Calponin control of cerebrovascular reactivity: therapeutic implications in brain trauma

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Abstract

Calponin (Cp) is an actin-binding protein first characterized in chicken gizzard smooth muscle (SM). This review discusses the role of Cp in mediating SM contraction, the biochemical process by which Cp facilitates SM contraction and the function of Cp in the brain. Recent work on the role of Cp in pathological states with emphasis on traumatic brain injury is also discussed. Based on past and present data, the case is presented for targeting Cp for novel genetic and pharmacological therapies aimed at improving outcome following traumatic brain injury (TBI).

Keywords: vascular reactivity • calponin • brain trauma

A role for calponin (Cp) in mediating smooth muscle (SM) contraction

The basic isoform of Calponin (henceforth denoted as Cp) is an actin-binding protein present in both the cytoskeleton and contractile machinery of SM cells [1]. It has been shown also to be synthesized in endothelial cells (EC) [2–4]. Originally purified from chicken gizzard SM by Takahashi et al. [5], Cp was subsequently identified as a 34-kD protein, which bound to tropomyosin [6]. Because of its selective localization, Cp has been deemed a critical protein in the regulation of SM contraction. Mezgueldi et al. [7, 8] identified an actin-binding site. This site also contains the part of Cp that, by binding to actin, inhibits myosin ATPase activity.

The mechanism by which Cp regulates SM contraction is somewhat controversial and may involve either direct inhibition of acto-myosin cross-bridge formation [8–9], thus ultimately preventing SM contraction. Worth et al. [10] demonstrated increased Cp protein expression in SM’s contractile state. Two other in vitro works showed that application of Cp in a dose-dependent fashion increase SM contraction [11, 12]. In contrast in whole muscle preparations, Cp appeared to have somewhat different effects. Jaworowski et al. [13], using intact skin SM from guinea pig, reported that while Cp inhibited maximal shortening velocity, it had only a minor influence on the force of contraction (~10% reduction). They further suggested that Cp may modulate the rate of acto-myosin cross-bridging. Similarly, Obara et al. [14] presented data supporting the concept that rather than directly inhibiting acto-myosin, Cp modulated the velocity of cross-bridging.

Alternate lines of evidence suggested that Cp involvement in SM contraction could be related to signalling rather than to a direct acto-myosin interaction. In a set of compelling studies, Morgan and colleagues showed that basic Cp not only interacts with PKC alpha and epsilon, but also with ERK [15]. They further showed that Cp is bound directly to the regulatory domain of PKC. Their data, combined with further work showing that during SM contraction ERK redistributed from cytoskeleton to contractile

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domains [16], support a Cp signalling role in PKC and ERK-mediated SM contraction.

It has been suggested that any conflicting data regarding a putative Cp role in regulating SM contraction may be due to either differences in Cp expression, the use of isolated SM cells versus intact muscle [17], or both. For example, components of SM cytoskeleton, including Cp, were shown to rapidly down-regulate when SM cells are placed in culture [18–20]. Given that Cp levels in SM may be critical for contractility, such decreased expression may confound interpretation of the in vitro data. Nonetheless, the consensus of the literature supports a regulatory role of Cp in mediating contraction of vascular SM.

Cp phosphorylation and vascular contractility

PKC-dependent Cp phosphorylation has been linked to SM contraction. PKC application to cultured ferret aortic SM cells results in enhanced vascular contractility [21, 22]. In contrast, application of staurosporine, a PKC inhibitor, prevents vascular SM contraction [23]. It should be pointed out, however, that results may be difficult to interpret, here, since staurosporine has relatively poor selectivity. Nonetheless, these results provide further evidence that PKC is critical for vasoconstriction. Cp is known to contain five potential phosphorylation sites, serine (SER)-175 and threonine (THR)-170, -180, -184 and -259 [24]. Ser-175 and Thr-184 are PKC-phosphorylation sites. Early on Naka et al. [25] showed that Cp is phosphorylated via protein kinase C (PKC). Subsequent in vitro studies demonstrated that upon phosphorylation, Cp becomes disassociated with acto-myosin, allowing for cross-bridging [26, 27]. Cp dephosphorylation via a Cp-specific phosphatase is thought to restore Cp-inhibition of acto-myosin cross-bridging, thus returning SM to its relaxed state [28]. In addition only THR-184 was shown to significantly affect (i.e. enhance) vasoconstriction [29]. Based on these findings, it can be concluded that PKC phosphorylates at the Cp THR-184 site, which results in vasoconstriction. However, two other groups have provided evidence to the contrary by showing that Cp phosphorylation does not affect SM contraction [17, 30, 31]. Again, this discrepancy could be the result of studying isolated SM cells versus intact muscle [17].

Cp localization and function in the brain

Much of the characterization of Cp has been carried out in peripheral tissue (i.e. non-CNS) preparations with little work done in brain. In fact, the role of Cp in controlling brain vascular tone has only been recently studied [32]. Furthermore, more fundamentally, whether Cp is found in brain has been the source of controversy.

When first purified, Takahashi’s laboratory reported that Cp did not exist in either chicken or bovine brain [32, 33]. In 1994, Applegate and colleagues identified an mRNA analogue to Cp in rat brain. However, this pertained to a novel acidic isoform, which did not interact with calcium-calmodulin, suggesting a differential role from that of the basic isoform. Subsequent studies have shown both the acidic and basic isoforms of Cp in various brain regions [34–37]. Using immunofluorescent techniques, we have localized the basic isoform of Cp in SM of reacting microvessels (i.e. terminal and precapillary arterioles) from brain regions such as the sensorimotor cortex (smCx) and dorsal hippocampus (hipp) [50–52]. In addition to being located in SM of cerebral blood vessels, Cp has been localized to neurons [35, 36, 41–43]. Ferhat et al. [38] demonstrated that Cp is most prevalent in hippocampal neurons during development. Plantier et al. [41] further showed that Cp is highly expressed in growth cones in the developing brain. Bannai et al. [42] demonstrated a decrease in the number of neurons located in the paraventricular nucleus of Cp gene-deficient mice. These studies, combined with evidence that Cp is up-regulated during dendritic spine plasticity [39], suggest that in addition to its vascular function, Cp may also modulate neuronal growth and development. Therefore, in general, the role of Cp in the brain appears to be heterogeneous; supporting not only vascular contractility but also a broad spectrum of neuronal functions in both the developing and adult brains.

Does Cp play a role in pathologic states?

While the mechanism by which Cp normally regulates SM contraction may not yet be entirely elucidated, Cp’s role in vascular contractility (or vasoreactivity) has been recently implicated in several pathologic states, including renal glomerular nephritis [46], ischaemia/reperfusion [47], hypertension [48], subarachnoid haemorrhage [49, 50], vasospasm [51, 52], and more recently by us in traumatic brain injury (TBI) [38–40]. While there are several experimental models of TBI documenting alterations in vasoreactivity and the cerebral microcirculation, very little work with these models has focused on the pathophysiologic mechanism underlying these alterations. Because of the seminal role of Cp in SM contraction and vasoreactivity as explained above, we focus here on the role of Cp in vasoreactivity in a rodent model of diffuse TBI where one of this model’s pathologic events is a persistent state of enhanced vasoreactivity and decreased cerebral blood flow (CBF) [53].

Cp and TBI

TBI results in a chronic state of enhanced vasoreactivity of reacting microvessels, which leads to hypoperfusion (decreased CBF) and hypoxia of the brain parenchyma [53–56]. Because these resulting pathophysiologies from brain primary injury are thought
to contribute to the development of secondary injury (i.e. nerve cell injury/death), our laboratory has dedicated the last decade to understanding the molecular mechanisms that underlie enhanced vasoreactivity following TBI. To date, research relating to disruption of the brain microcirculation following TBI has focused primarily on receptors [57–59], growth factors [60, 61] and neurochemicals [54, 62–64]. However, none of these investigations have studied the signal transduction mechanism underlying either control of normal vascular tone or the dysfunctional sustained SM contraction that follows after TBI. Related to these issues and at a more fundamental level, little work has been done to demonstrate the role of contractile proteins in mediating vasoconstriction in the normal and injured brain. Because of the above Cp work supporting a role in SM contraction, we undertook to study the spatial and temporal patterns of Cp expression in brain reacting microvessels that may underlie enhanced vascular reactivity and decreased CBF as observed after TBI.

We first sought to determine whether TBI had an effect on Cp brain cellular expression and found significant increases in Cp immunoreactivity (IR) in SM of reacting microvessels from smCx and hpp as early as 4 hrs after injury. These increases were sustained up to 48 hrs after TBI [38] and correlated temporally with the previously observed enhanced vascular reactivity and decreased CBF [53]. Only trace amounts of Cp IR were detected in endothelial cells at all time points of the study which is consistent with the findings by Birukov et al. [65]. Analysis of single SM cells revealed a Cf shift from the cytosol towards plasma membrane of SM during sustained vasoreactivity after TBI (Fig. 1) [38, 39]. This finding is in concert with the in vitro work by Morgan and colleagues who also showed Cp migration during SM contraction [15]. The significance of the Cp migration is not fully understood at this point. One explanation is that Cp migration facilitates ERK or PKC translocation prior to various phosphorylation events [66–68]. Alternatively, Cp migration may be important in maintaining cytoskeletal integrity of SM during its contraction. Cp has been shown to interact with caldesmon (Cd) [69], another cytoskeletal protein known to be located in endothelium and at the SM plasma membrane [38]. As such, Cp’s migration and its molecular linkage with Cd could provide overall SM structural integrity during its contraction.

Because Cp phosphorylation could modulate SM contraction, we undertook a study of such process and its association with sustained vasoreactivity and CBF alteration after TBI. Cp was phosphorylated from 4 to 48 hrs after TBI [40], this phosphorylation temporally coinciding with enhanced vasoreactivity. This provided in vivo evidence that Cp phosphorylation was temporally associated both with the SM sustained contraction and hypoperfusion of the cerebral microcirculation after TBI. In order to further test these last conclusions, we designed an experiment in which a peptide that bound to the THR184 phosphorylation site on Cp (177FASQOQMTA185, Invitrogen, Carlsbad, CA, USA) was injected intracerebroventricularly (ICV) prior to TBI [70]. Subsequently assessment of Cp phosphorylation and CBF were carried out by Western immunoblotting and arterial spin labelling magnetic resonance imaging (ASL-MRI), respectively. A single bolus injection of 20 nmol of the peptide effectively blocked Cp phosphorylation (Western) and ameliorated the TBI-induced hypoperfusion in smCx and hpp as detected by ASL-MRI (Fig. 2).

We then asked the question whether the observed molecular and functional changes correlated with alterations in cognitive behaviours, which are also known to occur after TBI. As such, our rodent model of diffuse TBI causes significant food acquisition and retention memory losses performed in an automated radial arm maze, with such deficits persisting up to 27 days after impact (Fig. 3). Using the same behaviour paradigm, we found that ICV peptide administration improved performance in the radial arm maze compared to that of animals that only received vehicle injections (Fig. 3). More recently, we are exploring the feasibility of using Cp gene therapy as a therapy for improving CBF and cognitive outcome following TBI.

**Endothelin (ET)-1, its receptors (ETrA, ETrB) and Cp-mediated vasoreactivity: therapeutic implications**

Because our work and that of others support a pivotal role of ET-1 and its receptors ETrA and ETrB in the control of vascular tone in normal and injured brains, we have included here, a brief discussion on these substances. Likewise, the causal association between ET-1 receptor-mediated vasoreactivity and Cp-mediated SM contractility is also explained.

ET-1 is a powerful vasoconstrictor, which exerts vasoactive actions through its two G-protein-coupled receptors, A and B [71]. Activation of either receptor is coupled with activation of PKC. However activation of ETrA results in vasoconstriction, Whereas that of ETrB causes vasodilation [72, 73]. Beginning in the early 1990s, clinical trials using ETrA/B antagonists were introduced for its potential role in ameliorating vasospasm in several pathologic states [74, 75]. Since then, ET-1 has been a target for improving blood flow to several organs in a number of pathologic states including hypertension [76] hepatorenal syndrome [77], heart failure [78] and decreased cerebral blood flow and hypoxia [79]. In 1995, Luscher and Wenzel published one of the first reviews where ET-1 receptor antagonists were identified as therapeutic agents to treat vascular disorders in clinical settings [80]. In their 1999 review, Benigni and Remuzzi [81] summarized data from pre-clinical and clinical studies, which showed promise for the use of specific ETrA antagonists in controlling hypertension.

While clinical trials using pharmacological blockade of ET-1 receptors for a variety of peripheral tissue pathologies have increased in recent years, until recently few experimental studies have addressed the mechanism by which these therapies may be working. Therefore our laboratory has concentrated on elucidating...
the mechanism by which ET-1 and its receptors may, in part, underlie enhanced vasoactivity and hypoperfusion of the brain parenchyma following TBI. Using experimental animal models, we and others have shown that ET-1 and ET-1 receptor signalling may underlie vascular dysfunction following TBI [82, 83]. Specifically, we have reported that ET-1, ETRa and ETRb are up-regulated following TBI [84, 85]. Further, we have demonstrated that by blocking the ETRa receptor, we can block the hypoperfusion that follows brain injury [86].

How Cp may be implicated in the signal transduction of ET-1 and its receptors in normal and injured brain was further demonstrated by us. As such, ETRa antagonism also blocked Cp phosphorylation and improved hypoperfusion and CBF after TBI [85]. In order to further establish a cause-effect relationship between Cp phosphorylation and ET-1 receptor signalling, we blocked PKC-induced phosphorylation of Cp by application of chelerythrine. It is known that ETRa is coupled to PKC signalling [72, 73]. Therefore, when we injected chelerythrine to block PKC prior to TBI, this intervention
resulted in a reduction both of Cp phosphorylation, as detected by 2-dimensional gel electrophoresis, and cerebral hypoperfusion (i.e. enhanced CBF) as detected by ASL-MRI after TBI [70]. Taken together, we propose the following mechanism leading to vasoconstriction (Fig 4): ET-1 binds to its receptor, ETrA, which activates PKC, leading to phosphorylation of Cp and ultimately leading to vasoconstriction of reacting microvessels. Following TBI, we posit that this signal transduction cascade may be enhanced, leading to an increase in vasoconstriction. Future studies will aim to control the persistent vasoreactivity and decreased CBF by pharmacologic intervention of the same signal transduction cascade, that is, by blocking either at the ET-1 receptor level using selective and non-selective inhibitors, Cp phosphorylation level, or both.

**Conclusion**

Cp is a critical component of SM contractile machinery leading to vasoconstriction. While the precise molecular mechanism by which Cp regulates SM contraction is somewhat controversial,
past and current work support the notion that Cp phosphorylation is essential to mediate this contraction. While Cp has been implicated in several pathologic states, our work supports a role for Cp in mediating both enhanced brain vasoreactivity and hypoperfusion (i.e. decreased CBF) resulting from TBI. Because these pathophysiological effects of primary brain injury in turn are likely to lead to the development of secondary injury (i.e. nerve cell injury/death), understanding the signal transduction cascade via ET-1 and its receptors, as well as their modulation of Cp action in SM contraction are paramount to design rational therapies that can be implemented effectively to improve TBI-induced neurological deficits in the clinical setting.

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