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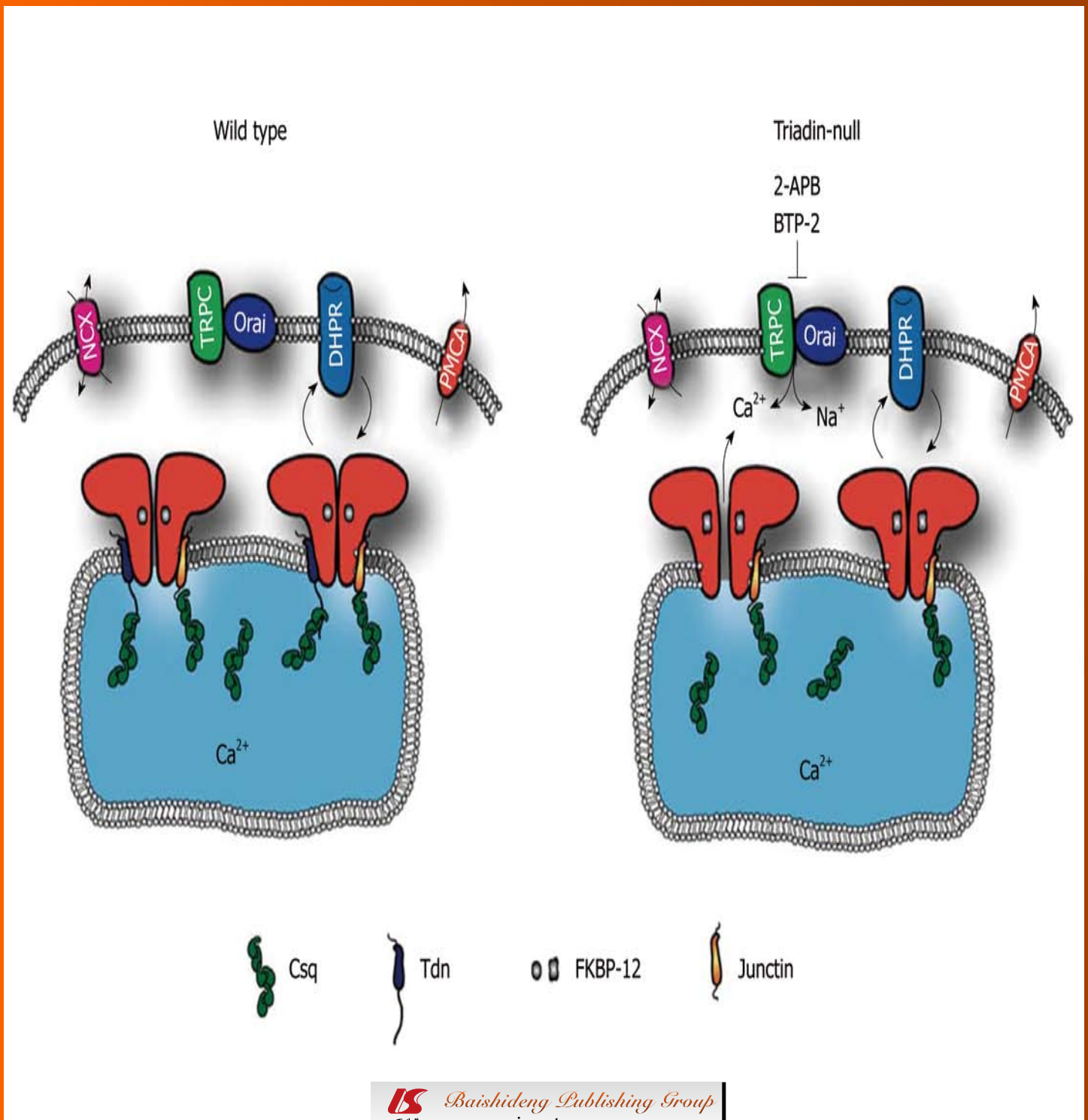

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EDITORIAL

On the footsteps of Triadin and its role in skeletal muscle

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Abstract

Calcium is a crucial element for striated muscle function. As such, myoplasmic free Ca^{2+} concentration is delicately regulated through the concerted action of multiple Ca^{2+} pathways that relay excitation of the plasma membrane to the intracellular contractile machinery. In skeletal muscle, one of these major Ca^{2+} pathways is Ca^{2+} release from intracellular Ca^{2+} stores through type-1 ryanodine receptor/Ca^{2+} release channels (RyR1), which positions RyR1 in a strategic cross point to regulate Ca^{2+} homeostasis. This major Ca^{2+} traffic point appears to be highly sensitive to the intracellular environment, which senses through a plethora of chemical and protein-protein interactions. Among these modulators, perhaps one of the most elusive is Triadin, a muscle-specific protein that is involved in many crucial aspect of muscle function. This family of proteins mediates complex interactions with various Ca^{2+} modulators and seems poised to be a relevant modulator of Ca^{2+} signaling in cardiac and skeletal muscles. The purpose of this review is to examine the most recent evidence and current understanding of the role of Triadin in muscle function, in general, with particular emphasis on its contribution to Ca^{2+} homeostasis.

INTRODUCTION

More than two decades after its discovery, and in spite a significant number of studies, our understanding of the role of Triadin in muscle function has remained, for the most part, unclear and elusive. This family of proteins, which are highly abundant and specific to striated muscle, have garnered a significant level of attention fuelled primarily by their ability to interact with the ryanodine receptor (RyR), a Ca^{2+} release channel that plays a preponderant role in skeletal and cardiac muscle function.

The multiple isoforms of Triadins currently identified in muscle cells seem consistent with the multiplicity of roles credited to these proteins, which include among others, modulation of RyR activity, excitation-contraction (EC) coupling, and Ca^{2+} homeostasis. The recent development of Triadin-null mouse models have provided us with a critical tool to understand the role of these proteins and have revealed important new insights into the mechanisms that regulate Ca^{2+} homeostasis in striated muscles.

PROTEIN HETEROGENEITY AND GENE STRUCTURE

Triadin was originally identified by Caswell *et al*[1] and

Kim *et al*[2] as a highly enriched 95-kDa protein of the junctional sarcoplasmic reticulum (jSR) in rabbit skeletal muscle. The primary sequence and structure of skeletal Triadin was later deduced from its cDNA sequence, which predicted a 705-amino-acid intrinsic membrane protein containing a short cytoplasmic N terminus, a single membrane-spanning domain, and a long intraluminal C-terminal domain[3,4]. Subsequent studies in rabbit hearts identified three unique cardiac Triadin isoforms with molecular mass of 35, 40 and 92 kDa[5]. Given that all isoforms, skeletal and cardiac, share identical sequences between amino acids 1-264 but have a unique C-terminal region, it appears that all Triadin proteins are products from alternative splicing of a single Triadin (*Trdn*) gene. Recent sequencing of the whole mouse genome has confirmed this perception (Figure 1). Subsequent studies in other species have revealed that similar tissue-specific patterns of Triadin expression are also present in mouse, canine, rat and human cardiac and skeletal muscle[6-9]. A summary of all Triadin genes currently cloned from cardiac and skeletal muscle is presented in Table 1.

Cardiac muscle appears to express a major Triadin isoform of 32 kDa (CT1 or MT1), which is susceptible to glycosylation and migrates as a secondary 38-kDa band[5,6,10]. A 92-kDa isoform has also been reported in rabbit and canine hearts but its expression is much less prominent than the 32-38-kDa doublet[5,6]. Similarly, it appears that in skeletal muscles Triadin is expressed predominantly as a 95-kDa isoform[1,3,4,6,8,11-13]. However, recent studies in rat skeletal muscle have also identified, and cloned, several shorter Triadin isoforms of 32, 49 and 51 kDa (Trisk-32, Trisk-49 and Trisk-51, respectively)[7,9]. Using the same antibodies generated against the rat skeletal Triadin, the expression of these shorter isoforms has recently been confirmed in mouse skeletal muscle[14], suggesting that multiplicity of isoforms may be a common feature of skeletal Triadin. Whether or not this multiplicity of Triadins is associated with specific functional roles for each isoform is still unknown[15,16]. However, the wide array of Triadin-protein interactions currently reported and the diversity of functional effects directly and indirectly associated with exogenous manipulation of Triadin expression levels seem to support this hypothesis.

PROTEIN-PROTEIN INTERACTIONS

Direct molecular interactions between Triadin and several protein components of the jSR, including the L-Type Ca^{2+} channel (dihydropyridine receptor, DHPR), RyRs and calsequestrin (Csq), among others, have been consistently reported in skeletal and cardiac muscle. As a result of the importance of many of these components for Ca^{2+} regulation, it is not surprising that alteration of these interactions has visible functional consequences for Ca^{2+} homeostasis.

DHPR α_{1S}

In skeletal muscle, early overlay experiments have suggested that Triadin has structural and functional interactions with both the DHPR α_{1S} subunit and RyR1[17,18]. However, with the elucidation of the primary sequence and topological structure of Triadin[4,11], it has become apparent that the DHPR-Triadin interaction involves intraluminal domains of Triadin that are unlikely to be accessible *in situ*. To date, there has been no new evidence that either supports or rules out direct structural or functional interactions between the cytoplasmic domain of Triadin and the DHPR complex.

Table 1 Triadin isoforms cloned from cardiac and skeletal muscle

Species	Cloned	Isoform	Mol. mass (kDa)		Ref.
			Predicted	Observed	
Cardiac muscle					
Rabbit	3	*CT1*	32	35	[5]
		CT2	34.6	40	[5]
		CT3	75	92	[5]
Canine	2	*CT1*	30.7	35/40[1]	[6]
		CT3	64.8	92[2]	[6]
Mouse	3	*MT1*	31.4	35/38[1]	[10]
		MT2	33	35.5	[10]
		MT3	34.3	40	[10]
Skeletal muscle					
Rabbit	1		79.1	94	[4]
Rat	4	*Trisk 32*	32.1	32	[9]
		Trisk 49	49.5	45	[9]
		Trisk 51	51.3	51	[7]
		Trisk 95	77.2	95	[7]
Canine	1		78.3	95	[6]
Human	3		81.5	117	[8]
		Trisk 51	51.5	51	[13]
		Trisk 95	95	95[2]	[13]

[1]Glycosylated isoform; [2]Marginally expressed or not detected.

RyRs

Immunohistochemistry studies in adult muscles have localized Triadin at the jSR in the vicinity of RyRs, revealing the close association of these two proteins in both skeletal[4,19] and cardiac[20] muscles. However, in skeletal muscle, co-localization with RyR1 only involves the 95-kDa isoform of Triadin, because the lower molecular weight isoforms (Trisk-32 and Trisk-49) appear to be segregated to non-jSR regions of the muscle[9]. Direct RyR1-Triadin interactions have been confirmed using glutathione-S-transferase/Triadin fusion proteins, which suggest that there is a stable multi-protein complex involving Triadin, RyR1 and the Ca^{2+} binding protein Csq[21]. More recently, mutagenesis analysis has suggested that the Triadin-binding site of RyR1 may reside within the negatively charged residues Asp^{4878}, Asp^{4907} and Glu^{4908} of RyR1[22]. Likewise, the corresponding RyR1-binding site of Triadin has been mapped to amino acids 200-232 within the intraluminal domain[22], a region rich in multiple clusters of alternating Lys and Glu residues, known as KEKE motif. This motif is common to all skeletal and cardiac isoforms of Triadin[22-24]. The direct functional effect of Triadin-RyR1 interaction on RyR1 channel activity is discussed below.

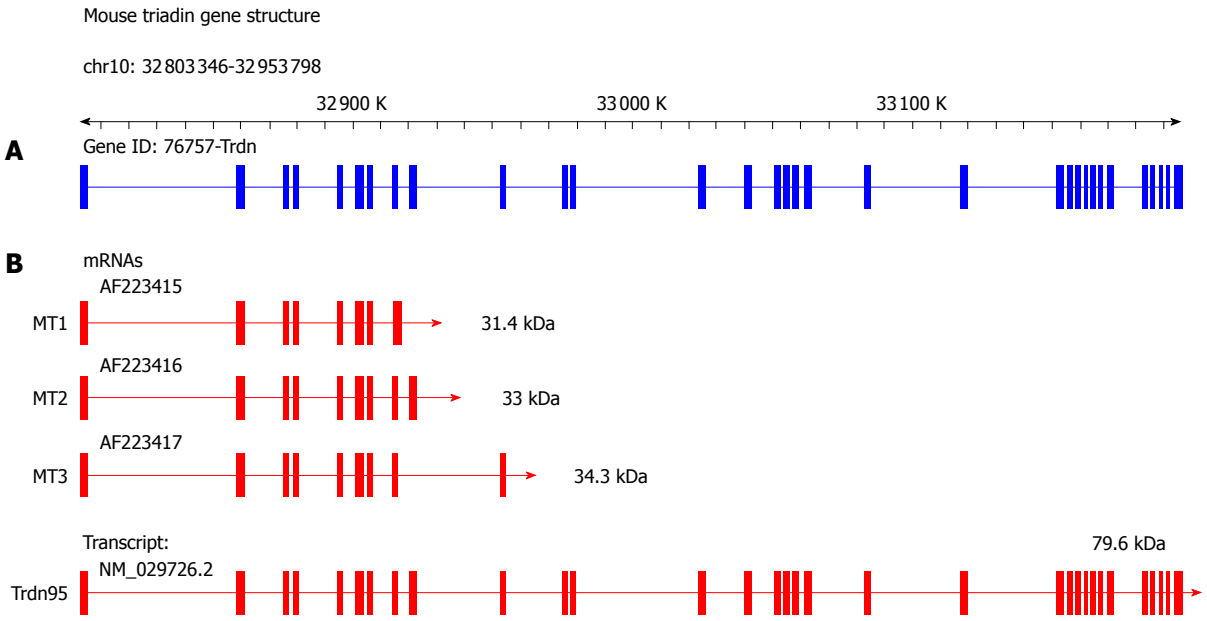

Figure 1 Genomic structure of mouse *Trdn* gene. A: Schematic representation of mouse Triadin cDNA structure within the context of the Triadin genomic locus according to the Mouse Genomic Informatics (*MGI*) gene model[60]; B: Splicing patterns of Triadin. Exons are shown as boxes and introns as lines. The exon splicing pattern that gives rise to the three cardiac Triadin isoforms currently cloned (MT1, MT2 and MT3) as well as the predicted full-length skeletal isoform (Trdn95) are indicated. Size and number of exon boxes in the genomic locus (blue) are not showed in actual scale.

Csq and junctin

In addition to binding RyRs, Triadin has been shown to directly interact with Csq and Junctin. Csq, an intra-SR Ca^{2+} binding protein is thought to be the main Ca^{2+} buffer protein of the SR[25-27], whereas Junctin is an SR integral membrane protein that shares structural and amino acid sequence similarity with Triadin[10,23,28]. Currently, there seems to be a consensus that Junctin and Triadin interact directly in the jSR membrane and stabilize a quaternary complex that anchors Csq to the RyR, probably through their shared KEKE motifs[21,23,29]. These quaternary complexes have been identified biochemically in both skeletal[21,30] and cardiac[23,24] muscles.

Based on the large Ca^{2+} binding capacity of Csq and its ability to undergo significant conformational changes over the physiological range of intra-SR Ca^{2+} concentrations[27,31], Csq has been proposed as an intraluminal Ca^{2+} sensor that plays a significant role in the ability of RyRs to sense and respond to changes in SR Ca^{2+} content. Even though *in vitro* studies in skeletal muscle using purified proteins have suggested that Csq and RyR1 can engage in direct structural/functional interaction[32-35], it is likely that *in vivo*, this functional crosstalk is primarily mediated through their interaction with Triadin and Junctin[36-38]. Even though the use of purified RyR1 in artificial bilayer membrane (BLM) studies to test the role of Triadin and Junctin in intraluminal Ca^{2+} regulation of skeletal muscle has recently suggested that Junctin may be the only protein involved in mediating signaling between Csq and RyR1[38], our studies have indicated that this may not be the predominant interaction *in vivo*. Indeed, Ca^{2+} imaging studies in Junctin-null mice suggest that, unlike in Triadin-null myotubes that show significant dysregulation of Ca^{2+} homeostasis, Junctin-null myotubes have a nearly wild-type phenotype, with no significant alteration in SR Ca^{2+} content or $[Ca^{2+}]_{rest}$ (unpublished data). These results strongly support the idea that Triadin is a key functional component of Ca^{2+} homeostasis in skeletal muscle.

Histidine-rich Ca^{2+}-binding protein

Histidine-rich Ca^{2+}-binding protein (HRC) is a Ca^{2+}-binding protein found in small amounts in the SR lumen, and shares many biochemical and structural features with Csq[39-42]. Biochemical studies in skeletal muscles have found that HRC can bind to Triadin in a Ca^{2+}-sensitive manner through the same KEKE motif involved in the binding of Triadin to Csq[40,43,44]. Therefore, it is not unlikely that binding of HRC to Triadin could affect RyR activity by disrupting the Triadin/Junctin/RyR/Csq Ca^{2+} release complex. Although the role of HRC in Ca^{2+} homeostasis in skeletal muscles is unknown, studies in cardiac cells have suggested that HRC is important for Ca^{2+} regulation. In the heart, overexpression of HRC is associated with alteration of both SR Ca^{2+} release and contractility, which coincidently is associated with reduction in Triadin and Junctin expression[41,45,46]. Conversely, HRC-null mice exhibit a significant increase in Triadin expression[47]. However, because cardiac and skeletal muscles express different isoforms of Triadin, the possibility that disruption of the HRC/Triadin interaction in skeletal muscle results in a different functional outcome than that observed in cardiac tissues should not be ruled out. In this regard, our own studies have indicated that, unlike HRC-null mice in which HRC and Triadin expression seem to be interlocked, in Triadin-null mice, the lack

of Triadin expression does not seem to affect HRC expression levels (unpublished data).

Overall, these studies suggest that Triadin is positioned to engage in meaningful structural/functional interactions with key modulatory components of Ca^{2+} release, and thus, seems poised to play a pivotal role in Ca^{2+} regulation in muscle cells.

EC COUPLING

RyR/DHPR interaction is key for EC coupling. Since the early biochemical studies in rabbit skeletal muscle[1,2,17] showing that Triadin binds to both DHPR α_{1S} and RyR1 and the proposed ternary complex between the proteins, there have been many studies that have linked Triadin to EC coupling in skeletal muscles.

Although the direct interaction between Triadin and DHPR α_{1S} has proven difficult to confirm, the evidence supporting a role for Triadin in modulating depolarization-induced Ca^{2+} release has been somewhat consistent. Early stopped-flow studies in triad vesicles have demonstrated that the use of an anti-Triadin antibody significantly inhibited depolarization-induced Ca^{2+} release[48], which supports the idea that Triadin may be involved in the functional coupling between DHPR and RyR1. More recently, in a series of functional studies, with overexpression of different isoforms of Triadin in cultured myotubes, it has been shown that Trisk-95, but not Trisk-55, significantly inhibits depolarization-induced Ca^{2+} release in rat[49] and C2C12[50] cells, strengthening the idea that Triadin, in particular the 95-kDa isoform, plays a critical regulatory role in skeletal-type EC coupling. Supporting this line of reasoning, Goonasekera *et al*[51] have shown that expression of mutant RyRs that lack Triadin-binding ability in dyspedic myotubes dramatically impairs electrically evoked Ca^{2+} transients, nearly ablating skeletal-type EC coupling without noticeable effects on other RyR1 functions. Similarly, Wang *et al*[52] have shown that the use of siRNAs to knockdown expression of Triadin in cultured myotubes led to a significant reduction in amplitude of K^+-induced Ca^{2+} transients, suggesting that Triadin may play a role in facilitating depolarization-induced Ca^{2+} release. However, with the recent development of Triadin-null mice, the idea of Triadin playing a critical or direct role in skeletal-type EC coupling has been challenged. Indeed, despite the lack of Triadin expression, homozygous Triadin-null (*Trdn*$^{-/-}$) mice do not exhibit embryonic or birth lethality nor demonstrate an obvious gross functional phenotype[12,14] as has been reported for dyspedic[53] and dysgenic mice[54,55], two other mouse models that bear significant disruption of the EC coupling signaling. Triadin-null skeletal muscles, however, have shown a significant decay in strength that confirms the general thought that Triadins are important modulatory components of skeletal muscle function[14].

Interestingly, Ca^{2+} imaging studies in Triadin-null myotubes have revealed that the absence of Triadin expression results in a noticeable reduction in peak amplitude of depolarization-induced Ca^{2+} transients[12]. Although, whole-cell patch clamp studies of Triadin-null myotubes have demonstrated that null cells display almost normal bidirectional signaling, with no changes in DHPR Ca^{2+} current densities and strong voltage-dependent Ca^{2+} release activity, they do have a moderate reduction in voltage-dependent Ca^{2+} release amplitude, and therefore, reduced orthograde signaling[56].

Triadin-null myotubes also display a significant alteration of the overall Ca^{2+} homeostasis driven primarily, but not exclusively, by the disruption of the RyR1/FKBP12 interaction[57]. Overexpression of FKBP12.6 can overcome this faulty interaction and almost completely reverse the effects of lack of Triadin expression on Ca^{2+} homeostasis. More importantly, overexpression of FKBP12.6 also is sufficient to erase all of the differences in depolarization-induced Ca^{2+} release observed between wild-type and Triadin-null myotubes[56]. The full restoration of EC coupling signals of Triadin-null myotubes by FKBP12.6 strongly suggests that the effects of Triadin on the orthograde signal are not directly but indirectly mediated by its side effects on the RyR1/FKBP12 interaction. Thus, further supporting the idea that skeletal Triadins are not involved in the bidirectional coupling between DHPR and RyR1.

MYOPLASMIC Ca^{2+} REGULATION

The well-documented interaction between Triadin and RyR1 has a significant impact on the Ca^{2+} channel behavior, and consequently, Ca^{2+} regulation in skeletal muscle cells. The first indication of a direct effect of Triadin on RyR1 function came from studies of Ohkura *et al*[35], who have reported that purified Triadin has an inhibitory effect on both ^{3}H-ryanodine binding to solubilized RyR1s, and on Ca^{2+} channel activity of purified RyR1 fused into BLMs. At the same time, Groh *et al*[58] have shown that a peptide containing a short fragment of the cytoplasmic domain of Triadin not only reduced the open probability of native and purified RyR1 channels in BLMs, but also inhibited the overall Ca^{2+} release from SR vesicles, identifying one of the first discrete domains of Triadin directly involved in a functional interaction with RyR1.

Our studies on native RyR1 channels reconstituted from Triadin-null skeletal muscles have revealed that the absence of Triadin significantly increases sub-conductance states of RyR channels, which in turn result in elevation of overall open probability. This enhanced channel activity seems to be directly associated with loss of FKBP12 binding capacity of RyR1, because the addition of exogenous FKBP12.6, that has a higher affinity for RyR1 than FKBP12, significantly reduces channel activity[57]. However, in a recent study, Wei *et al*[38] have found that, unlike previous studies, addition of purified skeletal Triadin had instead an activating effect on the channel activity of purified RyR1 fused into BLMs. It is still unclear whether these differences in functional effect account for differences in experimental protocols or actual functional

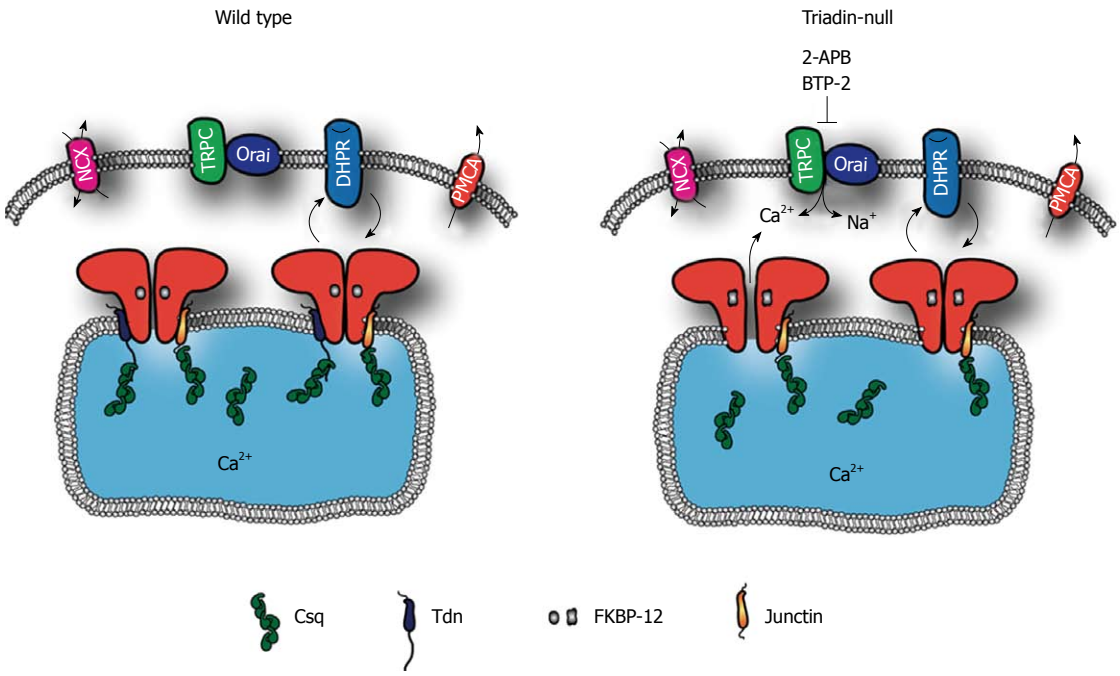

Figure 2 Proposed model of Ca^{2+} regulation by Triadin in wild-type and Triadin-null skeletal muscle. Lack of Triadin binding to type-1 ryanodine receptor (RyR1) indirectly affects FKBP12/RyR1 interaction causing, on the one hand, an increase in RyR1 channel gating and, on the other hand, a weakening of the DHPRα_{1S}/RyR1 orthograde signaling. Dysregulation of RyR1 activity of Triadin-null cells leads to enhanced SR Ca^{2+} leakage and subsequent reduction in SR Ca^{2+} content. In addition, lack of Triadin expression activates Ca^{2+} entry pathways that are both store-dependent and store-independent (sensitive to TRPC/Orai-1 inhibitors). Ca^{2+} entry and SR Ca^{2+} leakage could contribute independently to elevate myoplasmic $[Ca^{2+}]_{rest}$.

differences in Triadin-binding sites at the intraluminal and cytoplasmic domain of RyR1. However, what all these reports seem to agree on is the idea that changes in Triadin expression result in modulation of RyR1 Ca^{2+} channel activity.

Consistent with the enhanced basal activity observed in RyR channels from Triadin-null muscles, *Trdn*$^{-/-}$ myotubes are characterized by reduced Ca^{2+} release response to caffeine[12,14,57] and the sarcoplasmic/endoplasmic reticulum calcium pump inhibitor thapsigargin[12,14], both suggestive of alterations in the SR Ca^{2+} content. Similar observations have been reported in Triadin-knockdown myotubes, where in addition to reduced SR Ca^{2+} load, there is an increased frequency in Ca^{2+} spark activity[50]. In agreement with these reports, reduced caffeine-induced Ca^{2+} release responses are also observed in dyspedic myotubes expressing Triadin-binding-deficient RyRs[51,59]. Overall, these results seem to support the idea that loss of Triadin expression leads to loss of negative regulation on RyR channels, which in turn, results in enhanced SR Ca^{2+} leakage. Accordingly, overexpression of Triadin in skeletal myotubes, a condition that should increase the negative regulation on RyR1 and suppress SR Ca^{2+} leakage, appears not to have a detrimental effect on caffeine-induced Ca^{2+} release and SR Ca^{2+} load[49,50].

Myotubes and adult muscle fibers from Triadin-null mice also show chronically elevated $[Ca^{2+}]_{rest}$[12,50,57]. This elevated resting myoplasmic $[Ca^{2+}]$ is partially reversed by inhibitors of RyR1 activity (ryanodine and FKBP12.6) and RyR1 leakage (bastadin-5). Similar effects have been observed with Ca^{2+} entry blockers (Cd^{2+} and La^{3+}) and TRPC/Orai-1 blockers (2-APB and BTP-2)[57]. This pharmacological profile is consistent with the idea that elevated resting Ca^{2+} in Triadin-null muscle cells involves both RyR-mediated SR Ca^{2+} leakage and enhanced extracellular Ca^{2+} entry at rest[57]. The effect of 2-APB and BTP-2 on $[Ca^{2+}]_{rest}$ in addition to the elevated intracellular $[Na^+]$ observed in Triadin-null cells (unpublished data) strongly suggests that the extracellular Ca^{2+} entry pathway activated by the lack of Triadin may be, at least partially, mediated by TRP channels or Orai-1.

In summary, the current accumulated data from Triadin-null muscle cells support the molecular model depicted in Figure 2 in which the lack of Triadin expression significantly destabilizes the FKBP12/RyR1 interaction, causing increased basal activity of RyR Ca^{2+} channels. This in turn results in increased SR calcium leakage, which contributes to elevate the myoplasmic resting free Ca^{2+}. On the other hand, this increased Ca^{2+} leakage leads to partial depletion of SR calcium stores, which drives TRPC- and/or Orai-1-mediated Ca^{2+} entry, further contributing to elevation of resting Ca^{2+}.

Although many questions remain, a clear picture of Triadins playing a relevant modulatory role in Ca^{2+} homeostasis of skeletal muscle has emerged. Triadin control of RyR Ca^{2+} channel activity has the potential to unravel a cascade of events that can ultimately adjust Ca^{2+} flux equilibrium in muscle cells, resulting in permanent modification of $[Ca^{2+}]_{rest}$. Hence, it appears that although not directly involved in Ca^{2+} transport Triadins may contribute a significant role to fine-tuning Ca^{2+} homeostasis in skeletal muscles.

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REVIEW

Methionine sulfoxide reductase A: Structure, function and role in ocular pathology

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Abstract

Methionine is a highly susceptible amino acid that can be oxidized to S and R diastereomeric forms of methionine sulfoxide by many of the reactive oxygen species generated in biological systems. Methionine sulfoxide reductases (Msrs) are thioredoxin-linked enzymes involved in the enzymatic conversion of methionine sulfoxide to methionine. Although MsrA and MsrB have the same function of methionine reduction, they differ in substrate specificity, active site composition, subcellular localization, and evolution. MsrA has been localized in different ocular regions and is abundantly expressed in the retina and in retinal pigment epithelial (RPE) cells. MsrA protects cells from oxidative stress. Overexpression of MsrA increases resistance to cell death, while silencing or knocking down MsrA decreases cell survival; events that are mediated by mitochondria. MsrA participates in protein-protein interaction with several other cellular proteins. The interaction of MsrA with α-crystallins is of utmost importance given the known functions of the latter in protein folding, neuroprotection, and cell survival. Oxidation of methionine residues in α-crystallins results in loss of chaperone function and possibly its antiapoptotic properties. Recent work from our laboratory has shown that MsrA is co-localized with αA and αB crystallins in the retinal samples of patients with age-related macular degeneration. We have also found that chemically induced hypoxia regulates the expression of MsrA and MsrB2 in human RPE cells. Thus, MsrA is a critical enzyme that participates in cell and tissue protection, and its interaction with other proteins/growth factors may provide a target for therapeutic strategies to prevent degenerative diseases.

Key words: Methionine sulfoxide reductases; Hypoxia; Protein interaction; α crystallins; Neuroprotection

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INTRODUCTION

Molecular oxygen is indispensible for survival of aerobic organisms, which use oxygen for energy pathways in mitochondria and numerous other processes, but the use of oxygen is also associated with the generation of reactive oxygen species (ROS)[1]. According to free radical theory, ROS promote oxidative damage to many cellular

constituents, including amino acids, lipids, and nucleic acids[2]. Oxidative damage to proteins and other biomolecules by ROS has been implicated in a variety of diseases and in aging and senescence-associated disorders[3]. The modifications caused by oxidation may or may not be reversible. Oxidized proteins may become non-functional as a result of structural changes and catalytic malfunction. The sulfur-containing amino acids methionine and cysteine are the major targets of ROS in proteins and are also the amino acids most susceptible to oxidation[4]. As a universal initiating amino acid for protein synthesis, methionine has additional importance for cellular functions. Both free methionine and protein-based methionine are readily oxidized by ROS to form methionine sulfoxide, which could alter protein structure and function[5]. To counteract ROS damage, organisms have evolved multiple defense systems, including low molecular weight compounds and antioxidant enzymes that protect against oxidative stress. The antioxidant system includes glutathione peroxidase[6,7], superoxide dismutase[8,9], catalase[10,11], thioredoxin reductase (TR)[12], methionine sulfoxide reductase (Msr)[13-17] and several other proteins, including but not limited to small heat shock proteins, particularly α-crystallins[18-21]. Msrs are prominent among these antioxidant enzymes because of their roles as repair enzymes and indirect scavengers of ROS[4,22]. Although different ROS scavenging systems/enzymes are present in the cells, in the case of methionine oxidation, a more energy-efficient mechanism involves the action of the Msr enzymes, MsrA and MsrB[23,24]. MsrA and MsrB can catalyze the reversion of the methionine *S*-sulfoxide and the methionine *R*-sulfoxide, respectively, to the reduced form of methionine within proteins[25]. Methionine can be easily oxidized into methionine sulfoxide, and its reduction by Msr could represent an efficient antioxidant system because, in proteins, the surface-exposed methionine residues can act as scavengers of a variety of oxidants[26]. Methionine oxidation denatures proteins and converts the hydrophobic properties of Met into hydrophilic properties, resulting in structural alterations[27]. Although MsrA and MsrB enzymes have the same function of methionine sulfoxide reduction, they differ, not only in substrate specificity, but also in active site composition, protein folding, subcellular localization, and evolution[28,29]. Msrs have wide tissue distribution and are present in multiple sites in the eye[14-17]. Msrs interact with other proteins and protect cells from oxidative-stress-induced cell injury[14-17]. In this review, we address specifically the role of MsrA in protecting the eye against various types of oxidative injury. Furthermore, the regulation of MsrA and MsrB is illustrated in chemically induced hypoxia in retinal pigment epithelial (RPE) cells *in vitro*.

Msrs: GENE STRUCTURE AND ISOFORMS

Msrs are expressed in most organisms and catalyze the thioredoxin-dependent reduction of free and protein-bound methionine sulfoxide to methionine[29-31]. Msrs, as well as glutathione and TRs, are ubiquitously expressed in cells[29,32] and, acting together with their substrates and co-factors, form repair systems that protect cells from oxidative stress and maintain cellular redox homeostasis[29,33,34]. According to current literature, there are three types of Msr: MsrA, MsrB and frMsr[10]. However, frMsr distribution is limited to unicellular organisms; multicellular organisms, including mammals, lack this protein[35]. The human MsrA gene is located on chromosome 8 and is coded by one gene regulated by two distinct promoters resulting in different isoforms: the long form and short form[36]. The long form is localized to the cytosol, mitochondria, and nucleus, while the short form is localized to the nucleus and cytosol[28,37,38]. The long form of MsrA encodes a peptide containing an N-terminal mitochondrial targeting sequence, a catalytic cysteine containing sequence, and a C-terminal thioredoxin-binding domain. The short form of MsrA lacks the mitochondrial sequence, but the cysteine-containing sequence and thioredoxin domain are active. Expression of MsrA is found in almost all human tissues with the exception of leukemia and lymphoma cell lines[39]. MsrA is specific for the reduction of free and protein-based methionine-S-sulfoxide in mammals[10,40]. MsrA can also reduce compounds such as N-acetylmethionine-S-sulfoxide, dimethyl sulfoxide, and ethionine-S-sulfoxide[22]. However, the functional differences between the long and short forms of MsrA have not yet been characterized.

Three mammalian MsrBs exist[10]. MsrB1 is present in the cytosol and nucleus and exhibits the highest catalytic activity because of the presence of selenocysteine in its active site. MsrB1 occurs in two forms, 14 kDa and 5 kDa, in mouse tissues and human HEK 293 cells, and both forms are selenoproteins[10,41,42]. MsrB is encoded by three different genes and their products are: MsrB1, a selenoprotein found in the nucleus and cytosol; MsrB2, which is present in the mitochondria; and MsrB3, which encodes for two splice variants, MsrB3A and MsrB3B, localized to the endoplasmic reticulum (ER) and the mitochondria, respectively[37,43]. The MsrB enzymes are present in all eukaryotic tissues, but their level of expression varies[37]. MsrB is specific for the reduction of protein-based methionine-R-sulfoxide, and reduces free methionine-R-sulfoxide with very low efficiency[10]. Among the three MsrBs, MsrB1 has the highest catalytic activity because of the selenocysteine in its active site. MsrB2, also known as CBS1, is targeted to mitochondria with the guidance of its N-terminal signal peptide and has cysteine as the catalytic residue. It shows high activity with methionine-R-sulfoxide but is inhibited by elevated concentrations of the substrate[43-45]. The two forms of MsrB3, MsrB3A and MsrB3B, are generated by alternative first exon splicing in humans. MsrB3A is targeted to the ER with the N-terminal ER signal peptide and an ER retention signal at the C terminus, whereas MsrB3B is targeted to mitochondria by its N-terminal mitochondrial signal peptide. Interestingly, mouse MsrB3 also has the ER and mitochondrial signal peptides located consecutively at the N terminus, but it is

targeted only to the ER because the mitochondrial signal is masked by the upstream ER signal[10].

CATALYTIC MECHANISM OF Msrs

Although structurally distinct, MsrA and MsrB share a common three-step catalytic mechanism[46,47]. Enzymatic studies have shown that the MsrA domain possesses two essential cysteine residues to carry out its function[48]. The catalytic mechanism of MsrA and MsrB is based on three steps. In the first step, the Msr catalytic cysteine residue interacts with the methionione sulfoxide substrate, which leads to the product release and formation of the sulfenic acid. In the second step, an intramolecular disulfide bridge is formed between the catalytic cysteine and the regenerating cysteine. In the final step, the disulfide bridge is reduced by an electron donor, the NADPH-dependent thioredoxin/TR system, leading to the regeneration of the Msr active site. The catalytic mechanism varies between different Msrs, especially in the number of recycling cysteines[49,50]. However, the selenoprotein MsrB1 is believed to use an alternative recycling cysteine residue located in a different position[28].

COMPARTMENTAL DISTRIBUTION OF MsrA IN THE EYE

The bulk of the work in the eye on MsrA has dealt with its function in the lens[51]. MsrA has been found to be highly expressed in the lens epithelium and fiber cells[14]. Its expression in the photoreceptor inner segments and in the inner nuclear layer of the retina and in the RPE has also been characterized[16,30,36,52]. In the monkey retina, MsrA gene expression is detected mostly in the macular RPE-choroid region, whereas its activity is detected mainly in the soluble fractions of neural retina and RPE-choroid[30]. MsrA protein is distributed throughout the retina but is abundant at the photoreceptor synapses and in the ganglion and Müller cells. MsrA expression is higher in macular RPE cells than in the peripheral RPE cells of the retina[30]. Our work has shown that MsrA is localized to sub-RPE macular drusen from patients with age-related macular degeneration (AMD)[16]. Our subcellular localization studies have revealed that MsrA is expressed in the cytosol and in mitochondria of human RPE cells[16].

MsrA IN OXIDATIVE STRESS

Cells and tissues possess a number of antioxidant systems to prevent oxidative damage that causes protein aggregation and cell death. The enzyme MsrA plays an important role in the antioxidant response by reducing the *S*-stereoisomer of methionine sulfoxide (MetSO) to methionine[4]. Accordingly, cells with increased or decreased levels of MsrA are highly resistant or vulnerable to oxidative stress, respectively[14,16,17]. MsrA not only protects cells from oxidative stress by repairing proteins damaged by methionine oxidation, but it also functions by engaging in a sequence of methionine oxidation and reduction cycles that eventually results in ROS scavenging[17]. The MsrA activity is determined by the two active-site cysteine residues: 72 and 218[48]. Cysteine 72 carries out a nucleophilic attack at the sulfur atom of the methionine sulfoxide substrate, leading to the formation of a covalent intermediate, whereas cysteine 218 attacks cysteine 72 to trigger breakdown of the covalent complex[48]. Evidence that MsrA has a role in protecting cells against oxidative damage was first shown in *Escherichia coli* where MsrA mutants are more sensitive to H_2O_2[53]. Overexpression of the MsrA gene predominantly in the nervous system markedly extends the lifespan of the fruit fly *Drosophila* by 70%[54]. In addition, MsrA transgenic flies are more resistant to paraquat-induced oxidative stress, and the onset of senescence-induced decline in the general activity level and reproductive capacity is delayed markedly[54]. MsrA null mutants of yeast[55] and mice[56] are more sensitive to oxidative stress than wild-type organisms, and their lifespans are shortened by about 26% in yeast[57] and 40% in mice[56]. Compared with the wild type, MsrA mutant mice exhibit enhanced sensitivity under hyperoxia and have a shorter lifespan under both normal and hyperoxic conditions. Mutants also accumulate higher tissue levels of oxidized protein under oxidative stress, and ae unable to upregulate expression of TR under oxidative stress[56]. Adenovirus-mediated overexpression of MsrA significantly diminishes the hypoxia-induced increase in ROS and facilitates cell survival in neuronal cells by preserving mitochondrial membrane potential and apoptotic events[15]. MsrA is protective against hypoxia/reoxygenation stress in cardiomyocytes, suggesting that it may be an important therapeutic target for ischemic heart disease[58]. The resistance in MsrA-overexpressing human fibroblasts is accompanied by a decrease in intracellular ROS and is partially abolished when cells are cultured with suboptimal concentrations of methionine[17]. These results indicate that MsrA could play an important role in cellular defense against oxidative stress by catalytic removal of oxidant through the reduction of methionine sulfoxide and in protection against death by limiting, at least in part, the accumulation of oxidative damage to proteins.

Our laboratory examined the protective role of MsrA in human fetal RPE cells[16]. Oxidative stress from H_2O_2 exposure results in the generation of ROS and activation of caspase-3 in RPE cells. In addition, an increase in MsrA expression in cytosol and mitochondria was also observed. Silencing of MsrA resulted in further induction of caspase-3 and accentuated cell death from oxidative stress[16]. Similar results have been reported in ARPE-19 cells in which MsrA gene-silenced cells were susceptible to oxidative stress[30]. Kantorow *et al*[14] have shown that overexpression of MsrA protects lens cells against H_2O_2-induced oxidative stress, whereas decreased expression of MsrA results in increased sensitivity to oxidative stress and decreased lens cell viability. This is attributed to the increased lens ROS levels and loss of mitochondrial func-

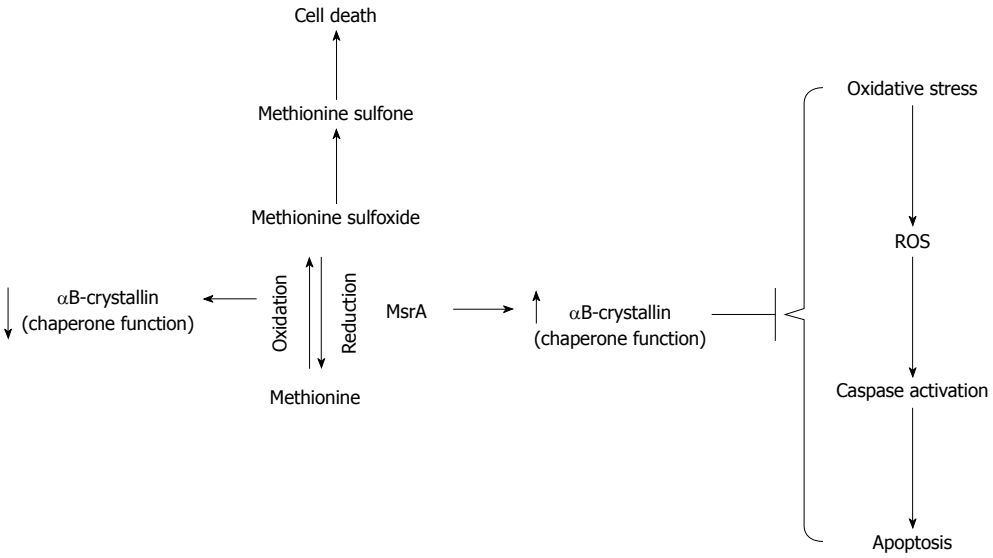

Figure 1 Scheme depicting the role of methionine sulfoxide reductase A and α-crystallin in methionine metabolism. Note the reversible nature of oxidation-reduction of methionine and its influence on the chaperone properties of α-crystallin. The inhibition of the apoptotic events from oxidative stress by αB crystallin is also presented in the figure. ROS: Reactive oxygen species.

tion. Furthermore, severe cytochrome c oxidation and lens cataract have been reported in hyperbaric oxygen-treated MsrA deficient mice by the same laboratory[59]. It is of interest that the isoforms of MsrB have also been shown to prevent oxidative damage to lens cells and RPE cells[60,61].

Thus, the protective effect of MsrA seems to result, at least in part, from an antioxidant mechanism, by preserving mitochondrial functions and inhibiting subsequent activation of caspases as seen during its deficiency[16]. Indeed, other studies have pointed out the protective role of MsrA against the deleterious effects of ROS in *Drosophila* and mammalian cells, emphasizing the important role of this enzyme in both maintenance of proteins under oxidative stress and overall redox cellular homeostasis[15,54]. Moreover, oxidized methionine may also be a critical component in redox signaling. For example, sulfiredoxin and sestrins that repair cysteine-sulfinic acid in peroxiredoxins are likely important, not only for their antioxidant function, but also in signaling pathways sensitive to peroxiredoxin hyperoxidation[62-64]. Similarly, MsrA could modulate signal transduction through the regulation of methionine oxidation/reduction within specific proteins, and MsrA modulation would expect to affect such redox-sensitive signaling pathways.

INTERACTION OF MsrA WITH OTHER PROTEINS

MsrA is involved in the repair of methionine residues in several proteins. α-crystallin and cytochrome C have been identified as major targets of MsrA in the lens[59,65]. This finding is of significance because of emerging evidence about the role of α-crystallins in ocular health. A direct link has been identified between α-crystallin methionine oxidation and age-related cataract formation[65]. α-crystallins are major proteins of the small heat shock protein family and are expressed in several tissues[66-68]. α-crystallins have been studied extensively in the lens for their chaperone function, but α-crystallins are now generally understood to have additional non-lens roles[18-21,69,70]. In addition to being a molecular chaperone, α-crystallin functions in cell death inhibition, neuroprotection, proteosomal interactions, and regulation of angiogenesis[18-21,70,71]. αB crystallin is more abundant in the RPE cells where it provides neuroprotection. α-crystallins are predominantly cytosolic proteins; however, mitochondrial and nuclear localization has also been reported[19,72,73].

Methionines of αA- and αB-crystallins are oxidized in human lenses between the ages of 45 and 65 years[74,75]. The methionine in αA-crystallin is oxidized to methionine sulfoxide in rat hereditary cataracts[76], and interestingly, substitution of methionine 68 in αB-crystallin with a less hydrophobic residue (Thr) results in loss of chaperone activity[77]. Development of cataract could be the result of protein aggregation caused by loss of α-crystallin chaperone function. Methionine oxidation damages α-crystallin chaperone activity, but MsrA can also repair oxidized methionines in α-crystallin and restore the chaperone function (Figure 1)[65]. Therefore, loss of MsrA activity upon aging or oxidative stress could result in loss of α-crystallin chaperone function and contribute to the development of cataract and other age-related oxidative stress-associated disorders, such as AMD[78], Parkinson's disease[79], Alzheimer's disease[80] and desmin-related myopathy[81]. α-crystallins also possess antiapoptotic properties[18-20]. Whether methionine oxidation could also affect the antiapoptotic function remains to be established.

We and others have shown that α-crystallins are localized in different retinal layers and in drusen of AMD samples[20,78,82]. We have further found that MsrA is local-

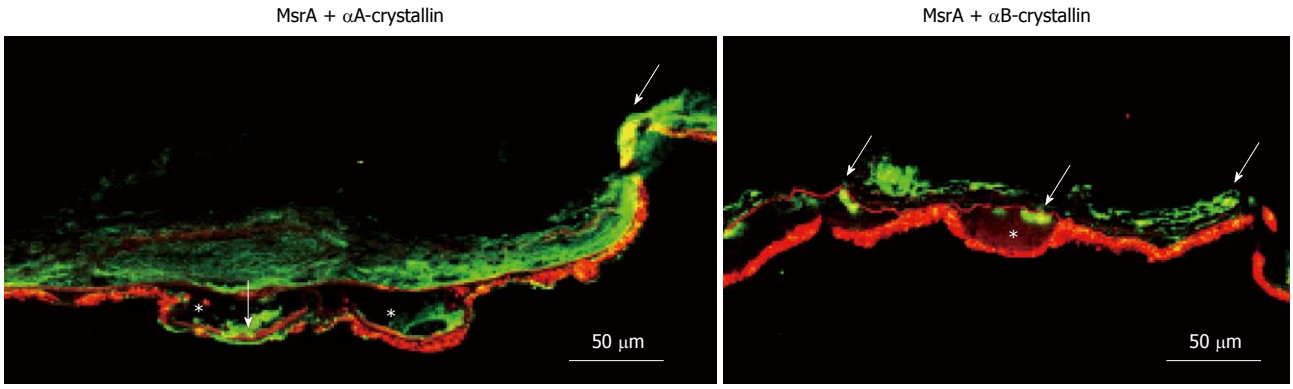

Figure 2 Immunohistochemical localization of αA-crystallin (red), αB-crystallin (red) and methionine sulfoxide reductase A (green) in the retina of patients with AMD. Retinal cryosections were air-dried, fixed, and processed as described[18] using αA-crystallin and αB-crystallin rabbit polyclonal antibodies (Stressgen, Ann Arbor, MI, USA), and mouse monoclonal methionine sulfoxide reductase (Msr) A antibody (Novus Biologicals, Littleton, CO, USA). Sections were viewed under a confocal microscope (Carl Zeiss, Thornwood, NY, USA). Arrows indicate co-localization (yellow) of αA-crystallin or αB-crystallin and MsrA. AMD:Age-related macular degeneration; *: Drusen; Scale bar = 50 μm.

ized as a component of drusen samples[16]. Our immunofluorescence data, shown in Figure 2, indicate that both αA and αB crystallins co-localize with MsrA in the AMD retinas, especially in the drusen, suggesting potential interaction between the two types of proteins. This observation is consistent with the phenomenon described above that methionine residues of crystallins undergo oxidation and lose chaperone function with aging and oxidative stress. MsrA is involved in the repair process, so that the interaction or association of both these proteins in the AMD retina is a critical process for further investigation at the molecular level.

In summary, repair of oxidized methionines in α-crystallin is necessary for the maintenance of chaperone function. MsrA is required in eyes and other tissues for maintenance and repair of α-crystallin chaperone function. The chaperone activity is essential for a number of significant functions ranging from protein folding to cytoskeletal remodeling, apoptotic control, neuroprotection, and angiogenesis regulation[18,21,70]. Loss of α-crystallin chaperone function probably results in protein aggregation, which may account for the cataract formation found in the absence of MsrA in mice[65]. Given the role of MsrA and α-crystallin in the health and disease of retinal and other tissues, these results are probably applicable to our understanding of the oxidative-stress-associated and aging disease mechanisms, and these results may provide a basis for the development of well-designed interventions for these conditions.

Msr REGULATION UNDER HYPOXIC CONDITIONS IN RPE CELLS

Msrs are able to protect cells against oxidative damage, thus as discussed above, and because of the importance of oxidative damage in retinal tissues after hypoxia/reoxygenation[83], we wished to study the regulation of Msrs (MsrA and MsrB2) in RPE cells in hypoxia. The retina is known to be the most metabolically active tissue in the body, and it is highly sensitive to reduction in oxygen tension[84]. Therefore, the role of the oxygen microenvironment in the retina may be of importance in understanding AMD and other retinal degenerative diseases. RPE cells were subjected to chemically induced hypoxia (100 μmol/L $CoCl_2$, 4 h) in serum-free medium[20]. $CoCl_2$ stimulates the hypoxia responsive pathways and has been shown to induce apoptosis by mitochondrial pathways and hypoxia-inducible factor (HIF)-1α-dependent and -independent mechanisms[85,86]. $CoCl_2$-induced hypoxia in RPE induced upregulation of HIF-1α and vascular endothelial growth factor (VEGF) (Figure 3A-C). Gene expression analysis of MsrA and MsrB2 showed initial upregulation for up to 1 h with hypoxia and a decline thereafter (Figure 3D and E), without affecting cell viability. Our findings in RPE cells are in accordance with several previous studies. For example, Msrs are reported to behave in the same manner in mouse embryonic cells under anoxia and acidosis[87]. MsrA is protective against hypoxia/reoxygenation stress in multiple cell types. Adenovirus-mediated overexpression of MsrA in primary neonatal rat cardiomyocytes subjected to hypoxia/reoxygenation reduced apoptotic cell death by > 45%[58]. Further support comes from studies using PC12 cells, in which overexpression of MsrA lowered the level of ROS[15], and in lens cells, in which depletion of MsrA using siRNA resulted in increased levels of ROS[14,16]. The lower ROS level conferred by MsrA overexpression in PC12 cells was associated with greater cell viability[15] after hypoxia/reoxygenation. As discussed above, MsrA interacts with α-crystallins, and we have shown that under prolonged hypoxic conditions, α-crystallins in RPE also showed a significant decrease, at both the transcriptional and translational levels[20]. It should be noted that retinas of αA or αB crystallin knockout mice are highly susceptible to hypoxia-induced cell death and that this could partly be due in part to decreased Msr expression. The finding that overexpression of MsrA reduces hypoxia-mediated apoptosis suggests that the endogenous antioxi-

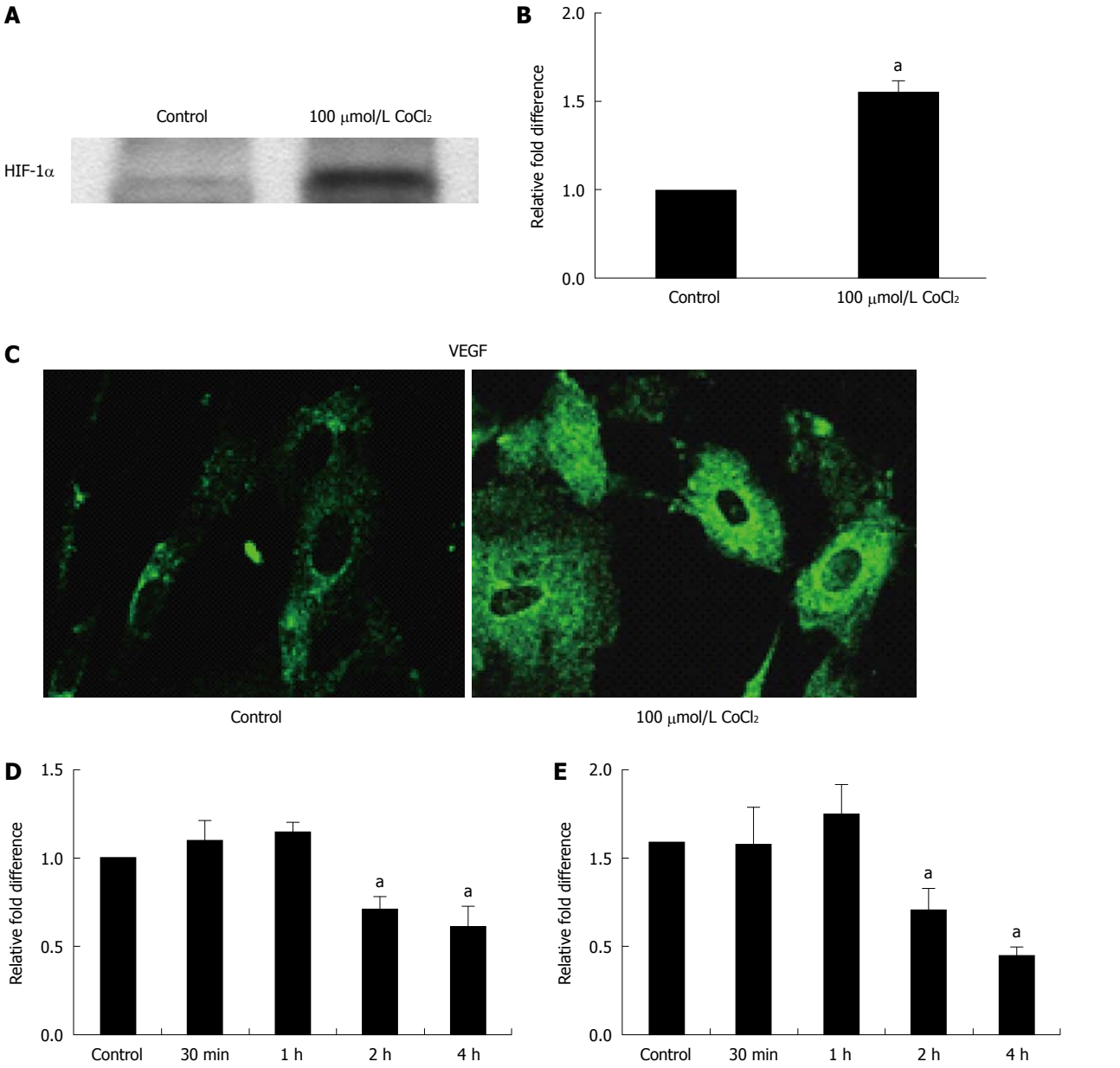

Figure 3 Regulation of methionine sulfoxide reductase A and B2 in retinal pigment epithelial subjected to chemically induced hypoxia. Hypoxia-inducible factor (HIF)-1α (A) and vascular endothelial growth factor (VEGF) (B and C) were used to validate hypoxia from $CoCl_2$. Confluent human fetal retinal pigment epithelial (RPE) cells were serum starved overnight and treated with 100 μmol/L $CoCl_2$ for 4 h. Western blot analysis shows HIF-1α (mouse monoclonal, Novus Biologicals) upregulation in nuclear extracts of $CoCl_2$-treated RPE cells (A). VEGF expression was significantly upregulated both at the mRNA (B) and protein levels (C). Real-time polymerase chain reaction was performed as described[19] in a light cycler (Roche, IN, USA) using β-actin as normalizing gene. A polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used for the detection of VEGF. Methionine sulfoxide reductase (Msr) A and MsrB showed initial upregulation but, at 4 h, showed significant downregulation (D and E). Data presented are mean ± SE. $^aP < 0.05$ *vs* control.

dant mechanism is not sufficient to counteract the oxidative stress caused by hypoxia. Thus, MsrA and MsrB, which reduce the S and R forms of methionine sulfoxide in proteins, respectively, may be viable therapeutic targets.

FUTURE PERSPECTIVES

A great deal of attention to MsrA has been directed towards its functional properties in the lens. Detailed studies on the action and importance of MsrA in the retina are scarce. As an important reductant, MsrA is likely to have a beneficial role in retinal diseases such as AMD, diabetic retinopathy and oxygen-induced retinopathy, which are associated with oxidative stress. The emerging role of other proteins such as the chaperone αB crystallin in retinal as well as choroidal angiogenesis[21] suggests that interaction of MsrA with such proteins may prove to be a critical area of investigation. α-crystallins are crucial molecules with multiple functions, and whether MsrA has a direct role in protecting their chaperone function will be worthy of study *in vivo*. Both MsrA and α-crystallins share antiapoptotic properties and both are present or translocated to the mitochondria during stress, therefore, it is hypothesized that their interaction occurs in mito-

chondria which is the major source of endogenous ROS. Recent findings indicate that, in AMD, both MsrA and α-crystallin accumulate in the drusen, and the nature of their physical and molecular interactions under pathological conditions is not known. Further, α-crystallins are neuroprotective in multiple neurodegenerative diseases[71,88-92], and given the fact that oxidation of the methionine residues inhibits the chaperone function of crystallins, it is plausible that modulation (by overexpression) of the Msr activity may be beneficial in the treatment of these diseases. In addition, aging contributes to increased oxidation of methionine in proteins so that MsrA alone or in combination therapy could offer significant and long-term protection.

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February 13-16
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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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