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Oxidant stress promotes disease by activating CaMKII

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Abstract

CaMKII is activated by oxidation of methionine residues residing in the regulatory domain. Oxidized CaMKII (ox-CaMKII) is now thought to participate in cardiovascular and pulmonary diseases and cancer. This invited review summarizes current evidence for the role of ox-CaMKII in disease, considers critical knowledge gaps and suggests new areas for inquiry.

Introduction

Enhanced oxidant stress is an important feature of cardiovascular diseases. Indeed, a working concept for many chronic diseases is that increased oxidant stress is maladaptive and promotes disease progression and severity. Despite this, trials of untargeted antioxidant agents have been disappointing. If increased oxidant stress is a factor for disease severity why would these clinical trials be unsuccessful? Some possibilities include that widely available antioxidant agents (e.g. vitamins) are inadequate therapeutic agents because of unfavorable pharmacokinetics, lack of antioxidant potency in specific subcellular compartments and/or because oxidant signaling is simultaneously beneficial and detrimental, making a net benefit of antioxidant agents difficult to demonstrate [1, 2, 3]. Our group hypothesized that discovery of concise disease promoting molecular pathways, downstream to pathologically increased oxidant stress, could provide mechanistic insights into the relationship between oxidation and illness and suggest new and effective antioxidant therapies.

CaMKII is activated by ROS

Our lab has focused on the role of CaMKII (the multifunctional Ca²⁺ and calmodulin-dependent protein kinase II) for much of the past two decades. We were the first to present evidence that CaMKII is a pathological, proarrhythmic signal [4–6] and that CaMKII inhibition could protect against heart failure [7]. We and others observed that CaMKII activity is increased in myocardium under stress conditions, such as hypertrophy and

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Disclosures

MEA is a cofounder of Allosteros Therapeutics, a company aiming to develop CaMKII inhibitor therapeutics.

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infarction, where oxidation is also elevated. Furthermore, we found that myocardial CaMKII inhibition was sufficient to protect against myocardial death and adverse left ventricular remodeling after ischemia-reperfusion injury and chronic myocardial infarction, conditions marked by elevated oxidant stress, suggesting CaMKII inhibition could counteract pathological oxidation and be a new and successful approach to treating injured myocardium. These and other findings led us to test the hypothesis that CaMKII might be activated by reactive oxygen species (ROS). Here I will focus on the role of CaMKII as a downstream signal activated by ROS, based on findings published since the initial description of redox activated CaMKII by our group in 2008 [8].

The balance between kinase and phosphatase activity is known, in several instances, to be influenced by ROS. Inactivating oxidation of a metal core in some phosphatases can tip the balance of activity in favor of protein kinase catalyzed phosphorylation. However, other kinases may be directly activated or inactivated by oxidation. We initially determined that CaMKII activity, measured against an artificial substrate, was enhanced in cell free conditions, where phosphatase activity was absent, by H₂O₂. In order to determine the critical activating epitopes oxidized under these conditions, we first focused on the CaMKII regulatory domain because this domain was recognized to be a target for an activity enhancing post-translational modification called threonine 287 (T287) autophosphorylation [9,10]. The regulatory domain consists of two defined regions: a calmodulin binding region and an autoinhibitory region (Fig 1). We developed a panel of mutant CaMKII proteins to test for the role of ROS modifiable amino acids that were conserved across the four CaMKII isoforms (Fig 2). Although initially suspecting a role for a cysteine at position 290 (C290), we found that H₂O₂ enhanced CaMKII activity was lost when we modified a methionine pair (281/282) by valine replacement (MMVV). This oxidation resistant mutant MMVV CaMKII retained normal activation by calcified calmodulin (Ca²⁺/CaM) and Ca²⁺/CaM-autonomous activity by T287 autophosphorylation, strongly suggesting that CaMKII was activated by ROS as a consequence of M281/282 oxidation. Since the time of this observation, other activating post-translational modifications have been identified in the CaMKII regulatory domain (Fig 1). Serine 280 (S280) can be modified during hyperglycemia by UDP-*N*-acetylglucosamine [11]. NO can activate CaMKII by nitrosylating C290 [12, 13, 14, 15]. Furthermore, NO can prevent CaMKII activation by nitrosylating C273, potentially allowing NO to confer coincidence detection properties that enhance spatial resolution for Ca²⁺ and NO signaling [15]. These modifications suggest that CaMKII is a nodal signal where multiple upstream inputs (e.g. ROS, hyperglycemia, uncoupled nitric oxide synthase) converge to convert CaMKII to a hyperactivated, Ca²⁺/CaM-independent enzyme with the capacity to participate in disease progression in myocardium. Later studies, discussed below, expanded the role of methionine 281/282 oxidized CaMKII (ox-CaMKII) and suggested ROS mediated pathological signaling in heart failure, arrhythmias, vascular injury, asthma and cancer involve ox-CaMKII. It is interesting to note that the methionine pair is the newest activation site, emerging in vertebrate evolution (Fig 2), whereas S280, T287 and C290 are older sites also present in invertebrate organisms. This suggests that more complex animals may deploy M281/282 for a physiological advantage related to redox signaling but potentially suffer disadvantage under conditions of excessive ROS where ox-CaMKII contributes to disease.

In humans there are four CaMKII isoforms (α , β , γ and δ), each encoded by a different gene on different chromosomes. These isoforms operate similarly, with initial activation upon Ca^{2+} /CaM binding and the capacity for sustained, Ca^{2+} /CaM independent activity upon regulatory domain post-translational modifications. The CaMKII holoenzyme consists of twelve monomeric subunits that assemble as a pair of stacked hexamers (Fig 1) [16]. It is likely that the CaMKII holoenzyme can form from an aggregation of various isoforms (i.e. as a heteromer). However, isoform expression varies by cell type and CaMKII δ is most clearly linked to myocardial disease [17, 18]. CaMKII δ is the most highly represented in myocardium with a smaller amount of CaMKII γ and perhaps miniscule expression of other isoforms [19]. The paired methionines (281/282, numbering varies slightly between isoforms) are conserved in the autoinhibitory regions of the regulatory domain of all the isoforms except for CaMKII α , which has a cysteine-methionine pair that is capable of conferring Ca^{2+} /CaM autonomous activity upon oxidation (Fig 2) [8]. Under resting conditions, in the absence of bound Ca^{2+} /CaM, CaMKII is largely inactive because the autoinhibitory region of the regulatory domain occupies an energetically favorable conformation preventing activity of the N terminus catalytic domain. Ca^{2+} /CaM binding allosterically distorts autoinhibition leading to transient CaMKII activation. Oxidation of M281/282 converts these hydrophobic residues into hydrophilic species that are disinclined to engage the hydrophobic pocket of the substrate binding site, thus disabling autoinhibition, even after Ca^{2+} /CaM unbinding. The first oxidation state of methionine is methionine sulfoxide. Methionine sulfoxide reductase A (MsrA) dynamically reduces ox-CaMKII; similar to phosphatase inactivation favoring net kinase activity, MsrA knock out leads to increased ox-CaMKII,[8, 20] whereas MsrA transgenic over-expression prevents ox-CaMKII accumulation and protects against myocardial disease [21, 22]. The second oxidation state of methionine, methionine sulfone is not reversible by MsrA, so conditions permissive for sulfone formation may not occur to a large extent in these models. At this time the relative contribution of H_2O_2 and superoxide ($\text{O}_2^{\bullet-}$) for oxidizing CaMKII in situ are uncertain. However, NADPH oxidases [8, 20, 21, 22] and mitochondria [23, 24] are both sources of ROS for ox-CaMKII. Various factors, such as hyperglycemia, [23] intracellular Na^+ overload, [25] cardiac glycoside toxicity, [26] cytokines, endotoxin,[27]angiotensin II[8, 21, 28] and aldosterone[22] are capable of increasing ox-CaMKII in myocardium. Thus, multiple signaling pathways converge on processes that generate ROS leading to increased ox-CaMKII. Net ox-CaMKII expression appears to be an integrated outcome of CaMKII oxidation and ox-CaMKII reduction by MsrA.

Ox-CaMKII promotes cardiomyopathy and death

Excessive CaMKII activity promotes cardiomyocyte death, likely by mitochondrial and extramitochondrial pathways. Similarly, elevated ROS contributes to cardiomyocyte death. Based on these ideas we asked if ox-CaMKII was a necessary or important connection between ROS and cardiomyocyte death. We initially reported that ox-CaMKII levels were increased after myocardial infarction or angiotensin II injection but not after isoproterenol [8]. ox-CaMKII expression was further increased by myocardial infarction and accompanied by greater myocardial death, dysfunction, adverse left ventricular remodeling and mortality in mice lacking MsrA [8]. These findings supported a view that ox-CaMKII was a biomarker

for myocardial infarction and angiotensin II infusion, conditions favoring increased myocardial ROS and that ox-CaMKII levels were a net consequence of upstream signals favoring ROS and MsrA catalyzed reduction of Met 281/282 oxidation. We found that aldosterone infusion also increased myocardial ROS and in the setting of myocardial infarction led to high mortality due to left ventricular rupture [22]. Excessive myocardial rupture was a consequence of enhanced myocardial expression of matrix metalloproteinase 9 (MMP9) due to ox-CaMKII mediated activation of myocyte enhancer factor 2 (MEF2). Taken together, these findings showed that ox-CaMKII was increased by angiotensin II and aldosterone, two neurohumoral agonists with well-established roles in heart failure, and suggested that therapeutic benefit of angiotensin II and aldosterone antagonist therapeutics may be partly related to inhibition of ox-CaMKII.

Myocardial ox-CaMKII is significantly increased in the first 24 hours after myocardial infarction; this early increase may be at least partially dependent on ROS generated by activation of a toll like receptor 4 (TLR4) and myeloid differentiation factor 88 (MyD88) pathway [27]. Mice lacking MyD88 had reduced ox-CaMKII after myocardial infarction or lipopolysaccharide challenge, reduced myocardial hypertrophy, improved mortality after myocardial infarction and decreased transcriptional activity by the nuclear factor κ light chain enhancer of activated B cells (NF- κ B). CaMKII appears to influence a substantial range of the gene expression changes resulting from myocardial infarction, including genes dependent upon NF- κ B signaling [29]. CaMKII activation of NF- κ B after myocardial infarction [29] and ischemia-reperfusion injury, [30] conditions marked by elevated ROS, contributes to a narrative suggesting that ox-CaMKII is a molecular link between pathological oxidative stress and myocardial inflammation. Pathologically stressed myocardium expresses an array of inflammatory gene products, including complement factor B (CFB), which is under control of NF- κ B. We found that CaMKII activation was required for myocardial CFB expression after myocardial infarction or lipopolysaccharide exposure and that CaMKII/NF- κ B mediated CFB expression was sufficient to cause myocardial cell membrane injury by participation in the complement membrane attack complex [29]. The role of CaMKII in CFB and MMP9 expression by myocardium was surprising and provided evidence that pathologically stressed myocardium contributed directly to 'extramyocardial' disease manifestations. Overall, these early studies provided clear evidence for a role of ox-CaMKII in myocardial injury and showed that diverse upstream pathways contributing to increased myocardial ROS had access to CaMKII, producing elevated ox-CaMKII expression in response to injury.

ox-CaMKII in arrhythmias and oxidation resistant CaMKII δ MMVV mice

Since the time of our discovery that CaMKII activation was proarrhythmic by favoring early [4, 6] and delayed [5] afterdepolarizations, we and many others have established a proarrhythmic role for CaMKII and an antiarrhythmic effect of CaMKII inhibition in atrial and ventricular arrhythmias. While not all of this work is related to the role of ROS and/or ox-CaMKII, a convincing body of evidence now supports a role for ox-CaMKII in a wide range of arrhythmias. Myocardial infarction is a critical predisposing circumstance that increases the likelihood of sudden death due to ventricular tachycardia and fibrillation [31]. Mice deficient in CaMKII δ and γ isoforms are protected against myocardial scar formation

after ischemia-reperfusion injury, due to decreased myocardial expression of macrophage inflammatory protein-1 α [32]. In the peri-infarct period the borderzone region, forming the boundary between dead and normal myocardium, is a hot spot for increased ROS and reentrant arrhythmias. ox-CaMKII expression is increased in the infarct borderzone compared to remote, uninjured myocardium and leads to loss of Na⁺ current availability, conduction slowing and block, whereas CaMKII inhibition is predicted to restore borderzone conduction velocity toward physiological levels [33]. Thus, one proarrhythmic action of ox-CaMKII appears to be promotion of reentrant ventricular arrhythmias by actions in the infarct borderzone.

Advanced age, a condition of accumulated ROS, is a risk factor for arrhythmias and sudden death. Aged rat and rabbit hearts, challenged with H₂O₂, were highly susceptible to afterdepolarization induced ventricular tachycardia and fibrillation, while CaMKII inhibition by a small molecule (KN-93) or peptide (AIP) inhibitor prevented these arrhythmias [34, 35]. Excess ROS enabled afterdepolarizations by actions at voltage-gated Na⁺ and Ca²⁺ currents and the ryanodine receptor type 2 (RyR2) intracellular Ca²⁺ release channel [35]. ox-CaMKII enhances a persistently non-inactivating component of the main voltage-gated Na⁺ current in heart that contributes to afterdepolarizations [36]. Angiotensin II stimulation of NADPH oxidase leads to increased ROS, increased ox-CaMKII [8] and afterdepolarizations, at least in part by actions at voltage-gated Ca²⁺ channels and RyR2 [37]. Intracellular Na⁺ overload contributes to elevated subsarcolemmal [Ca²⁺] and reduced intramitochondrial [Ca²⁺]. The reduction in mitochondrial [Ca²⁺] leads to increased ROS, likely by decreased generation of reducing equivalents NADH/NADPH, increased ox-CaMKII and afterdepolarizations by CaMKII-mediated RyR2 Ca²⁺ leak [25]. In aggregate, these findings show that ox-CaMKII contributes to afterdepolarization initiated arrhythmias by pathways that involve RyR2 and voltage-gated Ca²⁺ and Na⁺ channels. Because repetitive afterdepolarizations can engage reentrant circuits, also potentially facilitated by ox-CaMKII, [33] it seems likely that elevated myocardial ox-CaMKII can contribute to most or all types of rapid (tachy) arrhythmias.

In contrast to tachyarrhythmias that can arise from almost any region of atrial or ventricular myocardium, rhythm disorders where heart rate is too slow to sustain physiological requirements mostly are due to defective performance of the sinoatrial node (SAN) pacemaker cells or the specialized conduction system (i.e. the atrial-ventricular node and His-Purkinje fibers). SAN dysfunction causes bradycardia while disease of the specialized conduction system, which connects the lower atrium and ventricles to SAN impulses, leads to impaired conduction velocity or conduction failure (i.e. 'heart block'). Most SAN dysfunction occurs in the setting of hypertension and heart failure, conditions with elevated myocardial ROS. We next considered that the role of ox-CaMKII to promote cardiomyocyte death [8] could also contribute to SAN dysfunction under conditions of elevated myocardial ROS. We found ox-CaMKII was elevated in SAN myocardium from patients with heart failure who required an artificial pacemaker compared to patients who had heart failure but did not require a pacemaker and compared to healthy donor hearts, suggesting ox-CaMKII was a biomarker for patients with heart failure and SAN dysfunction. ox-CaMKII was also increased in the vicinity of the SAN in dogs with pacing induced heart failure and SAN dysfunction and in mice with SAN dysfunction because of angiotensin II infusion at a rate

sufficient to achieve plasma levels measured in patients with heart failure. These findings indicated concordant elevation in SAN ox-CaMKII between patients with SAN defects and animal models of heart failure and SAN dysfunction. Angiotensin II infused mice showed increased SAN cell death, while mice with transgenic or gene-targeted SAN CaMKII inhibition were resistant to angiotensin II infusion mediated SAN cell death and dysfunction [38]. Mathematical modeling suggested SAN dysfunction would emerge after sufficient SAN cell death led to a source-sink mismatch between SAN and surrounding atrial myocardium that prevented reliable atrial depolarization. Taken together, these data supported a view that ox-CaMKII was a mediator of bradyarrhythmias due to SAN dysfunction in the setting of heart failure and/or increased angiotensin II.

Up until this point we had shown oxidation of methionines 281/282 generated a form of CaMKII (ox-CaMKII) with constitutive enzymatic activity, persisting in the absence of Ca^{2+} /CaM binding, and that myocardial ox-CaMKII was increased in response to upstream signals associated with pathological stress and heightened ROS. Furthermore, we found loss of MsrA increased ox-CaMKII while MsrA over-expression decreased ox-CaMKII. We interpreted an extensive body of data from us and from others to corroborate the hypothesis that ox-CaMKII contributed to various forms of myocardial disease. However, to test this hypothesis more rigorously and more directly, we needed to generate an animal model with targeted loss of ox-CaMKII. To achieve this goal we designed knockin mice where CaMKII δ , the isoform most linked to myocardial disease, [17, 18] methionines 281/282 were replaced with valines. The MMVV mutant CaMKII δ is resistant to ROS activation but retained other features of wild type CaMKII, including Ca^{2+} /CaM autonomous activity due to threonine 287 autophosphorylation [8]. Thus, the MMVV δ mice provided a definitive tool to directly test if CaMKII oxidation, at methionines 281/282, was required for pathological, downstream responses to excessive ROS.

We first used these MMVV δ mice to interrogate the role of ox-CaMKII in atrial fibrillation [21]. Atrial fibrillation is the most common sustained and serious arrhythmia and markers of ROS are elevated in patients with atrial fibrillation [39, 40]. Furthermore, elevated angiotensin II is increased in patients with hypertension and heart failure, which are risk factors for atrial fibrillation [41]. CaMKII expression is increased in fibrillating atria from patients [42] and promotes atrial fibrillation, at least in part, by increasing RyR2 Ca^{2+} leak to trigger afterdepolarizations [43]. We found ox-CaMKII was increased in atrial tissues obtained at the time of cardiac surgery from patients with atrial fibrillation compared to patients in sinus rhythm, suggesting ox-CaMKII was a consequence of increased ROS and a myocardial marker of atrial fibrillation. Interestingly, the elevated ox-CaMKII levels were not seen in patients with atrial fibrillation who were taking angiotensin II antagonist drugs, consistent with the connection between ox-CaMKII and angiotensin II and aldosterone signaling we had previously established in animal models [8, 22, 28]. We next infused mice with angiotensin II, according to the method we developed to achieve circulating levels that induced SAN dysfunction [38]. The angiotensin II infused mice exhibited more atrial ox-CaMKII and developed atrial fibrillation more often than saline infused wild type mice in response to rapid atrial stimulation. In contrast, angiotensin II did not increase atrial fibrillation, compared to saline infusion, in MMVV δ mice, in mice lacking a critical component of NADPH oxidase (p47) deprived of the ability to mount a normal increase in

myocardial ROS in response to angiotensin II, in mice with transgenic myocardial over-expression of MsrA nor in mice with knockin replacement of a CaMKII targeted serine (2814) on RyR2 that promotes sarcoplasmic reticulum Ca^{2+} leak. Notably, atrial myocytes isolated from the MMVV6 mice were resistant to angiotensin II induced increases in RyR2 Ca^{2+} leak and afterdepolarizations. The resistance of MMVV6 and these other genetic models to angiotensin II biased atrial fibrillation induction occurred despite a normal hypertensive and myocardial hypertrophic response to angiotensin II infusion, suggesting hypertension was not a risk factor for atrial fibrillation in the absence of ox-CaMKII δ . This study [21] provided the first evidence that targeted loss of ox-CaMKII δ was sufficient to prevent proarrhythmic responses to angiotensin II in a mouse model of atrial fibrillation.

Atrial fibrillation and SAN dysfunction commonly occur together in patients [44] and our studies suggested that ox-CaMKII could participate in both atrial fibrillation and SAN disease. Furthermore atrial fibrillation and SAN dysfunction are associated with increased mortality, [45] but up until this point the potential for ox-CaMKII to increase mortality had not been directly tested. We turned our attention to diabetic cardiomyopathy because diabetes is a growing public health problem and most diabetic patients die from cardiovascular complications where excessive ROS is hypothesized to contribute to disease outcomes. We first identified increased myocardial ox-CaMKII in tissues obtained from diabetic patients after myocardial infarction compared to myocardium from patients who had suffered from myocardial infarction but were not diabetic. Diabetic patients have approximately double the mortality rate of non-diabetic patients after myocardial infarction and this increased mortality persists even after factoring out common clinical variables, such as the extent of epicardial coronary disease and left ventricular dysfunction [46]. In order to test for a role of ox-CaMKII in worsened clinical outcomes of diabetics after myocardial infarction, we turned to an established mouse model of type I diabetes due to exposure to a pancreatic β cell toxin, streptozotocin (STZ).

First we measured ox-CaMKII in myocardium from diabetic and non-diabetic patients after myocardial infarction. We found that myocardium from diabetic patients exhibited significantly more ox-CaMKII than myocardium from non-diabetic patients, [23] suggesting that ox-CaMKII was elevated as a consequence of diabetes. STZ-treated mice reliably developed elevated blood glucose and weight loss, consistent with loss of β cell function. Similar to diabetic patients, these diabetic mice had substantially higher mortality after myocardial infarction surgery, compared to saline injected non-diabetic controls, and excess mortality was prevented by insulin therapy, confirming hyperglycemia or another insulin-dependent process was directly responsible for increased death. Remarkably, diabetic MMVV6 mice were protected from excessive mortality caused by diabetes, strongly supporting the concept that ox-CaMKII participated in excess mortality in the setting of diabetes and myocardial infarction. We found that hyperglycemia increased myocardial ROS generation by mitochondria. Mitochondrial ROS appeared to be critical for elevated mortality in STZ treated mice after myocardial infarction because infusion with a mitochondrial-targeted antioxidant (mito-TEMPO) protected against increases in mortality after myocardial infarction surgery due to diabetes. Diabetes conferred elevated mortality because it induced profound sinus bradycardia and loss of normal heart rate variability due to increased SAN cell death and fibrosis. SAN cells were protected in MMVV6 mice and in

wild type mice after mitoTEMPO infusion but not in $p47^{-/-}$ mice, suggesting that excessive ROS was a by product of mitochondrial activity rather than NADPH oxidase, in contradistinction to our observations in the setting of angiotensin II infusion [8, 20, 22, 38]. Loss of normal heart rate variability is a marker for increased mortality and diabetic patients with myocardial infarction exhibit impaired heart rate acceleration compared to non-diabetic counterparts [47]. Our study showed that ox-CaMKII was elevated in myocardium from diabetic patients and is essential for increased mortality after myocardial infarction in mice with STZ-induced diabetes. However, recent work has shown that *O*-GlcNacylation of serine 280 on CaMKII δ also contributes to hyperactivation of CaMKII and RyR2 receptor leak in the setting of hyperglycemia [11]. Thus, it will be interesting and important to learn how ox-CaMKII and *O*-GlyNacylated CaMKII may collaborate to promote diabetic cardiomyopathy, arrhythmias and sudden death.

Taken together, these studies make a strong case that ox-CaMKII participates in myocardial disease where it promotes arrhythmias and heart failure. The MMVV δ mouse provides a model to directly probe the role of ox-CaMKII in physiology and disease. To date, we have shown that selective loss of methionines 281/282 on CaMKII δ protect against downstream consequences of angiotensin II and type I diabetes. We recently developed a similar model of ROS-resistant CaMKII γ to test if or how oxidative activation of CaMKII δ and CaMKII γ contribute to transduction of ROS-signaling.

Vascular Disease

Atherosclerotic vascular disease is a major cause of death worldwide and myocardial infarction is a watershed event leading to death in atherosclerotic disease. Atherosclerotic plaques in diseased coronary arteries can become unstable and rupture leading to compromised blood flow and infarction under conditions that favor death of plaque resident macrophages [48]. The two best understood pathways for triggering cell death involve activation of the Fas receptor and mitochondrial inner membrane depolarization and persistent opening of the mitochondrial permeability transition pore; the Fas receptor and mitochondrial pathways are associated with increased ROS. In collaborative work led by the Tabas group we found that elevated cholesterol induced ROS in macrophages and oxidation of CaMKII γ promoted macrophage death by activation of the Fas and mitochondrial death pathways [49]. CaMKII was activated by a variety of 'upstream' agonists (e.g. LPS, thapsigargin, tunicamycin), highlighting the nodal role of CaMKII signal transduction for promoting cell death. Furthermore, ox-CaMKII facilitated death in a variety of cell types, suggesting these pathways may participate in diverse diseases where excess cell death contributes to disease progression. CaMKII δ over-expression leads to increased myocardial and mitochondrial ROS, [49, 50] suggesting that CaMKII may participate in a feed forward circuit contributing to elevated ROS and ox-CaMKII. The role of ox-CaMKII in activating a mitochondrial death response is consistent with our finding that mitochondrial-targeted CaMKII inhibition protected against myocardial death and dysfunction after ischemia-reperfusion injury and myocardial infarction, conditions marked by excessive ROS [51]. These findings show ox-CaMKII participates in cell death responses that may contribute to atherosclerotic plaque destabilization and infarction. I interpret the widespread role of

CaMKII in cell death under high ROS conditions to suggest that ox-CaMKII could contribute to many diseases.

Vascular smooth muscle cells participate in developmental and pathological remodeling and may contribute to hypertension and vascular occlusion. Using an elegant genetic mouse model with vascular smooth muscle targeted CaMKII inhibition, the Grumbach group found that CaMKII activity contributes to the angiotensin II mediated hypertensive response in vivo, where it influences a complex and interconnected series of central and peripheral activities [52]. CaMKII is known to promote vascular smooth muscle hypertrophy [53] but this remodeling process is also affected by vascular smooth muscle death and migration. In collaborative work led by the Grumbach laboratory, we found that vascular smooth muscle cells isolated from MMVV8 mice were highly resistant to H₂O₂ induced death and migrated less rapidly than wild type control cells in vitro [54]. Interestingly, the MMVV8 vascular smooth muscle cells exhibited reduced ROS, reduced expression of p47 and increased expression of superoxide dismutase, an antioxidant enzyme that catalyzes the reaction converting superoxide into H₂O₂. These findings suggested that ox-CaMKII contributes to a yet to be defined pathway for regulating redox balance. Furthermore, CaMKII γ and CaMKII δ mRNA expression were significantly increased in MMVV8 compared to wild type vascular smooth muscle cells, suggesting that CaMKII expression was controlled by ROS via ox-CaMKII. These findings show ox-CaMKII participates in vascular smooth muscle responses to injury and suggest ox-CaMKII may provide feedback information for cellular redox balance.

Flow-mediated vascular remodeling occurs in response to shear stress, leading to blood vessel expansion. Increased flow promotes dilation and matrix remodeling [55]. One established animal model of flow-mediated vascular remodeling involves ligation of a carotid artery leading to enhanced flow and expansion of the contralateral, non-ligated carotid artery. In collaborative work led by the Grumbach group, we discovered that CaMKII δ was required for flow mediated vascular remodeling because compensatory expansion of the non-ligated artery was nearly absent in CaMKII $\delta^{-/-}$ mice but readily observed in wild type controls [56]. ox-CaMKII was critical because MMVV8 mice also lacked this vascular remodeling response. MMP9 derived from adventitial macrophages and the non-remodeling phenotype in MMVV8 mice was rescued by transplantation with wild type bone marrow. These findings added to our earlier work in myocardium [22] and expanded the cell types where ox-CaMKII contributed to tissue matrix remodeling by enhancing MMP9 expression. Interestingly, the MMVV8 mice did not exhibit a change in threonine 287 autophosphorylated CaMKII, suggesting that ox-CaMKII and autophosphorylated CaMKII were modulated independently in this model. All together, new findings in MMVV8 mice reveal a critical role for ox-CaMKII in multiple aspects of vascular biology relevant to disease and suggest that CaMKII inhibition may be a new and successful antioxidant therapy for common, acquired forms of vascular disease.

Asthma

Asthma has emerged as a major public health problem and many patients are resistant to current therapies [57]. Asthma has multiple, complex phenotypes including airway

hyperreactivity, increased proinflammatory cytokines, airway epithelial hyperplasia and excessive airway mucus secretion. Despite this complexity, there is a clear and established connection of elevated oxidation, for example in exhaled gas, and asthma [58]. However, the potential mechanisms and pathways for increased ROS to affect asthma have remained uncertain. In 2008 our group won an award from the Sandler Foundation and the American Asthma Foundation that was designed for investigators with no expertise in asthma. We fit this category. Based on our then nascent, collaborative work with ox-CaMKII in vascular smooth and our finding that CaMKII contributed to inflammatory gene expression in the setting of elevated oxidant stress, we proposed to test the hypothesis that ox-CaMKII in airway smooth muscle contributed to asthma by promoting hypertrophy and inflammation. While we did find that ox-CaMKII was present in airway smooth muscle, ox-CaMKII expression did not appear to be different in biopsy specimens obtained from normal volunteers or asthmatic subjects. Instead, we found that ox-CaMKII in human airway epithelium obtained at autopsy was augmented in step with increasing severity of asthma and, more importantly, using paired samples, ox-CaMKII was increased over baseline in airway epithelium biopsied from mildly asthmatic patients twenty-four hours after exposure to an inhaled allergen [20]. These findings led us to revise our hypothesis and focus on the potential for ox-CaMKII to promote asthma by actions in airway epithelium.

We next turned to established mouse models of allergic asthma, induced by exposure to ovalbumin or *Aspergillus fumigatus*. Similar to allergen-challenged asthma patients, 'asthmatic' mice exhibited increased airway epithelial ox-CaMKII expression. In order to directly test the role of epithelial CaMKII in asthma phenotypes, we developed a mouse model with targeted expression of a CaMKII inhibitory peptide (AC3-I) [7] in airway epithelium [20]. These mice were resistant to clinically important asthmatic responses to allergen challenge, including airway hyperreactivity (induced by metacholine), increased airway epithelial proliferation and mucus secretion. The benefits of CaMKII inhibition in pulmonary epithelium appeared to arise from reduction in a Ca^{2+} -activated Cl^- current known to augment airway mucus secretion in asthma [59] and decreased NF- κ B activity with loss of cytokine expression that is thought to sensitize airway smooth muscle and promote bronchospasm. We also found that CaMKII inhibition reduced proliferation of airway epithelial cells that contribute to airway narrowing. A p47 containing form of NADH oxidase was one critical source of ROS for oxidizing CaMKII because *p47*^{-/-} mice were resistant to asthma. However, a more recent study from our group suggests that epithelial mitochondria are also an important source of ROS for increasing TGF β 1 expression and activity that contributes to pulmonary fibrosis after ovalbumin exposure [60]. Taken together, these studies suggest that CaMKII is an important pathological ROS signal transduction molecule in airway epithelium and that ox-CaMKII triggers diverse phenotypes that define allergic asthma.

Cancer

CaMKII hyperactivation has emerged as a signaling node for promoting a variety of cancers. CaMKII actions are varied and may be cancer type specific, but in general operate by targeting proteins important for cell cycle control, cell survival, metastasis and metabolism [61]. The Warburg effect is a fundamental feature of many cancers whereby a metabolic

preference for glycolysis enables survival in low O₂ environments [62]. Increased expression of MnSOD and activation of AMPK are present in highly aggressive cancers and AMPK activation by phosphorylation, in response to increased H₂O₂ and/or reduced ATP, at threonine 172 promotes glycolysis. Recent evidence suggests that CaMKII is the critical ROS sensor and that ox-CaMKII catalyzes the AMPK activating phosphorylation at threonine 172 [24]. Inhibition of CaMKII, knockdown of MnSOD or inhibition of AMPK reduced survival in aggressive breast cancer cells and KN-93, a small molecule CaMKII inhibitor, was more effective than conventional chemotherapeutic agents paclitaxel and fluoruracil for killing breast cancer cells. These data identify ox-CaMKII as a key signal for enabling the Warburg effect in breast cancer cells and suggest the exciting possibility that CaMKII inhibition could have therapeutic benefit in cancers relying on glycolysis for selective advantage over non-tumor cells.

Open questions and next steps

It has been thrilling to witness the expanding role of CaMKII in the cardiovascular system. The concept that CaMKII is activated by ROS suggests a potentially wider role for CaMKII in physiological and pathological responses to oxidant stress. Since our initial discovery of ox-CaMKII in 2008, new investigations have provided improved understanding of how ox-CaMKII can connect excess ROS with pathological outcomes in cardiovascular, pulmonary disease and cancer. These studies have mostly considered ox-CaMKII to be downstream to ROS, but some evidence is emerging that excessive CaMKII activity may contribute to ROS generation [49, 50]. Thus, it is possible that CaMKII is part of a pro-oxidant circuit that constitutes a feed forward, positive feedback pathway. If true, it would suggest that CaMKII inhibition, per se, could be a therapeutic antioxidant strategy in diverse diseases.

The ability of CaMKII to sense ROS through paired regulatory domain methionines is a relatively modern invention, compared to all other known regulatory domain post-translational modifications. I assume that methionines provided an evolutionary advantage over more ancient forms of CaMKII, suggesting the possibility that ox-CaMKII enhances performance of critical tissues and organs. It will be important to understand if and how ox-CaMKII participates in physiological adaptations. It is possible that evolution favors an ox-CaMKII mediated physiological enhancement but is indifferent to the later life, pathological consequences of excessive CaMKII activity due to ox-CaMKII and ROS accumulation. Work over the past 20 years has identified several post-translational modifications in the CaMKII regulatory domain that confer Ca²⁺/CaM autonomous, constitutive activity. However, there is a lack of understanding of how these post-translational modifications may interact to sculpt CaMKII activity in various physiological and pathological environments. The MMVV mice and other tools that will no doubt follow are likely to provide new information to address these and other important issues relating to the continually fascinating role of CaMKII in health and disease.

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Highlights

- CaMKII is activated by ROS modification of regulatory domain methionines. Oxidized CaMKII activity persists in the absence of calmodulin binding.
- The capacity for CaMKII methionine oxidation first appears in vertebrates and is reversed by methionine sulfoxide reductase A.
- Oxidized CaMKII contributes to downstream disease phenotypes in heart, blood vessels, asthma, diabetes and cancer.
- CaMKII inhibition and knockin replacement of ROS activating methionines in genetic mouse models protects against ROS induced disease.
- CaMKII appears to be an important for transduction of pathological ROS levels into diverse forms of disease, and CaMKII inhibition could represent a novel form of anti-oxidant therapy.

Figure 1.

CaMKII structure and regulatory domain post-translational modifications. Top left panel – known and putative posttranslational modifications capable of activating CaMKII independent of Ca^{2+} and calmodulin ($\text{Ca}^{2+}/\text{CaM}$) binding. Top right panel – schematic map of CaMKII domains and expanded view of the autoinhibitory (left) and calmodulin binding (right) regions. Bottom panel – Schematic depiction of the CaMKII holoenzyme in a compact, inactive conformation (left most), in an extended, active conformation after $\text{Ca}^{2+}/\text{CaM}$ binding (second from left), in an extended, active conformation with $\text{Ca}^{2+}/\text{CaM}$ binding and post-translational modification to the autoinhibitory region (second from right) and in an extended, constitutively active conformation after $\text{Ca}^{2+}/\text{CaM}$ unbinding due to persistent post-translational modification to the autoinhibitory region (right most).

Organism	281	282	Regulatory domain
Flat worm (Schistosoma haematobium) CaMKII	KNCDRVAS	SVHRQET	IVVLRKFNARRKLKAAVHTALLVTKRSS
Sponge (Suberites domuncula) CaMKII alpha-like	CQERVAS	SLVHRQET	VECLKKFNARRKLKGAILTTMLATRNFS
Nematode (Caenorhabditis elegans) CaMKII	CNRERVAS	SAIHRQET	VDCLKKFNARRKLKGAILTTMIATRNLS
Segmented worm (Capitella teleta) CaMKII	CQERVAS	SVHRQET	VDCLKKFNARRKLKGAILTTMLCSRNF
Pacific oyster (Crassostrea gigas) CaMKII-like	CQERVAS	SVVHRQET	VDCLKKFNARRKLKGAILTTMLATRNFS
Sea urchin (Strongylocentrotus purpuratus) CaMKII	QNRURYAS	SAHHRQET	VDCLKKFNARRKLKGAILTTMLATRNFS
Horseshoe crab (Limulus polyphemus) CaMKII	CQERVAS	TLHRQET	VECLKKFNARRKLKGAILTTMLATRNFS
Fruit fly (Drosophila melanogaster) CaMKII	CQERVAS	SVVHRQET	VDCLKKFNARRKLKGAILTTMLATRNFS
Honey bee (Apis mellifera) CaMKII	CQERVAS	SVVHRQET	VDCLKKFNARRKLKGAILTTMLATRNFS
Frog (Xenopus tropicalis) CaMKIIa	SHRSTVAS	CHHRQET	VDCLKKFNARRKLKGAILTTMLATRNFS
Frog (Xenopus tropicalis) CaMKIIb	CQRSTVAS	MHHRQET	VECLKKFNARRKLKGAILTTMLATRNFS
Frog (Xenopus tropicalis) CaMKIIg	CQRSTVAS	MHHRQET	VECLKKFNARRKLKGAILTTMLVSRNFS
Frog (Xenopus tropicalis) CaMKIId	CQRSTVAS	MHHRQET	VDCLKKFNARRKLKGAILTTMLATRNFS
Lizard (Anolis carolinensis) CaMKIIa	SHRSTVAS	CHHRQET	VDCLKKFNARRKLKGAILTTMLATRNFS
Lizard (Anolis carolinensis) CaMKIIb	CQRSTVAS	MHHRQET	VECLKKFNARRKLKGAILTTMLATRNFS
Lizard (Anolis carolinensis) CaMKIIg	CQRSTVAS	MHHRQET	VECLKKFNARRKLKGAILTTMLVSRNFS
Lizard (Anolis carolinensis) CaMKIId	CQRSTVAS	MHHRQET	VDCLKKFNARRKLKGAILTTMLATRNFS
Chicken (Gallus gallus) CaMKIIa	SHRATVAS	CHHRQET	VDCLKKFNARRKLKGAILTTMLATRNFS
Chicken (Gallus gallus) CaMKIIb	CQRSTVAS	MHHRQET	VECLKKFNARRKLKGAILTTMLATRNFS
Chicken (Gallus gallus) CaMKIIg	CQRSTVAS	MHHRQET	VECLKKFNARRKLKGAILTTMLVSRNFS
Chicken (Gallus gallus) CaMKIId	CQRSTVAS	MHHRQET	VDCLKKFNARRKLKGAILTTMLATRNFS
Zebra fish (Danio rerio) CaMKIIa	SHRSTVAS	CHHRQET	VECLKKFNARRKLKGAILTTMLATRNFS
Zebra fish (Danio rerio) CaMKIIb	CQRSTVAS	MHHRQET	VECLKKFNARRKLKGAILTTMLVSRNFS
Zebra fish (Danio rerio) CaMKIIg	CQRSTVAS	MHHRQET	VECLKKFNARRKLKGAILTTMLVSRNFS
Mouse (Mus musculus) CaMKIIb	CQRSTVAS	MHHRQET	VECLKKFNARRKLKGAILTTMLATRNFS
Mouse (Mus musculus) CaMKIIg	CQRSTVAS	MHHRQET	VECLKKFNARRKLKGAILTTMLVSRNFS
Mouse (Mus musculus) CaMKIId	CQRSTVAS	MHHRQET	VDCLKKFNARRKLKGAILTTMLATRNFS
Human (Homo sapiens) CaMKIIa	SHRSTVAS	CHHRQET	VDCLKKFNARRKLKGAILTTMLATRNFS
Human (Homo sapiens) CaMKIIb	CQRSTVAS	MHHRQET	VECLKKFNARRKLKGAILTTMLATRNFS
Human (Homo sapiens) CaMKIIg	CQRSTVAS	MHHRQET	VECLKKFNARRKLKGAILTTMLVSRNFS
Human (Homo sapiens) CaMKIId	CQRSTVAS	MHHRQET	VDCLKKFNARRKLKGAILTTMLATRNFS
Bovine (Bos taurus) CaMKIIa	SHRSTVAS	CHHRQET	VDCLKKFNARRKLKGAILTTMLATRNFS
Bovine (Bos taurus) CaMKIIb	CQRSTVAS	MHHRQET	VECLKKFNARRKLKGAILTTMLATRNFS
Bovine (Bos taurus) CaMKIIg	CQRSTVAS	MHHRQET	VECLKKFNARRKLKGAILTTMLVSRNFS
Bovine (Bos taurus) CaMKIId	CQRSTVAS	MHHRQET	VDCLKKFNARRKLKGAILTTMLATRNFS

Figure 2.

The regulatory domain paired methionines are a modern invention. Aligned sequences for the regulatory domain showing the highly conserved nature of all post-translational modification sites (upper left panel, Fig 1) except for paired methionines, which first appear in vertebrates. CaMKIIa has a cysteine methionine pair that allows for ROS mediated CaMKII activation, similar to oxidation of the paired methionines.[5]