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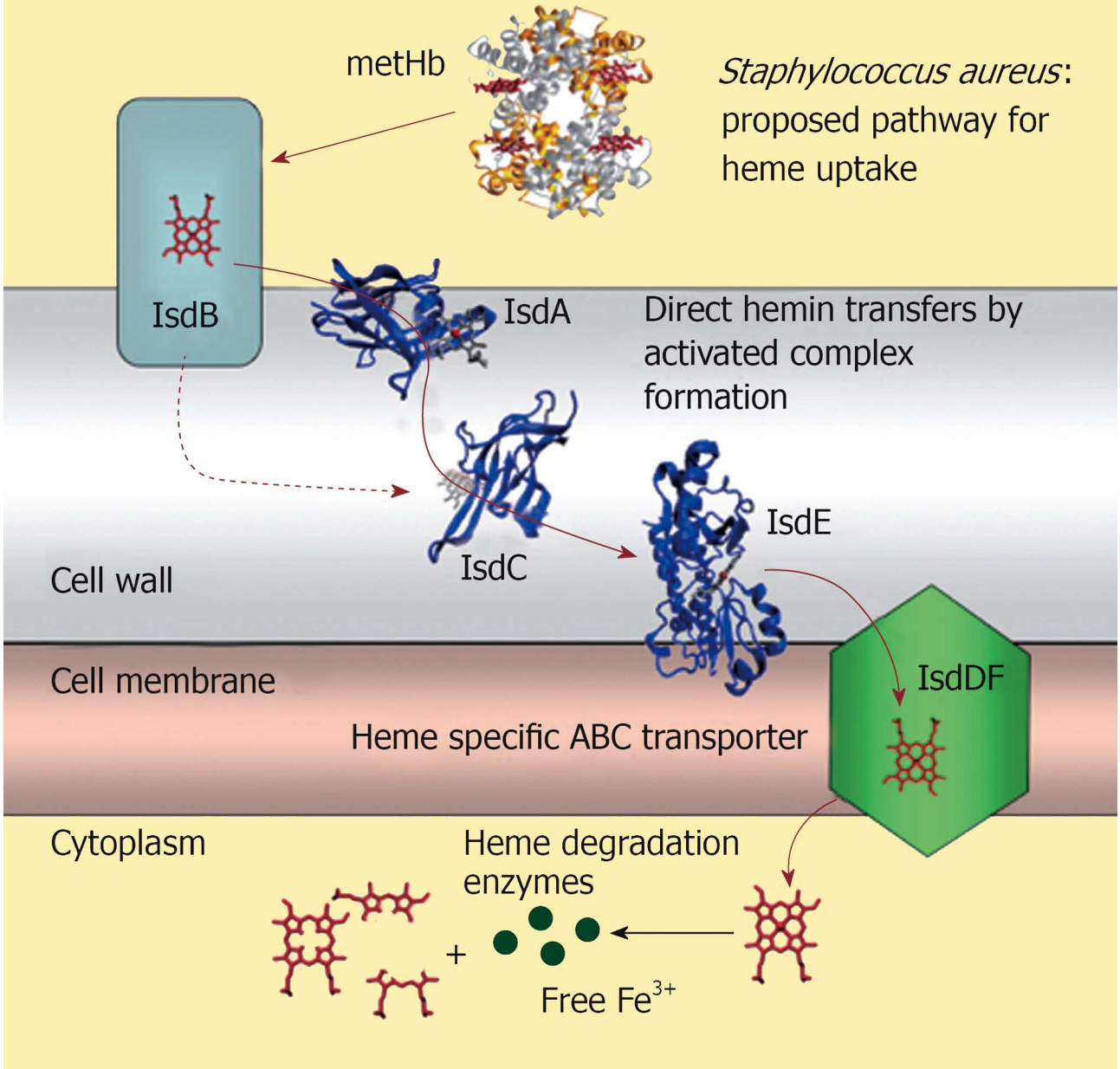
Cartoons for the proposed pathway of heme acquisition from metHb by the *Staphylococcus aureus* Isd system.



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TOPIC HIGHLIGHT

Emanuel E Strehler, PhD, Professor, *Series Editor*

Role of platelet plasma membrane Ca^{2+}-ATPase in health and disease

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Abstract

Platelets have essential roles in both health and disease. Normal platelet function is required for hemostasis. Inhibition of platelet function in disease or by pharmacological treatment results in bleeding disorders. On the other hand, hyperactive platelets lead to heart attack and stroke. Calcium is a major second messenger in platelet activation, and elevated intracellular calcium leads to hyperactive platelets. Elevated platelet calcium has been documented in hypertension and diabetes; both conditions increase the likelihood of heart attack and stroke. Thus, proper regulation of calcium metabolism in the platelet is extremely important. Plasma membrane Ca^{2+}-ATPase (PMCA) is a major player in platelet calcium metabolism since it provides the only significant route for calcium efflux. In keeping with the important role of calcium in platelet function, PMCA is a highly regulated transporter. In human platelets, PMCA is activated by Ca^{2+}/calmodulin, by cAMP-dependent phosphorylation and by calpain-dependent removal of the inhibitory peptide. It is inhibited by tyrosine phosphorylation and calpain-dependent proteolysis. In addition, the cellular location of PMCA is regulated by a PDZ-domain-dependent interaction with the cytoskeleton during platelet activation. Rapid regulation by phosphorylation results in changes in the rate of platelet activation, whereas calpain-dependent proteolysis and interaction with the cytoskeleton appears to regulate later events such as clot retraction. In hypertension and diabetes, PMCA expression is upregulated while activity is decreased, presumably due to tyrosine phosphorylation. Clearly, a more complete understanding of PMCA function in human platelets could result in the identification of new ways to control platelet function in disease states.

Key words: Plasma membrane Ca^{2+}-ATPase; Human platelets; Ca^{2+} transport; Signaling; Cytoskeleton; Phosphorylation; PDZ domain

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INTRODUCTION

The normal physiological function of platelets is to maintain hemostasis, which is accomplished by platelet aggregation and initiation of clot formation at the site of damage to a blood vessel. Aberrant platelet function has severe pathological consequences because platelet-mediated thrombus formation leads to heart attacks and strokes. An increase in intracellular Ca^{2+} is a major signaling event in the activation of platelets, the plasma membrane Ca^{2+}-ATPase (PMCA) prevents inappropriate activation by maintaining low cytoplasmic Ca^{2+}. This review describes

the role of PMCA in resting and activated platelets in health and disease.

PLATELET Ca^{2+} SIGNALING

An increase in intracellular calcium ($[Ca^{2+}]_i$) is a major signal for platelet activation and accompanies activation by all agonists under physiological conditions[1]. Ca^{2+} is first released from intracellular stores termed the dense tubular system in response to formation of inositol trisphosphate from plasma membrane phosphatidyl inositol-(4,5)-bisphosphate. This step is mediated by activation of G-protein-coupled receptors and activation of phospholipase C. Release from intracellular stores is followed by influx of extracellular Ca^{2+} *via* store-mediated Ca^{2+} entry and the P2X receptor. The increase in $[Ca^{2+}]_i$ is very rapid (milliseconds to seconds) followed by a slower return to lower levels brought about by PMCA and SERCA (Ca^{2+}-pump located in the dense tubular system). In platelets, the plasma membrane Na^+/Ca^{2+} exchanger and mitochondria do not contribute significantly to reduction in $[Ca^{2+}]_i$[1], although the exchanger contributes to calcium influx in collagen-activated platelets[2]. Modulation of $[Ca^{2+}]_i$ has been shown to be an important regulator of platelet structural changes following the initial aggregation process[3], and of thrombus growth[4]. Jackson and colleagues have demonstrated that prolonged elevation of $[Ca^{2+}]_i$ and resultant calpain activation causes platelet fragmentation and formation of microbodies that limits the growth of the thrombus in collagen-activated platelets under flow conditions. PMCA is regulated by calpain, as described in more detail below. Furthermore, platelet-platelet interactions in the growing thrombus lead to Ca^{2+} signaling throughout the thrombus, which is required for individual platelets to remain bound to the thrombus. Thus, the rate at which PMCA pumps Ca^{2+} from the platelets after activation probably has important functional consequences for platelet activation, thrombus formation and thrombus maintenance.

PMCA IN HUMAN PLATELETS

PMCA is a P-type plasma membrane Ca^{2+}-pumping ATPase that contains 10 transmembrane sequences. Most of its protein mass is in the cytoplasm. Importantly, PMCA is stimulated by Ca^{2+}/calmodulin and therefore responds directly to $[Ca^{2+}]_i$[5-7]. The accepted function of PMCA is to maintain low $[Ca^{2+}]_i$ by catalyzing ATP-dependent Ca^{2+} efflux, although new roles have recently been identified such as organization of protein complexes at the plasma membrane[8]. There are four independent genes from which alternatively spliced isoforms are expressed, which results in a total of approximately 30 varieties of PMCA[6,7]. Isoforms PMCA1b and PMCA4b have both been detected in human platelets[9,10], although the expression of PMCA1b appears to be very low[11]. Thus, PMCA4b is the major isoform that provides for Ca^{2+} efflux in the human platelets. Johansson *et al*[12] and more recently Rosado *et al*[13] have demonstrated the prominent role of PMCA in Ca^{2+} extrusion in human platelets and the lack of contribution of Na^+/Ca^{2+} exchange. It has been shown that PMCA in human platelets is regulated by cAMP-dependent phosphorylation[9], tyrosine phosphorylation[9,14-17], and blood pressure[16-19], as shown in Table 1. It is clear that PMCA plays an important role in regulating platelet $[Ca^{2+}]_i$ and that control of PMCA activity has significant functional consequences[9,13] that are discussed in more detail.

Table 1 Modes of regulation of plasma membrane Ca^{2+}-ATPase in human platelets

Type of regulation	Effect on PMCA	Ref.
Ca^{2+}/calmodulin	Stimulation	[20]
Protein-kinase-A-dependent phosphorylation	Stimulation	[9,12]
Tyrosine phosphorylation	Inhibition	[9,13,16,21]
Calpain-mediated cleavage (124 kDa)	Stimulation	[22]
Calpain-mediated cleavage (100 kDa and smaller)	Inhibition	[22]
Transcriptional/translational	Up- or downregulation depending on isoform	[23,24]

PMCA: Plasma membrane Ca^{2+}-ATPase.

REGULATION OF PMCA BY PHOSPHORYLATION

cAMP

In 1992, Johansson *et al*[19] demonstrated that increased platelet cAMP led to an increased rate of Ca^{2+} extrusion, although direct phosphorylation of PMCA was not demonstrated. PMCA is indeed a substrate for protein kinase A, and phosphorylation results in activation of pump activity[25]. In 1997, Dean *et al*[9] demonstrated directly that treating platelets with prostaglandin E1, which raises platelet cAMP levels, resulted in direct phosphorylation of PMCA, which could explain the earlier observation of enhanced Ca^{2+} efflux[19].

Tyrosine phosphorylation

Dean *et al*[9] also have demonstrated that PMCA is phosphorylated at tyrosine residues during platelet activation by thrombin. The kinetics of tyrosine phosphorylation during platelet activation correspond with activation of focal adhesion kinase (FAK) and rearrangement of the cytoskeleton. Phosphorylation occurs on Tyr 1176 in PMCA 4b when purified PMCA is treated with the tyrosine kinase src *in vitro*, which results in significant inhibition of PMCA activity[9]. Rosado *et al*[13] have confirmed our observation of PMCA tyrosine phosphorylation during platelet activation and concomitant inhibition of Ca^{2+} efflux following agonist-mediated increases in $[Ca^{2+}]_i$. Wan *et al*[14] have shown that Tyr 1176 is the only tyrosine residue that is phosphorylated on PMCA4b during platelet activation *in vivo*, and that the probable kinase responsible for this phosphorylation is FAK, based on

the amino acid sequence that surrounds Tyr 1176 and the kinetics of FAK activation. Recently, Bozulic *et al*[21] have demonstrated that inhibition of PMCA4b tyrosine phosphorylation by introduction of an inhibitory peptide of the same sequence as the phosphorylation site on PMCA results in a significant decrease in $[Ca^{2+}]_i$ during platelet activation. Introduction of the peptide inhibited PMCA tyrosine phosphorylation by 60% and significantly delayed the onset of thrombin-mediated platelet aggregation. Taken together, these results demonstrate that tyrosine phosphorylation of PMCA4b during platelet activation inhibits PMCA-dependent Ca^{2+} efflux and provides a positive feedback loop mechanism for enhancing the increase in $[Ca^{2+}]_i$ during platelet activation.

REGULATION OF PMCA BY CALPAIN

It is well established that calpain is activated by the increase in $[Ca^{2+}]_i$ that accompanies platelet activation, and that the proteolytic events that follow calpain activation significantly affect signaling during activation[3]. PMCA has been demonstrated to be a substrate for calpain both *in vitro*[26,27] and in human erythrocytes[28]. Calpain-dependent cleavage of PMCA first removes the C-terminal auto-inhibitory domain that results in formation of a 124-kDa PMCA fragment, whereas further calpain-dependent cleavage catalyzes formation of 100-kDa and smaller fragments[26,28]. The 124-kDa fragment is fully active and no longer regulated by calmodulin, whereas the 100-kDa and smaller fragments are inactive. Thus, calpain has the potential to activate and inhibit PMCA irreversibly. Brown *et al*[22] have demonstrated that PMCA is cleaved to smaller fragments during platelet activation with a time course similar to src and SNAP 23 cleavage by calpain[29,30]. Approximately 60% of PMCA is cleaved within 18 min after activation with thrombin or collagen to 124-kDa and smaller species. The significance of this event during platelet activation is that it maintains a portion of PMCA in a form that is fully active even as $[Ca^{2+}]_i$ is decreasing. In contrast to the 124-kDa species, the 100-kDa form is probably inactive[26,27,31]. Furthermore, the 100-kDa species is also likely to have the C terminus removed and thus become unable to interact with the cytoskeleton (see below). However, it has not been established whether N- or C-terminal fragments are removed to form the 100-kDa species during platelet activation[22]. This regulation of PMCA probably has important consequences for later stages of platelet activation such as aggregation and clot retraction.

PMCA ASSOCIATION WITH THE CYTOSKELETON IN HUMAN PLATELETS

PDZ domains

Several types of protein-protein interactions lead to complexes of membrane proteins with cytoplasmic proteins. One of these interactions utilizes the PDZ domain, a structural domain that was initially discovered in proteins localized to postsynaptic structures in neural tissue[32]. The PDZ domain binds tightly to specific tetrapeptide sequences at the C terminus of membrane-associated proteins. Membrane proteins that exhibit this C-terminal motif include ion channels, neurotransmitter receptors and PMCA[32-34]. Strehler and coworkers have shown that PMCA isoforms 2b and 4b interact with guanylate kinase family proteins *via* their PDZ domains[25,34]. As described below, we have demonstrated that PMCA4b binds to the cytoskeleton in activated platelets *via* its PDZ binding motif[20]. Platelets contain several PDZ-domain-containing proteins including SAP97[35], CLP-36[35,36] and PDZ-GEF1[37]. SAP97 and CLP-36 possess multiple protein interaction domains and thus are able to connect PMCA to the cytoskeleton by interacting with other proteins that are capable of binding to the cytoskeleton, such as band 4.1/spectrin (binds SAP97) and α-actinin (binds CLP-36).

Cytoskeletal rearrangement in platelet activation

Resting platelets exhibit well-defined cytoskeletal structures: cytoplasmic actin filaments and a membrane skeleton located just under the plasma membrane that consists of both actin filaments and microtubules[38]. Upon activation, there is extensive rearrangement of the cytoskeleton; the proportion of total actin in filaments increases rapidly from 30% to 70%[39]. Prior to platelet aggregation, cytoskeletal changes result in altered platelet morphology such as formation of filopodia, aid in secretion of stored contents from granules, and are associated with activation of the fibrinogen receptor, αIIbβ3 integrin[40]. Activation of the fibrinogen receptor results in fibrinogen binding that leads to cell-cell interactions and platelet aggregation. Tyrosine phosphorylation is intimately involved in the process of platelet aggregation and cytoskeletal rearrangement. Seconds after platelets bind an activator such as thrombin, src and syk non-receptor tyrosine kinases are activated. This results in a wave of tyrosine phosphorylation and association of phosphorylated proteins with the cytoskeleton[41]. Activation of αIIbβ3 integrin and subsequent binding of fibrinogen results in a second wave of tyrosine phosphorylation, including activation of FAK and the binding of additional proteins to the cytoskeleton including src, FAK[42] and PMCA[20,43].

PMCA translocation to filopodia

Many platelet proteins become associated with cytoplasmic actin cytoskeleton during platelet activation[38,40,41]. We have also shown this to be true for PMCA[20]. We showed that approximately 75% of the PMCA becomes associated with the cytoskeleton and remains associated long after PMCA tyrosine phosphates are removed. PMCA associated with the cytoskeleton retains Ca^{2+}-ATPase activity which indicates that a change in cellular location could greatly affect local $[Ca^{2+}]_i$. Attachment of ion transporters to the cytoskeleton has been shown to occur in several cell types[44], but our work has provided the first example of PMCA association with the cytoskeleton. We also have shown that association with the cytoskeleton is inhibited

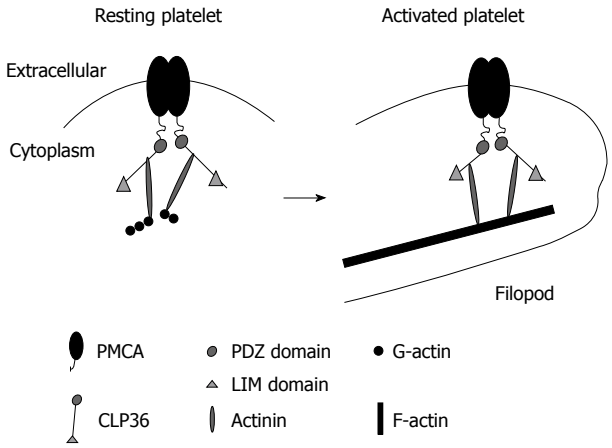

Figure 1 Model for association of plasma membrane Ca^{2+}-ATPase with the cytoskeleton and translocation to the filopodia during platelet activation. Plasma membrane Ca^{2+}-ATPase (PMCA) is associated with CLP-36, actinin and actin in the resting platelet in small complexes. Upon platelet activation (arrow), actin polymerizes into long fibers resulting in formation of filopodia and translocation of PMCA into the filopodia where it remains active in the plasma membrane. Taken from reference 33 with permission from Schattauer GmbH, Stuttgart.

by introduction of a PMCA4b C-terminal peptide into platelets. This peptide contains the PDZ-domain-binding motif described above.

In a more recent publication[43], we have demonstrated, using immunofluorescence microscopy, that PMCA is translocated to filopodia in activated platelets and that this translocation is blocked by introduction of the PMCA4b C-terminal peptide. Although incorporation of the peptide has no effect on the rate and extent of platelet activation, it significantly increases the rate of clot retraction. These results indicate that PMCA translocation to filopodia is important for regulating late events in platelet thrombus formation such as clot retraction. We have speculated[43] that clot retraction rate is enhanced when PMCA translocation is inhibited because Ca^{2+} levels in filopodia are elevated due to lack of PMCA, and that this enhances the retraction process.

Mechanism of PMCA association with the cytoskeleton

In order to understand the mechanism of PMCA association with the cytoskeleton in activated platelets, co-immunoprecipitation assays coupled with immunoblotting and electrospray ionization tandem mass spectrometry have been used to identify proteins that interact with PMCA in resting platelets[35]. Our results have indicated that the LIM family protein, CLP-36, binds to PMCA in resting platelets and mediates binding of PMCA to the cytoskeleton during platelet activation. In addition, PMCA is associated with α-actinin and γ-actin in resting platelets. This implies that PMCA is already associated with small actin complexes in resting platelets, by means of PDZ domain interactions. PMCA then associates with the actin cytoskeleton during cytoskeletal rearrangement upon platelet activation (Figure 1). These observations suggest complex regulation of PMCA by interactions with anchoring and cytoskeletal proteins in addition to the reversible serine/threonine and tyrosine phosphorylation events we have previously described in human platelets.

REGULATION OF PLATELET PMCA IN DISEASE

There is an extensive literature on changes in platelet $[Ca^{2+}]_i$ in a variety of diseases including hypertension, diabetes and coronary heart disease. In this review, I limit the discussion to effects of disease on platelet PMCA. In 1986, Resink *et al*[45] reported that a calmodulin-sensitive ATPase, presumably PMCA, had significantly increased activity in platelet membranes obtained from hypertensive individuals. A similar study published in 1992[46] reached the opposite conclusion, when it was shown that calcium efflux activity was significantly inhibited in hypertension. In 1994, Dean *et al*[15] reported that PMCA activity was decreased as a function of diastolic blood pressure in humans. We speculated that a factor present in the circulation of hypertensive individuals causes inhibition of PMCA and resultant increased $[Ca^{2+}]_i$. After demonstrating that PMCA is inhibited by tyrosine phosphorylation[9], it has become clear that tyrosine phosphorylation of PMCA could explain the inhibition of PMCA in hypertension. Therefore, we measured the levels of tyrosine phosphorylation of PMCA in healthy volunteers as a function of blood pressure[16]. Separation of volunteers into normotensive (diastolic < 85 mmHg) and hypertensive (diastolic > 85 mmHg) has revealed a significantly higher level of PMCA tyrosine phosphorylation in hypertensive individuals. These results suggest that PMCA in platelets of hypertensive individuals is inhibited because of tyrosine phosphorylation leading to elevated $[Ca^{2+}]_i$, hyperactive platelets, and enhanced risk of heart attack and stroke. A rat model of hypertension has indicated changes in the expression levels of PMCA1b and PMCA4b in rat platelets as a function of hypertension, but no activity measurements have been reported[10], therefore, the contribution to platelet calcium efflux in this model is unknown. Platelet $[Ca^{2+}]_i$ has also been reported to be increased in type 2 diabetes by several groups. Rosado *et al*[16] have shown that this increase in $[Ca^{2+}]_i$ is caused by tyrosine phosphorylation of PMCA, thus confirming our earlier work in hypertension. However, a more recent publication[23] has reached the conclusion that PMCA4b has enhanced expression in diabetes, although calcium transport studies were not undertaken, so that the overall effect on activity and $[Ca^{2+}]_i$ was not determined. The same group has published similar data with respect to PMCA expression in hypertension, which was increased, but in that study, PMCA activity was measured and found to be decreased[24]. The lack of correlation between protein expression and activity has been ascribed to tyrosine phosphorylation.

CONCLUSION

Ca^{2+} is a major second messenger in platelet activation, and elevated $[Ca^{2+}]_i$ leads to hyperactive platelets. Elevated

platelet $[Ca^{2+}]_i$ has been documented in hypertension and diabetes; both conditions increase the likelihood of heart attack and stroke. Thus proper regulation of platelet calcium metabolism is extremely important. PMCA is a major player in platelet calcium metabolism because it provides the only significant route for calcium extrusion. In keeping with the important role of calcium in platelet function, PMCA is a highly regulated transporter. In human platelets, PMCA is activated by Ca^{2+}/calmodulin, by cAMP-dependent phosphorylation and by calpain-dependent removal of the inhibitory peptide. It is inhibited by tyrosine phosphorylation and calpain-dependent proteolysis. In addition, the cellular location of PMCA is regulated by PDZ-domain-dependent interaction with the cytoskeleton during platelet activation. Rapid regulation by phosphorylation results in changes in the rate of platelet activation and secretion, whereas calpain action and interaction with the cytoskeleton appear to regulate later events in platelet function such as clot retraction. In hypertension and diabetes, PMCA expression is upregulated whereas activity is decreased, presumably due to tyrosine phosphorylation. Clearly, a more complete understanding of PMCA function in human platelets could result in identification of new ways to control platelet function in disease states.

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TOPIC HIGHLIGHT

Emanuel E Strehler, PhD, Professor, *Series Editor*

Plasma membrane Ca^{2+}-ATPases: Targets of oxidative stress in brain aging and neurodegeneration

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Abstract

The plasma membrane Ca^{2+}-ATPase (PMCA) pumps play an important role in the maintenance of precise levels of intracellular Ca^{2+} $[Ca^{2+}]_i$, essential to the functioning of neurons. In this article, we review evidence showing age-related changes of the PMCAs in synaptic plasma membranes (SPMs). PMCA activity and protein levels in SPMs diminish progressively with increasing age. The PMCAs are very sensitive to oxidative stress and undergo functional and structural changes when exposed to oxidants of physiological relevance. The major signatures of oxidative modification in the PMCAs are rapid inactivation, conformational changes, aggregation, internalization from the plasma membrane and proteolytic degradation. PMCA proteolysis appears to be mediated by both calpains and caspases. The predominance of one proteolytic pathway *vs* the other, the ensuing pattern of PMCA degradation and its consequence on pump activity depends largely on the type of insult, its intensity and duration. Experimental reduction of PMCA expression not only alters the dynamics of cellular Ca^{2+} handling but also has a myriad of downstream consequences on various aspects of cell function, indicating a broad role of these pumps. Age- and oxidation-related down-regulation of the PMCAs may play an important role in compromised neuronal function in the aging brain and its several-fold increased susceptibility to neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, and stroke. Therapeutic approaches that protect the PMCAs and stabilize $[Ca^{2+}]_i$ homeostasis may be capable of slowing and/or preventing neuronal degeneration. The PMCAs are therefore emerging as a new class of drug targets for therapeutic interventions in various chronic degenerative disorders.

Key words: Calcium; Neurons; Plasma membrane Ca^{2+}-ATPase; Calmodulin; Oxidative stress; Excitotoxicity; Brain aging; Neurodegeneration

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INTRODUCTION

Neuronal stimulation induces transient changes in the levels of free intracellular calcium $[Ca^{2+}]_i$ which in turn plays an important regulatory role in crucial nerve cell functions such as the release of neurotransmitters, signal transduction, induction of gene expression, synaptic plasticity, and learning and memory formation[1]. Follow-

ing the transduction of the Ca^{2+} signal, neurons instantaneously return the $[Ca^{2+}]_i$ to baseline levels in order to allow cells to respond to a new stimulus and to prevent the cytotoxicity associated with prolonged exposure to elevated Ca^{2+} levels[2]. The various mechanisms for restoring baseline resting $[Ca^{2+}]_i$ following neuronal excitation include sequestration into the endoplasmic reticulum and mitochondria, buffering by Ca^{2+} binding proteins, and extrusion across the plasma membrane by the Na^+/Ca^{2+} exchangers (NCXs) and the plasma membrane Ca^{2+}-ATPases (PMCAs)[3,4]. The PMCA pumps are regulated by the Ca^{2+} sensor protein calmodulin (CaM), which binds to the C terminus, resulting in conformational changes that displace the autoinhibitory domain away from the catalytic site, thus relieving autoinhibition and causing several-fold stimulation[5,6]. The PMCAs represent the major transport system at the plasma membrane responsible for the long term regulation of resting free $[Ca^{2+}]_i$ and counteracting transient increases that occur during Ca^{2+} signaling[7]. The PMCAs are encoded by four different genes that give rise to four distinct isoforms PMCA 1-4; further specialization is achieved by alternative splicing of the primary mRNA transcripts. Neurons are unique in that they express an unusually wide array of PMCA subtypes, including all four isoforms and multiple splice variants, attesting to the complexity of Ca^{2+} handling in these cells.

DISRUPTION OF CALCIUM HOMEOSTASIS IN THE AGING BRAIN: CONTRIBUTION OF THE PMCAs

The hypothesis that brain aging is linked with alterations in neuronal Ca^{2+} homeostasis was first proposed by Khachaturian[8-11]. Since then, a wide constellation of experimental evidence has emerged supporting the assertion that a disruption in the precise regulation of $[Ca^{2+}]_i$ may be a final common pathway leading to altered neuronal function and cell death[12-16]. In the last decade, we have witnessed an extensive growth in the literature providing compelling evidence indicating that Ca^{2+} regulating systems in brain neurons are altered with increasing age[17-21]. Brain aging is associated with increased activation of the voltage-gated Ca^{2+} channels, altered Ca^{2+} transport across the mitochondria, and decreased activity of the NCXs and the sarco-endoplasmic reticulum Ca^{2+}-ATPases[22-25]. It is not clear whether any single event by itself has a greater impact, or the multiplicity of defects in Ca^{2+} regulating systems is required before the overall disruption of Ca^{2+} homeostasis is observed in aged neurons.

Age-related alterations in the PMCAs were first assessed by Michaelis and coworkers[26-30]. These authors demonstrated for the first time a significant age-associated decline in the PMCA enzyme activity and ATP-dependent Ca^{2+} transport activity in synaptic plasma membranes (SPMs) isolated from Fisher 344 rats. We further confirmed the age-dependent decrease in PMCA activity in the more hardy Fisher 344/Brown Norway rats[31], a hybrid strain with a longer average life span and remarkable resistance to a variety of age-related pathologies[32]. The decline in PMCA activity monitored in SPMs isolated from rats at five different ages (5, 14, 22, 30 and 34 mo) representing young adults, middle-aged and aged animals is progressive with increasing biological age and does not appear to be the result of the end-stage of the brain aging process. Decrease in PMCA catalytic activity is associated with a statistically significant decrease in maximum velocity (V_{max}) with no appreciable change in the affinity of the enzyme for Ca^{2+} (K_{act})[31]. Efforts to investigate the mechanism underlying lowered PMCA activity showed that the decrease is due in part to the reduction in PMCA protein levels present in the SPMs of aged rats. An approximately 20% reduction in PMCA protein is observed at 34 mo, the highest age we tested, compared to the 5 mo young adults[31]. Age-related reduction in the SPM PMCA may be attributed to a variety of reasons such as decreased synthesis, altered stability, abnormal targeting to synaptic membranes, structural alterations leading to enhanced removal from the synaptic terminals, or modifications leading to reduced immunoreactivity, although none of these possibilities have been experimentally validated. Given that the pan PMCA antibodies we used in these studies recognized all four isoforms, it is unclear if specific isoforms/splice variants of the PMCAs are selectively influenced by the aging process or the decrease is evenly represented across all four isoforms. Lowered PMCA activity and protein levels in SPMs as observed with increasing age is likely to contribute to the disruption of Ca^{2+} homeostasis, a hallmark of aged neurons.

A substantial body of evidence implicates a progressive reduction in antioxidant capacity as an important contributor to the subtle alterations in cellular function associated with aging[33]. Such an imbalance in the levels of cellular antioxidants, in conjunction with an accelerated rate of generation of free radicals, can lead to lipid peroxidation and/or post-translational modification of proteins resulting in their conformational instability, structural modification and often times the accumulation of inactive or less active forms of enzyme molecules[34,35]. The approximately 40% decrease in PMCA enzyme activity observed in aged SPMs may reflect both the presence of inactive PMCA molecules in the membrane as well as enhanced clearance of the damaged protein, whereas the approximately 20% lower immunoreactivity may reflect only the enhanced removal of the PMCAs in aged brain[31]. One potential source of modification in the activity of integral membrane proteins such as the PMCAs is the possibility of alterations in the membrane lipid environment in which the protein is embedded and/or the accumulation of structural changes to the protein itself, particularly in view of its unusually long half life[36]. The high abundance of oxidation-sensitive polyunsaturated fatty acyl chains in synaptosomal membranes makes it likely that the age-related loss in PMCA catalytic turnover may be due to

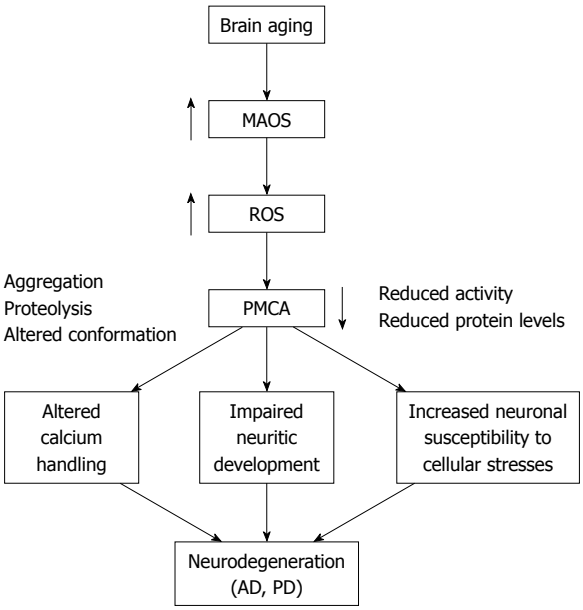

Figure 1 A schematic depiction of the cellular events that link brain aging and neurodegenerative disorders. MAOS: Membrane-associated oxidative stress; ROS: Reactive oxygen species; PMCA: Plasma membrane Ca^{2+}-ATPase; AD: Alzheimer's disease; PD: Parkinson's disease.

lipid peroxidation and consequent changes in the physicochemical properties of the membrane bilayer. Our studies, utilizing HPLC analysis of the 2, 4-dinitrophenylhydrazone derivatives present in synaptic membranes isolated from young and aged rat brain, reveal no significant change in the content of reactive aldehydes (malondialdehyde, formaldehyde, acetaldehyde or acetone), which would comprise potential breakdown products of lipid peroxidation[37]. Electron paramagnetic resonance measurements employing 5- and 12-stearic acid spin labels with the nitroxide reporter groups at two depths in the bilayer also show no significant changes in fatty acid chain dynamics or membrane fluidity in the synaptic membranes that could correlate with the observed age-associated inhibition of PMCA activity. The absence of any detectable end-products of lipid peroxidation or alterations in fatty acyl chain dynamics strongly indicate that the age-dependent changes in PMCA are likely the result of direct alteration of the protein and that the PMCAs may be targets of reactive oxygen and/or nitrogen species (Figure 1).

THE PMCAs: TARGETS OF OXIDATIVE STRESS

Biochemical studies

Free radical-induced oxidative damage to proteins is an important factor in the pathophysiology of several human diseases including Alzheimer's disease, Parkinson's disease, ischemia-reperfusion injury and also in normal biological aging. Oxidative modification of proteins may involve altered conformation, misfolding, enhanced propensity for aggregation and oxidation of amino acid residues[32,33]. To determine the susceptibility of the PMCAs to oxidative stress and to identify the molecular signatures of oxidative modification that may appear on the protein upon exposure to oxidants, we carried out a series of studies employing several experimental paradigms[38-40]. Exposure of SPMs to oxidants such as H_2O_2, peroxynitrite and the azo-initiators 2,2'-Azobis 2-aminopropane dihydrochloride and 4,4'-Azobis 14-cyanovaleric acid (peroxyl radical generating agents) for a short period of 10 min result in a rapid loss of Ca^{2+}-activated ATPase activity of the PMCAs[39]. As observed in aging brain SPMs, lowered PMCA activity is attributed to a diminished V_{max} with no significant change in K_{act}. Although all three oxidants led to significant loss of PMCA activity, the effect of peroxynitrite was the most potent, followed by peroxyl radicals and H_2O_2. The major structural change observed in the PMCA is the appearance of high molecular weight aggregates observed in immunoblots run under non-reducing conditions of electrophoresis. The PMCA adducts can be reversed by the addition of reducing agents and chaotropic agents and therefore appear to be generated by the oxidation of cysteine residues to form intermolecular disulfide bonds and increased hydrophobic interactions between PMCA molecules, respectively[39,41].

Given that the studies described above were conducted in SPMs, it is not clear whether oxidant-mediated changes in PMCA are due to direct effects on the protein and/or due to secondary effects resulting from peroxidation of the polyunsaturated fatty acids abundant in SPMs. Thus, in the next series of studies, the erythrocyte PMCA was purified from its native membrane environment and reconstituted into mixed micelles made of phosphatidylcholine and functional and structural alterations in the isolated protein investigated following a brief exposure to H_2O_2[38]. The purified PMCA preparations allowed us to perform structural characterization of the protein (PMCA 4 being the predominant isoform present in erythrocyte membranes) and shed light on the molecular mechanisms involved in H_2O_2-mediated PMCA inactivation. Exposure of the protein to H_2O_2 (25-100 μmol/L, 10 min, 37℃) inhibits both basal and CaM-stimulated PMCA activity at nearly identical rates. Neither the concentration-dependent stimulation of PMCA activity by CaM nor the binding of CaM to the H_2O_2-exposed PMCA is disrupted suggesting that the inhibitory effect of the oxidant is apparently not mediated through a direct effect on the CaM binding domain. H_2O_2 quenches PMCA tryptophan fluorescence, an indicator of global conformational changes in the protein, with a rate similar to that of PMCA inactivation. A novel finding in these studies was the protection offered by CaM against the deleterious effects of H_2O_2. Pretreatment of PMCA with CaM prior to the addition of H_2O_2 completely protects the enzyme against oxidant-mediated inactivation suggesting the existence of a CaM-induced conformational state resistant to oxidation. As observed in SPMs, exposure of the purified PMCA to H_2O_2 also generates high molecular weight aggregates. Although the

PMCA adducts are significantly reversed by dithiothreitol, there is no recovery in PMCA activity suggesting that the oxidation-induced conformational changes are not fully reversed even when the disulfide bonds are reduced. Quantification of cysteine residues in the PMCA using the fluorescent maleimide probe ThioGlo-1[42] shows labeling of only 7 of the 21 known cysteines present in PMCA 4, presumably due to the existence of intramolecular disulfide bonds and/or mixed protein-glutathione disulfide bonds. As expected, exposure to H_2O_2 significantly reduces cysteine labeling (40% reduction). Amino acid analysis reveals no chemical change in any other amino acid except for methionine residues, a fraction of which are oxidized to methionine sulfoxide (0.06 mol methionine sulfoxide/mol PMCA or 0.002 methionine sulfoxide/mol methionine), as there are 28 methionine residues in PMCA 4[38].

Single molecule spectroscopy approaches

Given that one of the structural alterations observed in the PMCA following exposure to H_2O_2 is altered conformation, we used single molecule spectroscopy to interrogate conformational changes in oxidant-modified PMCA molecules to better understand the mechanism underlying its inactivation[43]. Single molecule spectroscopic strategies have been developed and successfully used by Johnson and coworkers to investigate conformational changes in the interaction between the PMCA and CaM at the molecular level, a feat that cannot be achieved by conventional ensemble kinetic and biochemical methods given the vast heterogeneity in PMCA conformational states[43-48]. Previous work by Yao *et al*[49] demonstrated that fluorescently labeled CaM bound to the autoinhibitory domain of PMCA exhibits a rotational correlation time much shorter than expected for the whole PMCA enzyme, consistent with segmental motion of the CaM binding domain. Subsequent polarization modulation studies on single molecule complexes of PMCA bound to tetramethylrhodamine-labeled CaM show the existence of two major conformational distributions, a high orientational mobility population present at 25 μmol/L Ca^{2+}, a concentration sufficient for full activation by CaM (attributed to PMCA with a fully dissociated CaM binding domain) and a second state with low orientational mobility which appears at a low Ca^{2+} concentration (0.15 μmol/L) (corresponding to autoinhibited PMCA-CaM complexes with a non-dissociated autoinhibitory domain)[45]. The existence of a PMCA-CaM state with the autoinhibitory domain not fully dissociated is indicative of a far greater complexity in PMCA-CaM interactions than previously realized. Binding and hydrolysis of the substrate ATP at the nucleotide-binding site of PMCA drive structural motions of the enzyme that result in the transfer of the terminal phosphate of ATP to a highly conserved aspartate residue in the phosphorylation domain. Consistent with this, addition of ATP (but not its non-hydrolysable analogs) to the PMCA-CaM complexes eliminates the low orientational mobility conformational state and abolishes the Ca^{2+} dependence in modulation depth. The ATP-dependence of the autoinhibitory domain mobility, a reflection of the structural interaction between the PMCA catalytic core and CaM binding domain, offers new insights into the functional relationships of the various PMCA-CaM ligands on the pump activity[43].

To investigate the conformational changes in PMCA-CaM complexes following oxidative modification by H_2O_2, we performed single molecule polarization modulation spectroscopy on PMCA exposed to 100 μmol/L H_2O_2 as described[38,43]. In the absence of ATP, the orientational mobility populations for native and oxidant-treated PMCA-CaM complexes are nearly the same, indicating that CaM binding and coupling of the autoinhibitory domain with the enzymatic core is unaltered by H_2O_2, observations that are consistent with our biochemical studies[38]. However, in the presence of 1 mmol/L ATP, marked differences are evident in the oxidant-treated PMCA molecules. The most interesting observation is the appearance of a population of PMCA-CaM complexes with low orientational mobility distribution present even at high Ca^{2+} (25 μmol/L), suggestive of PMCA molecules with autoinhibitory domain associated with the catalytic site[45]. Thus, H_2O_2 appears to disrupt the structural coupling between ATP binding and hydrolysis and the autoinhibitory domain, suggesting that the loss of PMCA enzyme activity resulting from oxidative damage is correlated with a reduced dissociation of the autoinhibitory domain from the nucleotide binding site. This interpretation is further substantiated by proteolysis studies showing reduced accessibility of chymotrypsin to the CaM binding domain of H_2O_2-treated PMCA[43]. Oxidative modification at or near the nucleotide binding site of the PMCA such as the formation of disulfide bonds may alter the structural geometry of ATP binding and impair protein conformational changes associated with the productive utilization of ATP[43]. Interestingly, the only chemical change observed in H_2O_2-treated PMCA is cysteine oxidation[38]. Of the twenty-one known cysteine residues present in PMCA 4, ten are located in the cytoplasmic loop between transmembrane four and five, a region that contains the active site of the enzyme. Of these, cysteine 537 is located on the stretch of residues comprising the receptor region that interacts with the CaM-binding domain[50,51], while cysteine 601 is a few residues away from the ATP binding site at lysine 591. Oxidative modification of one or more of these crucial cysteines is likely to alter conformational interactions between the catalytic site and the CaM binding domain, resulting in nonproductive binding of ATP and subsequent enzyme inactivation[43].

Effects of oxidative stress on the PMCAs in neurons

While *in vitro* studies on the PMCAs present in SPMs and purified protein preparations provide valuable information on oxidation-induced alterations in PMCA activity, structure and conformation, they do not address the sensitivity of the PMCA pumps to oxidative stress as it would

occur in intact cells with its array of antioxidant enzymes that can counteract the deleterious effects of oxidants. A number of studies have indeed addressed this issue and investigated the effects of neurotoxins, excitotoxic insults, and reactive oxygen species (ROS) on the PMCAs present in neurons[40,52-56], and in non-neuronal cells[57-59]. Acute exposure of cerebrocortical neurons to N-methyl D-aspartate (NMDA), kainate, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, okadaic acid or maitotoxin results in accelerated cell death, which correlates with the activation of the Ca^{2+}-dependent protease calpain and subsequent PMCA proteolysis[54]. PMCA degradation in cells is consistent with earlier observations showing its susceptibility to *in vitro* proteolysis by exogenous calpain, which cleaves the protein at the autoinhibitory CaM binding domain resulting in irreversible activation[53,60,61]. Although the studies by Hajimohammadreza *et al*[54] did not assess the consequence of calpain-mediated cleavage on PMCA activity, they were the first to report PMCA degradation in degenerative neuronal cultures.

The response of the PMCAs (PMCA 2 and PMCA 4, in particular) to excitotoxic agents was further highlighted in hippocampal neurons exposed to glutamate[55]. This manipulation reduces PMCA activity as evidenced by a significantly lower rate of PMCA-mediated Ca^{2+} efflux[55]. Interestingly, lowered PMCA function correlates with a loss of the protein from the plasma membrane. Calpain inhibitors abrogate these effects signifying the role of this Ca^{2+}-activated protease in glutamate-mediated PMCA inactivation and clearance from the plasma membrane. In contrast to *in vitro* studies showing irreversible activation of the purified PMCA upon exposure to exogenous calpain, *in situ* activation of the protease in cells reduces both PMCA function and protein levels. Calpain-triggered down-regulation of PMCA may be due to degradation, endocytosis or possible internalization of the protein en route to degradation in lysosomes[55]. Although not experimentally proven, the possibility of PMCA internalization/recycling being part of a physiological Ca^{2+} signaling cascade was suggested by the relatively small degree of PMCA internalization observed in healthy cells in response to a non-excitotoxic concentration of glutamate. It is not clear if the effects of calpain are due to direct proteolysis of the PMCA protein or mediated by some other cytoskeletal component(s) involved in its retrieval from the membrane. PMCA down-regulation and internalization are also seen in the cell body and neurites of hippocampal neurons treated with H_2O_2[56] suggesting that this may be part of a concerted response mounted by cells in response to conditions of excitotoxicity and oxidative stress.

The above mentioned series of studies demonstrate the sensitivity of the PMCAs to exogenously added excitotoxic agents and oxidants. In a recent study, we assessed the effects of ROS generated within neurons using the cells own machinery as would likely occur *in vivo*[40]. Primary cortical neurons were treated with paraquat, a redox cycling agent that utilizes the cellular microsomal cytochrome P450 enzyme system to generate intracellular superoxide free radicals[62]. Exposure of neurons to paraquat results in an almost instantaneous generation of superoxide free radicals as monitored by the inactivation of aconitase, an enzyme with iron-sulfur centers believed to be one of the most sensitive targets of ROS[63]. A 24 h exposure of primary cortical neurons to paraquat (5-100 μmol/L) results in marked alterations in PMCA activity which exhibits a biphasic response[40]. While low concentrations of paraquat (5-25 μmol/L) stimulate CaM-independent PMCA activity by approximately two-fold and abolish its sensitivity to CaM, higher concentrations (50-100 μmol/L) inhibit both basal and CaM-stimulated PMCA activity. As observed before with exogenously added toxic agents[54-56], paraquat treatment also leads to calpain-mediated PMCA proteolysis[40]. It is notable that PMCA stimulation and loss of sensitivity to CaM occur under conditions that precede its proteolysis leading us to speculate that low concentrations of paraquat may cause structural changes in the PMCA that promote the dissociation of the autoinhibitory CaM binding domain, resulting in PMCA stimulation. Paraquat-induced conformational changes may expose the calpain cleavage site on the PMCA thus making it more accessible to proteolytic degradation. Paraquat-treated cells also exhibit PMCA aggregation, a consistent signature of PMCA oxidation observed in SPMs and the purified protein[38,39].

Increase in oxidative stress and elevations in $[Ca^{2+}]_i$ have also been linked to the activation of caspases which mediate programmed cell death or apoptosis[64]. The link between the PMCAs and apoptosis was discovered when the PMCA 4b subtype was shown to be a substrate of caspase 3 in the early stages of apoptosis[65-67]. In contrast to calpain, which can form several products of different sizes depending on the site of cleavage[53,60,68], cleavage by caspase occurs precisely at an aspartate (*consensus site* $^{1077}DEID^{1080}$), a few residues upstream of the CaM binding domain. The single 120 kDa proteolytic fragment is fully active even in the absence of CaM[65,66], a condition that would help the cell to respond more efficiently to an increased Ca^{2+} load[67]. The effects of caspase-mediated PMCA cleavage on its activity and function appear to yield mixed results. In a study by Schwab *et al*[67], 2002, PMCA proteolysis by caspases inactivated PMCA 2 and 4 in both neurons and non-neuronal cells undergoing apoptosis, resulting in impaired $[Ca^{2+}]_i$ handling and consequent Ca^{2+} overload. Expression of non-cleavable PMCA mutants prevents the disruption in Ca^{2+} handling, slows down the kinetics of apoptotic cell death, and significantly delays necrosis. A number of cellular conditions, such as exposure to excitotoxins, oxidative stress and ischemic injury, may activate both calpains and caspases. The predominance of one proteolytic pathway *vs* the other and ensuing pattern of PMCA proteolysis and effects on pump activity appear to depend largely on the type of insult and its intensity and duration. If the stress is mild, increase in PMCA activity may counteract the increased Ca^{2+} load

and protect cells against death. However, under severe and more chronic conditions, the PMCAs may be down-regulated further impairing Ca^{2+} homeostasis and promoting cell death (Figure 1).

FUNCTIONAL EFFECTS OF ALTERED PMCA EXPRESSION

Given the technical challenges associated with culturing adult and aged neurons and the dearth of an appropriate technology that allows the assessment of PMCA function in live animals, there is limited information on the functional consequences of altered PMCA levels such as those that occur in the synaptic terminals in the aging brain[31]. A number of approaches have been made by various laboratories (including ours) to experimentally manipulate PMCA expression and determine the downstream consequences on various aspects of cell function. This is especially pertinent given the critical role of the PMCAs in maintaining neuronal $[Ca^{2+}]_i$ and also its newly assigned role in modulating cellular signaling pathways[69]. The PMCAs have been shown to interact with a number of signaling proteins such as nNOS, calcineurin, and various members of the membrane microdomain organizing proteins of the membrane-associated guanylate kinase family[70-77]. More recently, the PMCAs have been shown to be localized in neuronal lipid rafts, cholesterol-enriched microdomains in the plasma membrane[78,79], believed to be local centers for cell signaling events. PMCA activity is significantly reduced in response to cellular cholesterol depletion suggesting the possibility of local regulation of the pump activity in lipid rafts[78].

Antisense-(AS) plasmid-mediated reduction of specific isoforms has yielded valuable information on the role of individual PMCA subtypes in regulating the dynamics of cellular Ca^{2+} handling and also their contribution to a diverse array of neuronal functions[80-84]. For example, blockade of PMCA 1 causes no change in the levels of resting free $[Ca^{2+}]_i$ or its release from intracellular stores but results in a significantly slower rate of Ca^{2+} clearance following release from intracellular stores[80]. However, the most striking effect of lowered PMCA 1 in PC6 cells is the impairment in neurite extension elicited by nerve growth factor (NGF)[80]. Cells with reduced PMCA 1 levels have fewer and shorter neurites and a conspicuous absence of defined growth cones in response to NGF. Interestingly, these effects are not mediated by a loss of NGF signaling but are rather attributed to downstream consequences of altered cellular Ca^{2+} transients. Further clues regarding the role of PMCA 1 in neuritic development were unraveled in a subsequent study showing a significant down-regulation in the expression of the integrin receptor alpha subunit in cells with blocked PMCA 1[81]. These studies indicate a close relationship between PMCA 1 and the regulation of cell-extracellular matrix attachment and contact-dependent growth.

Transient reduction of PMCA 2 using AS techniques prolongs the effects of NMDA and increases the sensitivity to inhalational anesthetics[83]. We utilized an RNA interference strategy to experimentally lower PMCA 2 expression[82]. Short interference RNA treatment led to 80% reduction of PMCA 2 expression, which remained suppressed throughout a 6 d period. siRNA-treated cells exhibit marked changes in total PMCA activity, with a shift from Michaelis-Menten kinetic properties, a three-fold increase in K_{act} for Ca^{2+} and 22% suppression of V_{max}[82]. Ca^{2+} imaging studies using Fura 2 show that neurons with lowered PMCA 2 have (1) elevated resting $[Ca^{2+}]_i$; (2) a significantly slower recovery following depolarization; and (3) inability to return to their own resting levels present prior to depolarization, signifying the importance of PMCA 2 in maintaining resting $[Ca^{2+}]_i$. Cells with elevated Ca^{2+} levels would likely exhibit an increased vulnerability to various metabolic and oxidative insults. Consistent with this idea, PMCA 2 deficient cells are more susceptible to cellular stresses particularly those involving Ca^{2+} overload[82].

Although experimental lowering of the PMCAs in cultured cells cannot be extrapolated to the *in vivo* situation that exists in brain, they greatly emphasize the importance of maintaining normal levels of PMCA protein in neuronal survival and growth. Given that lowered PMCA expression increases the vulnerability of cells to various insults, the reverse condition, i.e. increased PMCA levels, would likely offer neuroprotection. Consistent with this notion, cells over-expressing PMCA 2 and 4 protect cells against Ca^{2+}-mediated cytotoxicity[85,86]. Coincidently, PMCA levels are up-regulated during the maturation of hippocampal neurons[87] and in neuroblastoma cells undergoing differentiation[88]. Analysis of PMCA expression profile in the developing brain shows that distinct PMCA subtypes are expressed at different levels in various brain regions, with a noticeably higher expression in cellular compartments characterized by a greater number of synapses, suggesting a key role of the PMCAs in synaptogenesis and in the maturation of neuronal electrophysiological properties[89-91].

THE PMCAs AND NEURODEGENERATION

A role for neuronal Ca^{2+} dysregulation in age-related neurodegenerative disorders was suggested almost three decades ago[8,92] and has been experimentally validated since then by a large body of literature[20,93-95]. However, the contribution of the PMCAs in neurodegeneration is just beginning to be elucidated. There are two principal means by which the PMCAs may either impact or be impacted by neurodegenerative diseases. Altered PMCA function may disrupt neuronal Ca^{2+} homeostasis and increase cellular Ca^{2+} load which may in turn influence the metabolism and production of pathological peptides/proteins such as the amyloid beta peptide in Alzheimer's disease and alpha synuclein in Parkinson's disease. Conversely, events downstream from the accumulation of the

pathological forms of these peptides/proteins may disrupt energy homeostasis, increase membrane excitability, elevate membrane-associated oxidative stress, and activate proteolytic enzymes which may consequently have an inhibitory effect on PMCA function (Figure 1). Convincing experimental support for either one or both of these possibilities is still lacking.

The PMCAs have been found to be significantly downregulated in models of global ischemia-reperfusion injury and seizures[65,73-77]. Evidence suggests that the suppression of PMCA activity is not simply due to the disruption of ion gradients and lowered ATP levels associated with these conditions, but rather due to oxidative modification and degradation of the PMCA protein[96]. The relationship between the PMCAs and Alzheimer's disease, the most common age-dependent neurodegenerative disorder, was first suggested by a reduction in PMCA activity in neurons exposed to the amyloid beta peptide[97-99]. Recent studies have further established the involvement of the PMCA in Alzheimer's disease[100]. PMCA activity in human brain tissue from Alzheimer's disease patients shows an altered dependence on Ca^{2+} compared to control brain with stimulation of activity at lower concentrations and less inhibition at high Ca^{2+} concentrations[100] suggesting that the affinity of the stimulatory site for Ca^{2+} increases and that for the inhibitory site decreases in Alzheimer's brain. More interestingly, addition of amyloid beta peptide to control brain altered its Ca^{2+} dependency to resemble that of Alzheimer's disease brain[100]. In preliminary studies on human brain tissue from Parkinson's disease, we have observed a significant reduction in PMCA activity compared to age-matched controls[101]. PMCA enzyme and protein levels are also lowered in SH-SY5Y neuroblastoma cells exposed to the Parkinsonian mimetics methyl phenyl pyridinium[101] and 6-hydroxydopamine (our unpublished observations). Overall, the findings presented here suggest that the PMCAs are altered in a number of neurodegenerative conditions. Further studies are needed to elucidate the underlying mechanisms and determine whether the observed changes in the PMCA pumps are a cause or consequence of disease progression. Therapeutic approaches that can protect the PMCAs and stabilize $[Ca^{2+}]_i$ homeostasis may be capable of slowing or even preventing neuronal degeneration. The PMCAs are therefore emerging as a new class of drug targets for therapeutic interventions in various chronic degenerative disorders.

CONCLUSION

The PMCA pumps are critical to the maintenance of precise levels of intracellular Ca^{2+}, quintessential to the functioning of nerve cells. The PMCAs in the SPMs diminish progressively with increasing age. This may be due to elevated levels of oxidative stress present in aged neurons. Lowered PMCA expression disrupts neuronal Ca^{2+} handling and increases the vulnerability of nerve cells to stresses involving Ca^{2+} overload. Age- and oxidation-related downregulation of the PMCAs may play an important role in compromised neuronal function in the aging brain and its several-fold increased susceptibility to age-associated neurodegenerative disorders.

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BRIEF ARTICLE

Regeneration and DNA demethylation do not trigger PDX-1 expression in rat hepatocytes

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Abstract

AIM: To explore the possibility that PDX-1 gene is reactivated as a consequence of molecular events that occur during liver regeneration.

METHODS: Rat hepatocytes were maintained in DMEM-F12, 10% fetal bovine serum (FBS), penicillin/streptomycin and geneticin when applicable. Rat insulinoma RIN 1046-38 cells were maintained in M-199-10% FBS and penicillin/streptomycin. The final concentration of glucose was 11.1 mmol/L. During regeneration, lateral and medial liver lobes of adult male Wistar rats were surgically removed, with up 70% loss of liver mass. In methylation experiments, 5-aza-deoxycytidine (5-aza-dC) was used. Primer3 software was used for polymerase chain reaction (PCR). Quantitative real time PCR (qRT-PCR) was performed using SYBR Green technology; primers were designed by Beacon Designer 6 software. Western blotting and SDS-PAGE were performed according to standard procedures. Antibodies were purchased from commercial suppliers.

RESULTS: We explored the possibility that liver regeneration could trigger PDX-1 expression, and hence insulin production. Twenty-four hours after surgical liver removal, regeneration was active as demonstrated by the increased proliferating cell nuclear antigen; however, all the other checked genes (involved in insulin gene expression): PC-1, Ngn3, NeuroD1, Btc, PDX-1 and Ins-1, were not related to the molecular events caused by this process. The only marker detected in regenerating liver was E47: a transcription factor of the the basic helix-loop-helix family known to be expressed ubiquitously in mammalian cells. In the rat pancreas, almost all of the tested genes were expressed as shown by RT-PCR, except for Ngn3, which was silenced 2 d after birth. Therefore, the molecular events in liver regeneration are not sufficient to promote PDX-1 expression. DNA methylation is a known mechanism to achieve stable repression of gene expression in mammals: Hxk 2 gene is silenced through this mechanism in normal hepatocytes. The administration of 5-aza-dC to cultured cells is in fact able to upregulate Hxk 2 mRNA. We investigated whether PDX-1 silencing in liver cells could be exerted through methylation of CpG islands in both the promoter and the gene coding regions. The results show that the drug increased the expression level of the Hxk 2 control gene but failed to rescue the expression of PDX-1, thus DNA demethylation is not sufficient to override repression of the PDX-1 gene.

CONCLUSION: During liver regeneration, PDX-1 gene is not reactivated. Demethylation does not de-repress PDX-1 gene expression. Therefore gene silencing is not achieved through this epigenetic mechanism.

Key words: Transcription factor PDX-1; Hepatectomy; Liver regeneration; Quantitative real time polymerase chain reaction; DNA methylation

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INTRODUCTION

Diabetes mellitus is a metabolic disease that is characterized by persistent hyperglycemia resulting from defects in insulin secretion and/or action[1,2]. When the amount of glucose in the blood increases, the release of insulin from the pancreas is triggered. This hormone removes glucose from the blood and stimulates the liver to metabolize glucose, thus controlling the level of sugar in the organism. In diabetic patients, the blood sugar levels remain high. This might derive from lack of insulin production, from insufficient levels of the hormone or from its diminished effectiveness. This peptide hormone is primarily involved in the glucose metabolism, and is produced by the pancreatic β cells that, also due to autoimmune responses, are destroyed in type 1 diabetes[3]. Insulin is a very potent regulator because it can exert its specific action at a blood concentration as low as 10^{-8} mol/L[4].

A number of different transcription factors are involved in the control of insulin expression and/or regulation[5]. The better studied is possibly PDX-1[6], which acts synergistically with E47, a member of the basic helix-loop-helix (bHLH) family of transcription factors: synergism requires DNA binding and activation domains of both PDX-1 and bHLH proteins. It has also been shown that synergistic transactivation results from the co-expression of E47, PDX-1 and NeuroD1[7]. The PDX-1 protein contains 283 amino acids with a predicted molecular weight of 31 kDa. Like most other transcription factors, PDX-1 is characterized by a modular architecture with separate functional domains. However, in spite of the vast body of literature on the function of PDX-1, its actual role in the activation/regulation of the insulin gene is controversial. In any case, it is clear that post-translational modification of the PDX-1 gene products is necessary for its full function. In particular, sumoylation has been reported to mediate PDX-1 translocation to the nucleus and to stimulate insulin gene expression. The SUMO modification also accounts for the heterogeneity of PDX-1 molecular weight: this post-translational modification in fact shifts PDX-1 molecular mass from 31 to 46 kDa[8].

The effects of PDX-1 on each gene under particular conditions is dependent on a subtle interaction between transcription factors (positively-acting and negatively-acting) that could also be activated under similar conditions. It is therefore difficult to correlate specific effects of PDX-1 expression with more general multi-faceted effects on gene expression.

In a recent study, we demonstrated that the PDX-1 transcription factor can significantly alter hepatocyte glucose metabolism by transcriptional regulation of at least one important gene of the glycolytic pathway[9]. These results should therefore be taken into consideration when using PDX-1 as a key factor for approaches based on gene therapy. Hence, Pdx-1 overexpression is likely to affect negatively the metabolic function also in animal models. The liver is considered a good candidate for the expression of insulin in patients with type 1 diabetes. However, this transcription factor is not expressed in the liver, and therefore, transformation of hepatocytes with exogenous PDX-1 gene copies is necessary. The rationale of this work was to assess whether endogenous PDX-1 expression could be spontaneously reactivated during liver regeneration in partially hepatectomized rats, and to verify whether PDX-1 silencing in normal hepatocytes might be achieved through DNA methylation.

MATERIALS AND METHODS

Clone-9 rat hepatocytes (American Type Culture Collection, ATCC No. CRL-1439) were maintained in DMEM-F12 (Gibco) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomicin and geneticin (100 μg/mL) when applicable. Cells were split every 3-4 d at a 1:10 ratio for no more than 10-15 times. Rat insulinoma RIN 1046-38 cells were maintained in M-199 supplemented with 10% FBS, penicillin and streptomycin. Glucose was added to a final concentration of 11.1 mmol/L. Cells were split every 4-5 d at a ratio of 1:5. In regeneration studies, the lateral and medial liver lobes of adult male Wistar rats (200-250 g) were surgically removed, which caused a 70% loss of liver mass[10]. In methylation experiments, 5-aza-deoxycytidine (5-aza-dC) was used at a final concentration of 5 μmol/L and treatment was performed as described by Goel and collaborators[11]. Total RNA purification was achieved using the Nucleospin RNA extraction kit (Macherey-Nagel). cDNA synthesis was performed using random primers, 1 μg total RNA as template and 200 U Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen). Polymerase chain reaction (PCR) was performed using a Geneamp 2400 (Applied Biosystems) and ExTaq DNA polymerase (Takara). PCR primers were designed using the Primer3 online software. Quantitative real time PCR (qRT-PCR) was performed using a Biorad iCycler and the SYBR Green technology; primers for qRT-PCR were designed using the Beacon Designer 6 software. All PCR primers have already been validated and used in a previous study[9]. SDS-PAGE and western blotting were performed according to standard procedures. Antibodies were purchased from Sigma (β-actin, cat. No. A-4700) and Santa Cruz Biotechnology (proliferating cell nuclear antigen; PCNA, cat No. SC-25280).

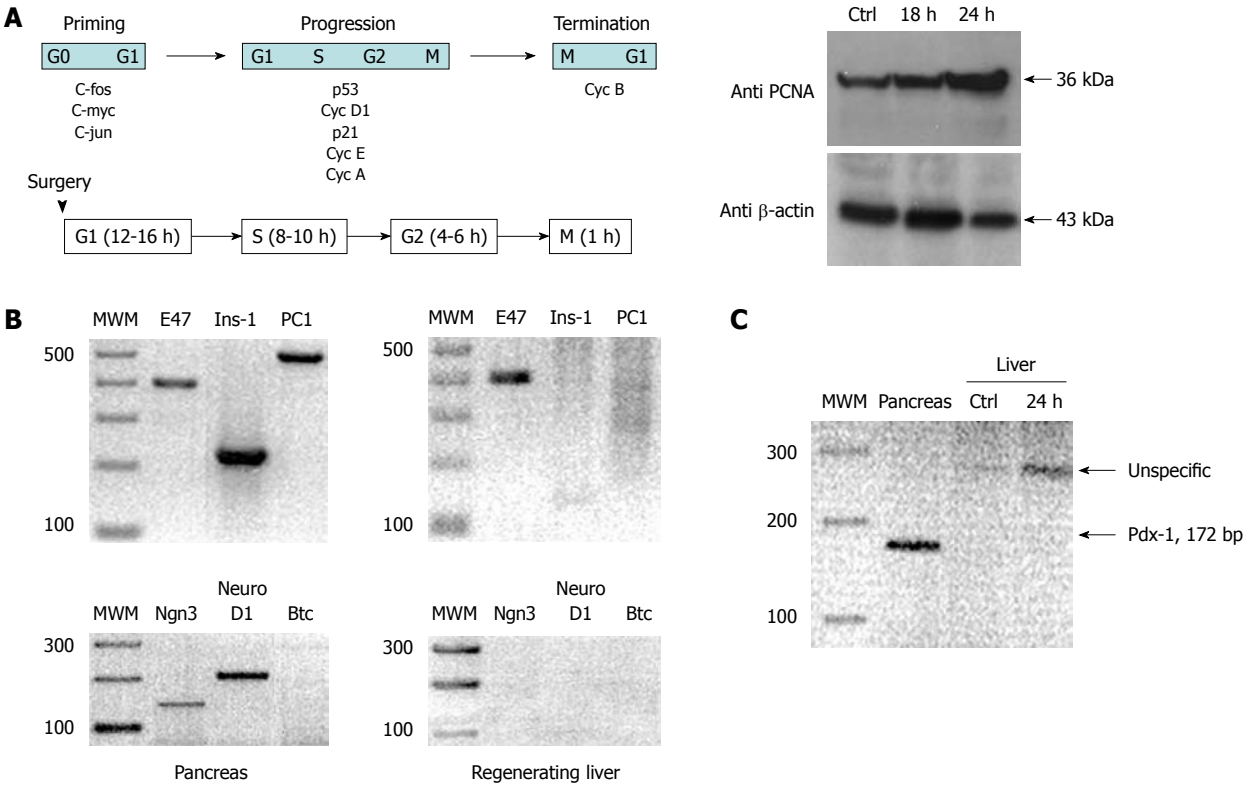

Figure 1 Schematic representation of the timing and molecular events that occur during liver regeneration. A: Schematic representation of the timing and molecular events that occur during liver regeneration. In the right panel, proliferating cell nuclear antigen immunodetection confirmed that, 24 h after hepatectomy, cell proliferation was reactivated; B: Real time polymerase chain reaction (RT-PCR) amplification of some important genes involved in insulin synthesis from pancreas and regenerating liver total RNA; C: RT-PCR amplification of the PDX-1 gene from pancreas and liver total RNA. PC-1: Pro-hormone convertase 1; Ins-1: Insulin 1; Ngn3: Neurogenin 3; Btc: β-cellulin.

RESULTS

PDX-1 and the regenerating liver

Figure 1 panel A shows that 24 h after surgical resection of the liver, regeneration was fully operational. This was demonstrated by the increased levels of PCNA shown in panels B and C. All the other genes involved in insulin gene expression that were checked (PC-1, Ngn3, NeuroD1, Btc, PDX-1 and Ins-1) did not show any relationships with the molecular events caused by this process. In fact, the only marker that could be detected in the regenerating liver was E47; a transcription factor that belongs to the bHLH family that is known to be expressed ubiquitously in mammalian cells. As a control, in the pancreas, almost all of the tested genes were expressed and could be identified by a band after RT-PCR; the only exception was Ngn3 that was silenced 2 d after birth in rats.

DNA methylation

As reported by Goel and collaborators[11], DNA methylation is known to be a mechanism that is commonly used to achieve stable repression of gene expression in mammalian cells: the Hxk 2 gene is silenced through this mechanism in the normal hepatocyte cell line Clone-9 (K-9). The administration of 5 μmol/L 5-aza-dC to K-9 cells in culture is in fact able to upregulate Hxk 2 mRNA transcription by about threefold compared to untreated cells. This drug can be incorporated into nascent DNA but cannot be methylated by cellular methylases; therefore, the gene expression pattern is altered and the expression of silenced genes is reactivated. As a result, we decided to check whether PDX-1 silencing in liver cells could be exerted through methylation of CpG islands in both the promoter and the gene coding regions. We cultured wild-type K-9 cells in the presence or absence of 5 μmol/L 5-aza-dC for 4 d and prepared cDNA from total RNA to be used for QRT-PCR; the Hxk 2 gene was used as a positive control and GAPDH as a reference gene. Figure 2 shows that, as expected, the drug treatment increased the expression level of the Hxk 2 control gene, but failed to rescue the expression of PDX-1, which indicated that the DNA demethylation status induced by the drug was not sufficient to override repression of the PDX-1 gene.

DISCUSSION

Liver regeneration is a complex process that involves reactivation of cell proliferation and expression of fetal markers such as α-fetoproteins that are normally silent in adult tissue[12-15]. The protein PDX-1 is an important transcription factor that is expressed during gut endoderm differentiation and organ formation. This was the rationale to explore the possibility that liver regeneration could also trigger PDX-1 expression, and hopefully, insulin production.

Regeneration was fully operational after partial surgical

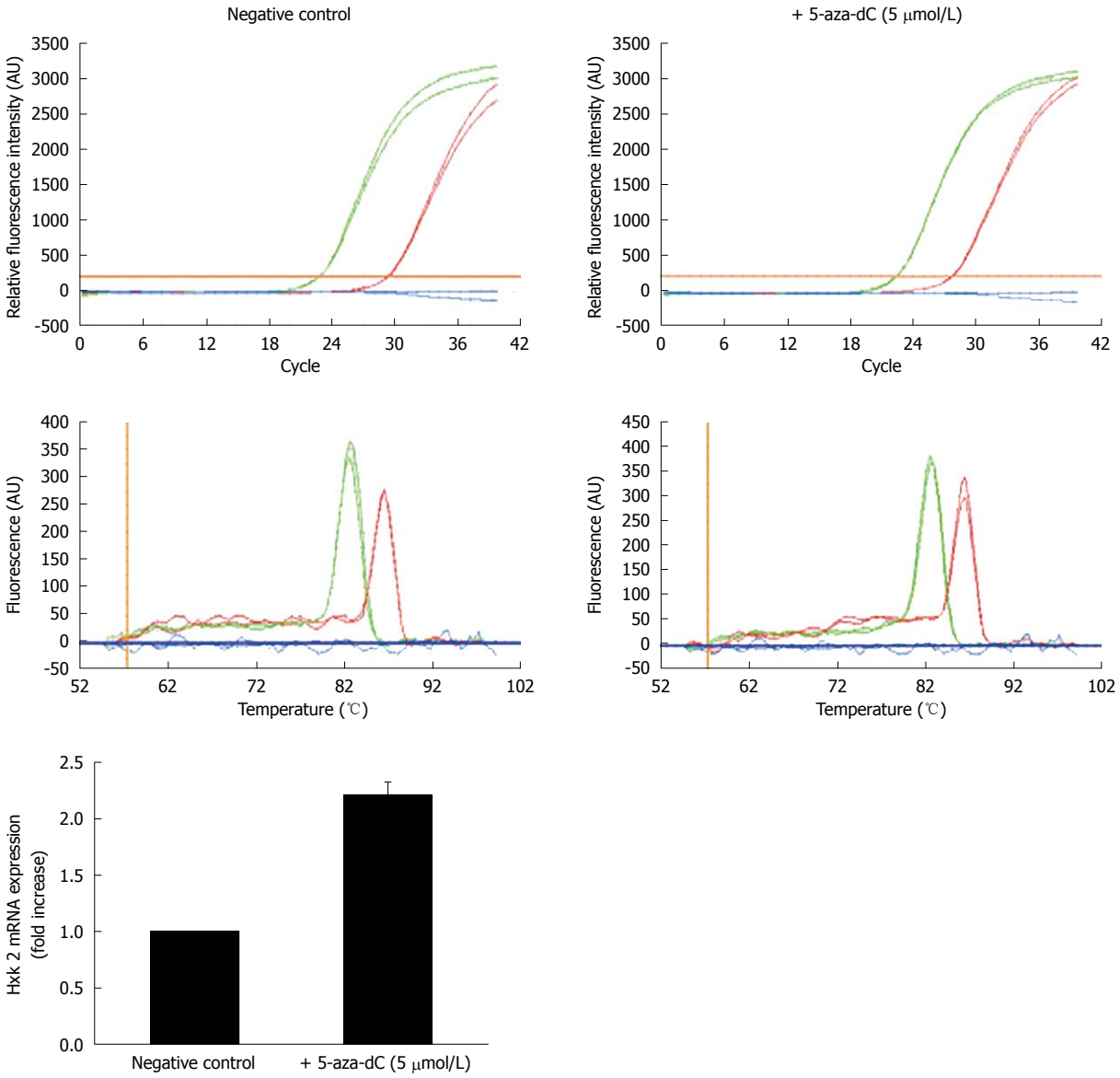

Figure 2 Quantitative real time polymerase chain reaction amplification profiles of GAPDH (green line), Hxk 2 (red line) and PDX-1 (blue line), and relative melting curves from total RNA of Clone-9 hepatocytes grown with or without 5 μmol/L 5-aza-deoxycytidine for 4 d. In the graphic representation below, data from at least three independent experiments that used different RNA preparations were normalized *vs* the negative control (mean ± SD); the Hxk 2 mRNA levels increase when cells are cultured in the presence of the drug, as expected, but no increase in the PDX-1 mRNA was observed. 5-aza-dC: 5-aza-deoxycytidine.

ablation of the liver; this was demonstrated by the increased levels of PCNA. Other genes involved in insulin expression did not show any correlation with the molecular events that derived from the enhancement of tissue proliferation. In fact, the only marker that could be detected in the regenerating liver was E47; a transcription factor that belongs to the bHLH family, which is known to be expressed ubiquitously in mammalian cells. In the pancreas of the same animals used as controls, on the contrary, almost all tested genes were expressed and could be identified by RT-PCR. The only gene that was not expressed is Ngn3 but it is known that this gene is silenced 2 d after birth in rats. Therefore, in the light of the results reported in this brief communication we can confidently state that the molecular events that occur during liver regeneration are not *per se* sufficient to promote PDX-1 expression; a key transcription factor in insulin regulation.

DNA methylation is a commonly accepted mechanism for the stable repression of gene expression in mammalian cells. The Hxk 2 gene is silenced through this mechanism in the normal hepatocytes. To explore the possibility that the expression of PDX-1 might also be blocked through DNA methylation, we grew K-9 cells in the presence of 5-aza-dC; a de-methylating agent that is incorporated into nascent DNA but cannot be methylated by cellular methylases. Treatment with this drug upregulates Hxk 2 mRNA transcription, thus, the gene is actually repressed by methylation, but its function can be rescued after exposure to 5-aza-dC. We checked whether PDX-1 silencing in liver cells could be exerted through methylation of CpG islands in both the promoter and the gene coding regions. We cultured wild-type K-9 cells in the presence or absence of 5 μmol/L 5-aza-dC; the Hxk 2 gene was used as positive control and GAPDH as a reference gene. Treatment with

5-asa-dC increased expression of the Hxk 2 control gene but failed to rescue the expression/function of PDX-1. The overall meaning of this result is that DNA demethylation induced by the drug is not sufficient to override repression of the PDX-1 gene.

The data presented in this short paper clearly indicate that the molecular events that occur during liver regeneration are not sufficient to reactivate spontaneously PDX-1 gene expression. In addition, the use of a demethylating agent in cultured rat hepatocytes is not able to de-repress PDX-1 gene expression, which indicates that silencing of the gene is unlikely to be achieved through this epigenetic mechanism. In conclusion, the results presented here clearly suggest that, because neither molecular mechanism is able to re-activate PDX-1 gene function, the repression of this gene must be ascribed to a different and possibly more complex phenomenon. The peptide hormone insulin is primarily involved in glucose metabolism, and is produced by pancreatic β cells that, also due to autoimmune responses, are destroyed in type 1 diabetes. However, the reactivation at liver level of the enzyme PDX-1 for therapeutic purposes does not seem to be an immediately attainable target.

COMMENTS

Background

Diabetes mellitus derives from an insulin deficiency; this hormone is produced by the pancreatic β cells, which are destroyed in type 1 diabetes. A number of different transcription factors are involved in the control of insulin expression and/or regulation and the best studied is possibly PDX-1. The actual role of this transcription factor in the activation/regulation of the insulin gene is controversial and the effects of PDX-1 on each gene depend on a fine interaction between transcription factors. We recently demonstrated that the PDX-1 transcription factor can significantly alter hepatocyte glucose metabolism. In this study, we assessed whether PDX-1 expression could be spontaneously reactivated during liver regeneration in partially hepatectomized rats; also, we verified whether PDX-1 silencing in the normal hepatocytes might be achieved through DNA demethylation.

Research frontiers

The transcription factor PDX-1 is a key molecule for approaches based on gene therapy of type 1 diabetes. The liver is considered a good candidate for the expression of insulin in patients affected by this disease. However, this transcription factor is not expressed in the liver, therefore, transformation of hepatocytes with exogenous PDX-1 gene copies is necessary. Thus, we explored the possibility that PDX-1 might be re-activated in regenerating liver, and if its silencing is caused by DNA methylation.

Innovations and breakthroughs

The molecular events that occur during liver regeneration are not sufficient to reactivate spontaneously PDX-1 gene expression. In addition, the use of a demethylating agent in cultured rat hepatocytes is not able to de-repress PDX-1 gene expression, which indicates that silencing of the gene is unlikely to be achieved through this epigenetic mechanism. These are new significant data that might have an important consequence on the activation of PFX-1 gene at the liver level for clinical/therapeutic purposes.

Applications

The results of this work suggest that neither addition of exogenous PDX-1 genes nor demethylation of the resident gene function can be used as an approach to gene therapy of type 1 diabetes. Therefore, reactivation PDX-1 enzyme at liver level for therapeutic purposes does not seem to be an immediately attainable target.

Peer review

This short paper describes some interesting findings that PDX gene is not involved in liver regeneration of rats. However,major revision is needed for publication.

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Benfang Lei's research on heme acquisition in Gram-positive pathogens and bacterial pathogenesis

Benfang Lei

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Abstract

Benfang Lei's laboratory conducts research on pathogenesis of human pathogen Group A *Streptococcus* (GAS) and horse pathogen *Streptococcus equi* (*S. equi*). His current research focuses on heme acquisition in Gram-positive pathogens and molecular mechanism of GAS and *S. equi* pathogenesis. Heme is an important source of essential iron for bacterial pathogens. Benfang Lei and colleagues identified the first cell surface heme-binding protein in Gram-positive pathogens and the heme acquisition system in GAS, demonstrated direct heme transfer from one protein to another, demonstrated an experimental pathway of heme acquisition by the *Staphylococcus aureus* Isd system, elucidated the activated heme transfer mechanism, and obtained evidence for a chemical mechanism of direct axial ligand displacement during the Shp-to-HtsA heme transfer reaction. These findings have considerably contributed to the progress that has been made over recent years in understanding the heme acquisition process in Gram-positive pathogens. Pathogenesis of GAS is mediated by an abundance of extracellular proteins, and pathogenic role and functional mechanism are not known for many of these virulence factors. Lei laboratory identified a secreted protein of GAS as a CovRS-regulated virulence factor that is a protective antigen and is critical for GAS spreading in the skin and systemic dissemination. These studies may lead to development of novel strategies to prevent and treat GAS infections.

Key words: Bacterial pathogenesis; Virulence factor; Innate immunity; Group A *Streptococcus*; *Streptococcus equi*; Esterase; Heme acquisition; Heme binding protein

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INTRODUCTION AND EDUCATIONAL EXPERIENCE

Dr. Benfang Lei (Figure 1) is an Associate Professor of Bacteriology in the Department of Veterinary Molecular Biology at Montana State University, USA. He received his Bachelor's degree in Chemistry from Wuhan University, China in 1982 and Master's degree in Chemistry from Beijing Institute of Chemical Technology, China in 1985. After performing research at the Institute of Photographic Chemistry, Academia Sinica for 3 years, he went to USA and received his Master's degree in chemistry from the University of Texas at El Paso in 1989 and PhD. in Biochemistry from the University of Houston in 1993. Prior to receiving his training in bacterial pathogenesis at Rocky Mountain Laboratories, the National Institute of Allergy and Infectious Diseases, NIH, he worked at a biotech company and then at the University of Houston as a Research Assistant Professor. In 2003, Dr. Lei was recruited to the Faculty of Montana State University. His research was supported by grants from The National Institutes of Health, US Department of Agriculture (USDA), and the Montana Board of Research and Commercialization Technology, USDA Animal Health Formula Funds, and the Montana State Agricultural Experiment Station.

ACADEMIC STRATEGY AND GOALS

The primary goals of Benfang Lei's research are to elucidate molecular mechanisms of Group A *Streptococcus* (GAS) and *Streptococcus equi* (*S. equi*) pathogenesis and to develop vaccines and therapeutic strategies to prevent and treat infections caused by both pathogens. To achieve these goals, Lei group uses multidisciplinary approach to identify novel protective antigens and virulence factors, investigate their functions, regulation, and role in pathogenesis and virulence, and explore applications of novel virulence factors in vaccine and therapeutics. Using this strategy, Lei group has identified a secreted protein as a novel virulence factor that is regulated by the two-component regulatory system CovRS (Control of Virulence), is a protective antigen, is critical for GAS dissemination, and has esterase activity. He is currently examining the role of the esterase in GAS evasion of the innate immunity. His laboratory also conduct detailed mechanistic studies on the GAS and *Staphylococcus aureus* (*S. aureus*) heme acquisition systems to understand how Gram-positive pathogens acquire heme from host. They are particularly interested in determining how heme is extracted from hemoglobin by its surface receptors Shr and IsdB and how heme is rapidly transferred from one protein to another. To this end, they will continue to examine the biochemical and/or chemical mechanisms, thermodynamics, and structural basis of the hemoglobin-to-Shr, hemoglobin-to-IsdB, and Shp-to-HtsA heme transfer reactions using biochemical, biophysical, structural, and spectroscopic approaches.

PRE-INDEPENDENT ACADEMIC ACHIEVEMENTS

Flavin-dependent two-component monooxygenase systems with potential application in biodesulfurization of fossil fuels

The flavin-dependent two-component monooxygenase systems are composed of a flavin reductase and a monooxygenase. The flavin reductase generates reduced flavin ($FMNH_2$), which is used as a co-substrate of the monooxygenase to oxidize another substrate. We cloned the *Vibrio harveyi* (*V. harveyi*) flavin reductase P (FRP), which reduces FMN using NADPH as the electron donor and provides it as a co-substrate of *V. harveyi* luciferase[1]. FRP was the first cloned flavin reductase of the two-component flavin monooxygenase systems. Another contribution to the field is that we established Sox/DszC, a component of the *Rhodococcus* organic sulfur oxidization system, as a FMN-dependent sulfide/sulfoxide monooxygenase[2]. A critical question unique to these systems is how $FMNH_2$ is transferred from the donor to the acceptor to avoid its rapid autooxidation when it is free. We conducted a detained kinetic analysis of $FMNH_2$ transfer in the FRP/luciferase reaction and found that $FMNH_2$ is directly channeled from FRP to luciferase[3]. This is the first and the most thorough study on the mechanism of $FMNH_2$ transfer in the field. These studies conducted during the early stage of the field are well recognized in the field, which is evident in a recent review[4]. In addition, these studies have had impact on developing biotechnology for biodesulfurization of fossil fuels.

Action and resistance mechanisms of antitubercular isoniazid

Tuberculosis due to *Mycobacterium tuberculosis* (*M. tuberculosis*) infection is the leading cause of death worldwide among known infectious diseases. Isoniazid has been the cornerstone in tuberculosis chemotherapy. Isoniazid is a prodrug and requires *in vivo* activation by the catalase/peroxidase KatG, and the activated compound inhibits the enoyl reductase InhA, resulting in inhibition of the synthesis of mycolic acid, a long chain fatty acid-containing component of the mycobacterial cell wall. We characterized the KatG-catalyzed isoniazid activation, isolated the resulting InHA inhibitor, and developed an inhibition assay[5]. We subsequently demonstrated that the common KatG mutations present in isoniazid-resistant clinical *M. tuberculosis* isolates abolish the ability of KatG to activate isoniazid[6]. High citations of these studies indicate that they had significant impact on studies on the mechanisms of isoniazid action and resistance and search for inhibitors of InhA for treating tuberculosis caused by isoniazid-resistant *M. tuberculosis*.

Identification of dominant antigens, the novel virulence factor Mac, and potential new vaccine candidates of GAS

Using contemporary investigative methods in the post-

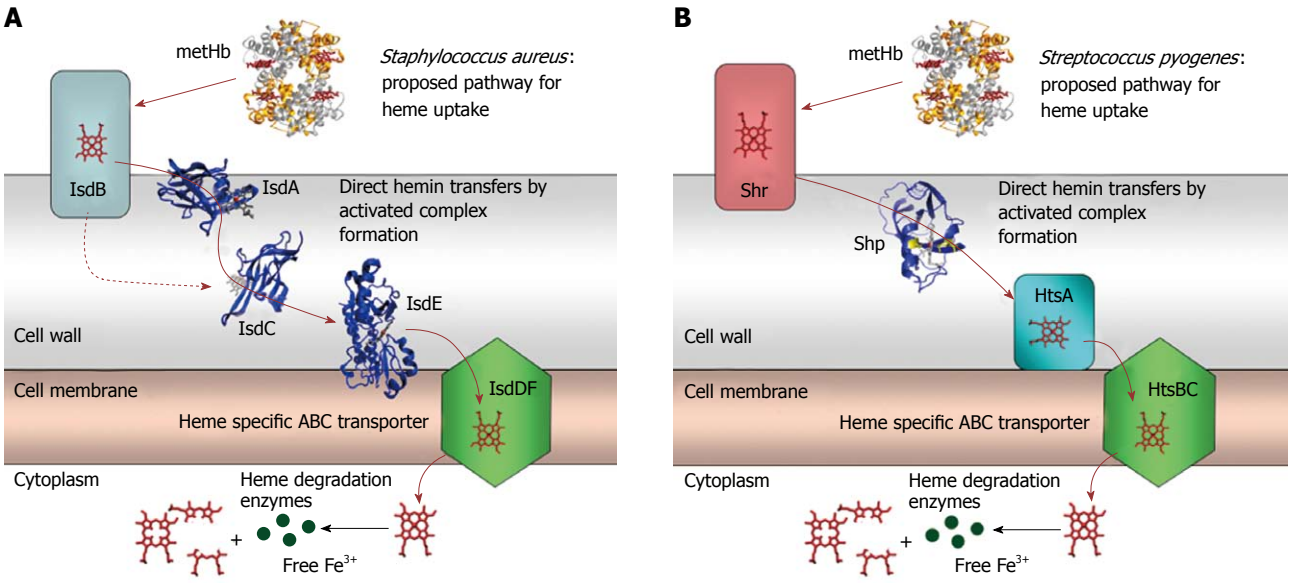

Figure 2 Cartoons for the proposed pathway of heme acquisition from metHb by the *Staphylococcus aureus* Isd (A) and Group A *Streptococcus* Shr/Shp/HtsABC (B) systems. The arrows indicate the direction of direct heme transfer. Heme transfer from IsdB to IsdC represented by the dotted arrow may be prevented *in vivo* by their physical locations in the cell wall. The structure models of the proteins were from the coordinates 2Q8Q, 2ITF, 2O6P, 2Q7A, and 1HHO. This figure was originally published in *J Biol Chem*. Zhu H, Xie G, Liu M, Olson JS, Fabian M, Dooley DM, Lei B. Pathway for heme uptake from human methemoglobin by the iron-regulated surface determinants system of Staphylococcus aureus. *J Biol Chem* 2008; 283: 18450-18460[23].

genomic era[7], we conducted the first systematic analysis of GAS culture supernatant proteins, identifying eight novel dominant extracellular antigens[8]. We subsequently found that one of the novel antigens, Mac, is a novel virulence factor that inhibits opsonophagocytosis of GAS by human neutrophils[9]. We found that there are two major Mac variants[10] that block immunoglobulin recognition by Fc receptors and degrade immunoglobulins, thereby enhancing survival of the pathogen through the inhibition of phagocytosis, endocytosis of IgG-opsonized particles, and antibody-dependent cell-mediated cytotoxicity[11]. These studies advanced the understanding of GAS pathogenesis and interactions with host. In another systematic study using the *in silico* analysis of the GAS genome, we identified all putative lipoproteins of GAS and then evaluated them for the potential as new vaccine candidates[12]. Further evaluation of these potential new vaccine candidates may develop an efficacious GAS vaccine.

INDEPENDENT ACADEMIC ACHIEVEMENTS

In the past 7 years, Dr. Lei's laboratory has contributed considerably to the literature in understanding heme acquisition in Gram-positive pathogens at the machinery, pathway, and kinetic and molecular mechanisms and pathogenesis or bacteriology of GAS and *S. equi*.

Iron acquisition and regulation in GAS

An *in silico* analysis of a GAS genome sequence identified 19 putative cell surface proteins, and one of them was identified as a novel heme-binding protein (Shp)[13]. The *shp* gene is co-transcribed with eight downstream genes, including three genes encoding an ATP-binding cassette transporter, HtsABC, and an upstream gene encoding another surface protein, Shr. We subsequently found that Shr and HtsA, the lipoprotein component of the HtsABC transporter also bind heme[14,15]. These studies suggest that Shr, Shp, and HtsABC constitute a heme acquisition machinery in GAS. Shp is the first cell surface heme binding protein identified in Gram-positive pathogens, which indicates that the surface proteins, in addition to ABC transporters, are required for heme acquisition by Gram-positive bacteria. We then found that the *ftsABCD* locus encodes a ferric ferrichrome transporter[16]. Thus, we contributed to discovery of two of the three known iron transporters in GAS. Interestingly, we found that the metalloregulator MtsR displays a different metal iron specificity in regulating the expression of iron- and manganese-specific MtsABC and heme-specific HtsABC transporters[17].

The molecular mechanism of heme transfer among the components of the GAS heme acquisition machinery

We found that Shp rapidly and efficiently transfers heme to HtsA[18], the first example of heme transfer from a cell surface protein to the lipoprotein component of a heme-specific ABC transporter in Gram-positive pathogens. Subsequently, we found that Shr efficiently transfers its heme to Shp but not to HtsA[15]. These findings led us to propose a model in which Shr acquires heme from methemoglobin and Shp relays heme from Shr to HtsA of HtsABC, which brings heme across the cytoplasmic membrane (Figure 2).

We chose to further study the Shp/HtsA reaction as a model to understand how heme is transferred from

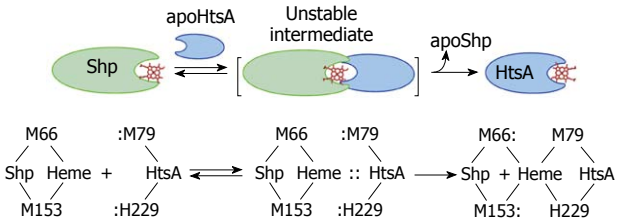

Figure 3 A direct heme axial ligand displacement model for the holoShp-holoHtsA reactions. The side chains of the heme axial ligands, M79 and H229, in apoHtsA are proposed to be inserted into the axial positions of heme in Shp, simultaneously displace M66 and M153 of Shp, and extract heme from the donor. M66/M153 and M79/H229 are the axial ligand residues of the heme iron in Shp and HtsA, respectively.

one protein to another during heme acquisition. We first demonstrated direct and rapid heme transfer from Shp to HtsA and elucidated the kinetic mechanism of the transfer reaction[19]. This is the first example of direct heme transfer from a surface protein to ABC transporter and the first detailed kinetic mechanism of heme transfer from one protein to another in bacterial heme acquisition processes. More importantly, this study demonstrated an activated heme transfer mechanism, that is, the heme donor Shp and acceptor HtsA form an activated complex to facilitate heme transfer. In addition, we developed a kinetic approach to distinguish direct and indirect heme transfer reactions. In a following publication[20], we demonstrated the unexpected importance of the axial ligands of the Shp heme iron on the transfer reaction and detected donor-heme-acceptor ternary complexes in the reactions of the Shp axial ligand mutants with wild-type HtsA. The significant advancement of this study is that a reaction mechanism model we called "plug-in" mechanism was derived. In this model, the side chains of the axial ligands of the empty heme pocket in apo-HtsA are inserted along the two axial sides of the Shp heme plane and displace the Shp axial residues to pry the cofactor out of Shp (Figure 3). We recently generated and characterized the axial ligand mutants of HtsA[21], which will be used to further test the plug-in model.

Heme acquisition pathway in S. aureus

The heme acquisition system of *S. aureus* consists of the surface proteins IsdA, IsdB, and IsdC and the ABC transporter IsdDEF. Using the expertise we developed from the studies of heme acquisition in GAS, we first examined the reaction of holo-IsdA and apo-IsdC and found that heme transfer from IsdA to IsdC is very efficient[22]. The significance of this work resides that these results provide the first example of heme transfer from one surface protein to another surface protein in Gram-positive bacteria and, perhaps most importantly, indicate that the mechanism of activated heme transfer, which we previously demonstrated in the Shp/HtsA reaction, may apply in general to all bacterial heme transport systems. Next, we found that methemoglobin directly transfers its heme to IsdB, but not to IsdA, IsdC, nor IsdE, that IsdB directly transfers its heme to IsdA and IsdC, and that IsdC, but not IsdB and IsdA, directly donates its heme to IsdE[23]. Taken together, these findings enable us to demonstrate an experimental model for heme acquisition in *S. aureus* (Figure 2). The most important achievement of this study is the establishment of the pathway for heme uptake from methemoglobin through the Isd surface proteins to the ABC transporter. In addition, this study provides the first example of direct and rapid heme transfer from methemoglobin to a bacterial protein. Furthermore, these findings also suggest that there are parallel functions of the components in the GAS and *S. aureus* heme uptake systems.

Discovery of the secreted esterase as a novel virulence factor

GAS produces a large number of extracellular proteins to mediate its pathogenesis, and the functions and functional mechanisms of most of these proteins are unknown. A secreted antigenic protein was found to have esterase activity[24]. Immunization with this protein protects mice against subcutaneous infection of GAS strains of more than one serotype[24]. Another significant observation in this publication is that the bacteria failed to spread from the infection site in the immunized mice, suggesting that the esterase is involved in the dissemination of the pathogen in the soft tissue. Consistent with the immunization and challenge results, a mutant defective in this esterase gene is attenuated in virulence and is unable to spread in the skin and disseminate into organs and blood[25]. Furthermore, we found that the esterase gene is regulated by the two-component regulatory system CovRS[25], which regulates many known virulence factors. The *S. equi* homologue of the GAS esterase has optimal activities against acetyl esters[26]. We are in the process to determine how this protein contributes to GAS pathogenesis and virulence.

Studies on bacteriology and pathogenesis of S. equi

S. equi is a horse pathogen causing strangles. Although *S. equi* and GAS have different host specificity, they have similar genetic make-up. We have taken a comparative approach in our studies on bacteriology and pathogenesis of both organisms. The two-component regulatory system VicRK is important to virulence and growth of both *S. equi* and GAS[27,28]. The secreted esterases produced by the two organisms have similar substrate specificity[26]. *S. equi* also has the Shp/HtsABC machinery for heme uptake[29]. However, the homologue of the GAS Mac protein does not inhibit opsonophagocytosis of *S. equi* by horse neutrophils[30]. These studies highlight the similarity and difference between the two pathogens.

CONCLUSION

Heme is a major source of essential iron for many bacterial pathogens, and the machineries for heme acquisition are potential targets for prevention and treatment of bacterial infections. Dr. Lei's laboratory has made significant contributions to the understanding of heme acquisition in Gram-positive pathogens regarding the machinery, pathway, reaction, kinetic and biochemical mechanisms,

and structural basis of heme transfer along the pathway. His studies have the potential to provide clinically relevant antibiotic strategies to inhibit the heme acquisition process for treating bacterial infections. Dr. Lei's studies on pathogenesis of GAS identified the secreted esterase as a CovRS-regulated virulence factor that is a protective antigen and is critical for GAS spreading in the skin and systemic dissemination. Further studies on the esterase will reveal novel mechanism of GAS pathogenesis.

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Meetings

Events Calendar 2010

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4th annual Stem Cells World Congress and exhibition

January 29-31
Cape Town, South Africa
International Conference on Chemical and Biomolecular Engineering

February 11-12
Barcelona, Spain
7th annual Screening Europe conference and exhibition

February 14-16
Lorne, Australia
31st Lorne Genome Conference on the Organization and Expression of the Genome

February 26-27
Manchester, United Kingdom
The 5th Annual Biomarkers Congress

February 27-March 5
Innsbruck, Austria
3rd FEBS Special Meeting on ABC Proteins-ABC2010

March 4-5
London, United Kingdom
3rd annual Advances in Synthetic Biology conference and exhibition

April 8-9
Qingdao, Shangdong, China
The 4th Annual China Chemical Focus 2010

April 24-28
Montreal, Canada
2010 2nd ASM Conference on Mobile DNA

May 5-7
Boston, MA, United Kingdom
4th annual RNAi and miRNA World Congress

May 25-26
Dublin, Ireland
4th annual Lab-on-a-Chip European Congress

June 8-9
Berlin, Germany
3rd annual Cancer Proteomics conference and exhibition

June 20-27
Novosibirsk, Russia
The Seventh International Conference on Bioinformatics of Genome Regulation and Structure\ Systems Biology (BGRS\SB-2010)

June 27-30
Washington, DC, United States
The World Congress on Industrial Biotechnology and Bioprocessing

July 4-8
Lyon, France
Society for Molecular Biology and Evolution-SMBE 2010

July 14-16
London, United Kingdom
International Conference on Chemical, Biological and Environmental Engineering

August 8-11
Durham, NC, United States
The 13th Biennial Molecular and Cellular Biology of the Soybean Conference

September 22-25
Heidelberg, Germany
EMBO Conference Series on Chemical Biology

September 26-October 1
Melbourne, Australia
OzBio2010: The Molecules of life: Discovery to Biotechnology

October 28-29
San Diego, CA, United States
2nd annual Microarray World Congress and 3rd annual Molecular Diagnostics World Congress

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Instructions to authors

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In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

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No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

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Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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