HENRIETTA CSERNE SZAPPANOS (Orcid ID : 0000-0003-0063-257X)

Received Date : 01-Sep-2016

Revised Date : 16-Feb-2017

Accepted Date : 07-Mar-2017

Article type : Special Issue

The cardiac L-type calcium channel alpha subunit is a target for direct redox modification during oxidative stress – the role of cysteine residues in the alpha interacting domain

Padmapriya Muralidharan[1], Henrietta Cserne Szappanos[1], Evan Ingley[2] and Livia C Hool[1,3*]

[1]School of Anatomy, Physiology and Human Biology, University of Western Australia, Crawley, WA, Australia, [2]Harry Perkins Institute of Medical Research, QEII Medical Centre, Nedlands and Centre for Medical Research, The University of Western Australia, Crawley, Western Australia, 6009, Australia.

[3]Victor Chang Cardiac Research Institute, Sydney, NSW, Australia.

Type: Review

Short title: $Ca_v1.2$ direct redox modification during oxidative stress

*Corresponding author: Professor Livia Hool

Address: M311, School of Anatomy, Physiology and Human Biology, University of Western Australia, 35 Stirling Highway, Crawley, WA, 6009, Australia.

Phone: 61 8 6488 3307, Facsimile: 61 8 6488 1025, Email: livia.hool@uwa.edu.au

Disclosure: All the authors declare no conflicts of interest, potential conflicts DO NOT exist.

Abstract

Cardiovascular disease is the leading cause of death in the Western world. The incidence of cardiovascular disease is predicted to further rise with the increase in obesity and diabetes and with the ageing population. Even though the survival rate from ischemic heart disease has improved over the past 30 years, many patients progress to a chronic pathological condition, known as cardiac hypertrophy that is associated with an increase in morbidity and mortality. Reactive oxygen species (ROS) and calcium play an essential role in mediating cardiac hypertrophy. The L-type calcium channel is the main route for calcium influx into cardiac myocytes. There is now good evidence for a direct role for the L-type calcium channel in the development of cardiac hypertrophy. Cysteines on the channel are targets for redox modification and glutathionylation of the channel can modulate the function of the channel protein leading to the onset of pathology. The cysteine responsible for modification of L-type calcium channel function has now been identified. Detailed understanding of the role of cysteines as possible targets during oxidative stress may assist in designing therapy to prevent the development of hypertrophy and heart failure.

Key words

$Ca_v1.2$ protein, cysteine, glutathionylation, heart, hypertrophy, L-type calcium channel, oxidative stress, structure-function relationship

Introduction

The term cardiovascular disease refers to the disease of the heart and blood vessels. Cardiovascular disease is the leading cause of death in the Western world. Approximately 17.3 million people died from cardiovascular disease in 2013, accounting for 30 per cent of all deaths globally, of which 7.3

million people died due to ischemic heart disease (1). Ischemic heart disease is the leading cause of mortality and morbidity. It occurs due to the inadequate supply of myocardial oxygen and blood (2). Survival rates from ischemic heart disease have improved over the past decades, but many survivors progress to a chronic pathological condition, known as cardiac hypertrophy that is an independent risk for increased mortality according to the Framingham Heart Study (3-5).Previous studies have shown that reactive oxygen species (ROS) and calcium play a significant role in mediating the onset of cardiac hypertrophy (6). ROS are recognised as activators of various signalling pathways associated with the development of pathophysiologic cardiac conditions (7). ROS can alter numerous cellular functions by altering the redox state of key proteins (8), such as ion channels.

Oxidative stress is associated with an increase in intracellular Ca^{2+}, a second messenger, that activates transcription factors, resulting in increased protein synthesis, thereby leading to cardiac myocyte remodelling and hypertrophy (9-13). The L-type Ca^{2+} channel is the main route for Ca^{2+}-influx into the cardiac myocytes, and it has been proposed to play a significant role in the development of cardiac hypertrophy. This review discusses the contribution of ROS and Ca^{2+} in cardiac physiology and in the development of pathological conditions of the heart with particular reference to the role of cysteines on the cardiac L-type Ca2+ channel in mediating oxidative stress responses.

Role of reactive oxygen species in cardiac function

Reactive oxygen species such as superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$) are extremely reactive molecules because of their unpaired electrons. Superoxide is the primary form of ROS, formed by the one electron reduction of molecular oxygen. Mitochondria are a significant source of superoxide production in mammalian cardiac myocytes (7, 14), because ROS are produced as a by-product of mitochondrial oxidative phosphorylation (15). ROS can also be produced outside the mitochondria from a number of cytosolic enzymes including xanthine oxidase and NAD(P)H oxidase and cytochrome P450-dependent oxygenases. $O_2^{\bullet-}$ can be formed, when a single electron is directly

transferred to oxygen by reduced coenzymes or in prosthetic groups (for example, flavins or iron sulphur clusters). Peroxides and hypochlorous acid (HOCl) are reduced forms of superoxide (16). Hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$/ONOOH) are more stable, nonradical forms of ROS, they lack the free unpaired electron and thus possess oxidizing properties. H_2O_2 is produced from $O_2^{\bullet-}$ by dismutation, which can occur spontaneously at low pH or can be catalysed by manganese superoxide dismutase (MnSOD) in mitochondria and in the cytosol. H_2O_2 can be partially reduced to hydroxyl radicals ($^{\bullet}OH$) and HOCl, through Fenton or Haber-Weiss reactions, in the presence of metal ions, or completely reduced to water. Superoxide can also react with nitric oxide ($^{\bullet}NO$) to form peroxynitrite ($ONOO^-$/ONOOH) (17, 18). Reactive oxygen and nitrogen species are key elements of many cell signalling pathways.

Under physiological conditions, ROS concentrations are tightly controlled by antioxidants. The low intracellular concentration of ROS in the range of 0.001-0.5 μM enables their role as second messengers in signal transduction and physiological cell signalling (19, 20). Oxidative stress is defined as an imbalance between ROS production and the cell's antioxidant defence, leading to an altered redox status (16, 21). It is caused by diminished levels of antioxidants and co-factors or by increased production of ROS (22).

A slight increase in the intracellular concentration of ROS (1-10μM) causes mild oxidative stress (a sub-lethal concentration) where the cell can adapt to the changed environment. A concentration of ROS exceeding 10-100μM damages molecular targets, such as lipids, proteins and carbohydrates, leading to impaired cell function and apoptosis or necrosis (19, 23). ROS activates numerous cell-signalling pathways that trigger transcription factors and enhance the expression of specific genes responsible for growth, proliferation and apoptosis of cells (15, 24), oxidising cysteine rich motifs on many proteins, such as growth factors and cytokine receptors, further activating signalling pathways (25, 26).

Ca^{2+} as a second messenger

Calcium ions play an essential role in many cellular processes. Calcium is vital to excitation, contraction and relaxation of the cardiac muscle. Since cardiac sarcoplasmic reticulum is less extensive and less precisely arranged in relation to the cross-banded pattern of the myofibrils than it is in skeletal muscle, cardiac muscle is reliant on Ca^{2+} influx for contraction. Depolarisation of the membrane of cardiomyocytes stimulates opening of voltage-gated Ca^{2+} channels (27). The L-type Ca^{2+} channel is the primary route for Ca^{2+} influx into the cardiac myocytes (28). Influx of calcium ions through the L-type Ca^{2+} channel triggers Ca^{2+} release from the sarcoplasmic reticulum (SR) stores through the inositol 1,4,5-triphosphate or ryanodine receptor 2 (RyR2). The amplifying process is termed Ca^{2+}-induced Ca^{2+} release. An increase in intracellular concentration of calcium ions shapes the long plateau phase of the ventricular action potential, along with the upstroke of the atrial pacemaker action potential, regulating the electrical signal that determines the cardiac rhythm (10, 29, 30).

The cardiac muscle consists of orderly arranged contractile proteins composed of thick myosin filaments and overlapping thin actin and tropomyosin filaments. Increased Ca^{2+} concentration facilitates the binding of Ca^{2+} to troponin C, thereby inducing the contraction of cardiac muscle (31, 32). The removal of cytosolic calcium facilitates the relaxation of cardiac muscles and precisely controls the intracellular Ca^{2+} concentration crucial for many signal transduction pathways and cellular activities. This occurs by the uptake of calcium into the sarcoplasmic reticulum via the Ca^{2+}-ATPase, extrusion from the cell through the Na^{+}/Ca^{2+} exchanger or by uptake into mitochondria through the mitochondrial calcium uniporter. The entry of calcium ions through the mitochondrial calcium uniporter is an important step in mitochondrial ATP production (33, 34), which determine the excitation and relaxation cycle of the heart. Although calcium ions can be sourced from internal calcium stores such as endoplasmic and sarcoplasmic reticulum, voltage-gated Ca^{2+} channels are a rapid mechanism of Ca^{2+} influx and Ca2+ signalling in excitable cells (35).

Ca^{2+} and ROS together play a role in the development of cardiac hypertrophy

ROS triggers the development of myocardial hypertrophy, extracellular matrix remodelling and cellular dysfunction. Various hypertrophic signalling kinases and transcription factors are stimulated by ROS (36). A consistent increase in ROS results in pathological cardiac remodelling and myocardial dysfunction (37). During mild oxidative stress, ROS at concentrations of <10μM can activate hypertrophic signalling kinases and transcription factors such as serine-threonine kinases, tyrosine kinases, Ca^{2+}/calmodulin-dependent protein kinases (CaMK), mitogen-activated protein kinases (MAPK) and nuclear factor of activated T cells (NFAT). ROS and Ca^{2+} signals are strongly linked to the development of cardiac hypertrophy. In cardiac tissue, an increase in mitochondrial calcium results in increased production of ROS from mitochondria (38). In addition, an increase in ROS from Q_o site of complex III in the mitochondria mediates a further increase in intracellular Ca^{2+} via activation of the L-type Ca^{2+} channel (7, 9, 10, 12). This process known as "ROS-induced ROS-release" has been shown to activate hypertrophic signalling in cardiac myocytes (9, 12, 39, 40) (Fig 1) and is recognised as a mechanism for inducing oxidative stress in vascular smooth muscle (9, 41, 42) and in neurons (43, 44).

An increase in the diastolic Ca^{2+} is sufficient to contribute to phenotypic remodelling that leads to the development of hypertrophy (45-48). Alterations in calcium influx as a result of overexpression of the L-type Ca^{2+} channel is sufficient to initiate the development of hypertrophy (49). This leads to activation of calcium-calmodulin-CaMKII-HDAC (histone deacetylase) and calcium-calmodulin-calcineurin-NFAT that are recognised as two major Ca^{2+}-dependent hypertrophic signalling pathways (31).

Voltage gated calcium channels in the heart

Two types of voltage-gated calcium channels are observed in cardiac myocytes (50). The T-type calcium channel is expressed in embryonic heart but has limited expression in adult cardiac myocytes. It plays a significant role in controlling the pacemaker activity of the sinoatrial node. The abundant L-type Ca^{2+} channel is the main route for Ca^{2+} influx required for excitation-contraction coupling. Large conductance L-type Ca^{2+} channel is activated by high depolarization pulses allowing calcium ion entry into the cytosol (51). They possess selective dihydropyridine sensitivity, activated by the dihydropyridine Ca^{2+} channel agonist (S)-(−)-Bay K8644, and inhibited by dihydropyridine Ca^{2+} channel antagonists nisoldipine, nifedipine and nitrendipine. The L-type Ca^{2+} channel is a heterotetrameric polypeptide complex consisting of a pore forming α_1 subunit (~170–240kDa), an intracellular β subunit (~55kDa), and extracellularly located disulphide linked α_2/δ subunit (~170kDa) (52). The α_1, α_2/δ and β_2 subunits are mostly found in excitable cell types (53). The α_1 subunit of the Ca^{2+} channel has four homologous motifs (I–IV), each composed of cytoplasmic NH_3 and COOH terminal domains, six membrane spanning α-helices, termed S1 to S6, linked by variable length cytoplasmic loops (linkers) (54). The S5–S6 is the ion selective pore region, S4 is the voltage sensor segment, and binding site for drugs altering the function of the channel (55). To date, 10 genes have been identified coding α_1 subunit proteins classified into four classes: $Ca_v1.1$ (α_{1S}), 1.2 (α_{1C}), 1.3 (α_{1D}) and 1.4 (α_{1F}). The α_{1C} subunit is mainly expressed in cardiac muscle, encoded by *CACNA1C* gene (53). The β subunit is a supplementary subunit, which is strongly bound to cytoplasmic linkers between motifs I and II of cardiac α_{1c}, also known as the alpha interacting domain (AID). The β subunit plays a crucial role in controlling activation and inactivation kinetics and open probability of the channel. It is also important in the trafficking of the α_{1c} subunit into cell membrane (56). The N terminus of α_{1c} plays a role in channel voltage-gating (57-59) and protein kinase C (PKC) modulation (60). The long intracellular C terminus is responsible for Ca^{2+}- and voltage-dependent inactivation (52, 59), protein kinase A (PKA)-dependent modulation (although the exact site for PKA phosphorylation has been disputed in part because the complex beta-adrenergic signaling pathway is not yet fully understood

and also because heterogeneous expression systems used to study responses have resulted in conflicting findings) and transient anchoring of the protein in plasma membrane. Truncation of the C-terminus results in increased Ca^{2+}-currents relieving an inhibitory role of the tail (61, 62).

Effect of altered redox state on the function of the cardiac L-type Ca^{2+} channel

In a similar manner to heterologously expressed rabbit smooth muscle L-type Ca^{2+} channel and the human cardiac Na^{+} channel (63), the cardiac L-type Ca^{2+} channel (64-68) is responsive to changes in cellular redox state. In ferret and guinea pig ventricular myocytes, channel function can be enhanced by the thiol specific oxidising agent 5,5-dithio-bis[2-nitrobenzoic acid] (DTNB) and inhibited by the thiol reducing agent dithiothreitol (DTT) (69). Ferret ventricular myocytes exposed to 200 μM DTNB demonstrate increased calcium currents, whereas the addition of 1mM DTT inhibits the calcium currents (70). Acute exposure of the channel to H_2O_2 or thiol oxidising agents results in increased channel activity, whereas subjecting the channel to thiol reducing agents or hypoxic conditions inhibits channel activity (65, 71-73). In human cardiomyocytes, glutathione is considered the main cytosolic redox buffer. Glutathione exists in a reduced state under normal physiological conditions. Glutathione reportedly modulates the redox state of the L-type Ca^{2+} channel and the $Ca_v1.2$ channel protein has been shown to be persistently glutathionylated in ischemic human heart (68, 74). These studies demonstrate that the L-type Ca^{2+} channel protein is responsive to changes in the cellular redox state, and the subsequent alteration in channel function and calcium influx is sufficient to lead to the development of pathology associated with oxidative stress.

As stated, increased calcium influx through the L-type Ca^{2+} channel can contribute to pathology and the redox sensitivity of the L-type Ca^{2+} channel is especially important in ischemia/reperfusion injury. Activation of the channel by voltage or application of dihydropyridine agonists exacerbates “ROS-induced ROS-release” (75) and mitochondrial activity in cardiac myocytes (11, 37, 76) as well as in non-excitable cells, e.g. renal tubular cells (77). Since the auxiliary beta subunit tethers cytoskeletal proteins, it has been demonstrated that the channel can alter mitochondrial function as a result of

movement of the beta subunit through cytoskeletal proteins following activation of the channel (37, 76, 78), in addition to the increase in intracellular calcium concentration (12, 79, 80).

Role of cysteines in the altered redox state of proteins

Cysteines are unique amongst the amino acids because they contain a reactive sulphhydryl or thiol (SH) group. Cross-linking (creation of a disulphide bond) between two cysteine residues may form between various parts of the same protein or between two separate polypeptide chains (81). The formation of a disulphide link is associated with the folding of a protein (and alteration in structure) and is assisted by the enzymes thioldisulphide oxidoreductase and protein disulphide isomerise (68). Cysteine residues are the preferred targets for redox or nitrosylation modification on proteins as free thiols can easily react with ROS or Reactive Nitrogen Species (RNS) (70). Changes in the redox state of the protein results in formation or disruption of disulphide bonds between the thiol/SH groups of cysteines. Protein sulphhydryls can be oxidised to protein disulphides, sulphenic acids and highly oxidised states such as sulphinic and sulphonic acid forms (82). In cysteine-rich proteins, the oxidation state of sulphhydryl groups of cysteine residues determines the structure and function of proteins. The reduction-oxidation state of proteins plays a significant role in ionic homeostasis, cellular host defence and cellular metabolism. Thiol oxidising and reducing agents are used to detect the redox sensitivity of proteins (68, 83-85)

Understanding the structure-function relationship through redox modification of cysteines

Intramolecular disulphide bonds preserve the structural integrity of cysteine-containing proteins, determining the folding and structural balance of ion channels, ion regulatory proteins, transporters and pumps (86). Under physiological reducing conditions, cytoplasmic cysteine residues rarely form disulphide bonds. In oxidative stress conditions disulphide bonds can be formed between adjacent cysteines in close proximity. While the structure of the $Ca_v1.2$ protein (and the full native channel protein) have not been fully elucidated, many site-directed mutagenesis studies have been

performed to understand the functional significance of cysteines in the altered redox state of ion channels (83), including the voltage-dependent potassium (Kv) channels (87), the ligand-gated cation channel P2X receptor (88), ryanodine receptor from skeletal (89) and cardiac muscle(90). Mutation of cysteines can disrupt the folding of ion channels e.g. inwardly rectifying K^+ channels Kir2.1 (91), in addition to altering channel function. The substituted cysteine accessibility method introduces new cysteines into the amino acid sequence or substitutes the existing cysteines with non-reactive amino acids, usually adenine or serine, allowing the systematic investigation of the residues in a specific region of a protein, such as a membrane-spanning segment, residues forming a ligand binding site or ion pore. While cysteine substitution usually does not cause disruption of protein folding/expression, it may cause alterations in channel function that depend on the specific site of the substitution. Cysteine substitution technique has an advantage over site directed mutagenesis, in that thiol modifying reagents can be used to investigate the role of cysteines in the structure and function of proteins. Functional changes could arise from structural changes on cysteine pairs in close proximity with minimal perturbation of the local or global protein structure. Thiol groups on the water accessible protein surface are ionized to thiolate anion, the most accessible to disulphide crosslinking or thiol modification. Therefore studying the reactivity of the engineered cysteine residues can reveal the relationship between structure and function of proteins, especially in the case of membrane proteins in different functional states, such as closed, open or desensitized/inactivated states. It is especially useful in the study of complex membrane proteins where high resolution structures are not available (83).

High resolution analysis of a three-dimensional protein structure is usually determined by X-ray crystallography or nuclear magnetic resonance (NMR) techniques. Circular dichroism spectroscopy is also used in protein structural studies, to identify small peptides, or specific parts of more complex proteins, such as α-helix, ß-sheet and random coil, using their characteristic circular dichroism spectra. Although these methods have been successfully used for determining the structure, stability and ligand binding of many proteins, the size, solubility, and low expression level of a

protein can limit the use of these techniques for studying large molecular complexes, especially membrane proteins (92). The three-dimensional structure of many mammalian calcium channels remains unknown.

Fluorescent spectroscopy is an alternative method for examining protein structure detecting autofluorescent amino acids such as tryptophan/tyrosine or using specific fluorescent dyes such as 8-anilino-1-naphthalene sulphonate (ANS) (93), SYPRO Orange (94), 4,4'-bis (1-anilinonaphthalene 8-sulphonate) (bis-ANS) (95), or N-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide (CPM) (96). These fluorescent dyes either bind covalently to the thiol group of cysteines, α-amino group of the N-terminus, ε-amino group of lysines or non-covalently to the hydrophobic and electrostatic regions (97). A change in the three dimensional structure of the protein results in a change of the fluorescence spectra. Mass spectrometry and fluorescent studies using the thiol-specific probe monobromobimane have been used to identify redox-sensitive cysteines of RyR1. Point mutations of single cysteines or groups of cysteines have resulted in altered redox responses (89).

The thermal stability assay can provide further information on the stability of protein structures. It uses the protein melting method, where an increase in temperature results in the unfolding of proteins. This increases the available binding sites for the fluorescent dye, so the fluorescence signal increases. At higher temperatures fluorescence decreases when unfolded proteins gradually begin to aggregate. The midpoint of the temperature corresponding to the maximum fluorescence obtained on the unfolding of the protein induced by a high temperature is characteristic and denotes the stability of the protein (97, 98). The thermal stability assay is used to study the structural stability of proteins, coupling of functional domains, stability of ligand binding, and the communication between agonists and receptors. It can therefore identify free cysteines as possible targets for redox modification (96, 99-101).

A direct method for studying the redox modification of channel function

Reconstituting the heterologously expressed, then purified His-tagged channel protein into artificial liposomes has provided an opportunity to examine the function of a membrane protein at the molecular level (74, 102). It enables direct assessment of the effects of modification of cysteines on the channel function. The purity of channel protein is assessed by mass spectrometry. Contrary to other methods, such as heterologous expression systems or native cells there are only channel protein and lipid present, therefore the direct effect of redox modifications of cysteines can be observed in the absence of regulatory proteins.

The α_{1c} subunit of the long N Terminal (LNT) isoform of the cardiac L-type Ca^{2+} channel protein ($Ca_v1.2$) contains 42 cysteines in total, 15 cysteines are extracellular, 8 are transmembrane and 19 are cytosolic (103). Under native conditions the interior of the cell is highly reduced and the cytoplasmic region of the channel is more likely to be targeted by redox agents under oxidative stress conditions. To examine this directly the reactive cysteines from different regions of the $Ca_v1.2$ channel protein were mutated to a serine or alanine and changes in open probability of single channel currents were assessed in the absence and presence of oxidised and reduced glutathione. Reconstitution of $Ca_v1.2$ protein alone is sufficient to form an ion conducting channel with single channel characteristics similar to native L-type calcium channels and sensitivity to the dihydropyridine blocker nisoldipine.(74) Mutation of cysteines in the intracellular Repeat I-II linker, the AID region (cysteines 519, 543 and 547 in the human LNT isoform) attenuated the effects of oxidising and reducing agents. More precisely C543 was critical for altering channel function in the presence of redox reagents and glutathione. It was also demonstrated that a point mutation of cysteine 543 to serine altered protein folding assessed using the thermal stability assay (104). These data confirm that redox modification of $Ca_v1.2$ channel protein alone is sufficient to alter structure and function of the channel under conditions of oxidative stress.

In physiological conditions the normal function of the $Ca_v1.2$ subunit requires the auxiliary α2δ and β2 subunits. Since the β2 subunit modifies inactivation kinetics of the native L-type channel and the cysteine at 543 lies within the alpha interacting domain where the β2 subunit binds to the alpha subunit, redox modification of the $Ca_v1.2$ subunit could alter the behaviour of the β2 subunit. Reactive oxygen species can alter the activity of many proteins including those closely associated with $Ca_v1.2$ subunit and further influence the function of the voltage gated calcium channel in vivo.

Conclusion and future directions based on identification of the critical cysteine

Average lifespan is continuously increasing as well as the occurrence of age-associated diseases. As the Framingham Heart Study and the Baltimore Longitudinal Study on Aging demonstrated, the heart is particularly affected by aging (4, 5, 105, 106). The health burden associated with patients living with heart disease is increasing with the ageing population the development of new therapies for the prevention of cardiac hypertrophy and heart failure is critical to reduce the significant cost to individuals and to society.

Cardiac aging is associated with declined cardiac function, apoptosis, cardiac remodelling, making the heart more vulnerable to stress. Many studies demonstrated mitochondrial dysfunction and as a consequence altered ROS production in aging heart (107, 108).

The L-type calcium channel is the main route for calcium influx into cardiac muscle cells and is essential for excitation and contraction. It is well recognised that the native channel can respond to changes in cellular redox state and this mediates changes in intracellular calcium that are associated with the development of heart failure in the ischemic human heart. However it was unknown whether this was due to direct modification of thiol groups on the channel protein since contradictory responses have been reported in channels in cardiac myocytes or in expression systems. Recent studies have identified that the function of the purified ion conducting α_{1C} subunit of the L-type Ca^{2+} channel is altered by thiol reducing and oxidising agents and the responsible cysteine has now been identified. During alterations in cellular redox state (hypoxia or oxidative

stress), direct regulation of the $Ca_v1.2$ channel protein is sufficient to alter calcium influx and facilitate rapid physiological responses (64-67, 109). Since glutathionylation of $Ca_v1.2$ occurs in the ischemic human heart, and increased calcium influx through $Ca_v1.2$ is sufficient to induce hypertrophic growth, direct glutathionylation of $Ca_v1.2$ channel protein during reperfusion may contribute to the pathology associated with ischemic heart disease (74).

At the cellular level the L-type Ca^{2+} channel exists as a multimolecular complex, and its function is modified by auxiliary subunits and communication with many other regulatory proteins. Many proteins can modulate the function of the $Ca_v1.2$ subunit. The site of the critical cysteine on the $Ca_v1.2$ subunit is located at close proximity to the alpha-interacting domain where the β subunit binds to the pore forming α-subunit. Therefore further studies would be useful to determine how redox modification of cysteines can alter subunit interactions between the α_{1c} and β subunits, channel complex formation and compartmentalization. This may help us to further elucidate the mechanisms that lead to cardiac remodelling, and shape the development of therapy targeting the calcium channel.

Acknowledgment

The authors thank other laboratory members for support and helpful discussions. The author's work reported herein was supported by grants from the National Health and Medical Research Council of Australia and Australian Research Council. Livia Hool was supported by an Australian Research Council Future Fellowship (FT100100756) and National Health and Medical Research Council Senior Research Fellowship (APP1002207) and Project Grant (APP1010726). Evan Ingley received support from the Cancer Council of Western Australia, The Harry Perkins Institute of Medical Research, Sock-it-to-Sarcoma and the Hollywood Private Hospital Research Foundation and NHMRC grants (513714 and 634352).

Disclosure

The authors declare no conflicts of interest.

References

1. World Health Organisation. 2013 [Available from: http://www.who.int/mediacentre/factsheets/fs317/en/.
2. Pepine CJ, Nichols WW. The pathophysiology of chronic ischemic heart disease. Clinical cardiology. 2007;30(2 Suppl 1):I4-9.
3. Stork T, Mockel M, Danne O, Voller H, Eichstadt H, Frei U. Left ventricular hypertrophy and diastolic dysfunction: their relation to coronary heart disease. Cardiovascular drugs and therapy / sponsored by the International Society of Cardiovascular Pharmacotherapy. 1995;9 Suppl 3:533-7.
4. Kannel WB, Gordon T, Offutt D. Left ventricular hypertrophy by electrocardiogram. Prevalence, incidence, and mortality in the Framingham study. Ann Intern Med. 1969;71(1):89-105.
5. Levy D, Garrison RJ, Savage DD, Kannel WB, Castelli WP. Prognostic implications of echocardiographically determined left ventricular mass in the Framingham Heart Study. N Engl J Med. 1990;322(22):1561-6.
6. Takimoto E, Kass DA. Role of oxidative stress in cardiac hypertrophy and remodeling. Hypertension. 2007;49(2):241-8.
7. Hool LC. Reactive oxygen species in cardiac signalling: from mitochondria to plasma membrane ion channels. Clin Exp Pharmacol Physiol. 2006;33(1-2):146-51.
8. Fearnley CJ, Roderick HL, Bootman MD. Calcium signaling in cardiac myocytes. Cold Spring Harb Perspect Biol. 2011;3(11):a004242.
9. Viola HM, Arthur PG, Hool LC. Transient exposure to hydrogen peroxide causes an increase in mitochondria-derived superoxide as a result of sustained alteration in L-type Ca2+ channel function in the absence of apoptosis in ventricular myocytes. Circ Res. 2007;100(7):1036-44.
10. Hool LC, Corry B. Redox control of calcium channels: from mechanisms to therapeutic opportunities. Antioxid Redox Signal. 2007;9(4):409-35.
11. Seenarain V, Viola HM, Ravenscroft G, Casey TM, Lipscombe RJ, Ingley E, et al. Evidence of altered guinea pig ventricular cardiomyocyte protein expression and growth in response to a 5 min in vitro exposure to H(2)O(2). J Proteome Res. 2010;9(4):1985-94.
12. Viola HM, Hool LC. Qo site of mitochondrial complex III is the source of increased superoxide after transient exposure to hydrogen peroxide. J Mol Cell Cardiol. 2010;49(5):875-85.
13. Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J, et al. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell. 1998;93(2):215-28.
14. Chen YR, Zweier JL. Cardiac mitochondria and reactive oxygen species generation. Circ Res. 2014;114(3):524-37.
15. Thannickal VJ, Fanburg BL. Reactive oxygen species in cell signaling. Am J Physiol Lung Cell Mol Physiol. 2000;279(6):L1005-28.
16. Taverne YJ, Bogers AJ, Duncker DJ, Merkus D. Reactive oxygen species and the cardiovascular system. Oxidative medicine and cellular longevity. 2013;2013:862423.
17. Turrens JF. Mitochondrial formation of reactive oxygen species. J Physiol. 2003;552(Pt 2):335-44.
18. Papaharalambus CA, Griendling KK. Basic mechanisms of oxidative stress and reactive oxygen species in cardiovascular injury. Trends Cardiovasc Med. 2007;17(2):48-54.
19. Stone JR, Yang S. Hydrogen peroxide: a signaling messenger. Antioxid Redox Signal. 2006;8(3-4):243-70.
20. D'Autreaux B, Toledano MB. ROS as signalling molecules: mechanisms that generate

specificity in ROS homeostasis. Nat Rev Mol Cell Biol. 2007;8(10):813-24.
21. Ray PD, Huang BW, Tsuji Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. Cellular signalling. 2012;24(5):981-90.
22. Rahman K. Studies on free radicals, antioxidants, and co-factors. Clinical interventions in aging. 2007;2(2):219-36.
23. Halliwell B, Whiteman M. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? Br J Pharmacol. 2004;142(2):231-55.
24. Burgoyne JR, Mongue-Din H, Eaton P, Shah AM. Redox signaling in cardiac physiology and pathology. Circ Res. 2012;111(8):1091-106.
25. Finkel T. Signal transduction by reactive oxygen species. The Journal of cell biology. 2011;194(1):7-15.
26. Son Y, Cheong YK, Kim NH, Chung HT, Kang DG, Pae HO. Mitogen-Activated Protein Kinases and Reactive Oxygen Species: How Can ROS Activate MAPK Pathways? J Signal Transduct. 2011;2011:792639.
27. Nerbonne JM, Kass RS. Molecular physiology of cardiac repolarization. Physiol Rev. 2005;85(4):1205-53.
28. Hool LC. What cardiologists should know about calcium ion channels and their regulation by reactive oxygen species. Heart Lung Circ. 2007;16(5):361-72.
29. Marks AR. Cardiac intracellular calcium release channels: role in heart failure. Circ Res. 2000;87(1):8-11.
30. Bers DM. Cardiac excitation-contraction coupling. Nature. 2002;415(6868):198-205.
31. Berridge MJ, Bootman MD, Roderick HL. Calcium signalling: dynamics, homeostasis and remodelling. Nat Rev Mol Cell Biol. 2003;4(7):517-29.
32. Bodi I, Mikala G, Koch SE, Akhter SA, Schwartz A. The L-type calcium channel in the heart: the beat goes on. J Clin Invest. 2005;115(12):3306-17.
33. Santo-Domingo J, Demaurex N. Calcium uptake mechanisms of mitochondria. Biochim Biophys Acta. 2010;1797(6-7):907-12.
34. Raffaello A, De Stefani D, Sabbadin D, Teardo E, Merli G, Picard A, et al. The mitochondrial calcium uniporter is a multimer that can include a dominant-negative pore-forming subunit. The EMBO journal. 2013;32(17):2362-76.
35. Clapham DE. Calcium signaling. Cell. 2007;131(6):1047-58.
36. Murdoch CE, Zhang M, Cave AC, Shah AM. NADPH oxidase-dependent redox signalling in cardiac hypertrophy, remodelling and failure. Cardiovasc Res. 2006;71(2):208-15.
37. Viola HM, Hool LC. Cross-talk between L-type Ca2+ channels and mitochondria. Clin Exp Pharmacol Physiol. 2010;37(2):229-35.
38. Yan Y, Wei CL, Zhang WR, Cheng HP, Liu J. Cross-talk between calcium and reactive oxygen species signaling. Acta pharmacologica Sinica. 2006;27(7):821-6.
39. Zorov DB, Filburn CR, Klotz LO, Zweier JL, Sollott SJ. Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes. J Exp Med. 2000;192(7):1001-14.
40. Zhou L, Aon MA, Almas T, Cortassa S, Winslow RL, O'Rourke B. A reaction-diffusion model of ROS-induced ROS release in a mitochondrial network. PLoS Comput Biol. 2010;6(1):e1000657.
41. MacKay CE, Knock GA. Control of vascular smooth muscle function by Src-family kinases and reactive oxygen species in health and disease. J Physiol. 2015;593(17):3815-28.
42. Chaplin NL, Nieves-Cintron M, Fresquez AM, Navedo MF, Amberg GC. Arterial Smooth Muscle Mitochondria Amplify Hydrogen Peroxide Microdomains Functionally Coupled to L-Type Calcium Channels. Circ Res. 2015;117(12):1013-23.
43. Droge W. Free radicals in the physiological control of cell function. Physiol Rev. 2002;82(1):47-95.
44. Hidalgo C, Donoso P. Crosstalk between calcium and redox signaling: from molecular

mechanisms to health implications. Antioxid Redox Signal. 2008;10(7):1275-312.
45. Brookes PS, Yoon Y, Robotham JL, Anders MW, Sheu SS. Calcium, ATP, and ROS: a mitochondrial love-hate triangle. Am J Physiol Cell Physiol. 2004;287(4):C817-33.
46. Berridge MJ. Remodelling Ca2+ signalling systems and cardiac hypertrophy. Biochem Soc Trans. 2006;34(Pt 2):228-31.
47. Lou Q, Janardhan A, Efimov IR. Remodeling of calcium handling in human heart failure. Adv Exp Med Biol. 2012;740:1145-74.
48. Crossman DJ, Ruygrok PN, Soeller C, Cannell MB. Changes in the organization of excitation-contraction coupling structures in failing human heart. PLoS One. 2011;6(3):e17901.
49. Muth JN, Bodi I, Lewis W, Varadi G, Schwartz A. A Ca(2+)-dependent transgenic model of cardiac hypertrophy: A role for protein kinase Calpha. Circulation. 2001;103(1):140-7.
50. Bean BP. Classes of calcium channels in vertebrate cells. Annu Rev Physiol. 1989;51:367-84.
51. Arikkath J, Campbell KP. Auxiliary subunits: essential components of the voltage-gated calcium channel complex. Current opinion in neurobiology. 2003;13(3):298-307.
52. van der Heyden MA, Wijnhoven TJ, Opthof T. Molecular aspects of adrenergic modulation of cardiac L-type Ca2+ channels. Cardiovasc Res. 2005;65(1):28-39.
53. Yang L, Katchman A, Morrow JP, Doshi D, Marx SO. Cardiac L-type calcium channel (Cav1.2) associates with gamma subunits. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2011;25(3):928-36.
54. Catterall WA. Voltage-gated calcium channels. Cold Spring Harb Perspect Biol. 2011;3(8):a003947.
55. Jurkat-Rott K, Lehmann-Horn F. The impact of splice isoforms on voltage-gated calcium channel alpha1 subunits. J Physiol. 2004;554(Pt 3):609-19.
56. Pragnell M, De Waard M, Mori Y, Tanabe T, Snutch TP, Campbell KP. Calcium channel beta-subunit binds to a conserved motif in the I-II cytoplasmic linker of the alpha 1-subunit. Nature. 1994;368(6466):67-70.
57. Shistik E, Ivanina T, Blumenstein Y, Dascal N. Crucial role of N terminus in function of cardiac L-type Ca2+ channel and its modulation by protein kinase C. J Biol Chem. 1998;273(28):17901-9.
58. McHugh D, Sharp EM, Scheuer T, Catterall WA. Inhibition of cardiac L-type calcium channels by protein kinase C phosphorylation of two sites in the N-terminal domain. Proc Natl Acad Sci U S A. 2000;97(22):12334-8.
59. Kobrinsky E, Tiwari S, Maltsev VA, Harry JB, Lakatta E, Abernethy DR, et al. Differential role of the alpha1C subunit tails in regulation of the Cav1.2 channel by membrane potential, beta subunits, and Ca2+ ions. J Biol Chem. 2005;280(13):12474-85.
60. Liu W, Deng J, Wang G, Zhang C, Luo X, Yan D, et al. KCNE2 modulates cardiac L-type Ca(2+) channel. J Mol Cell Cardiol. 2014;72:208-18.
61. Gao T, Cuadra AE, Ma H, Bunemann M, Gerhardstein BL, Cheng T, et al. C-terminal fragments of the alpha 1C (CaV1.2) subunit associate with and regulate L-type calcium channels containing C-terminal-truncated alpha 1C subunits. J Biol Chem. 2001;276(24):21089-97.
62. Hulme JT, Yarov-Yarovoy V, Lin TW, Scheuer T, Catterall WA. Autoinhibitory control of the CaV1.2 channel by its proteolytically processed distal C-terminal domain. J Physiol. 2006;576(Pt 1):87-102.
63. Chiamvimonvat N, O'Rourke B, Kamp TJ, Kallen RG, Hofmann F, Flockerzi V, et al. Functional consequences of sulfhydryl modification in the pore-forming subunits of cardiovascular Ca2+ and Na+ channels. Circ Res. 1995;76(3):325-34.
64. Fearon IM, Palmer AC, Balmforth AJ, Ball SG, Varadi G, Peers C. Modulation of recombinant human cardiac L-type Ca2+ channel alpha1C subunits by redox agents and hypoxia. J Physiol. 1999;514 (Pt 3):629-37.
65. Fearon IM, Varadi G, Koch S, Isaacsohn I, Ball SG, Peers C. Splice variants reveal the region involved in oxygen sensing by recombinant human L-type Ca(2+) channels. Circ Res. 2000;87(7):537-9.

66. Hool LC. Hypoxia increases the sensitivity of the L-type Ca(2+) current to beta-adrenergic receptor stimulation via a C2 region-containing protein kinase C isoform. Circ Res. 2000;87(12):1164-71.
67. Hool LC. Hypoxia alters the sensitivity of the L-type Ca(2+) channel to alpha-adrenergic receptor stimulation in the presence of beta-adrenergic receptor stimulation. Circ Res. 2001;88(10):1036-43.
68. Zima AV, Blatter LA. Redox regulation of cardiac calcium channels and transporters. Cardiovasc Res. 2006;71(2):310-21.
69. Hool LC. The L-type Ca(2+) channel as a potential mediator of pathology during alterations in cellular redox state. Heart Lung Circ. 2009;18(1):3-10.
70. Campbell DL, Stamler JS, Strauss HC. Redox modulation of L-type calcium channels in ferret ventricular myocytes. Dual mechanism regulation by nitric oxide and S-nitrosothiols. J Gen Physiol. 1996;108(4):277-93.
71. Hool LC, Arthur PG. Decreasing cellular hydrogen peroxide with catalase mimics the effects of hypoxia on the sensitivity of the L-type Ca2+ channel to beta-adrenergic receptor stimulation in cardiac myocytes. Circ Res. 2002;91(7):601-9.
72. Hool LC, Di Maria CA, Viola HM, Arthur PG. Role of NAD(P)H oxidase in the regulation of cardiac L-type Ca2+ channel function during acute hypoxia. Cardiovasc Res. 2005;67(4):624-35.
73. Smani T, Hernandez A, Urena J, Castellano AG, Franco-Obregon A, Ordonez A, et al. Reduction of Ca(2+) channel activity by hypoxia in human and porcine coronary myocytes. Cardiovasc Res. 2002;53(1):97-104.
74. Tang ZZ, Sharma S, Zheng S, Chawla G, Nikolic J, Black DL. Regulation of the mutually exclusive exons 8a and 8 in the CaV1.2 calcium channel transcript by polypyrimidine tract-binding protein. J Biol Chem. 2011;286(12):10007-16.
75. Zorov DB, Juhaszova M, Sollott SJ. Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. Physiol Rev. 2014;94(3):909-50.
76. Viola HM, Hool LC. Role of the cytoskeleton in communication between L-type Ca(2+) channels and mitochondria. Clin Exp Pharmacol Physiol. 2013;40(4):295-304.
77. Tanaka T, Nangaku M, Miyata T, Inagi R, Ohse T, Ingelfinger JR, et al. Blockade of calcium influx through L-type calcium channels attenuates mitochondrial injury and apoptosis in hypoxic renal tubular cells. Journal of the American Society of Nephrology : JASN. 2004;15(9):2320-33.
78. Viola H, Johnstone V, Cserne Szappanos H, Richman T, Tsoutsman T, Filipovska A, et al. The L-type Ca(2+) channel facilitates abnormal metabolic activity in the cTnI-G203S mouse model of hypertrophic cardiomyopathy. J Physiol. 2016;594(14):4051-70.
79. Viola HM, Hool LC. How does calcium regulate mitochondrial energetics in the heart? - new insights. Heart Lung Circ. 2014;23(7):602-9.
80. Zinkevich NS, Gutterman DD. ROS-induced ROS release in vascular biology: redox-redox signaling. Am J Physiol Heart Circ Physiol. 2011;301(3):H647-53.
81. Miseta A, Csutora P. Relationship between the occurrence of cysteine in proteins and the complexity of organisms. Molecular biology and evolution. 2000;17(8):1232-9.
82. Thomas JA, Mallis RJ. Aging and oxidation of reactive protein sulfhydryls. Exp Gerontol. 2001;36(9):1519-26.
83. Akabas MH. Cysteine Modification: Probing Channel Structure, Function and Conformational Change. Adv Exp Med Biol. 2015;869:25-54.
84. Comini MA. Measurement and meaning of cellular thiol:disufhide redox status. Free Radic Res. 2016;50(2):246-71.
85. Laragione T, Bonetto V, Casoni F, Massignan T, Bianchi G, Gianazza E, et al. Redox regulation of surface protein thiols: identification of integrin alpha-4 as a molecular target by using redox proteomics. Proc Natl Acad Sci U S A. 2003;100(25):14737-41.
86. Kourie JI. Interaction of reactive oxygen species with ion transport mechanisms. Am J Physiol. 1998;275(1 Pt 1):C1-24.

87. Jerng HH, Pfaffinger PJ. S-glutathionylation of an auxiliary subunit confers redox sensitivity to Kv4 channel inactivation. PLoS One. 2014;9(3):e93315.
88. Keceli B, Kubo Y. Voltage- and ATP-dependent structural rearrangements of the P2X2 receptor associated with the gating of the pore. J Physiol. 2014;592(21):4657-76.
89. Petrotchenko EV, Yamaguchi N, Pasek DA, Borchers CH, Meissner G. Mass spectrometric analysis and mutagenesis predict involvement of multiple cysteines in redox regulation of the skeletal muscle ryanodine receptor ion channel complex. Research and reports in biology. 2011;2011(2):13-21.
90. Mi T, Xiao Z, Guo W, Tang Y, Hiess F, Xiao J, et al. Role of Cys(3)(6)(0)(2) in the function and regulation of the cardiac ryanodine receptor. Biochem J. 2015;467(1):177-90.
91. Cho HC, Tsushima RG, Nguyen TT, Guy HR, Backx PH. Two critical cysteine residues implicated in disulfide bond formation and proper folding of Kir2.1. Biochemistry. 2000;39(16):4649-57.
92. Seddon AM, Curnow P, Booth PJ. Membrane proteins, lipids and detergents: not just a soap opera. Biochim Biophys Acta. 2004;1666(1-2):105-17.
93. Schonbrunn E, Eschenburg S, Luger K, Kabsch W, Amrhein N. Structural basis for the interaction of the fluorescence probe 8-anilino-1-naphthalene sulfonate (ANS) with the antibiotic target MurA. Proc Natl Acad Sci U S A. 2000;97(12):6345-9.
94. Ke Y, Ng CA, Hunter MJ, Mann SA, Heide J, Hill AP, et al. Trafficking defects in PAS domain mutant Kv11.1 channels: roles of reduced domain stability and altered domain-domain interactions. Biochem J. 2013;454(1):69-77.
95. Takashi R, Tonomura Y, Morales MF. 4,4'-Bis (1-anilinonaphthalene 8-sulfonate) (bis-ANS): a new probe of the active site of myosin. Proc Natl Acad Sci U S A. 1977;74(6):2334-8.
96. Tol MB, Deluz C, Hassaine G, Graff A, Stahlberg H, Vogel H. Thermal unfolding of a mammalian pentameric ligand-gated ion channel proceeds at consecutive, distinct steps. J Biol Chem. 2013;288(8):5756-69.
97. Hawe A, Sutter M, Jiskoot W. Extrinsic fluorescent dyes as tools for protein characterization. Pharmaceutical research. 2008;25(7):1487-99.
98. Ericsson UB, Hallberg BM, Detitta GT, Dekker N, Nordlund P. Thermofluor-based high-throughput stability optimization of proteins for structural studies. Anal Biochem. 2006;357(2):289-98.
99. Alexandrov AI, Mileni M, Chien EY, Hanson MA, Stevens RC. Microscale fluorescent thermal stability assay for membrane proteins. Structure. 2008;16(3):351-9.
100. Soon FF, Suino-Powell KM, Li J, Yong EL, Xu HE, Melcher K. Abscisic acid signaling: thermal stability shift assays as tool to analyze hormone perception and signal transduction. PLoS One. 2012;7(10):e47857.
101. Branigan E, Pliotas C, Hagelueken G, Naismith JH. Quantification of free cysteines in membrane and soluble proteins using a fluorescent dye and thermal unfolding. Nature protocols. 2013;8(11):2090-7.
102. Kloda A, Petrov E, Meyer GR, Nguyen T, Hurst AC, Hool L, et al. Mechanosensitive channel of large conductance. Int J Biochem Cell Biol. 2008;40(2):164-9.
103. Viola HM, Macdonald WA, Tang H, Hool LC. The L-type Ca(2+) channel as a therapeutic target in heart disease. Current medicinal chemistry. 2009;16(26):3341-58.
104. Muralidharan P, Cserne Szappanos H, Ingley E, Hool L. Evidence for redox sensing by a human cardiac calcium channel. Sci Rep. 2016;6:19067.
105. Fleg JL, O'Connor F, Gerstenblith G, Becker LC, Clulow J, Schulman SP, et al. Impact of age on the cardiovascular response to dynamic upright exercise in healthy men and women. J Appl Physiol (1985). 1995;78(3):890-900.
106. Lakatta EG, Levy D. Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: Part II: the aging heart in health: links to heart disease. Circulation. 2003;107(2):346-54.
107. Dai DF, Rabinovitch PS. Cardiac aging in mice and humans: the role of mitochondrial

oxidative stress. Trends Cardiovasc Med. 2009;19(7):213-20.
108. Martin-Fernandez B, Gredilla R. Mitochondria and oxidative stress in heart aging. Age (Dordr). 2016;38(4):225-38.
109. Gaur N, Rudy Y, Hool L. Contributions of ion channel currents to ventricular action potential changes and induction of early afterdepolarizations during acute hypoxia. Circ Res. 2009;105(12):1196-203.

Figure legends

Fig 1. The cellular mechanisms leading to cardiac hypertrophy following an oxidative stress insult. During reperfusion following ischemia the myocyte is exposed to an increase in hydrogen peroxide that increases calcium influx through the L-type Ca^{2+} channel as a result of oxidation of C543 on $Ca_v1.2$ channel protein (9, 11, 74). The increased intracellular calcium concentration leads to further ROS production from the mitochondria as a result of increased mitochondrial calcium uptake (9, 39, 40) that maintains the L-type Ca^{2+} channel in an oxidised state. Increased levels of intracellular calcium ions and ROS simultaneously trigger many signal transduction pathways ultimately leading to hypertrophy.

Fig 2. Schematic diagram of the long N Terminal (LNT) isoform of $Ca_v1.2$ channel protein with cysteines highlighted. The position of cysteines in extracellular loops (magenta), transmembrane regions (yellow), intracellular loops (red) and mutated cysteines (purple) are shown. Mutation of all 3 cysteines in loop I-II linker attenuates oxidative stress responses but C543 is critical to mediating alterations in channel function following an oxidative stress insult (see text for explanation).