyclonal antibody against it. They then developed a sandwich type ELISA to detect PRCP in blood samples of 1004 obese, diabetic or obese diabetic patients and observed a positive association between plasma PRCP concentration and signs and symptoms of metabolic syndrome. Their study pointed to PRCP as a promising biomarker/screening tool for metabolic syndrome. We also observed an increased plasma PRCP activity in diabetic patients, using a different assay for plasma PRCP detection. In an enzymatic assay, plasma from 4 groups of patients (control, metformin-treated, insulin-treated and uncontrolled diabetic patients) were assessed for PRCP activity. We used H-Ala-Pro-pNA (APpNA), a sensitive, stable and commercially available chromogenic substrate of PRCP for our studies. The mechanism of increased PRCP activity in diabetes and its association with glycemic control is not yet understood. One possibility could be due to the lower blood pH of diabetic patients as a result of ketoacid accumulation, since PRCP's optimal activity is at acidic pH. However, we also observed an increased PRCP protein levels in diabetic plasma and therefore the enhanced activity is accompanied by increased plasma concentration of PRCP in diabetes.

There are two possible explanation behind elevated PRCP level and activity in diabetic plasma: Plasma PRCP is increased as (a) a defense mechanism; or (2) a consequence of altered metabolic balance seen in diabetes. As a line of defense, elevated PRCP could have several beneficial impacts (Mallela et al. [38]), especially in the cardiovascular system: (1) Angiotensin II inactivation; Ang II conveys multiple features of cardiovascular dysfunction

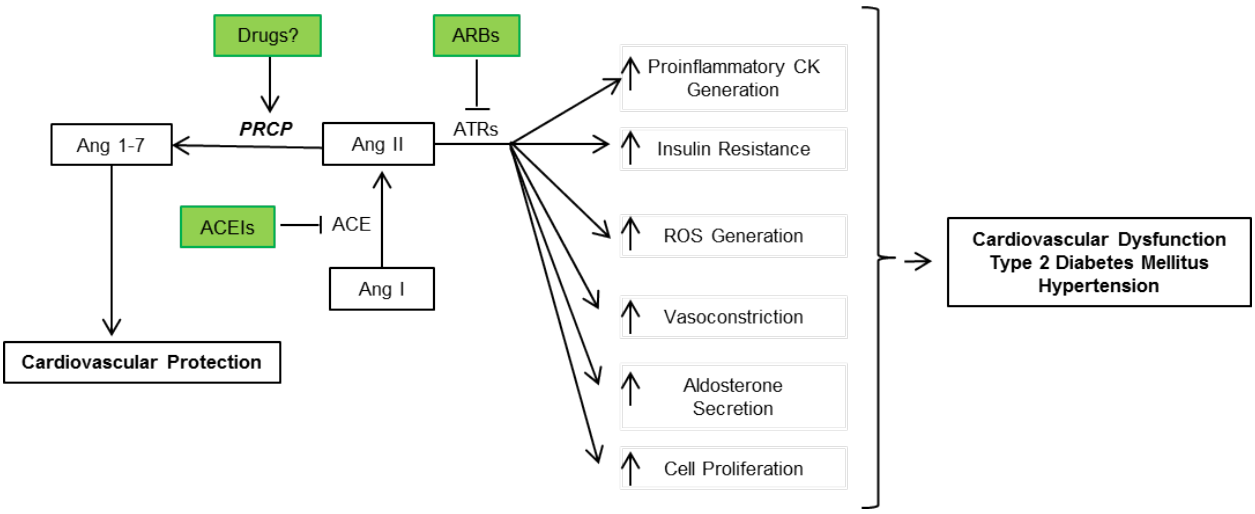


Figure 4.1. The Roles of Angiotensin II in Cardiovascular Dysfunction.
By promoting vasoconstriction, vascular smooth muscle proliferation, aldosterone secretion, ROS generation, insulin resistance and inflammatory cytokine production, Ang II can deteriorate insulin sensitivity and cardiovascular health. ACEIs and ARBs are the available drug groups administered to diabetic and cardiac patients. However, PRCP can also block Ang II-related deleterious side effects by metabolizing it to Ang 1-7, a vasodilator peptide that can itself increase insulin sensitivity. CK: Cytokine, ATR: Angiotensin II Receptor, ARBs: Angiotensin Receptor Blockers, ACEIs: Angiotensin-Converting Enzyme Inhibitors.

and insulin resistance (Fig. 4.1). Angiotensin converting enzyme 2 (ACE2) or PRCP could protect the peripheral tissues from detrimental effects of Ang II by metabolizing it. Interestingly, ACE2 has a basic optimal activity however PRCP is active at neutral and acidic pH. Moreover, metabolization of Ang II results in generation of Ang 1-7, that improves cardiac workload by maintaining coronary artery perfusion and endothelial function (Loot et al. [125]); (2) Angitensin III metabolization; Ang III has actions similar to Ang II, therefore its inactivation by PRCP leads to a decrease in blood pressure; (3) Increasing insulin sensitivity; PRCP can enhance insulin sensitivity by three mechanisms: (a) through inactivation of Ang II; (b) via stabilization of IRS-1 (Duan et al. [19]); and (c) by stimulating BK generation; (4) PRCP causes BK liberation from cell surface HK-PK complex. BK not only has vasodilator effects via increased nitric oxide generation (Shariat-Madar et al. [126]), and improves insulin sensitivity via promoting IRS-1 phosphorylation (Miyata et al. [127]), inhibiting hepatic gluconeogenesis (Barros et al. [128]) and directly enhancing Glut4

translocation to the cell membrane (Kishi et al. [129]), but it also causes a release of tissue plasminogen activator (tPA) which has fibrinolytic and anti-platelet aggregation roles (Pawluczyk et al. [130]). Thus, by increasing BK generation, elevated plasma PRCP in diabetes could have antithrombotic, hypotensive and insulin sensitizing effects; (5) Finally, PRCP decreases ROS generation (Adams et al. [54]), where increased ROS increases insulin resistance and cardiovascular dysfunction in nutrition overload conditions.

However, there are some downsides to the plasma PRCP elevation in diabetes: (1) PRCP metabolizes α-$MSH_{1\text{-}13}$. In the hypothalamus, α-$MSH_{1\text{-}13}$ acts via melanocortin receptors to suppress appetite and decrease inflammation. By inactivating α-$MSH_{1\text{-}13}$, PRCP can increase weight gain in already obese diabetic patients. Furthermore, diabetes is an inflammatory state, and decreased α-$MSH_{1\text{-}13}$ can deteriorate insulin resistance as a consequence of boosted inflammation; (2) Bradykinin generated by PRCP can deteriorate inflammatory response by stimulating prostacyclin (PGI2) generation; and (3) PRCP activates PK to kallikrein, initiating the contact activation of intrinsic coagulation cascade resulting in clot formation. Although, FXII can also activates PK, but increased plasma PRCP concentration and activity could further increase activation of PK toward a more thrombotic state and deteriorating micro and macrovascular complications of diabetes. Metabolic syndrome is a hypercoagulable state. Obesity increases plasma concentration of thrombotic factors FIX, FXI, FXII, vWF and PAI-1 (Plasminogen activator inhibitor-1) (De Pergola and Pannacciulli [131] and Kaye et al. [132]). In addition, there is an increased plasma coagulation factors and a decreased anti-coagulant protein C (Kim et al. [133]) in diabetes. On the other hand, insulin has atheroprotective roles via maintaining healthy endothelial function and increasing nitric oxide generation (Kim et al. [134]). To clarify the possible association between plasma PRCP and kallikrein activity, we investigated PK concentration and activity in diabetic plasma. The diabetes-induced increased plasma PK concentration in our study is supported by other investigators (Kedzierska et al. [83], Clermont et al. [86], Rothschild et al. [135] and Jaffa et al. [85]). We observed a 173% increase in plasma PK levels in uncontrolled diabetic pa-

tients, which was interestingly decreased to 128% and 113% in metformin and insulin treated patients, respectively. There are three possible causes of increased plasma PK in diabetes: (1) Increased urinary loss of c1-inhibitor, the endogenous kallikrein inhibitor, in patients with albuminuria as suggested by Jaffa et al. [85]; (2) Decreased PK activation; and the most likely reason: (3) An increased liver PK synthesis. We can not comment on the urinary loss of kallikrein inhibitor, because we do not have the data from the examined plasma samples; We rejected the second hypothesis as we observed a robust increase in kallikrein activity in uncontrolled diabetic patients, measured by S2302 hydrolysis. Moreover, increased PRCP activity as seen in our results, can further increase PK activation; PK is synthesized by the liver and studies suggest that maintained hyperglycemia stimulate hepatic PK synthesis (Jaffa et al. [85], Federspil et al. [84] and Kedzierska et al. [83]), unless the patient has liver failure. Moreover, Federspil et al. [84] observed that the higher the plasma glucose level the more hepatic PK synthesis. This is in agreement with our results since the uncontrolled and non-compliant diabetic patients had the highest plasma PK levels and activity. Alternatively, Jaffa et al. [85] found a positive association between plasma PK and A1c (glycated hemoglobin), which is an indicator of long term glucose control. Therefore, it is inferred that the higher plasma PK in diabetic patients can be due to an increased liver PK synthesis and that PK level is positively associated with plasma glucose levels. In addition, metformin and insulin treated patients had a significantly lower plasma PK concentration compared to the uncontrolled patients and more importantly the kallikrein activity in these groups were similar to the control. Metformin, the most widely used oral hypoglycemic agent, decreases plasma glucose by inhibiting hepatic gluconeogenesis, improving insulin sensitivity, attenuating plasma fatty acid concentration and maintaining pancreatic β-cells functions (Bailey [136], Bailey and Turner [137] and Cusi and DeFronzo [138]). In a study of 4075 diabetic patients in UK, metformin treatment decreased the risk of microvascular complications and mortality in diabetes (Group et al. [139]). The antithrombotic effects of metformin can be due to the inhibition of PAI-1 production from adipose tissue (He et al. [140]), however no

studies have investigated the influence of metformin on plasma PK. Based on these findings, we can speculate that: (1) Tight glucose control can decrease plasma PK levels and result in a decreased risk of microvascular complications of diabetes; or (2) Insulin and metformin might have the ability to alter hepatic PK synthesis and/or plasma kallikrein activity or their PK-lowering effects might be due to the decreased glycemia. Better understanding of PK alterations by metformin and insulin need further studies, however we can conclude that blood glucose control may be one underlying cause of decreased PK upon metformin and insulin treatment since Rothschild et al. [135] also observed a marked decrease of plasma PK as early as 4 hours after insulin treatment in streptozotocin-induced diabetic rats. FXI, a serine protease member of the intrinsic coagulation cascade, is also synthesized by liver. FXII and thrombin activate FXI to FXIa, which in turn promotes clot formation by activating FIX. Therefore elevated plasma PK activation to kallikrein can increase FXII activation, and result in an elevation of plasma FXI activation. Moreover, like PK, sustained hyperglycemia could be another reason of increased FXI synthesis by the liver. Several studies have linked the elevated plasma FXI in diabetic patients (Merlo et al. [141] and Mard-Soltani et al. [91]) to the increased risk of thrombosis (Yang et al. [142]) and coronary heart disease (Berliner et al. [143], Merlo et al. [141]). We found that both insulin and metformin treated patients had significantly lower plasma FXI, raising the possibility of the hyperglycemia-induced increased liver FXI synthesis in diabetes. Our findings emphasize on the importance of tight glycemic control for the prevention of diabetes vascular complications since PK and FXI are major prothrombotic factors. However, it is possible that insulin and metformin can alter plasma PK and FXI by other mechanisms rather than lowering glucose levels. Therefore, more studies are needed to characterize the molecular pathway of insulin and metformin-induced decrease in PK and FXI levels.

Alternatively, the increased plasma PRCP levels in diabetic patients could be a consequence of metabolic imbalance, either hyperglycemia, hyperlipidemia or generally insulin resistance conditions. Both hyperglycemia and hyperlipidemia promote oxidative stress in

the peripheral tissues. Elevated plasma fatty acid stimulates NADPH oxidase (NOX), induces ROS production and treatment with NADPH oxidase inhibitor significantly decreases ROS levels in obese animals (Furukawa et al. [144]). Enhanced ROS level lead to uncoupling of mitochondria and a decreased mitochondrial ATP production (Boudina and Abel [145]). Furthermore, elevated ROS can deteriorate peripheral tissues insulin sensitivity. High glucose concentration can also enhance ROS generation by several mechanisms. Advanced glycation end products (AGEs) through their specific receptors (RAGE), stimulate NOX and ROS release (Goldin et al. [146]). In addition, hyperglycemia raises inducible nitric oxide synthase (iNOS) expression and further ROS generation (Ceriello [147]). Increased ROS levels due to hyperglycemia and/or hyperlipidemia promotes organ failure resulting in exacerbation of metabolic syndrome complications such as renal failure, cardiomyopathies, atherosclerosis, etc. On the other hand, ROS generation is enhanced in PRCP deficient mice, implicating a role for PRCP as an anti-oxidant probably through increased BK-mediated eNOS activity (Adams et al. [54]). In addition, PRCP is expressed not only at the endothelial cell surface but also in the lysosomes. Therefore, it is possible that in nutrition excess conditions, more PRCP is released from lysosomes into the plasma as an anti-oxidative mechanism to protect ROS-sensitive tissues such as kidney, vasculature and heart. Insulin maintains vascular function by increasing endothelial nitric oxide bioavailability and regulating blood flow (Kim et al. [148]). However, insulin resistance and the accompanying ROS generation could damage endothelial cells. Thus, diseased endothelial cells can be another possible route for increased plasma PRCP in diabetes.

To characterize the consequences of nutrition imbalance on PRCP expression, we chose rat H9c2 cardiomyocyte cell line for our further studies, since: (1) Heart disease is the leading cause of death in metabolic syndrome; (2) In spite of medical treatment and life style changes, yet the mortality rate of metabolic syndrome tend to rise due to the diabetes and obesity epidemic in the United States; (3) PRCP is implicated in metabolism through inactivation of α-$MSH_{1\text{-}13}$ and regulation of thyroid axis; (4) PRCP expression is related

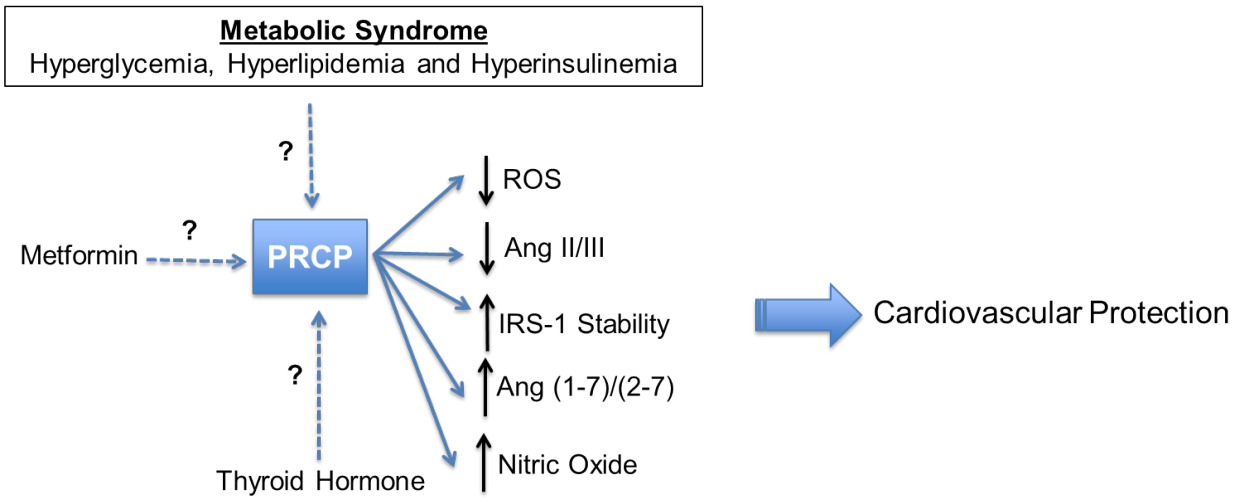


Figure 4.2. Alterations of Cardiac PRCP Expression in Metabolic Syndrome. PRCP protects the heart by inactivating Ang II and Ang III, stabilizing IRS-1, blocking ROS production and promoting nitric oxide generation. However, we propose that increased plasma concentrations of glucose, fatty acids and insulin (as seen in obesity and diabetes) as well as thyroid hormone or hypoglymeic agent, metformin may impair or restore cardiac outcome by altering PRCP expression in the heart.

to acute coronary syndrome in men (McCarthy et al. [35]); (5) PRCP polymorphisms have been linked to preeclampsia and essential hypertension (Zhang et al. [52], Wang et al. [50] and Wu et al. [51]); (6) PRCP deficient mice had a prothrombotic phenotype (Adams et al. [54]); (7) Plasma PRCP level is positively correlated with carotid plaque formation and posterior wall thickness of the heart; and finally, (8) PRCP is suggested as a cardioprotective enzyme (Mallela et al. [38]) via several mechanisms, therefore we hypothesized that an altered cardiac PRCP expression might be an underlying cause of nutrition overload-accelerated cardiovascular dysfunction (Fig. 4.2).

Although the main source of energy for cardiac muscle is fatty acid oxidation, but yet glucose provides 10%-20% of cardiac ATP. In diabetes and obesity, increased circulating fatty acid forces the heart into relying on FA for 100% ATP production, since the mitochondria is already packed with FAs and has no room for glucose oxidation. The fat accumulation in the cardiomyocytes then impairs insulin signaling by IRS-1 serine phosphorylation (Solinas et al. [149]) which further deteriorates glucose uptake by suppressing Glut4 translo-

cation to the cell membrane. Therefore the fat flooded cardiac cell enters a vicious cycle of insulin resistance and lipotoxicity. Finally, fatty acids induce the transcription of fatty acid metabolism genes (PPARα and CD36) (Asayama et al. [150] and Luiken et al. [151]), and proinflammatory mediators (TNF-α, MCP-1, VCAM, ICAM and IL-8) (Badimon et al. [152]). Prolylcarboxypeptidase can also promote inflammation by BK generation and inactivation of α-$MSH_{1\text{-}13}$. In our studies, 80 mM glucose increased PRCP expression in the H9c2 cells, however surprisingly we observed that 0.08 mM palmitate treatment suppressed cardiomyocyte PRCP expression and activity in a time- and dose-dependent manner. Wei et al. [101] observed that palmitate concentrations of less than 0.1 mM did not influence cell viability, however higher concentrations increase ROS levels and apoptosis in H9c2 cells. Our MTT results also showed that 0.08 mM concentration of palmitate did not alter cell viability. Therefore, palmitate-induced cardiac PRCP depletion was not due to palmitate toxicity. With the cardioprotective roles of PRCP in our mind, we concluded that palmitate-induced cardiomyocyte PRCP suppression may exacerbate cardiovascular complications in obesity and diabetes.

As discussed earlier, insulin has cardioprotective roles and insulin resistance is the core denominator of metabolic syndrome. Therefore, we explored the impact of insulin on palmitate-induced PRCP depletion and observed that insulin at 100 nM concentration can restore PRCP expression in H9c2 cardiomyocytes. Binding of insulin to its tyrosine kinase cell surface receptor will finally result in translocation of the major cardiac glucose transporter, Glut4, to the cell membrane and an increased glucose uptake. Although insulin also induces fatty acid uptake through CD36 translocation to the cell membrane, but in a high circulating fatty acid situation, CD36 is already permanently relocated to the cell surface at the expense of complete Glut4 internalization (Ouwens et al. [153] and Ros-Baro et al. [154]). Therefore, insulin stimulation of H9c2 decreases intracellular palmitate pool by tipping the balance in favor of a more glucose uptake instead of fatty acids, and probably affects PRCP expression. In addition, Laat et al. [155] showed that chronic insulin infusion downregulates

toll-like-receptor 4 (TLR4) expression in the heart. Thus another explanation for restoring PRCP expression in heart by insulin could be through negative regulation of TLR4, to which saturated fatty acids are ligands.

Thyroid hormone is another important regulator of cellular metabolism and energy balance. PRCP has also been shown to regulate HPT axis. Jeong et al. [156] observed that in fed state, the PRCP deficient mice had significantly higher plasma free-T4 compared to the wild type animal, however there were no differences in free-T3 levels between the groups. Therefore we characterized the influence of thyroxine (T4) on palmitate-induced H9c2 PRCP depletion and like insulin, we observed an elevation of palmitate inhibited cardiac PRCP expression after T4 treatment. Insulin resistance is closely related to thyroid dysfunction. In a study of 10,920 diabetic patients, there was an 11% increased risk of thyroid disease and type 1 and 2 diabetes had the same frequency of thyroid dysfunction (Perros et al. [157] and Kadiyala et al. [158]). Moreover, hypothyroidism increases the risk of insulin resistance in muscle and adipose tissue (Dimitriadis et al. [159]). Thyroid hormone also stimulates expression of glucose transporters (Glut) (Dimitriadis et al. [160]). Weinstein et al. [161] observed that T3 treatment increases basal and insulin stimulated Glut4 expression and glucose uptake in the skeletal muscle. In a clinical study of women with hypothyroidism, levothyroxine treatment significantly improved insulin sensitivity (Deyneli et al. [162]). Arioglu-Inan et al. [163] showed that T3 treatment improves pancreatic islets morphology, hyperglycemia and diastolic dysfunction in diabetic rats. Furthermore, in a streptozocin-induced diabetic rat thyroid hormone administration markedly surpassed several features of cardiac function post-infarction and this was mediated via increased AMPK and Akt activation, therefore it was suggested that thyroid hormone synergized insulin action and improved cardiac outcome in diabetes (Mourouzis et al. [164]). On the basis of the above evidence, we can conclude that T4-induced restoration of cardiac PRCP expression is a key to a better cardiac function in metabolic syndrome.

In the light of our plasma studies, the decreased PRCP activity in metformin-treated patients and the role of AMPK (the enzyme activated by metformin) as an energy sensor and regulator of metabolism, we next explored the PRCP expression upon metformin-palmitate co-treatment. However, we did not observe any alteration in cardiomyocyte PRCP expression with the addition of metformin. Thus, we concluded that although both metformin and PRCP promote cardioprotection, they may not share identical pathways toward enhancing cardiac health in metabolic syndrome. Moreover, it is possible that the metformin regulatory effect is not at the cardiac PRCP expression level, but metformin might impact PRCP expression or release in other tissues (liver, skeletal muscle, etc) rather than the heart.

For better understanding of the palmitate-accelerated cardiomyocyte PRCP depletion, a detailed molecular mechanism of fatty acid uptake and signaling appears to be necessary. The reason behind choosing palmitic acid for our studies was that palmitate is the most common (saturated) fatty acid found in the body. However to elucidate the specificity of palmitate-induced PRCP inhibition, we explored other saturated and unsaturated fatty acids effect on H9c2 PRCP expression. To our surprise, we found that only palmitate and not other fatty acids, suppresses cardiac PRCP expression. Studies have shown that unlike saturated fatty acids, unsaturated fatty acids (linoleic acid, arachidonic acid and docosahexanoic acid in our study) inhibit NFκB-induced expression of proinflammatory cytokines. Furthermore, unsaturated fatty acids inhibitory effect on inflammation is mediated through toll like receptors (TLR4 and TLR2) (Lee et al. [165]). TLRs are a family of pattern recognition receptors that recognize conserved regions of pathogens (such as LPS) and trigger immune response as well as inflammation. Of the 11 known TLRs (Feng and Chao [166]), all of them have been found in the heart, however TLR4, TLR3, and TLR2 has the highest expression in the heart (Nishimura and Naito [167]. Aside from microbial regions, TLRs have some non-pathogen ligands. For instance, saturated fatty acids (SFA) can activate TLR2 (Nguyen et al. [168]) and TLR4 (Lee et al. [169]), activating NFκB and JNK pathways, finally increasing expression of proinflammatory cytokines. Therefore, SFA-

activated TLR stimulation can enhance insulin resistance. Interestingly, docosahexanoic acid is shown to reduce TLR2 (De Boer et al. [124]) and TLR4 expression (Lee et al. [170]). In conclusion, evidence supports an anti-inflammatory role for unsaturated fatty acids and a pro-inflammatory role for saturated fatty acids both mediated through inhibition or activation of TLR2/TLR4-NFκB-pro-inflammatory cytokine pathway, respectively. Therefore we reasoned that palmitate-specific regulation of PRCP expression might be downstream to TLR-NFκB pathway. Previous studies suggested that saturated fatty acids like palmitic acid (16:0) and lauric acid (dodecanoic acid, 12:0) are the most potent inducers of COX-2, iNOS and IL-1 among all the saturated fatty acids (Lee et al. [165]), however we did not observe any alteration of PRCP expression upon dodecanoic acid treatment. One possible reason might be due to the difference between the cell line used in our study with other studies (using macrophage cell lines) or the expression pattern of TLRs in our cell line. Since some studies (Lee et al. [171]) pointed that lauric acid activates TLR2 dimerized with TLR1 or TLR6, and the dimerization is necessary for TLRs-induced downstream activation of NFκB, the responsiveness of a specific cell line depends on the expression of TLR4, TLR2 and TLR1/6. Yet, it is possible that the palmitate-inhibition of PRCP expression is not downstream to TLR pathway, since lauric acid also activates TLRs but it did not alter PRCP expression in our study.

Mechanisms other that TLR can also mediate fatty acid uptake into the cells. Although some studies suggest passive diffusion as a mechanism for cardiac lipid uptake, less that 20% of the long-chain-fatty-acids (LCFA) are passively diffused into the heart muscle (Glatz and Storch [172]), and the rest is mediated via protein transporters namely (1) FA translocase (FAT/CD36), (2) Plasma membranebound FA binding protein 1 (FABPpm), and (3) FA transporter protein family (FATP1) (Abumrad et al. [173]). Among which, CD36 is specially distributed in organs with highest fatty acid metabolism such as adipose tissue, heart and skeletal muscle (Abumrad et al. [174]). Angin et al. [116] showed that pharmacologic inhibition of cardiac CD36 decreased LCFA uptake and improved cardiac contractility

by switching the heart metabolism toward glucose rather than lipids. Thus, we SSO, a known inhibitor of CD36, and observed that PRCP expression is restored after palmitate-SSO treatment. This finding, helped us to confirm that blocking cardiac palmitate uptake and a consequent decrease in intracellular fat accumulation, positively impacts PRCP gene expression. In addition to LCFA uptake, CD36 can cooperate with TLR2-TLR6 to recognize bacterial ligands (LPS) and further promotes inflammation (Stuart et al. [175] and Hoebe et al. [176]). Therefore, we assume that blocking CD36 also influences downstream inflammation induced by elevated fatty acid-activation of toll like receptors (TLRs).

TLRs have been linked to cardiovascular disorders as well as metabolic syndrome. TLR4 pathway is activated during heart failure (Zhao et al. [177]) and TLR4 deficiency markedly improves insulin sensitivity and cardiac outcome post myocardial infarction (Riad et al. [178]). As well, TLR2 knock out mice have better contractile function after ischemia-reperfusion injury (Sakata et al. [179]). Moreover, TLR4 deficient mice are protected from high fat diet induced-insulin resistance and obesity (Pierre et al. [180]). Jang et al. [181] indicated that TLR2 knock down protects against 0.2 mM palmitate-induced increased expression of TNFα, IL-6 and IL-1β. TLR2 and TLR4 expression has been significantly elevated in T2DM and their expression was positively correlated with body weight (Dasu et al. [182]). With the implication of TLRs in cardiac pathologies and metabolic syndrome, and its activation by circulating fatty acids, we postulated that the effect of palmitate on PRCP expression might be downstream to TLR-MyD88 (myeloid differentiation factor 88)-IRAKs (IL-1R-associated kinases)-NFκB (nuclear factor-B) pathway. If so, NFκB inhibition should protect against palmitate-induced PRCP depletion. Thus, we examined PRCP activity after BAY 11-70820 (an inhibitor of NFκB) and palmitate co-treatment for 48 hours. The observation that 2 µM of BAY 11-7082 did not restore palmitate-induced decrease in PRCP activity along with the fact that lauric acid (another saturated fatty acid activator of TLR) did not alter PRCP expression lead us to the assumption that palmitate-induced regulation of PRCP expression might be through another mechanism and not TLR-NFκB pathway. Lee

et al. [170] observed that in a monocytic cell line, adding unsaturated fatty acids inhibited LPS-induced NFκB activation, however in our studies co-exposure of the H9c2 cell line to palmitate and increasing concentration of DHA (a n-3 polyunsaturated fatty acid) did not restore PRCP activity. Based on these findings, although we can conclude that palmitate-specific cardiomyocyte PRCP regulation is probably not through TLR-NFκB pathway, the exact molecular mechanisms need to be further studied.

In summary, our findings suggest that excess concentrations of glucose and fatty acid can regulate cardiomyocyte PRCP expression. Palmitate, a 16 carbon saturated fatty acid, can deplete cardiomyocyte PRCP, and lipid metabolism regulators, insulin and thyroxine can restore cardiomyocyte PRCP expression. Our findings can point to a possible mechanism for increased cardiovascular dysfunctions in nutrition overload conditions through altered PRCP expression. Moreover, our human plasma studies suggest PRCP as a possible screening tool for diabetes and a potential FXI/PK-induced increased risk of vascular complications in uncontrolled diabetic patients. Future investigations can help toward better understanding of the mechanistic regulation of PRCP by palmitate. Also, the possible impact of high blood glucose level on increasing PRCP release to the plasma need to be further characterized. Finally, our results highlight the importance of lipid/glucose control in protecting against metabolic syndrome-accelerated cardiovascular problems probably through PRCP regulation.

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