The Role of the Ubiquitin Proteasome System in Ischemia and Ischemic Tolerance

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Abstract

Ubiquitin modification targets a protein for rapid degradation by the proteasome. However, poly-ubiquitination of proteins can result in multiple functions depending on the topology of the ubiquitin chain. Therefore ubiquitin signaling offers a more complex and versatile biology compared to many other post translational modifications. One area of potential for the application of this knowledge is the field of ischemia-induced brain damage, as occurs following a stroke. The ubiquitin proteasome system may exert a dual role on neuronal outcome following ischemia. Harmful ischemia results in an overload of the ubiquitin proteasome system, and blocking the proteasome reduces brain infarction following ischemia. However, the rapid and selective degradation of proteins following brief ischemia results in endogenous protection against ischemia. Therefore further understanding of the molecular signaling mechanisms which regulate the ubiquitin proteasome system may reveal novel therapeutic targets to reduce brain damage when ischemia is predicted, or to reduce the activation of the cell death mechanisms and the inflammatory response following stroke. The aim of this review is to discuss some of the recent advances in the understanding of protein ubiquitination and its implications for novel stroke therapies.

Keywords

rapid ischemic tolerance; preconditioning; bcl-2; synapse; neuroprotection

Ubiquitin was discovered in the 1970's as a small (9 kDa) covalent post translational modification of proteins. A role for ubiquitination in the proteasomal degradation of short lived proteins was soon established (Ciechanover and others, 1984, Finley and others, 1984). The discovery of the role of the ubiquitin proteasome system in the anaphase promoting complex regulation of the cell cycle awakened many scientists to the potential for this system to regulate many biological processes (Pickart 2004). Although ubiquitin is best known as a prelude to proteasomal protein degradation, it also regulates protein-protein interactions and therefore numerous biological processes including protein translocation, signal transduction, gene transcription apoptosis and autophagic processes (Mukhopadhyay and Riezman 2007).

What makes ubiquitin particularly versatile as a post translational modification is the variety of modifications which it can form. Ubiquitin is conjugated to a lysine residue on the substrate protein (although N terminal linkage has also been reported). The availability of 7 lysine residues within the ubiquitin protein sequence enables the formation of poly-ubiquitin chains, each of which appears to encode different biological functions (Fig 1). The most common linkage is the Lys48 linkage, which is associated with protein degradation by the proteasome. In contrast, Lys63 linkages appear to play a role in protein-protein interactions mediating signal transduction, translocation and DNA repair (although see below). Ubiquitin also regulates lysosomal as well as proteasomal protein degradation. Lys63 linked poly-ubiquitin modified

proteins are targeted to the lysosome for processing (Ikeda and Kerppola 2008). The role of other poly-ubiquitin lysine linkages is not yet clear, and still subject to investigation (Ikeda and Dikic 2008). With so many functions available to different poly-ubiquitin additions, it is clear that the process of poly-ubiquitination must be tightly regulated at a number of points.

Ubiquitin is added and removed from a target protein by E3-ligase and de-ubiquitinating enzymes respectively, which function similarly to protein kinases and phosphatases regulating protein phosphorylation. The number of potential E3-ligases encoded in the genome is similar to the potential number of protein kinases, suggesting that ubiquitination may play a critical role in regulating as many biological processes as protein phosphorylation. The ubiquitin system requires a sequential process of E1-, E2- and E3-ligases to conjugate ubiquitin to a substrate (Hershko 1983) (Fig 1). Ubiquitin is synthesized as a precursor, and the C-terminus tail of the pro-protein is removed by a de-ubiquitinylating enzyme (DUB) to expose a C-terminal glycine residue (Lund and others, 1985). Ubiquitin forms a high energy thioester bond with an E1-ligase, in a reaction requiring ATP (Pickart 2001). This step is the only step of the conjugation process which requires ATP, and may account for the observation that ubiquitination may occur under conditions of low ATP levels such at ischemia (see later).

The ubiquitin is transferred from the E1-ligase to the E2-ligase (Fig 1). There are approximately 20 E2-ligases encoded by the human genome, in contrast to only one E1-ligase. Some E2-ligases are used for one ubiquitin family member, for example Ubc9 is an E2-ligase unique to sumoylation (Johnson and Blobel 1997). Different E2-ligases have specificity for different steps of the ubiquitination process; UbcH5a catalyzes the non-specific lysine linkage of ubiquitin, where as Ubc13 formed only Lys63 linked poly-ubiquitin chains (Windheim and others, 2008). In another recent report the rapid mono-ubiquitination of cell cycle regulatory proteins by the APC (anaphase promoting complex) was identified as being mediated by Ubc4, where as Lys 48 poly-ubiquitination by the APC was mediated by Ubc1 (Rodrigo-Brenni and Morgan 2007). Interestingly the E2-ligases interact with different domains on the E3-ligase (Windheim and others, 2008). The choice of E2-ligase may determine the topology of the substrate ubiquitination modification, and poly-ubiquitination of a target may require multiple E2-/ E3-ligase combinations.

In the final step of the process, the ubiquitin is transferred from the E2-ligase to the substrate by an E3-ligase. This reaction defines the target specificity of ubiquitination. There are two major E3-ligase families, the RING (really interesting new gene) and HECT (Homologous to the E6-AP Carboxyl Terminus) families. Additional E3-ligases have recently been identified including a novel zinc finger protein containing E3 ligase and U box domain containing proteins (Jiang and others, 2001, Wertz and others, 2004). However, U-box proteins may be involved with poly-ubiquitin chain extension / E4 ligase activity (see below). Furthermore E3-ligase independent mono-ubiquitination has been reported; however the significance of this observation is not yet clear (Hoeller and others, 2007). The RING and HECT E3-ligases have quite different mechanisms for transferring the ubiquitin to the target protein. RING E3-ligases mediate the direct transfer of the ubiquitin from the E2-ligase directly to the target protein. This may involve a conformational change in the E2-ligase once it has bound to the E3-ligase, to enable the access of the ubiquitin for transfer. In contrast, the HECT E3-ligases form an intermediate E3-ligase-ubiquitin complex prior to the transfer of the ubiquitin to the target protein.

Whether all E3-ligases mono-ubiquitinate or poly-ubiquitinate target proteins is not yet clear. A fourth enzyme class, the E4-ligase was recently described and shown to regulate poly-ubiquitin chain extension. Poly-ubiquitin chains in excess of four ubiquitin residues are required for proteasomal degradation of proteins (Thrower and others, 2000). Ubiquitin fusion degradation protein 2 (UFD2) functions with E1, E2 and E3-ligases to ubiquitinate a UFD

substrate (UFD4). Omitting UFD2 from the reaction resulted in termination of the poly-ubiquitin chain with only three ubiquitins (Koegl and others, 1999). In addition the first three ubiquitins were added via Lys 29 linkages whereas chain extension was Lys48 mediated linkages (as shown by incubation with K29R and K48R ubiquitin mutants respectively) (Koegl and others, 1999). This study also showed that the E4-ligase contains a poly-ubiquitin binding domain, although it is not clear whether the binding of the E4 changes the conformation of the already attached ubiquitin. The authors suggest that E4-ligases may promote the degradation of post-translationally activated protein, by extending the oligo-ubiquitin chain (Koegl and others, 1999).

UFD2 contains a U box domain, which may identify potential new E4-ligase members. An interesting member of this family is the E4-ligase CHIP (C terminus of Hsc70-interacting protein), which functions with the E3-ligase mutated in a familial form of Parkinson's disease, PARKIN, to regulate Pael receptor degradation (Hoppe 2005, Imai and others, 2002). However, other studies suggest that CHIP also acts as an E3 ligase (Windheim and others, 2008). Whether all proteins which contain a U-box are E4-ligases remains to be determined, but E4-ligase activitiy has been reported in proteins that do not contain a U-box domain (Hoppe 2005). The nuclear protein P300 recognizes MDM2 (Murine double minute 2) mediated mono-ubiquitination of p53 promoting further ubiquitin chain extension (Grossman and others, 2003). P300 does not contain a U-box domain in its sequence, suggesting that another domain on this protein may function as the E4-ligase or the P300 recruits another E4-ligase. The target specificity of E4-ligases is not yet clear, or whether this function is mediated by E2/ E3 ligase combinations (Windheim and others, 2008). The elucidation of proteins with E3- and E4-ligase activity for specific proteins may reveal additional targets for drug manipulation of this system.

One of the more perplexing questions which is currently being investigated by a number of groups is the mechanism(s) by which the ubiquitin chain on a protein substrate is extended. A number of complex models have been proposed (Fig 2), and yet a clear answer is not apparent (see review by Hochstrasser (Hochstrasser 2006)). Following addition of one or more ubiquitins to the substrate protein the E3 ligase, which interacts with the substrate protein will be too far from the end of the chain to mediate the conjugation of the next ubiquitin molecule. A number of models have been proposed to explain this issue. One suggested mechanism is that the poly-ubiquitin chains are synthesized prior to addition to the substrate, either by the E2 or E3-ligase. For example the Ube2g2/ gp78 mediated poly-ubiquitination involves the assembly of poly-ubiquitin chains on the E2-ligase, prior to transfer to the substrate (Li and others, 2007). This may be the reason a number of studies have reported auto-ubiquitination of E2- and E3-ligases. Alternatively, the initial E3-ligase mono-ubiquitinates the substrate and then either different E2 ligases regulate chain extension, or an additional factor (the E4 ligase) regulates extension (Hoppe 2005). Currently different E3-ligases are being studied in different systems and under different physiological conditions, which may account for the unclear picture from these recent studies.

Ubiquitin chains can be remodeled to change the function of the ubiquitin modification to regulate degradation (see Fig 3). Wertz and others showed how following Tumor Necrosis Factor (TNF) receptor activation, the signaling protein receptor interacting protein (RIP-1) is bound to the TNF receptor complex via a Lys63 linked poly-ubiquitin modification (Wertz and others, 2004). To turn down TNF receptor signaling, RIP-1 is deubiquitinated by the co-factor A20 (via its ovarian tumor domain (a DUB family domain)). Following de-ubiquitination, RIP-1 is Lys48 poly-ubiquitinated by A20 targeting it for proteasomal degradation and thereby turning off TNFR signaling. Interestingly, A20 contains a novel E3 ligase domain. The A20 zinc finger domain is structurally divergent from the RING zinc finger domain common to many E3 ligases (Wertz and others, 2004).

In addition to modification of the poly-ubiquitin chain prior to proteasomal trafficking, proteins at the proteasome are also subject to chain editing (Fig 3). The S26 proteasome contains multiple E3 ligases and DUB domains. Recent studies of the interplay between the ligase Hul5 and the DUB Ubp6, show that these enzymes can regulate substrate degradation by extending their ubiquitin chains and enhancing proteasome activity, or removing their ubiquitin modifications to reduce degradation (Crosas and others, 2006).

Taken together these studies show that the process of protein ubiquitination is a tightly regulated process. The degradation of proteins by the ubiquitin proteasome system and subsequent loss of function determines the consequence for the cell (i.e. degradation of cell death proteins promotes protective phenotypes (Meller and others, 2006). However, ubiquitin has multiple functions, not just protein degradation. Consistent with a role in poly-ubiquitin chains mediating protein-protein interactions, a family of ubiquitin-like modifier proteins has been identified.

Ubiquitin binding domains, protein interactions by ubiquitination

The binding of ubiquitin chains, which are associated with non-proteasome targeting, is accomplished by ubiquitin binding domains (UBD) on the acceptor proteins. There are a number (>30) of different ubiquitin binding domain motifs that can bind to mono- and poly-ubiquitin. Interestingly the affinity of a UBD and ubiquitin is low, typically in the low micromolar range (Ikeda and Dikic 2008). Since ubiquitin levels in cells are relatively high, this suggests that the regulation of binding is due to accumulation of UBD containing proteins in cellular compartments and the use of multiple UBDs within a protein/complex (Ikeda and Dikic 2008).

Further analysis of this issue is ongoing, and it was recently shown that the 3D topology of Lys63 and Lys48 linked poly-ubiquitin may offer some ideas as to how the binding domains specific to each are accomplished. X-ray crystallography of Lys48 linked di-ubiquitin show a compact arrangement of the ubiquitin moieties, in contrast Lys63 ubiquitin is more open and linear in shape (Varadan and others, 2004, Varadan and others, 2002). Therefore the structure of the Lys48 and Lys63 linkages are quite different. In a recent study of the binding affinities of different UBDs four classes were described, those which bound mono-ubiquitin, linkage selective and nonselective domains, and one class which did not bind any ubiquitin ligand (Raasi and others, 2005). Interestingly, the protein homologs containing UBDs show a remarkable conservation of ubiquitin chains specificity between species (Raasi and others, 2005).

Ubiquitin-like modifiers

Ubiquitin is a member of an ever growing family of ubiquitin-like modifiers, which include SUMO, NEDD8 and Atg. For more detailed information on these proteins the reader is referred to some recent reviews (Geiss-Friedlander and Melchior 2007, Kerscher and others, 2006). These proteins have a similar tertiary structure to ubiquitin, but have a lower amino acid homology to ubiquitin. A similar series of sequential reactions regulate the modification of proteins by ubiquitin-like modifiers, for example SUMO-ylation requires an E1, E2 and E3-ligase mediated reaction. Modification by ubiquitin-like modifiers results in the regulation of protein function and localization, rather than direct protein degradation. These additional protein modifiers also interact with the ubiquitin system to regulate protein function, as discussed below.

Mono-ubiquitination

Most research has focused on how poly-ubiquitination (Lys48) results in protein degradation by the proteasome. Mono-ubiquitination has also been shown to regulate a number of divergent cellular processes (Hicke 2001). One of the more commonly studied functions of mono-ubiquitination is the regulation of gene expression. Mono-ubiquitination of histone H2A and H2B is an important event for the elongation step of transcription. In addition, mono-ubiquitination functions as a signaling molecule for the assembly of transcription enzymes and unraveling of chromatin (see (Weake and Workman 2008) for a review of histone ubiquitination and gene expression).

Mono-ubiquitination can also affect protein function outside of the nucleus. One such example is the regulation of receptor endocytosis (Hicke 2001). Mono-ubiquitination of a receptor or ion channel promotes endocytosis and internalization. These proteins are either de-ubiquitinated and recycled, or subject to lysosomal degradation. In addition, the tumor suppressor protein p53 is exported from the nucleus to the mitochondria when it is mono-ubiquitinated (Marchenko and others, 2007), where as poly-ubiquitination results in its proteasomal degradation. p53 is then de-ubiquitinated by HAUSP at the mitochondria which enables its interaction with Bcl-2 family members (Marchenko and others, 2007).

Crosstalk between ubiquitin, phosphorylation and other ubiquitin-like modifiers

Recent studies suggest that post translational modifiers do not work alone, but rather form a complex system of signaling crosstalk to regulate biological processes(Hunter 2007). The ubiquitination of a substrate can be regulated by its phosphorylation, for example the phosphorylation of Bim on Ser 65 by ERK1/2 enables its interaction with its E3-ligase, resulting in ubiquitination and proteasomal degradation (Ley and others, 2003) (Fig 4). Interestingly the ubiquitination of multiple Bcl-2 family proteins are regulated by the phosphorylation of critical residues (Breitschopf and others, 2000a). In addition nitrosylation of Bcl-2 regulates its degradation by the proteasome (Chanvorachote and others, 2006). Some kinases are also regulated by ubiquitination, which can turn on or off a biological signal, for example RIP-1 is regulated by A20, which controls IKKβ function following TNF receptor signaling (Wertz and others, 2004).

The crosstalk between ubiquitin and the ubiquitin like modifier SUMO has been the focus of many studies. Under normoxic conditions, hypoxia induced factor (HIF) 1 is hydroxylated promoting its ubiquitination by the E3-ligase VHL(von-Hippel Lindau tumor factor) resulting in protein degradation. Hypoxia induced the sumoylation of HIF1α, which also enables its ubiquitination by VHL. De-sumoylation of HIF1α results in stabilization of the protein by preventing it's ubiquitination and promoting the expression of HIF1-regulated genes (Cheng and others, 2007). These data are in contrast to other reports which suggest that sumoylation promotes HIF1α gene expression (Bae and others, 2004), and suggest that either sumoylation/desumoylation may be involved in HIF1 degradation, perhaps similar to A20 mediated regulation of RIP-1, or additional signaling mediators may regulate HIF1α in response to hypoxia.

The tumor suppressor p53 is subject to complex regulation as signaling crosstalk, including phosphorylation, acetylation, nitrosylation, sumoylation, mono-ubiquitination and poly-ubiquitination (Lavin and Gueven 2006). The stability of the E3-ligase that regulates p53 ubiquitination MDM2, is regulated by the tumor suppressor p14ARF and other regulatory proteins (Zhang and others, 1998). In addition Arf can regulate protein sumoylation (Gallagher and others, 2006). Hence, one can determine that ubiquitination (and other ubiquitin-like

modifications of proteins) does not happen in isolation, but rather as part of a highly complex, intricately regulated signaling system. Given the specificity within the system and the remarkable variety of target proteins whose function and stability are regulated by the ubiquitin proteasome system, it is clear that further study of this system may reveal targets for therapeutic manipulation.

The proteasome

In order to be targeted to the proteasome, a protein requires a poly-ubiquitin tail of at least four residues, resulting in a high affinity for the proteasome and subsequent processing. The proteasome complex allows the rapid complete and selective degradation of substrates due to its structure. The physical tube like structure of the proteasome ensures that only targeted proteins are processed. The core is narrow, which requires the unfolding of a protein into a linear peptide sequence (Pickart and Cohen 2004). The importance of the proteasome is underlined by the fact that many knockouts of proteasome subunits result in lethal phenotypes (Heinemeyer and others, 1991, Orlowski 1999).

The S26 proteasome is made up of an S20 core and an S19 cap (the S denotes sedimentary co-efficient, the molecular weight of the core and cap structures are approximately 760 and 1 Mda respectively). The proteasome is structurally similar to the HsIV and CIsP proteases found in prokaryotic cells, however on an evolutionary basis these proteases are unrelated. The S20 proteasome shows little protease activity on its own and requires the S19 cap proteins for full function (Pickart and Cohen 2004). The catalytic core of the proteasome is formed by four 7-membered rings of alpha and beta subunits (Baumeister and others, 1998). The catalytic sites are on the β subunits, where as the α sub units control the passage of substrates to the catalytic sites.

The proteasome S19 cap is made up of approximately 17 subunits (designated RPN in *C. elegans* or yeast). The cap is the site of protein unfolding, a process which requires ATP. Indeed many of the cap subunits possess ATPase activity. The cap forms a ring and a lid type feature to regulate entry of the protein to the protease (Baumeister and others, 1998, Pickart and Cohen 2004). As well as unfolding the protein, the cap is the site for removal of the ubiquitin chain prior to degradation of the protein allowing ubiquitin to be recycled by the cell. Two types of de-ubiquitinating enzymes act on substrates, USP14 removes the proximal ubiquitin from a protein, where as UCH (ubiquitin C Terminal hydrolases) removes distal ubiquitins from the substrate. Recently, it was shown that the cap also contains E3-ligase activity. The role of this is not clear, but the E3-ligase Hul5 functions with the de-ubiquitinating enzyme USP14 to regulate protein degradation (Crosas and others, 2006).

The role of the ubiquitin-proteasome system in ischemia and ischemic tolerance

The ubiquitin proteasome system has been implicated in a number of pathologies which effect neuronal structure and function. Proteasome inhibitors when administered for long durations or at high concentrations induce neuronal cell death (Qiu and others, 2000). Blocking proteasome function delays axonal degeneration following cell injury or axon cutting (Wallerian degeneration) (Zhai and others, 2003). Interestingly, the wld[s] mutant mouse shows a slowing of the degeneration, and express a mutant form of the UFD2 E4-ligase lacking catalytic activity fused to nicotinamide mononucleotide transferase (Mack and others, 2001).

Ubiquitin-rich inclusions are a common feature of certain neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease. These plaques tend to be enriched in ubiquitin, and it has been suggested that they are deposits of mis-folded proteins. Accumulation of the

proteins into aggregates may overwhelm the proteasome system resulting in cell stress and neuronal death. For more details on the role of the ubiquitin proteasome system in these diseases the reader is referred to the following reviews ((Lim 2007,Oddo 2008)).

A number of studies have investigated protein ubiquitination and proteasome activity following ischemia (Fig 5). These studies suggest detrimental effects of the ubiquitin proteasome system following ischemia, resulting in damage to cell components, or mediating inflammatory responses and leukocyte infiltration to the brain. Ischemia in the brain is modeled by either a local reduction in blood flow to a discrete brain area (focal ischemia) or a complete reduction in blood flow to the entire brain (global ischemia) (Traystman 2003). Following global ischemia the ubiquitination of proteins which form aggregates has been reported (Liu and others, 2005b). These protein aggregates contain poly-ribosomes, translation associated proteins and the E3/ E4-ligase CHIP (Liu and others, 2005b). Following global ischemia, the prolonged accumulation of poly ubiquitinated proteins in the post synaptic density has been reported in the selectively vulnerable hippocampal regions, but were briefly found in the ischemia resilient dentate gyrus cells (Liu and others, 2005b,Liu and others, 2004). However, these studies did not identify which type of poly-ubiquitin linkage was added to the proteins. The proteasome is also affected by harmful ischemia resulting in cap disassembly and the trafficking of these cap subunits to protein aggregates and a reduction in proteasome function. The formation of aggregates by ubiquitinated proteins, due to impaired proteasome function, may contribute to cell stress following ischemia thereby amplifying the neuronal damage to the neurons.

Preconditioning neurons with brief ischemia resulted in a reduction in protein aggregation following ischemia (Liu and others, 2005a). Both the formation of triton-X100 insoluble ubiquitinated proteins and the depletion of free ubiquitin following harmful ischemia were reversed in ischemic tolerant (delayed) neurons. However, it is not clear whether the proteins are less ubiquitinated because tolerant cells are not damaged by the ischemia or whether the tolerance reduces protein aggregation, thereby protecting the cells. Interestingly, ischemic tolerance is associated with raised heat shock protein expression, and raised heat shock protein levels result in less ubiquitin redistribution in neurons following ischemia (Ouyang and others, 2005).

A number of protein substrates have been identified as being regulated by the proteasome following ischemia. Ubiquitination of survival proteins following ischemia has been reported in the retina. Analysis of a gracile axonal dystrophy mouse reveals an exon deletion of ubiquitin C-terminal hydrolase-L1. These mice show less ubiquitination of Bcl-2 and XIAP in response to ischemia, compared to wild type mice (Harada and others, 2004). In addition PKC gamma, type 2 iodinase and AKAP121 are degraded by the proteasome following ischemia (Carlucci and others, 2008, Lamirand and others, 2007, Matsumoto and others, 2004). Hence a number of proteins are subject to ubiquitination following ischemia, which may regulate cell fate.

Ubiquitination may regulate autophagic processing of proteins following ischemia as well as proteasomal protein degradation. Autophagy is a lysosomal-dependent mechanism of degrading proteins, and organelles. Autophagosomes form by the enveloping of the cytosol by a phagophore (a membrane sac) in a highly regulated process, which then fuses with a lysosome containing proteases that degrade the contents. Autophagy has divergent effects, which are relevant to ischemia and other neuropathological conditions. Autophagic neuronal death has been reported in response to ischemia (Adhami and others, 2006, Carloni and others, 2008, Koike and others, 2008, Qin and others, 2008, Uchiyama and others, 2008). However, whether autophagy is a survival or damaging response is not yet clear. While autophagic cell death has been reported since the 1960's, and is a mechanism of cell death which can proceed without the apparent biochemical hallmarks of programmed cell death (i.e. caspase cleavage and

activation), some studies suggest a protective role (Carloni and others, 2008). Hence the activation of autophagic processes following ischemia/ neuronal damage could be a double-edged sword (for a review on ischemia and autophagy see (Rami and Kogel 2008)). Therefore it is not yet clear whether the autophagic clearance of ubiquitinated proteins is a reserve for the proteasome, or a separate system for protein clearance which is activated in response to an overwhelming of the proteasome following ischemia.

Proteasome inhibitors reduce ischemia-induced brain injury

Further evidence implicating a role of the ubiquitin proteasome system in ischemia induced cell damage come from studies showing that proteasome inhibitors reduce brain infarction following ischemia in animal models. There are two classes of proteasome inhibitors. The first such as *clasto*-lactacystin-β-lactone (usually administered as a pro drug lactacystin) are derived from naturally occurring compounds from bacteria/ fungi. The second class of inhibitors are short modified peptides, such as the classical proteasome inhibitor MG132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) and bortezomib. It is noteworthy that some of these inhibitors can block other proteases such as caspases, calpains and cathepsin. For structures and pharmacology of proteasome inhibitors the reader is referred to the review of Wojcik and Napoli (Wojcik and Di Napoli 2004).

The proteasome inhibitor MLN519, which is structurally similar to lactacystin(Wojcik 1999), provides effective neuroprotection in models of focal ischemia (2 hours middle cerebral artery occlusion) when given up to 4 hours post ischemia (Williams and others, 2003). MLN519 treated rats show less neuronal damage, astrocytic damage, leukocyte infiltration and infarction compared to untreated rats subjected to injurious ischemia. The dose of MLN519 reduced blood proteasome activity by 80% and also decreased NFKB activation. Protection was not evident in this study at 8 hours post ischemia (Williams and others, 2003).

In follow up studies, MLN519 shows neuroprotective effects in behavioural assessment when administered up to 10–48 hours following ischemia (Williams and others, 2004, Williams and others, 2005). MLN519 increased survival and reduced ischemia induced weight loss. In addition animals subjected to ischemia and MLN519 also performed better in some neurological tests than ischemia treated controls. This exciting study suggests that proteasome inhibitors may be a useful strategy to reduce delayed post ischemic brain injury. Indeed there is a similar temporal profile of post ischemic brain injury and neuroinflamation (Dirnagl and others, 1999). This may be a more feasible therapeutic time point to investigate, given the failure of previous neuroprotective therapeutic strategies which targeted apical events following ischemia and hence had a narrow therapeutic time window (NMDA receptor antagonists and calcium channel blockers).

Bortezomib (Velcade), a di-peptide boronate derivative approved for multiple myeloma, also reduces ischemia induced brain injury. In a study of embolic focal ischemia, bortezomib induced protection up to 4 hours post ischemia, but enhanced the therapeutic time widow for Tissue plasminogen activator therapy to 6 h (Zhang and others, 2006b). In a separate study, bortezomib reduced brain injury in a permanent MCAO model and a second embolitic model (Henninger and others, 2006). These studies show that bortezomib reduces blood proteasome activity by 77% and also reduces NFKB and eNOS (nitric oxide synthase) expression following harmful ischemia (Henninger and others, 2006).

These studies suggest that as a post-stoke therapy, proteasome inhibitors may have therapeutic efficacy in reducing neuro-inflammation associated delayed cell death in the brain. This view is supported by the fact proteasome inhibitors do not protect neurons from *in vitro* modeled ischemia or glutamate toxicity (Meller and others, 2008, Waataja and others, 2008). The global blockade of the proteasome, however, may result in many unwanted side effects and as such

the future target for this research will be to identify critical molecular mechanisms that are blocked by the proteasome inhibitors to develop more specific therapeutics. In addition, the proteasome may also mediate protective events, which may be promoted to induce protection against ischemia. This mechanism is slowly being revealed by the study of the endogenous protective phenomenon of ischemic tolerance.

Protein ubiquitination and ischemic tolerance

Tolerance, induced by a preconditioning event, is a universal phenomenon and has been observed in many species and organs. A preconditioning agent is defined as an agent or stimuli, which at a low dose is not toxic but induces protection against a larger toxic dose (tolerance) (Dirnagl and others, 2003, Janoff 1964). The protection induced by preconditioning can render the organ/ system protected from other harmful stimuli (cross-tolerance), for example endotoxin (lipo-poly saccharide (LPS)), seizures or spreading depression all induce tolerance to ischemia (Ahmed and others, 2000, Kobayashi and others, 1995, Rosenzweig and others, 2007). Many early studies of ischemic tolerance focused on the protection of the myocardium following the induction of ischemic tolerance, however, ischemic tolerance was soon reported in the brain (Kitagawa and others, 1990). While many of the earlier heart studies have guided experiments in the brain, differences exist between the two systems (Tauskela and others, 1999).

Ischemic tolerance has been observed in murine, gerbil and rat models of ischemia, and following focal or global ischemia (Dirnagl and others, 2003). Ischemic tolerance has been modeled *in vitro* using oxygen glucose deprivation (OGD), hypoxia (low oxygen) chemical ischemia with metabolic inhibitors and glutamate/ N-Methyl D aspartate (NMDA) agonists, suggesting that the ischemic tolerance phenomenon is not just a blood reperfusion effect (Chen and Simon 1997, Meller and others, 2005). However, the ability of preconditioning in one body region to induce ischemic tolerance in another (remote preconditioning) suggests that in the whole body situation a vascular preconditioning mediator is released, which can induce tolerance in a different tissue (Ren and others, 2008).

Evidence from prospective and retrospective studies suggests a potential role of ischemic tolerance in humans (Schaller 2005, Schaller and Graf 2002, Wegener and others, 2004, Weih and others, 1999). Patients with prior transient ischemic attacks to the same territory of the brain affected by a subsequent stroke, have a reduced infarct volume and severity of symptoms. Hence, endogenous ischemic tolerance in the human brain exists.

There are two accepted time windows for ischemic tolerance following the preconditioning stimuli. A rapid time window has been shown to exist 30 minutes-1 hour following preconditioning. In contrast delayed tolerance is induced 24 hours following preconditioning, and can last up to 3 days. Although intermediate time windows have been reported (Ren and others, 2008). The difference in time windows of the two types of tolerance gives clues as to the biological mechanism of each. Delayed tolerance is mediated via new protein synthesis and a change in the genomic response to ischemia. The protein synthesis inhibitor cycloheximide blocks delayed tolerance in both *in vivo* and *in vitro* models (Barone and others, 1998, Meller and others, 2006, Meller and others, 2005). In contrast, cycloheximide did not block an *in vitro* rapid ischemic tolerance model in rat cortical neurons (Meller and others, 2006). Many studies of ischemic tolerance have focused on the gene based, *de-novo* protein synthesis dependent long-term/ delayed ischemic tolerance, for more detailed reviews (Chen and Simon 1997, Dirnagl and others, 2003, Obrenovitch 2008, Steiger and Hanggi 2007).

Studies of the molecular mechanism of delayed ischemic tolerance have identified a number of important genes whose neuroprotective effects may mediate tolerance to harmful ischemia, including Bcl-2, Bcl-w, heat shock proteins, Proliferating Cell Nuclear Antigen, glutamate

transport proteins EAAT2/ 3 and erythropoietin (Bossenmeyer-Pourie and Daval 1998, Currie and others, 2000, McLaughlin and others, 2003, Minami and others, 2000, Ruscher and others, 2002). More recently it has been proposed that a preconditioning event results in a re-programming of the genomic response of the brain to subsequent harmful ischemia, and has similarities to the natural protective state of hibernation (Stenzel-Poore and others, 2007, Stenzel-Poore and others, 2004, Stenzel-Poore and others, 2003). Gene-based and metabolic-based mechanisms of endogenous tolerance to ischemia are observed in a number of divergent species and scenarios including mammal hibernation, deep sea turtles, arctic tree squirrels, fish and beetle larvae (Frerichs and Hallenbeck 1998, Frerichs and others, 1994, Hoback and others, 2000, Lee and others, 2006, Podrabsky and others, 2007, Storey 2003, Storey 2007).

Rapid ischemic tolerance in animal and cell culture models of ischemia

In contrast to delayed ischemic tolerance, rapid ischemic tolerance has been less extensively studied. Rapid ischemic tolerance has been described in the heart (Eisen and others, 2004) and also occurs in brain. However, less is known about the molecular mechanisms by which rapid ischemic tolerance induces its neuroprotective effects in brain, compared to delayed tolerance. Indeed approximately 20 studies directly investigate the molecular mechanisms of rapid tolerance induced neuroprotection.

The first report of rapid tolerance to ischemia in the brain was by Schurr and others in 1986. They showed that prior exposure of rat hippocampal slices to anoxia 30 minutes prior to harmful ischemia (anoxia) maintained electrical responsiveness of the cells (Schurr and others, 1986). Rapid ischemic tolerance has been observed in primary cortical neuronal cultures, organotypic preparations or slices of hippocampus using anoxia, oxygen and glucose deprivation, NMDA and pharmacological preconditioning stimuli (Bandyopadhyay and others, 2002, Meller and others, 2006, Perez-Pinzon and Born 1999, Reshef and others, 1996, Schurr and others, 1986). Typically most studies use a one hour interval between preconditioning and harmful ischemia, although protection is also observed within a 30 min window (Perez-Pinzon and others, 1997). Rapid ischemic tolerance *in vitro* protects against oxygen glucose deprivation, chemical induced ischemia (iodoacetate), anoxia and NMDA excitotoxicity (Meller and others, 2008, Reshef and others, 1996)

Rapid ischemic tolerance has also been described in animal models of ischemia. The protection one observes depends on the duration of reperfusion following the second, harmful ischemia and the severity of the second ischemic insult. For example, rapid ischemic tolerance, using 3 $\times$ 5 minute periods of middle cerebral artery (MCA) occlusion in mice, 30 min prior to 60 min MCA occlusion (MCAO) or permanent MCAO, reduced infarction 24 hours following the final ischemic insult (Stagliano and others, 1999). In a global model of ischemia in rats, protection induced by 2 minutes bi-lateral carotid occlusion followed by 30 minutes of reperfusion resulted in protection to a 10 minute occlusion when assessed 3 but not 7 days later (Perez-Pinzon and others, 1997). Other studies have reported rapid ischemic tolerance in focal models of ischemia (Atochin and others, 2003, Nakamura and others, 2002). Preconditioning animals with 30 min MCAO resulted in protection from 180 min MCAO one hour later, and the protection was evident 7 day following the last ischemic insult. We observed similar protection in a focal model of rapid ischemic tolerance when we paired 30 min MCAO with a 60 min reperfusion followed by a 100 min harmful MCAO (Minami and others, 2003).

The concept of remote preconditioning to induce ischemic tolerance has been investigated (Ren and others, 2008). The authors showed that three cycles of occlusion of the femoral artery (left) resulted in protection from ischemia induced by permanent occlusion of the left distal MCA combined with a 30 min occlusion of the bilateral common carotid arteries. This suggests that rapid ischemic tolerance can be induced remotely, which has interesting clinical implications. Furthermore this suggests that endogenous rapid ischemic tolerance in the whole

animal is induced by the release of a mediator into the blood stream, as well as occurring in the tissue.

A number of mechanisms have been investigated with respect to rapid ischemic tolerance. Many studies have described a role of adenosine receptors in mediating tolerance induced by anoxia, isoflurane and adenosine receptor agonists (Liu and others, 2006, Perez-Pinzon and others, 1996, Reshef and others, 1996). In addition *in vitro* and *in vivo* rapid ischemic tolerance is blocked by the adenosine A1 receptor antagonists 8-Cyclopentyl-1,3-dipropylxanthine (DCPCX) (Nakamura and others, 2002, Perez-Pinzon and others, 1996). The source of the adenosine which is mediating these effects following the brief ischemic event is not yet clear, and potential mechanisms could include excytotoxic release of adenosine, transporter-mediated release or metabolism of ATP into adenosine (Parkinson and others, 2002). Other neurotransmitter receptors which may regulate rapid ischemic tolerance include the delta opioid receptor. Rapid ischemic tolerance to glutamate excitoitoxicity following hypoxic preconditioning is blocked by the delta opioid receptor antagonist naltrindole (Zhang and others, 2006a). The mechanisms which regulate delta opioid induced tolerance are not yet clear.

Rapid ischemic tolerance induced by preconditioning with anoxia and the metabolic poison iodo-acetate can be blocked by the inhibitor of the ATP-sensitive potassium (K_{ATP}) channel glibenclamide. The effect of adenosine in mediating protection against iodoacetate is also blocked by glibenclamide. A rapid tolerant state in neurons can be induced by incubation for 30–60 min with the K_{ATP} channel openers chromakalin, diazoxide and pinacidil (Perez-Pinzon and Born 1999, Reshef and others, 1998). Taken together, these data suggest that the activation of k_{ATP} channels following preconditioning via the increased adenosine A1 receptor activation plays a role in rapid ischemic tolerance.

The ubiquitin proteasome system and rapid ischemic tolerance

The most obvious difference between delayed and rapid ischemic tolerance is the time window for effect. As the name implies, rapid ischemic tolerance occurs quickly following the preconditioning stimuli and is not blocked by the protein synthesis inhibitor cycloheximide (1 µM) (Meller and others, 2006). This suggests that the neuroprotective mechanism of rapid tolerance occurs independently of new protein synthesis. Therefore, the protective mechanism of rapid ischemic tolerance would be predicted to result in the rapid post-translational modification of *insitu* proteins to mediate the neuroprotective event.

Consistent with this hypothesis, potential role for the ubiquitin proteasome system in regulating rapid ischemic tolerance both in brain and in heart was recently described (Cai and others, 2008, Meller and others, 2006, Meller and others, 2008). Rapid ischemic tolerance is blocked by multiple proteasome inhibitors (*clasto*-lactacystin β-lactone, MG132 (Z-Leu-Leu-Leu-CHO) and MG115 (Z-Leu-Leu-Nva-H), suggesting that following preconditioning ischemia proteins are degraded resulting in a neuroprotective phenotype (Meller and others, 2006, Meller and others, 2008). This concept of down-regulation or depletion of proteins to generate a protective phenotype has been suggested by others; gene transcriptional down-regulation is a common phenotype of delayed ischemic tolerance (Stenzel-Poore and others, 2003). In addition ischemic tolerance in a cardiac protection model was recently shown to be blocked in a transgenic mouse deficient for low molecular mass polypeptide 2 (LMP-2) (a subunit of the immunoproteasome). This mechanism of protection was attributed to activation of AKT, due to degradation of the repressor PTEN (phosphatase and tensin homologue deleted on chromosome 10) (Cai and others, 2008).

Ubiquitination of the pro-cell death protein Bim following preconditioning ischemia

The first protein shown to be ubiquitinated in a model of rapid ischemic tolerance was the pro-cell death member of the Bcl-2 protein family Bim. The balance between pro-survival and pro-apoptotic Bcl-2 family proteins regulate the initiation of intrinsic cell death signaling upstream of the mitochondria. Multiple Bcl-2 family proteins are regulated by the ubiquitin-proteasome system (Basu and Haldar 2002, Breitschopf and others, 2000a, Breitschopf and others, 2000b, Li and Dou 2000). Bim, a pro-cell death member of the bcl-2 family is a member of the BH3 only subgroup of proteins (O'Connor and others, 1998). Bim is commonly expressed as Bim_{EL} (a 23–26 kDa protein) but splice variants result in a truncated Bim_L or Bim_S, which are also potent cell death inducers (O'Connor and others, 1998). Additional splice variants have been identified in humans, but their function is not yet clear (Miyashita and others, 2001). Bim can induce apoptosis via the direct activation of Bax/ Bak, where as other BH3 only containing Bcl-2 proteins result in sensitization alone (Kuwana and others, 2005). Bim induction has been shown to be essential for neuronal cell death (Putcha and others, 2001), whereas reducing Bim protein levels reduces cell death in neurons (Strasser and others, 2000) and renders them resilient to harmful ischemia (Meller and others, 2006).

Our studies show that only Bim, but not Bax or Bid protein levels are rapidly decreased following preconditioning ischemia, and that this decrease is associated with an increase in ubiquitination of Bim (Meller and others, 2006). The effect on Bim was transient; ischemic tolerance is lost 4 hours following preconditioning coincident with Bim levels returning to normal (Meller and others, 2006). The decrease in Bim was blocked by the proteasome inhibitor MG132, which is consistent with preconditioning ischemia inducing Bim ubiquitination and proteasomal degradation that renders neurons protected against the harmful effects of ischemia.

Previous studies in MEF cells show that Bim ubiquitination is dependent on its phosphorylation of a Ser65 residue by p42/p44 MAPK (Ley and others, 2003, Ley and others, 2005). Furthermore, blocking p42/p44 MAPK activation blocks ischemic tolerance (Meller and others, 2006). Consistent with Bim ubiquitination playing a role in ischemic tolerance, blocking p42/p44 MAPK also prevents Bim ubiquitination and degradation (Fig 4). Given the promiscuous nature of p42/p44 MAPK being involved in many cell functions, it is reasonable to assume that this is not the only target for MAPK mediated phosphorylation following preconditioning ischemia.

What has yet to be identified is the E3-ligase responsible for ubiquitinating phosphorylated Bim following preconditioning ischemia. It has been suggested that the tyrosine kinase c-Cbl is a Bim E3-ligase in osteoclasts, (Akiyama and others, 2003). However, other studies failed to show an increase in Bim protein levels in the testis of c-Cbl knockout mice, which would be expected if the E3-ligase is absent (El Chami and others, 2005). Further evidence against a role of c-Cbl come from studies which show the biology of the interaction between c-Cbl and Bim does not match the known biology of Bim ubiquitination (Wiggins and others, 2007). For example, Bim ubiquitination is not affected by mutation of tyrosine residues on Bim, whereas mutation of serine residues (Ser 69) prevents Bim ubiquitination. Bim failed to interact with c-Cbl under conditions where Bim ubiquitination increased, Bim stability was not affected by knockout of c-Cbl in MEF cells, and finally p42/p44 MAPK-mediated Bim ubiquitination occurs in fibroblasts obtained from c-Cbl knockout mice. Recently the E3 ligase CIS has been suggested as a Bim E3-ligase (Zhang and others, 2008). The authors show how CIS is associated with the microtubule scaffold in cells, and that microtubule de-polymerizing agents release Bim and enable its association with CIS, resulting in its ubiquitination. However the authors did not investigate the regulation of this system by p42/p44 MAPK. This leads to an intriguing situation: what else is ubiquitinated following preconditioning ischemia? Is Bim ubiquitinated by multiple E3-ligases? And do different E3-ligases interact with Bim under different biological conditions?

Proteomic analysis of ubiquitinated proteins in rapid ischemic tolerance

We recently employed a non-biased proteomic approach to screen for ubiquitinated proteins in rapid ischemic tolerance (Meller and others, 2008). The agarose bound UBD of p62 was used to enrich ubiquitinated proteins from preconditioned tissue, which were subsequently identified by mass spectrometry. Our results show a higher pulldown/ identification of proteins following preconditioning ischemia compared to control. Some proteins were identified in both control and preconditioned tissue, and unfortunately the absolute levels could not be determined. It was not determined whether these proteins are regulated by the same or different E3-ligases. Whether these proteins are Lys63 or Lys48 liked is not clear. However, further analysis showed that the degradation of two candidate proteins (Marcks and fascin) was inhibited by the proteasome inhibitor MG132. This data would suggest that the ubiquitination of these proteins resulted in their proteasomal degradation.

Bioinformatic analysis of the proteins identified by our proteomic approach revealed an interesting function of the identified proteins, 16 out of 25 of the proteins had been identified to interact either directly or indirectly with proteins associated with the post synaptic density. This strongly suggested that the ubiquitin proteasome could be changing synaptic function, which may be associated with protection.

Actin is a major cytoskeletal component of the post synaptic density, so we focused our investigations on two actin binding proteins, fascin and MARCKS (Meller and others, 2008). In order to maintain the shape and function of the spine, actin forms filaments which are cross-linked by other proteins to provide strength, and one such protein is fascin. Fascin regulates shape and trajectory of neurites in drosophila neurons (Kraft and others, 2006). Actin does not directly anchor to the plasma membrane, but contacts intermediate proteins such as myristoylated, alanine-rich C-kinase substrate (MARCKS) (Hartwig and others, 1992, Sundaram and others, 2004). Interestingly, NMDA induces degradation of MARCKS but via the proteases cathepsin and calpain rather than the proteasome (Graber and others, 2004). Following preconditioning ischemia, proteasome-mediated degradation of MARCKS and fascin occurs, resulting in a decrease in the interaction of these proteins with actin (Meller and others, 2008).

In our study we show the proteasome dependent degradation of two actin binding proteins following preconditioning ischemia, which is associated with the solublization of actin filaments, and the loss of dendritic spines. The loss of spines was blocked by jasplakinolide and the proteasome inhibitor MG132, suggesting that the ubiquitination of proteins and actin depolymerization following preconditioning results in structural reorganization of the neuron (Meller and others, 2008). Since ischemic tolerance induced protection was also blocked by jasplakinolide and MG132, this suggests that these structural changes are critical for rapid ischemic tolerance (Meller and others, 2008).

The concept that ischemia can remodel the synapse is not new. Indeed in a series of studies Mark Goldberg's group showed that brief ischemia results in dendritic spine loss, where as longer ischemia results in permanent spine loss (Faddis and others, 1997, Hasbani and others, 2001a, Hasbani and others, 2001b). Dendritic spine loss following ischemia has also been reported following focal ischemia *in vivo* (Zhang and others, 2005). Dendritic spine loss is recoverable if the exposure to ischemia is transient and non-harmful, but is persistent when toxic levels of ischemia occur (Park and others, 1996, Zhang and others, 2005). Spine numbers recover approximately 2–4 hours following ischemia (Meller and others, 2008, Park and others, 1996). Interestingly, the recovery of the spines occurs at the same location on the dendrites that the spines were originally lost, both *in vitro* (Hasbani and others, 2001a) and *in vivo* (Zhang and others, 2005), suggesting a post synaptic remodeling event.

The ubiquitin proteasome system has also been implicated in synaptic reorganization in response to neuronal activity and in LTP (Dong and others, 2008, Ehlers 2003, Fonseca and others, 2006). The dendritic spine is the major site of post synaptic input to a neuron. The post synaptic density, a region of dense cellular material adjacent to the synapse, is rich in proteasome subunits and ubiquitin (Chapman and others, 1992). The development of synapses in a number of species is regulated by the ubiquitin proteasome system (Yi and Ehlers 2007). In addition, proteasome inhibitors can induce neurite outgrowth in PC12 cells treated with NGF (Maufroid and others, 1996), but inhibition of N-end ubiquitination result in reduced neurite outgrowth (Obin and others, 1999).

When neurons are activated, proteasome sub units enter the dendritic spine and are associated with the actin cytoskeleton (Bingol and Schuman 2006). Hence the proteasome is positioned to regulate (degrade) cytoskeleton proteins as reported following both ischemia and neuronal activation (Ehlers 2003, Meller and others, 2008). Activation of neurons resulted in remodeling of the synapse by the enhanced and decrease degradation of post synaptic associated proteins. The proteins regulated included neurotransmitter receptors and structural proteins (Ehlers 2003). These structural changes resulted in long lasting enhancement of NMDA mediated CREB signaling. Phosphorylation of the transcription factor CREB is associated with protection and survival in neurons following ischemia (Walton and others, 1999).

Stimulation of neurons can also change the receptor composition of the post synaptic density. PSD-95 levels decrease reducing AMPA receptor subunit levels at the membrane surface and endocytosis of GluR1 subunits (Bingol and Schuman 2004, Colledge and others, 2003). Further studies show that PSD-95 is ubiquitinated by MDM2 (which is normally associated with p53 regulation) (Colledge and others, 2003). In contrast activation of a neuron reduces GABA receptor ubiquitination enhancing their numbers at the membrane surface (Saliba and others, 2007) (in unpublished studies we have observed enhance GABA currents following preconditioning ischemia). Interestingly it has recently been shown that nicotine can inhibit proteasome function, resulting in the accumulation of ubiquitinated proteins (Rezvani and others, 2007). Nicotine had a direct binding ability with the proteasome, which may have many implications given the high social use of this drug.

These studies would suggest that, perhaps not surprisingly, one of the major consequences of ischemia induced protein ubiquitination and degradation is a change in synaptic function (Fig 6). Our studies have confirmed this hypothesis, in that we observe a reduction in NMDA currents and toxic NMDA signaling following preconditioning in a rapid ischemic tolerance model (Meller and others, 2008). The reduction in NMDA excitotoxicity following preconditioning was blocked by jasplakinolide and MG132, which suggests that the same mechanisms which regulate tolerance to ischemia, also result in tolerance to toxic levels of NMDA.

A loss of synaptic NMDA receptors has been shown to result in tolerance to OGD (Sattler and others, 2000) and disrupting post synaptic NMDA receptor-PSD-95 interactions also result in reduced toxicity following ischemia (Sattler and others, 1999). Waataja recently reported a study which correlates with our own observations, in that NMDA receptor activation resulted in PSD-95 degradation by the proteasome, and a loss in dendritic spines (Waataja and others, 2008). These changes were blocked by the proteasome inhibitor MG132 and over expression of p14ARF (which regulates MDM2 stability). Furthermore a loss of spines following NMDA exposure resulted in a loss of EPSPs (excitatory post synaptic potentials), which suggests that synaptic NMDA receptors were blocked or absent (Waataja and others, 2008). Glutamate toxicity in these cells was not mediated by the proteasome, similar to OGD mediated cell death in our experiments (Meller and others, 2008). However, toxicity to glutamate was enhanced in ARF overexpressing cells (Waataja and others, 2008). Furthermore the glutamate induced

loss of spines was similar in time course to the ischemia induced loss of spines, complete by one hour and recovering to control levels by 3–4 hours (Hasbani and others, 2001a, Meller and others, 2008). The study by Waataja did not study whether the glutamate response or excitoxicity was reduced in cells pretreated with glutamate (Waataja and others, 2008). Taken together, these two studies suggest that proteasome dependent removal of dendritic spines occurs following a preconditioning ischemia and that this is a protective event.

Translating the promise:- future targets for research

Following ischemia the pattern of activation of intracellular mechanisms denotes the potential therapeutic time windows to a given strategy. Ischemia induced cell death is a progressive event and requires 24–48 hours to fully develop (Dirnagl and others, 1999) (Fig 7). Studies on the role of the ubiquitin proteasome system in ischemia and rapid ischemic tolerance suggest two potential therapeutic strategies (Fig 7). Based on studies of rapid ischemic tolerance, acute neuroprotection may be an obtainable therapeutic goal. By further understanding the mechanism by which cell death mediating proteins are rapidly degraded by the ubiquitin proteasome system, one could devise therapies to stimulate their degradation. Bim degradation is rapid, occuring one hour following preconditioning. Given the established role of the Bcl-2 family of proteins in mediating cell death following ischemia and other brain injuries, a reduction in Bim may result in neuroprotection (Meller and others, 2006, Shinoda and others, 2004).

A second phase of therapy would involve the reduction in secondary brain injury and neuro-inflammation. These have been shown to be reduced by proteasome inhibitors. While acute therapy with these agents may be tolerated, proteasome inhibitors may aggravate part of the response to ischemia, by inducing accumulation of ubiquitinated proteins that must then be cleared by autophagic processes. By further understanding the molecular mechanisms that regulate neuro-inflammation a more specific target therapy may be devised. Indeed, the ubiquitin proteasome system contains many elements, not only the E1, E2 and E3 ligases, but additional modifying factors each of which are potential targets for molecular intervention. Furthermore knowledge of the mechanisms which regulate these processes may be transferable to other neurological diseases of which cell death is a feature. This remains an exciting time for ubiquitin research and therapies based on this versatile signaling system are no doubt being developed.

Acknowledgments

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Ubiquitin

Lys 6   Lys 27   Lys 33   Lys 63

Lys 11   Lys 29   Lys 48
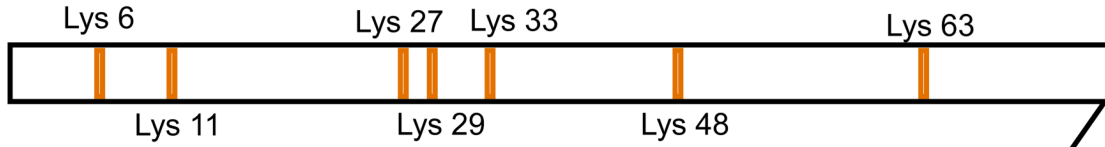
cleaved by dub to reveal C-terminus gly

Protein Ubiquitination

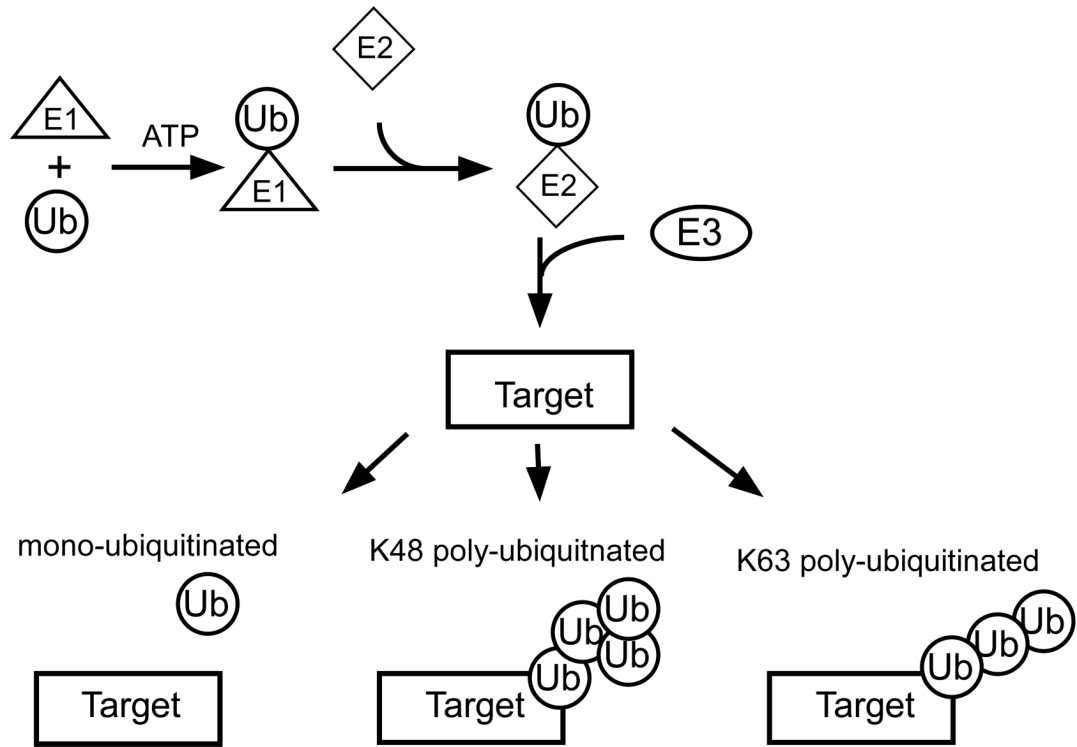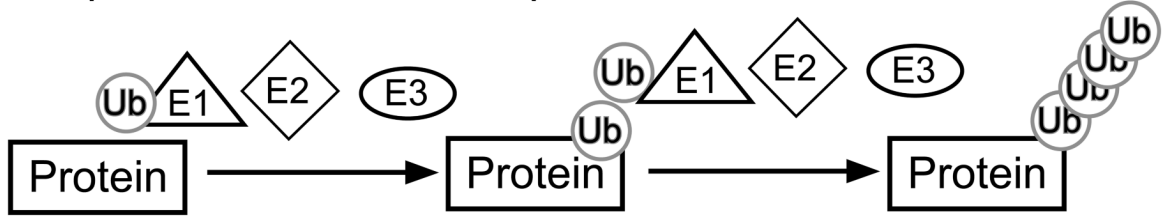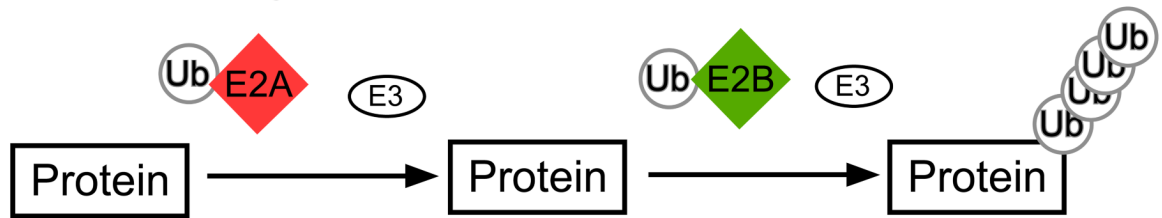mono-ubiquitinated   K48 poly-ubiquitnated   K63 poly-ubiquitinated

Figure 1.
Structure of ubiquitin. Upper) Ubiquitin is synthesized as a precursor protein, whose c terminus is cleaved by a de-ubiquitinating enzyme to reveal a C-terminus glycine residue. Seven lysine residues are present in the primary amino acid structure of ubiquitin, which allows multiple potential poly ubiquitin chain linkages. Lower) Ubiquitin is added to a substrate protein by the sequential action of an E1-ligase, E2-ligase and an E3- ligase. The different poly-ubiquitin chains have different topologies, which are associated with their function. Ubiquitin chains with a Lys 48 linkage usually result in proteasomal degradation of the protein. Lys 63 linked chains can target proteins to the proteasome, lysosome or play a role in protein-protein interactions.
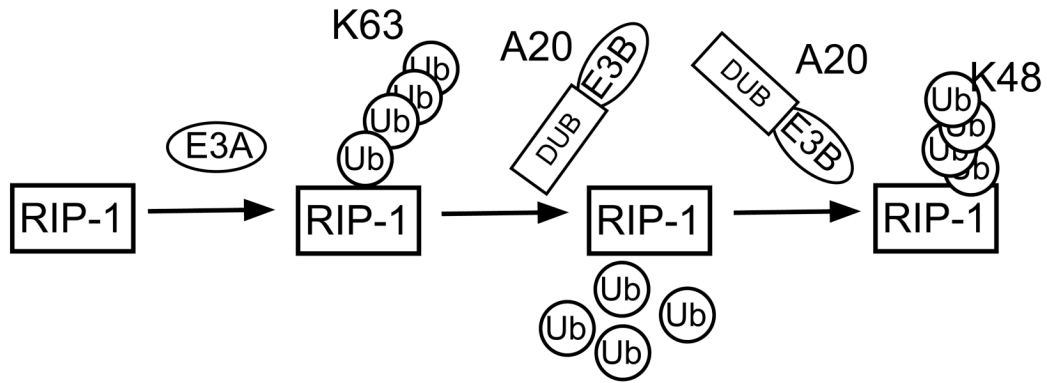
Figure 2.
Potential models of poly-ubiquitination. Sequential addition of ubiquitin refers to the process of ubiquitin transferring from the E1, to the E2 and then E3 ligase onto the substrate, this process would then be repeated for each additional ubiquitin added to the chain. However this model fails to describe how the E3-ligase which interacts with the substrate protein can physically reach the end of the ubiquitin chain. Some protein ubiquitination is mediated by multiple E3-ligases acting with the E3-ligase. The first E2-ligase mediates mono-ubiquitination, and the second E2-ligase promotes poly-ubiquitination. Some E2-ligases can assist in the transfer of pre-synthesized poly-ubiquitin onto the substrate. Finally, some proteins

have E4 ligase activity. When added to the reaction, E4 ligases promote poly-ubiquitin chain extension.

A20 mediated remodeling

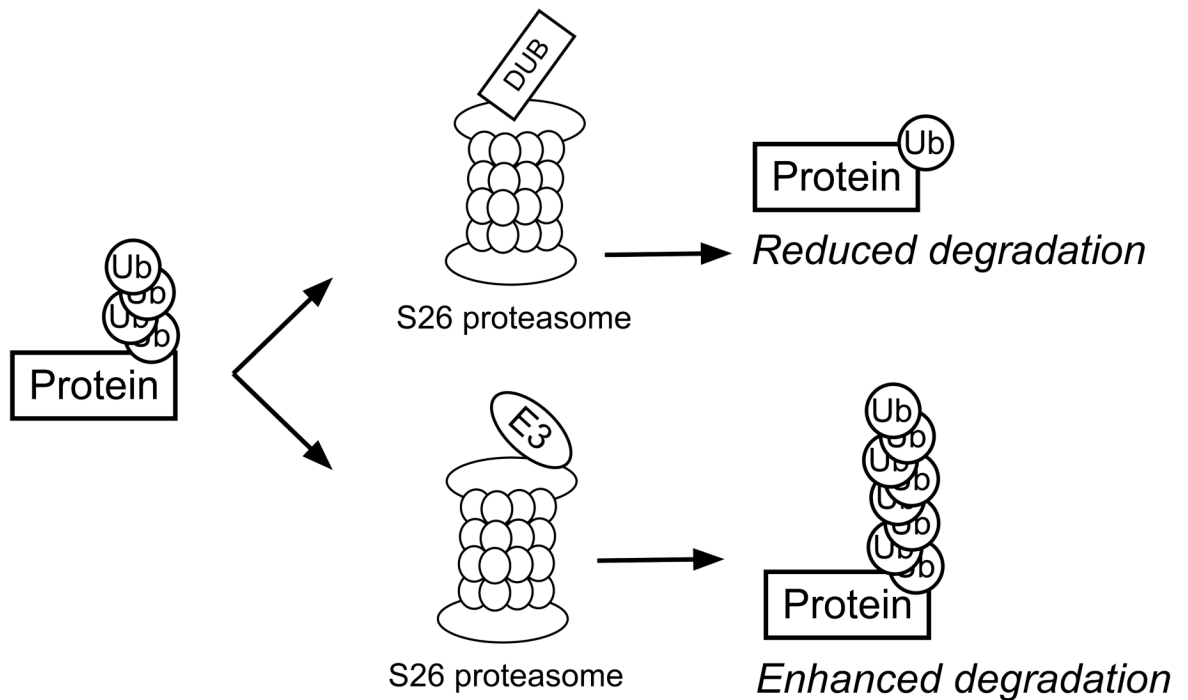## Proteasome remodeling of ubiquitin chain

Figure 3.
Examples of ubiquitin chain remodeling. A20 mediates de-ubiquitination of Lys63 poly-ubiquitinated RIP-1. A20 can also catalyze the Lys48 poly-ubiquitination of RIP-1. At the proteasome the actions of de-ubiquitinating enzymes and E3-ligases can prevent a substrate from proteasome degradation, or extend the poly-ubiquitin chain and therefore enhance degradation.
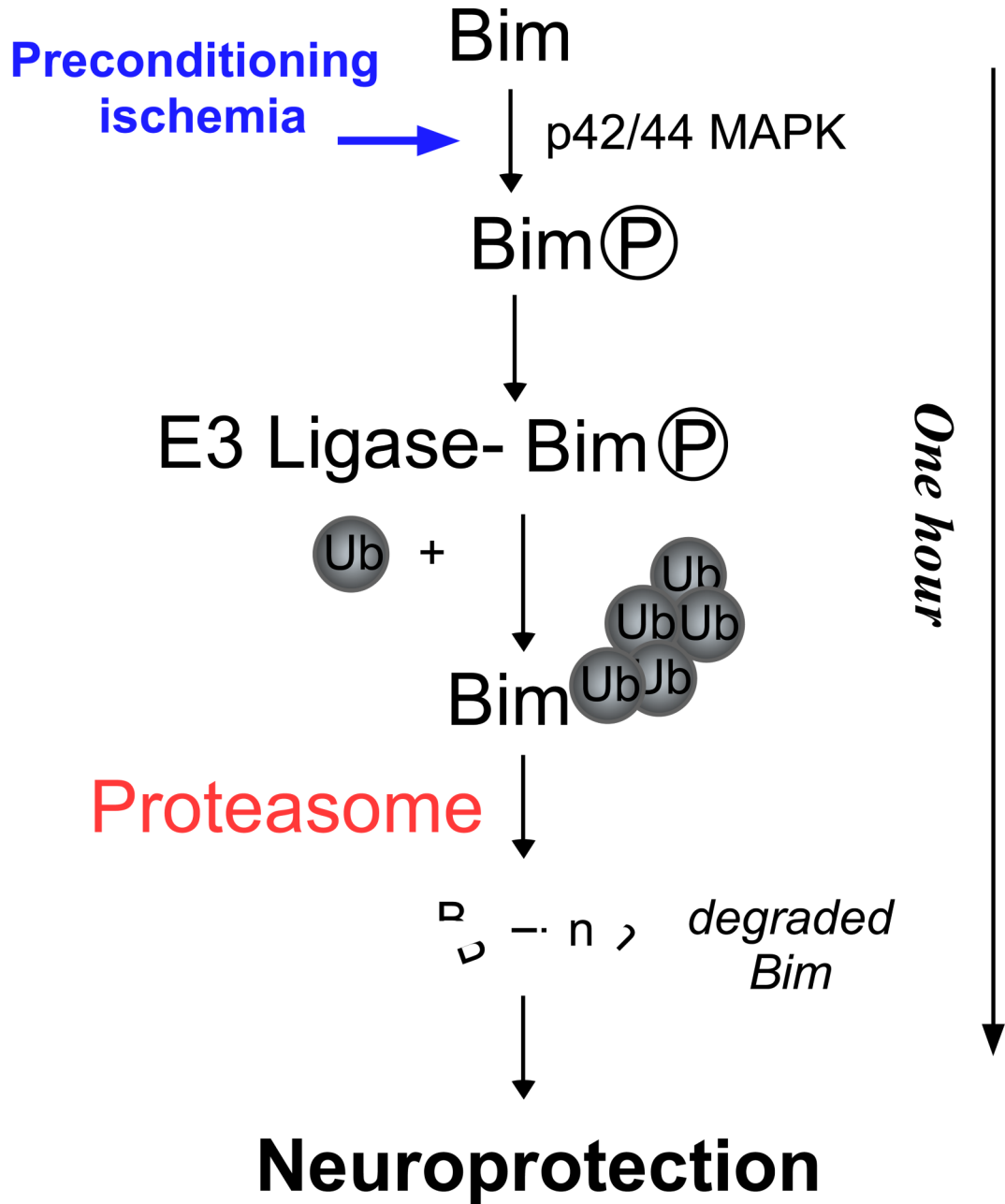
Figure 4.
Crosstalk between phosphorylation and ubiquitination promotes the degradation of Bim
following brief preconditioning ischemia. Following ischemia, activation of p42/p44 MAPK
promotes Bim phosphorylation. Phosphorylated Bim is ubiquitinated by its E3-ligase and
degraded by the proteasome. The loss of Bim results in protection against further ischemia.
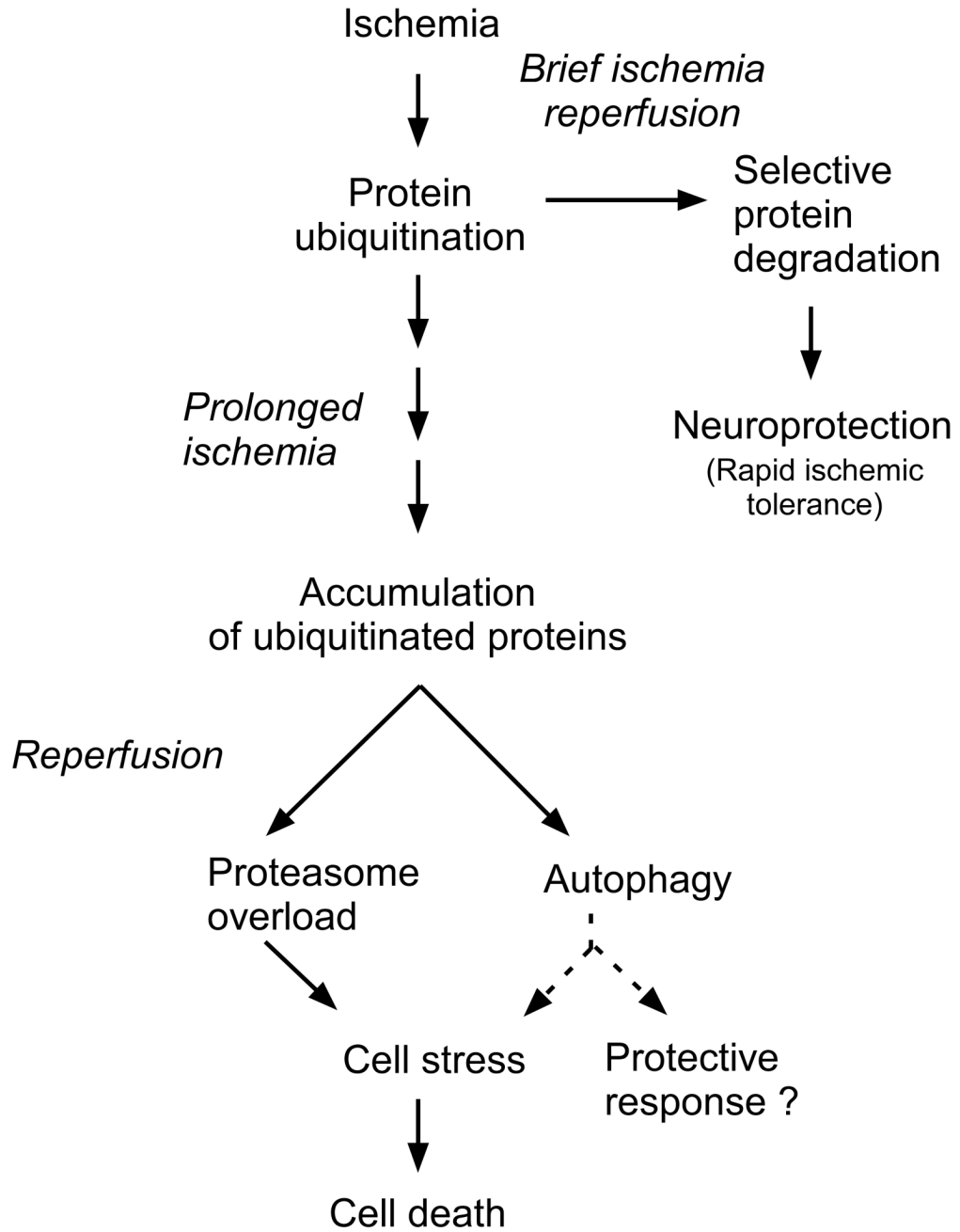What is unclear, is which E3-ligase mediates Bim ubiquitination following preconditioning
ischemia.

Figure 5.
Overview of potential role of ubiquitin proteasome system in mediating the cellular response to ischemia. Brief ischemia can result in the rapid degradation of proteins by the proteasome and tolerance to ischemia. In contrast following harmful ischemia the accumulation of ubiquitinated proteins may contribute to cell stress. Harmful ischemia reduces proteasome function. The ubiquitinated proteins form aggregates which are subject to autophagic processing. Whether autophagy is a protective response or promotes further cell stress following ischemia is unclear.

Overview of Rapid Ischemic Tolerance associated synaptic remodeling
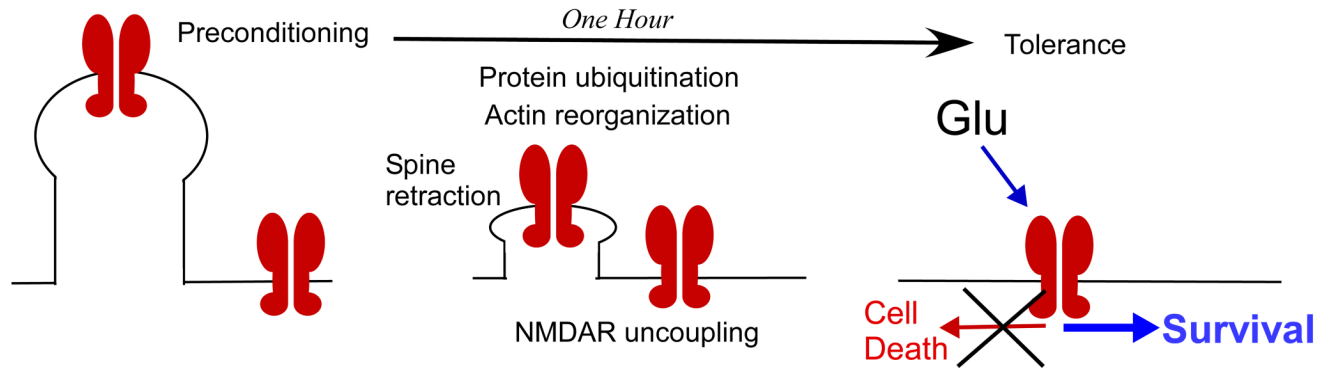

Figure 6.
Overview of preconditioning ischemia mediated morphological re-organization in dendrite and alterations in NMDA receptor mediated signaling. Following preconditioning ischemia, protein degradation and actin re-organization result in uncoupling of the NMDA receptor complexes from the actin cytoskeleton. The morphological and cytoskeleton changes results in an uncoupling of the toxic NMDA receptor mediated signaling but not physiological NMDA receptor function. As a result NMDA mediated excitotoxicity is reduced following preconditioning ischemia. This result may help identify how to switch off harmful NMDA receptor mediated signaling.
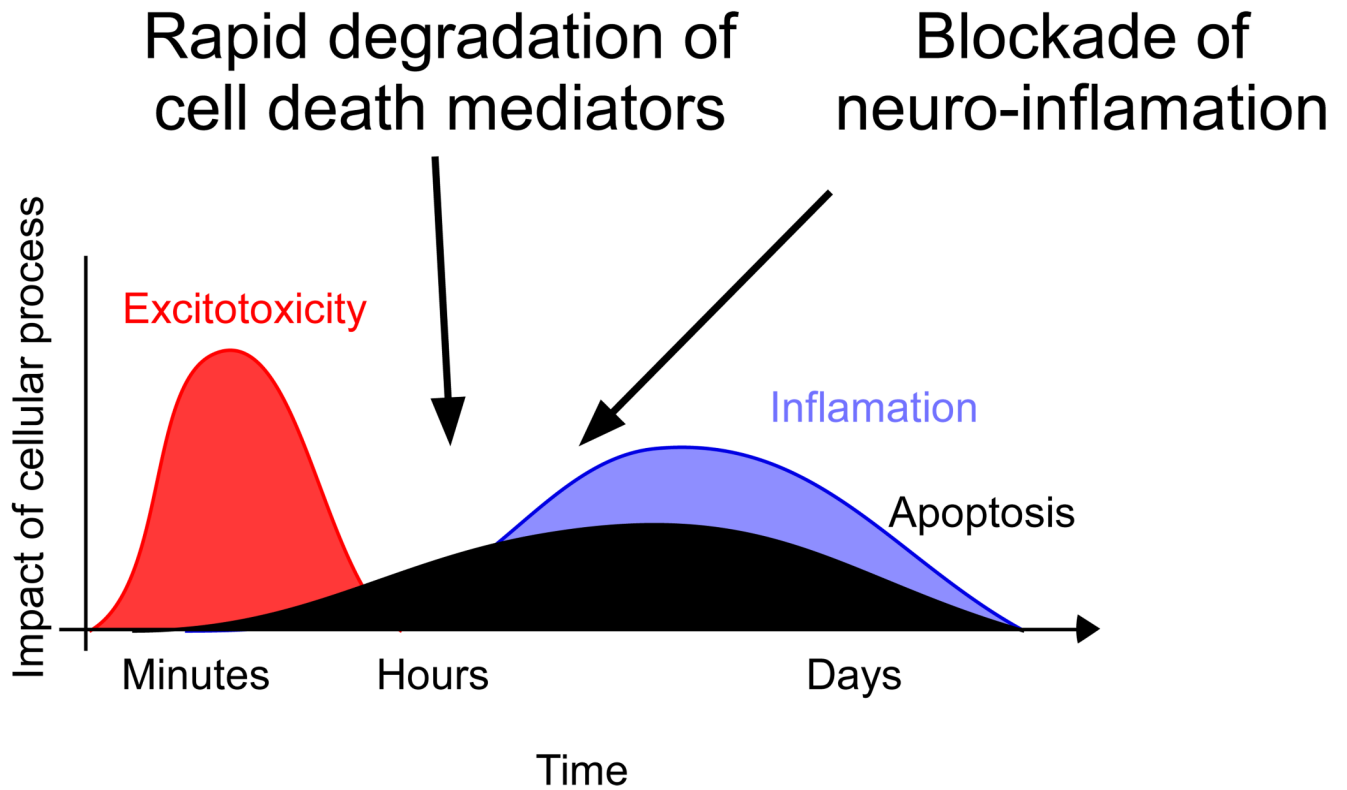
Figure 7.
Potential therapeutic sites of intervention based on ubiquitin proteasome mediated protection. Initial therapeutic strategies may target proteins whose rapid degradation is identified as being neuroprotective in rapid ischemic tolerance models. Delayed cell death has also been attributed to neuro-inflammation processes following ischemia. Proteasome inhibitors have shown to provide delayed therapeutic effects in reducing infarction and behavioral deficits by reducing neuro-inflammation. A combined therapeutic strategy targeting multiple processes which contribute to cell death following ischemia may effectively reduce brain injury following stroke. Time course figure adapted from (Dirnagl and others, 1999).