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Heat Shock Proteins in Brain: Role of Hsp70, Hsp 27 and HO-1 (Hsp32) and Their Therapeutic Potential

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Abstract

Heat shock proteins are induced by heat shock via HSF proteins binding to heat shock elements in their promoters. Hsp70 is massively induced in response to misfolded proteins following cerebral ischemia in all cell types, but is induced mainly in neurons in the ischemic penumbra. Over expression of Hsp70 via transgenes and viruses or systemic administration of Hsp70 fusion proteins that allow it to cross the blood brain barrier protect brain against ischemia in most reported studies. Hsp27 can exist as unphosphorylated large oligomers that prevent misfolded protein aggregates and improve cell survival. P-Hsp27 small oligomers bind specific protein targets to improve survival. In brain Protein Kinase D phosphorylates Hsp27 following ischemia which then binds ASK1 to prevent MKK4/7, JNK, Jun induced apoptosis and decrease infarct volumes following focal cerebral ischemia. Heme oxygenase-1 (HO-1) metabolizes heme to carbon monoxide, ferrous ion and biliverdin. CO activates cGMP to promote vasodilation, and biliverdin is converted to bilirubin which can serve as an anti-oxidant both of which may contribute to the reported protective role of HO-1 in cerebral ischemia and subarachnoid hemorrhage. However, ferrous ion can react with hydrogen peroxide to produce pro-oxidant hydroxyl radicals which may explain the harmful role of HO-1 in intracerebral hemorrhage. Heat shock proteins as a class have great potential as treatments for cerebrovascular disease and have yet to be tested in the clinic.

Keywords

Brain Ischemia; Hemorrhage; Heat Shock Proteins; Immune; Apoptosis; Heme

Introduction

Heat shock proteins (Hsps), originally described as proteins induced by heat, are defined by the presence of heat shock elements in their promoters and induction by transcriptional regulatory proteins called Heat Shock Factors (HSFs) [1]. It is now known Hsps respond to a wide variety of brain injuries including ischemia and hemorrhage. In this short review we examine the different functional roles of Hsp70, Hsp27 and Hsp32 (Heme Oxygenase, HO-1) and their potential as therapeutics. These three are high lighted because they have been the most studied, demonstrate the different functional roles of Hsps, and have different cellular localizations. Hsp70 is expressed at low levels in normal brain but is induced in all cells following ischemia, but particularly in neurons in the penumbra, and with ATP serves

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to refold misfolded or unfolded proteins (Figure 1) [1]. Hsp27 is expressed at low levels in normal brain, and is induced primarily in astrocytes, and in the absence of ATP binds misfolded proteins but does not refold them but may help solubilize misfolded proteins and prevent them from forming aggregates (Figure 4) [2]. Hsp32, more commonly known as heme oxygenase-1 (HO-1), is induced followed brain hemorrhage and brain ischemia mainly in microglia, and metabolizes heme to biliverdin, ferrous iron and carbon monoxide (Figure 6) [3]. This article is meant to be a very short synopsis, outline updates and briefly identify evolving therapeutic potential of each.

Heat Shock Protein 70 (Hsp70) – Protein ReFolding

The constitutive Hsp70 expressed in all cells is often referred to as Hsc70 or Hsp73. This protein is modestly inducible by heat shock and ischemia. It binds to all newly forming polypeptides on ribosomes in cells to keep them from folding inappropriately while they are being synthesized [4]. In addition, once fully synthesized, Hsc70 may help chaperone newly synthesized proteins to their intracellular targets, insert proteins into membranes, or help in folding proteins either alone or in conjunction with other protein complexes – for example helping in mitochondrial biogenesis or to form post synaptic glycine receptor clusters [5].

The inducible Hsp70 protein is also referred to as Hsp72 or simply Hsp70. In normal brain it is barely detectable, but following heat shock, ischemia and other injuries becomes the most abundant protein in the cell. This is because, among several different functions to be discussed, it helps to refold partially misfolded proteins or target them for degradation (Figure 1).

HSF1, one of the heat shock factors, are bound in normal cells to Hsp70, Hsp90 and other proteins. With the appearance of misfolded proteins in cells, HSF1 disassociates from these chaperones, enters the nucleus and binds to heat shock elements (HSE) in the promoter of Hsp70 and other Hsp70s (Figure 1). This results in increased transcription of hsp70 and eventually increased translation to more Hsp70 protein (Figure 1). Hsp70 protein, in conjunction with ATP, Hsp40 and Hsp90, then binds the misfolded proteins. This complex goes through several cycles of attempting to refold the proteins. This continues with binding of Hip to the N-terminus of Hsp70 and Hop to the C-terminus and continues with successful refolding of the protein (Figure 1) [6]. If refolding does not occur, then Bag-1 binds to the N-terminus of Hsp70, and the E3-ubiquitin ligase CHIP binds to the C terminus of Hsp70. This complex then targets the misfolded protein to the proteasome where it is ubiquitnated and degraded. Thus, Hsp70 serves to triage damaged proteins to either refolding or protein degradation [6]. Of interest, misfolded proteins directly activate Ire1 and initiate the unfolded protein response (UPR) [7]. All of these responses help cells survive stress and improve outcomes following cerebral ischemia as discussed below.

Heat Shock Protein 70 (Hsp70) – Apoptosis

Inducible Hsp70 not only binds abnormally folded proteins, but has been shown to bind and regulate the functions of an increasing number of proteins involved in apoptosis (Figure 2). Hsp70, with its co-chaperone Hsp40, can stabilize FANCC – an inhibitor of PKR (RNA-dependent protein kinase R), and inhibit TNF induced apoptosis. Hsp70 can also inhibit assembly of the death inducing signaling complex (DISC), inhibiting Fas, TRAIL and TNF induced apoptosis [8]. After TNF induced DISC formation and caspase 8 activation, Hsp70 can inhibit BID activation and apoptosis [8]. Hsp70 can bind factors upstream of mitochondria, including blocking JNK induced release of BAD from mitochondria, blocking JNK activation of caspase 8, and binding ASK1 and block MAPkinase induced apoptosis [8].

HSP70 can block BAX translocation, thus preventing mitochondrial outer membrane permeabilization. Hsp70 can bind Apaf-1 and prevent formation of the apoptosome (Apaf-1, cytochrome C, caspase 9) and activation of caspase 3 [8, 9]. Hsp70 can bind AIF (apoptosis inducible factor) and prevent mitochondrial mediated, caspase-independent apoptosis, and with Hsp40 it can form a complex with ICAD to prevent activation of CAD (caspase activated DNAase) [8]. Hsp70 can stabilize Mcl-1 which prevents BAX activation and mitochondrial mediated caspase-3 mediated apoptosis (Figure 2).

Though Hsp70 does not directly interact with caspases, we have shown that mutating the DEVD terminal sequence in Hsp70 to EEVD turns Hsp70 into a direct caspase 3 inhibitor [9]. It is notable that some of the above interactions appear to occur in some cell types and not others, and appear to be activated with certain types of pro-apoptotic stimuli and not others. Until it is clear what explains these differences, evidence for direct interactions of Hsp70 with a given pro- or anti-apoptotic molecule must be determined for each experimental paradigm.

Heat Shock Protein 70 (Hsp70) – Immune Pathways

Intracellular Effects of Hsp70 on Immune and Inflammatory Pathways

Hsp70 also has profound effects on various intracellular immune pathways and signaling. We have shown that Hsp70 interacts with the IKK complex to decrease NFkappaB signaling [10] and others have shown that Hsp70 can decrease NFkappaB signaling by binding IkB, p65, p50 and c-Rel, and preventing TRAF activation of IKK [11] [12] [13] (Figure 2). Though the specific Hsp70-NFkB binding partner may be cell and stimulus specific, this interaction likely accounts for studies that show Hsp70 can decrease levels of TNF, IL1, MMP9 and other pro-inflammatory mediators that are regulated by NFkB.

Hsp70 has also been reported to decrease iNOS activation in glia, and reduce NADPH oxidase activity and increase superoxide in neutrophils, all of which would decrease free radicals [11]. Hsp70 down regulates activity of MMP9 likely through NFkB. It can down regulate MMP2 as well, possibly through interactions of Hsp70 with STAT1, and may decrease MMP processing from inactive to active forms [12]. Overall, the intracellular effects of Hsp70 generally serve to suppress immune responses.

Extracellular Effects of Hsp70 on Immune Signaling

Hsp70, and other heat shock proteins, appear to be either released by stressed cells as free soluble proteins or in detergent soluble membrane vesicles (exosomes) [8]. Once released, they can affect both the innate and adaptive immune systems, and generally appear to be “pro-inflammatory.”

Hsp70 can stimulate TLR-2, TLR4 and CD14 pathways on monocytes/macrophages, dendritic cells and microglia and activate intracellular NFkappaB, IRF and Stat3 signaling [8]. Hsp70-peptide complexes can interact with macrophage and dendritic cells CD40, CD91 and LOX-1 and facilitate antigen presentation. In the adaptive immune system, Hsp70-peptide complexes can elicit CD8+ and CD4+ T cell responses [13, 11, 8]. Hsp70 activates cytolytic activity of Natural Killer Cells via the C-type lectin receptor CD94 and the CD56 adhesion molecule[11]. Tailored Hsp-tumor peptides are being developed as tumor vaccines that facilitate killing of tumor cells via the adaptive immune system [14]. The pro-inflammatory properties of extracellular Hsp70 might affect its use in acute disorders like stroke, and might be an even greater problem in chronic neurological diseases (see below). It is notable that an inverse correlation of Hsp70 protein levels in blood and atherosclerosis and risk of heart attack have been recently noted, and Hsp70 protein appears to be released into blood following myocardial infarction [11].

Hsp70 Defines the Brain Ischemic Penumbra and is Neuroprotective

There is little to no inducible Hsp70 heat shock protein expressed in normal brain (Figure 3A, left hemisphere; Figure 3C). However, following 10 minutes of focal cerebral ischemia Hsp70 protein is induced in the middle cerebral artery (MCA) distribution 24 hours later (Figure 3A, right hemisphere). Following a 1.5 hour MCAO there is infarction in much of the MCA distribution, but with some Hsp70 expression at the watershed zone between the middle and anterior cerebral arteries (Figure 3B, arrow). For the 10 minute occlusion, the penumbra represents the entire MCA distribution, and for the 2 hour occlusion the penumbra represented the border zone between middle and anterior cerebral arteries. Hsp70 protein induction in the penumbra occurs primarily in neurons (Figure 3D) [15]. In areas of infarction, or areas adjacent to the infarction, Hsp70 protein can be expressed in microglia, astrocytes and endothelial cells[15].

The roles of the induced Hsp70 protein within the cells likely include many of those discussed above. Moreover, this increased Hsp70 protein expression likely protects cells. Transfection and viral over expression of Hsp70 in neurons and glia protects against in vitro ischemia; knocking out Hsp70 worsens and transgenic over expression improves outcomes from focal and global cerebral ischemia; and pharmacological induction of Hsp70 protects against cerebral ischemia [1] [11] [12, 16] [17] [18] [19] [20].

Though the previous studies provide proof of principle, several recent studies have shown that modified Hsp70 proteins can protect against brain ischemia when administered intravenously. Intravenous TAT-Hsp70, a Hsp70 attached to a TAT motif to improve crossing the blood brain barrier, led to decreased infarct volumes, improved functional outcomes, and improved survival of neural progenitors in a mouse focal ischemia model [21]. We administered Fv-Hsp70, Hsp70 attached to a modified antibody to improve crossing the blood brain barrier, and showed decreased in infarct volumes and improved functional outcomes when given 2–3 hours after stroke in rats [22]. Notably, FvHsp70 appeared to cross the blood brain barrier in the ischemic hemisphere but not the normal hemisphere, possibly improving on target effects [22].

The intravenous administration of Hsp70, however, might be concerning since it might stimulate immune responses when given extracellularly and worsen injury – even if it protects when it becomes intracellular. Thus, other approaches might be better. Most recently neural precursor cells transduced with TAT-Hsp70 have been shown to protect against experimental focal cerebral ischemia better than the precursor cells alone [23]. In addition, a small molecule inducer of Hsp70 given after stroke has also been shown to be protective, avoiding the problem of extracellular Hsp70 administration [24]. Given the mounting literature on Hsp70 induced neuroprotection, one wonders if it is not time to begin to consider this approach for human stroke trials. However, the issues of exactly how to modulate Hsp70 to avoid peripheral immune stimulation and promote brain neuroprotection have yet to be resolved in animal stroke models, and require further investigation.

Heat Shock Protein 27 (Hsp27) – Unfolded and Misfolded Proteins

Hsp27, as typified by all members of the heat shock protein family, is inducible by heat shock, ischemia and other cellular stressors via HSF actions on its promoter. Hsp27, however, is quite distinct from Hsp70 since it does not require ATP for its actions, and though it binds misfolded proteins, it does not appear to function in protein refolding per se [2] [6] [25].

Dephosphorylation of Hsp27 favors large oligomer formation which bind to or form “reservoirs” for unfolded or misfolded proteins [6]. The large oligomers appear to perform

the “chaperone” functions attributed to Hsp27. Though the oligomers clearly bind unfolded and misfolded proteins, they do not appear to refold proteins. Instead, Hsp27 appears to hinder formation of misfolded protein aggregates and improve cell survival. Hsp27 associates with protein aggregates in many neurodegenerative diseases including Alzheimers, Huntingtons, amyotrophic lateral sclerosis, Alexanders and many others; and over expression of Hsp27 in animal models of these diseases decreases formation of aggregates and often improves cell survival [2] [6, 26] [25].

Hsp27 large oligomers also appear to interact with many cytoskeleton proteins including regulating actin polymerization and function of intermediate filaments (Figure 4) [25]. It has also been proposed that the dynamic regulation of Hsp27 oligomers may regulate ubiquitination of specific target proteins with Hsp27 small oligomers favoring ubiquitnation and proteasomal degradation of IkappB and p27 and other specific proteins, whereas large Hsp27 oligomers may favor sumolylation and prevention of proteasomal degradation of these proteins (Figure 4) [6].

Heat Shock Protein 27 (Hsp 27) – Role in Apoptosis

Hsp27 has been shown to decrease apoptotic cell death in a number of non-neural systems. It can diminish pro-caspase 9 activation by inhibiting interaction with cytochrome c and formation of the apoptosome and can inhibit release of cytochrome c from mitochondria (Figure 4) [2] [6]. Hsp27 can directly interact with pro-caspase 3 and decrease its activation and can indirectly suppress stress-induced Bax oligomerization and translocation to the mitochondria [2]. Hsp27 has also been reported to inactivate the pro-death JNK pathway and activate the pro-survival Akt/PKB pathway (Figure 4) [2]. A caveat about many of these findings is that they have not always been replicated, leading to the conclusion that the specific anti-apoptotic actions of Hsp27 may differ in different cell types and with different stressors and injuries.

Hsp27 Role in Ischemic Brain

An elegant study by Stetler et al from the Jun Chen group has recently defined the role of Hsp27 in neuroprotection in ischemic brain [27]. They showed that wild type Hsp27 and phosphorylatable Hsp27 (but not non-phosphorylatable Hsp27) protected brain in a mouse focal cerebral ischemia model. They showed that Hsp27 phosphorylation by protein kinase D (PKD) at serine 15 and serine 82 was necessary for Hsp27 induced neuroprotection. They went on to show that phosphorylated Hsp27 bound ASK1 and prevented ASK1 signaling via MKK4/7 to JNK and C-Jun which otherwise resulted in apoptosis (Figure 4) [27]. This is a seminal study in neuroprotection afforded by Hsp27 since it provides several potential targets for therapy – administering P-Hsp27, stimulating PKD with small molecule inhibitors, or testing ASK1 inhibitors.

Role of Hsp27 in Neuroprotection

Hsp27 might be an attractive target for neuroprotection. Since it does not require ATP for its actions it might work in ischemic tissue with low ATP levels. Of interest Hsp27 is expressed at very low levels in normal brain, though is highly expressed in normal motor neurons, dorsal root ganglia neurons, medial preoptic neurons and a subset of Purkinje cells [2]. Following ischemia, Hsp27 is induced almost entirely in astrocytes (Figure 5) in the region of ischemia, and presumably identifies both the ischemic brain destined to die, as well as the ischemic penumbra. Given the role of Hsp27 in stabilizing the cytoskeleton, it is interesting that Hsp27 is expressed in both dorsal root ganglia neurons and motor neurons with very long processes, but not in cortical pyramidal neurons that also have very long processes. The reason why Hsp27 is fairly astrocyte specific is also not known, though it might interact

with GFAP to stabilize the astrocyte cytoskeleton during periods of ischemic stress (Figure 5).

Viral transfection of Hsp27 *in vitro* and *in vivo* has been shown to protect against brain ischemia and kainate induced neuronal cell death [2]. As mentioned, infarct volumes are decreased in Hsp27 transgenic mice [28] but only with Hsp27 that can be phosphorylated by PKD[27]. Notably, this protection was achieved in mice where Hsp27 would have been expressed in all cell types. Hsp27 can also partially protect dorsal root ganglion neurons and motor neurons against different types of stressors/diseases; and Hsp27 can decrease aggregate formation and improve outcomes in mouse neurodegenerative disease models including Huntingtons, Parkinsons and Alzheimers Disease [2]. A direct test of Hsp27 in cerebral ischemia has also been performed where the PEP-1-Hsp27 fusion protein, designed to cross the BBB, was given intraperitoneally and protected hippocampal pyramidal neurons against death in the gerbil global ischemia model [29]. Hsp27 would appear to be an attractive target for neuroprotection in stroke because of its ATP-independence, modulatory functions, and absence of any untoward effects that might aggrevate ischemic brain injury.

Heme Oxygenase -1 (HO-1, Hsp 32) – Role in Heme Metabolism and ROS

HO-1 metabolizes heme to biliverdin, carbon dioxide and ferrous iron using NADPH as a cofactor (Figure 6). HO-1 is a heat shock protein family member since it has a HSF binding element in its promoter and is modestly inducible by heat shock [30]. However, HO-1 is induced by a large number of factors including Heme itself, Reactive Oxygen Species (ROS) via Nrf2 (ARE elements in the HO-1 promoter), Hypoxia Inducible Factor (HIF), NFkappaB, Jun/Fos, c/EBPa/Elk1, Jak/STAT, USF1/USF2 and other transcriptional activators (Figure 6) [3]. HO-1 in brain is induced primarily in microglial cells following hemorrhage (Figure 7), whereas the related non-inducible HO-2 protein that also metabolizes heme is expressed primarily in neurons.

HO-1 Can Mediate Cell Death or Cell Survival

The literature on the role of HO-1 in brain and various tissues is vast and often contradictory as to whether it promotes cell death or cell survival [30]. This likely relates to which cell types are being investigated and the nature of the specific stressor. Since HO-1 metabolizes heme to ferrous iron, the local ability of a cell or tissue to deal with iron will dictate the outcome. If hydrogen peroxide is abundant this will promote the Fenton reaction, formation of hydrogen peroxide and cause oxidative damage (Figure 6). If ferrous iron is rapidly sequestered by ferritin and other binding proteins then this harmful pathway will be circumvented. The protective actions of HO-1 include metabolizing heme which may be toxic on its own, and formation of biliverdin which is then metabolized to bilirubin which is a potent anti-oxidant (Figure 6). Moreover, HO-1 metabolizes heme to CO which acts on cGMP to promote vasodilation in vessels (Figure 6). Thus, it is understandable that HO-1 could be protective or harmful in different cellular contexts and with different stressors.

Indeed, knocking out HO-1 worsens outcomes in animal models of ischemic stroke but improves outcomes in animal models of intracerebral hemorrhage (ICH) [31] [32]. Why HO-1 might protect following ischemia and worsen damage following ICH has not been resolved, but could relate to acute iron overload in acute ICH. In addition, HO-1 appears to protect against EAE and specific brain infections [30].

Role of HO-1 in Subarachnoid Hemorrhage

Bleeding into the subarachnoid space occurs following aneurysm rupture. To model this we previously injected hemoglobin into the cisterna magna and found hemoglobin to slowly

enter the brain parenchyma over many hours to a day (Figure 7A). Concurrent with this was induction of HO-1 protein over 24 hours (Figure 7B), with the HO-1 induction occurring almost exclusively in cerebral blood vessels (not shown) and in brain microglia (Figure 7B,C) [33]. There is substantial evidence that induction of HO-1 or delivery of HO-1 to brain or subarachnoid space can protect against vasospasm that occurs following subarachnoid hemorrhage [34] [35] [36]. The neuroprotection afforded by HO-1 could relate to removal of heme and iron from around blood vessels, and production of bilirubin as an anti-oxidant. However, oxidation of bilirubin to BOXes has also been implicated in the production of vasospasm after SAH [37], so that HO-1 might have protective as well as injurious effects following SAH [38].

The possible protective role for HO-1 in subarachnoid hemorrhage (SAH) and ischemic stroke versus a possible harmful role in intracerebral hemorrhage certainly tempers enthusiasm for HO-1 as a therapeutic target in cerebrovascular disease. However, given the many known chemical inducers of HO-1, and the delay between SAH and onset of vasospasm, this could provide a novel approach to preventing ischemic neurological deficits post SAH.

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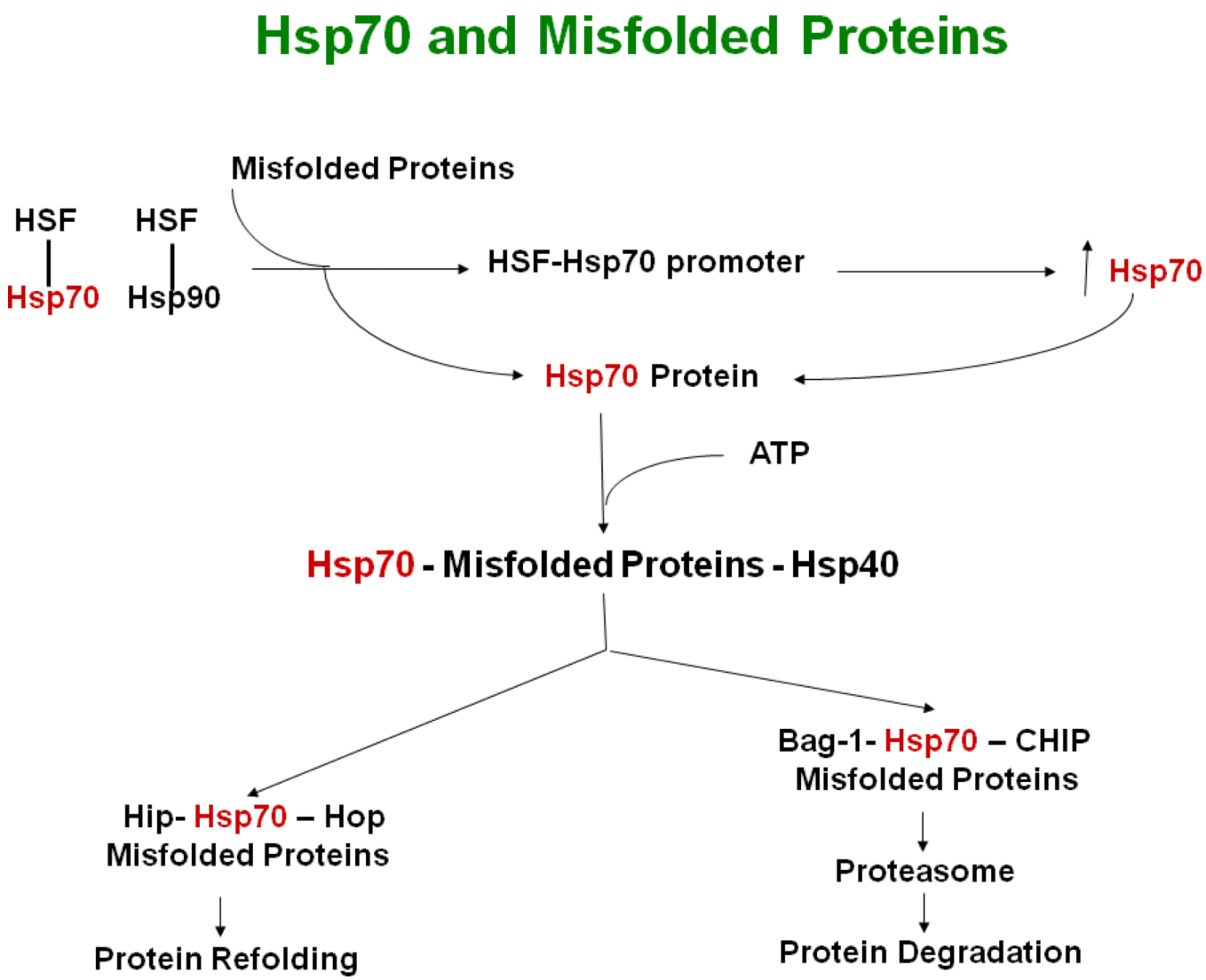

Figure 1.
Induction of Hsp70 protein by misfolded proteins. Misfolded proteins bind Hsp70 and Hsp90, with HSF being dissociated and binding to the promoters of both genes and inducing both. Hsp70, together with the Hsp40 co-chaperone and ATP, binds misfolded proteins. Hsp70 then triages the misfolded proteins. The Hip-Hsp70-Hop complex serves to refold misfolded proteins and restore function. The Bag-1 – Hsp70-CHIP complex targets misfolded proteins for ubiqutinization and processing in the proteasome for protein degradation. This figure is modified from Sharp et al [15].

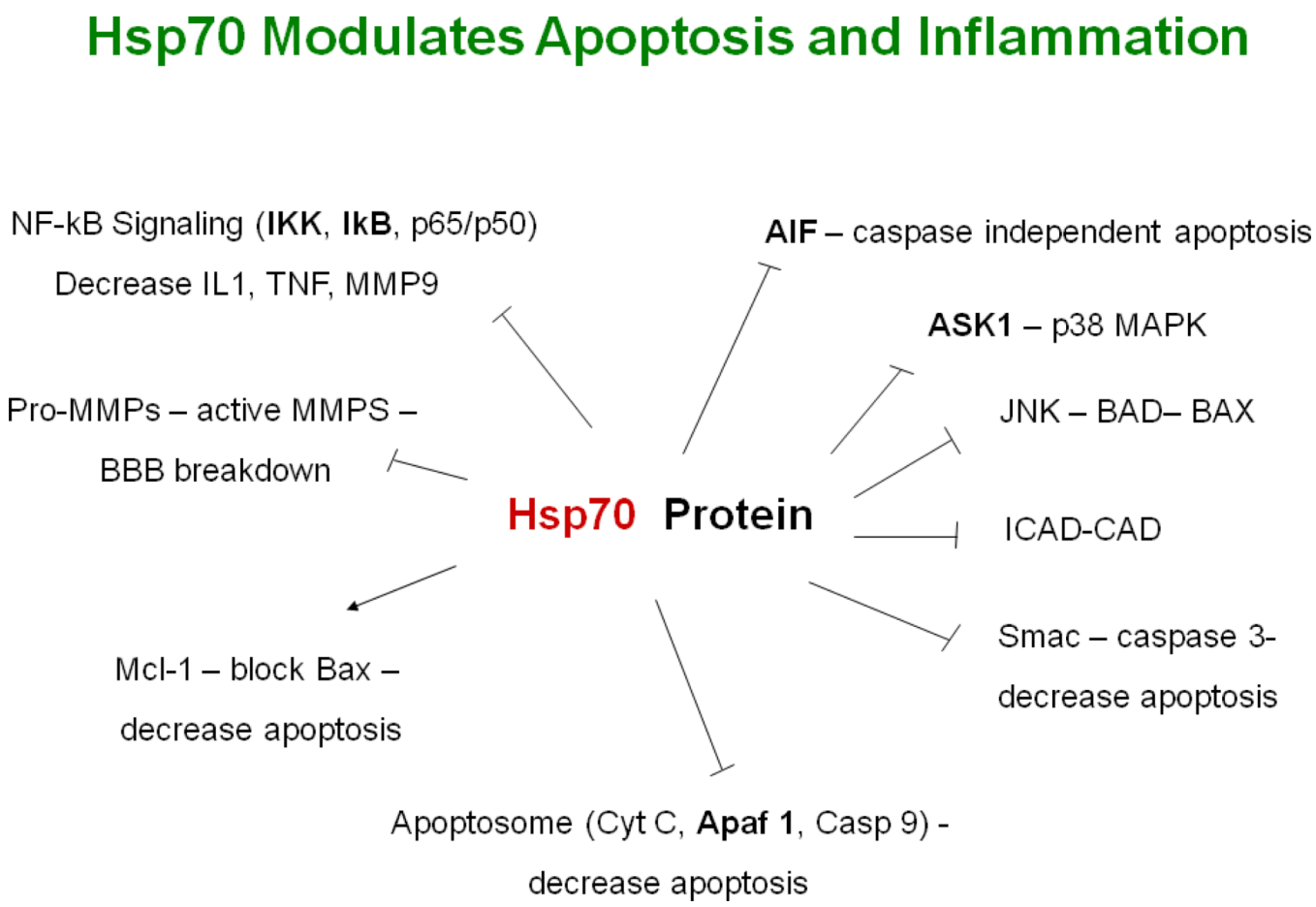

Figure 2.
Hsp70 protein also targets specific proteins that regulate apoptosis and inflammation. Some of the proteins bound by Hsp70 are cell specific, but the proteins in bold are those bound by Hsp70 in several cell types in several publications.

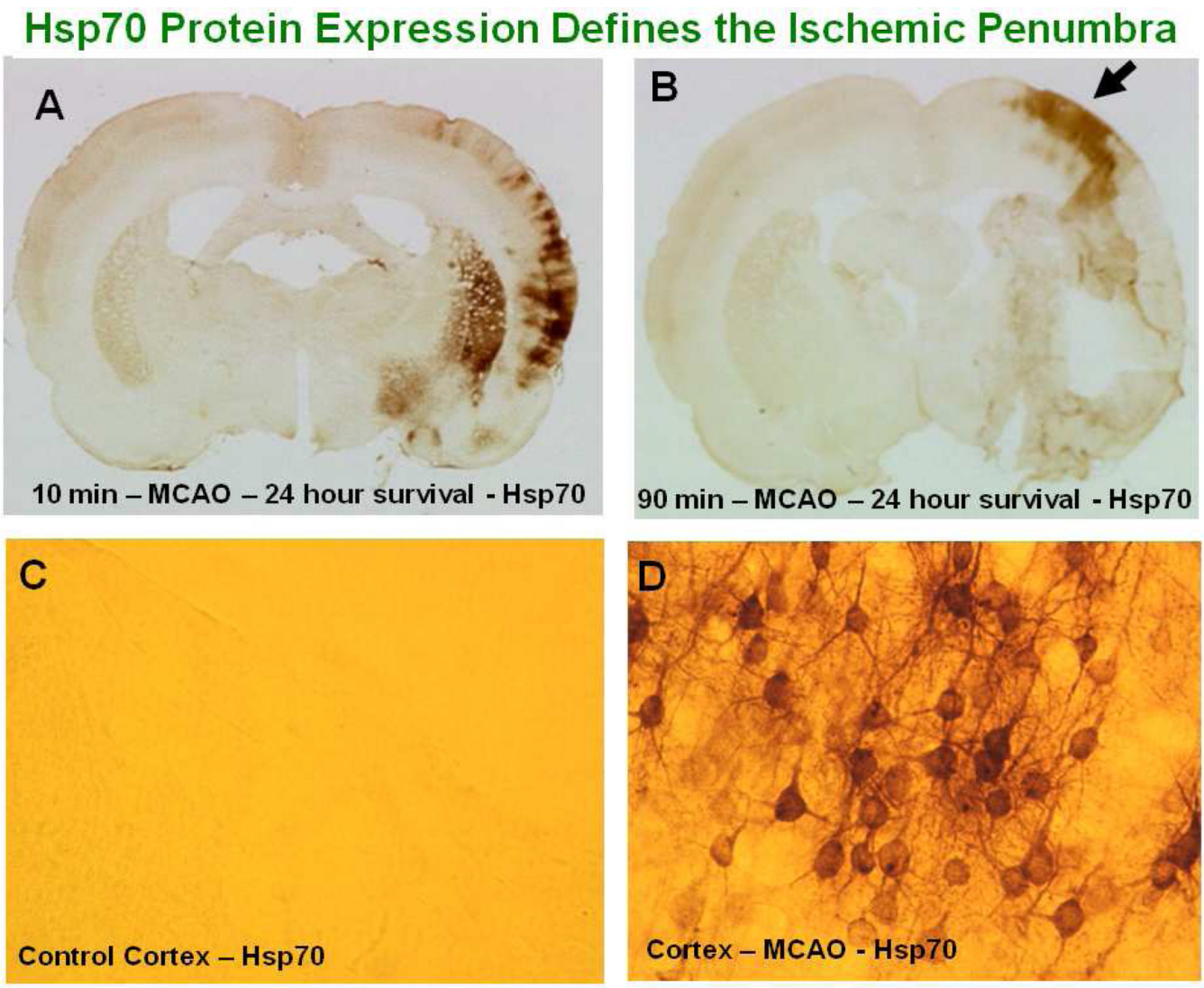

Figure 3.

Hsp70 protein expression in brain. Hsp70 protein is expressed at very low levels in normal brain (C). One day following 10 minutes of middle cerebral artery (MCA) ischemia Hsp70 protein is induced in neurons (D) throughout the MCA distribution (A). One day following 120 minutes of MCA ischemia, which produces a large infarction, Hsp70 protein is expressed in neurons at the watershed distribution/borderzone of the anterior and middle cerebral arteries (arrow, B). This figure is modified from Zhan et al. [39]

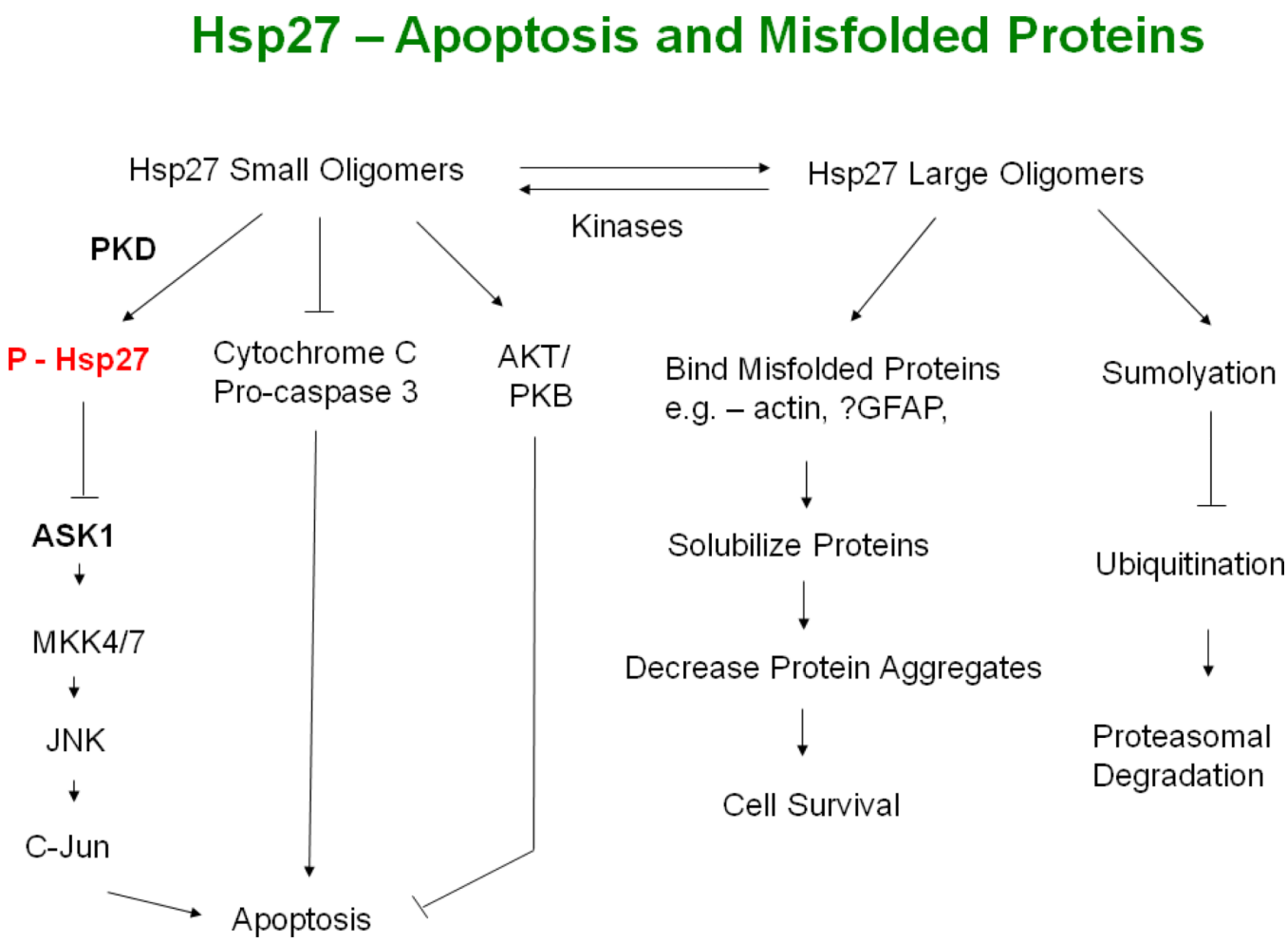

Figure 4.

Hsp27 protein interacts with misfolded proteins and is phosphorylated to small oligomers that regulate apoptosis. Stetler et al in the Chen group have shown that PKD phosphorylates Hsp27 which then binds ASK1 and prevents cellular apoptosis, and proteins brain against focal cerebral ischemia [27]. P-Hsp27 binds cytochrome c and AKT/PKB in some cell types to modulate apoptosis. Large unphosphorylated Hsp27 oligomers bind misfolded proteins and serve to help solubilize proteins and prevent protein aggregates and cellular inclusions and improve cell survival. Hsp27 also serves to modulate protein degradation via the proteasome. This figure is modified from Stetler et al and Lanneau et al [27] [6].

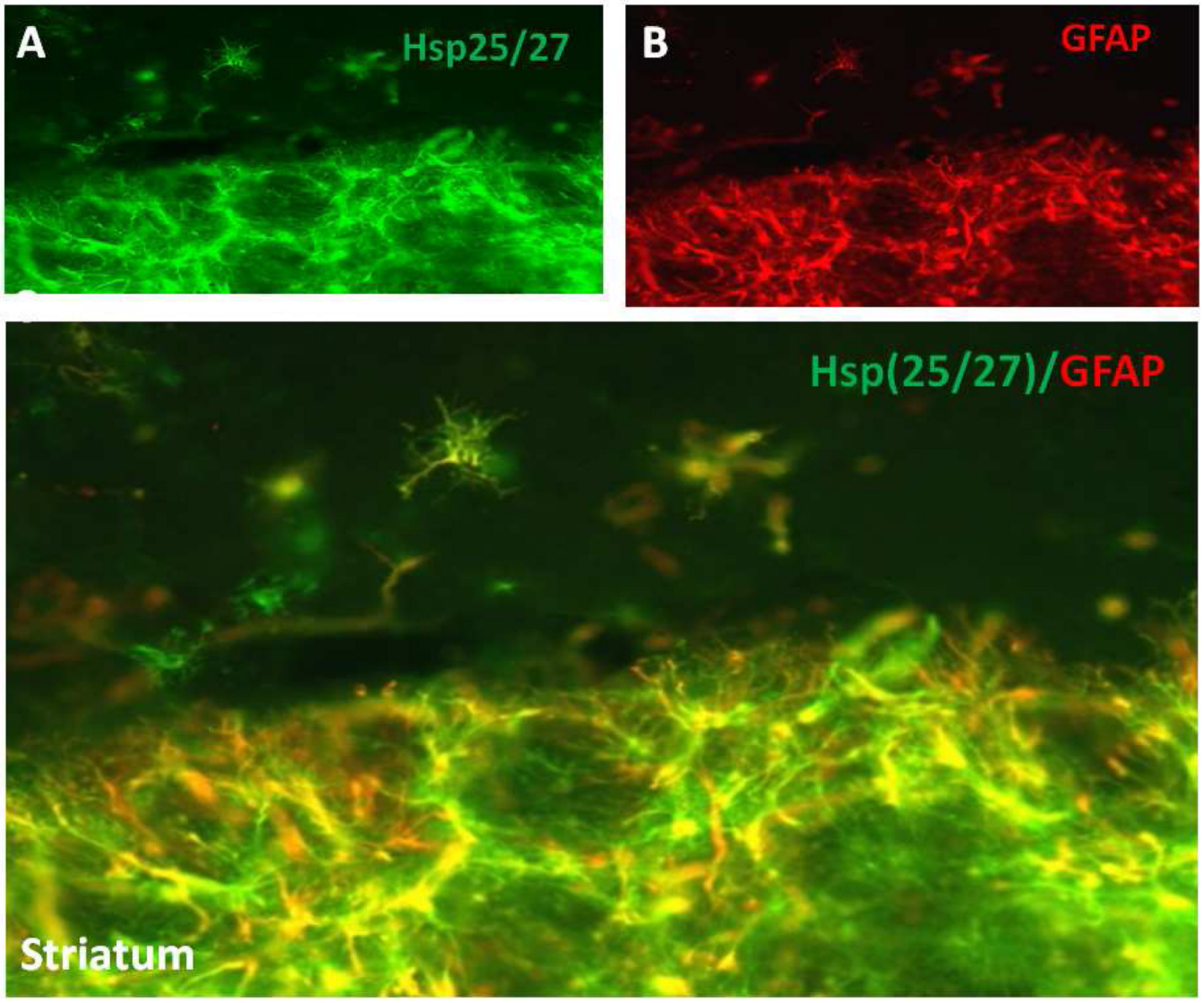

Figure 5.
Hsp27 protein expression in striatum following middle cerebral artery (MCA) ischemia. One day following 20 minutes of focal MCA ischemia Hsp27 protein is induced in striatum (A, C) in astrocytes (B). Panel C shows the co-localization of Hsp27 (A, green) and GFAP (B, red) in striatal astrocytes (C, yellow).

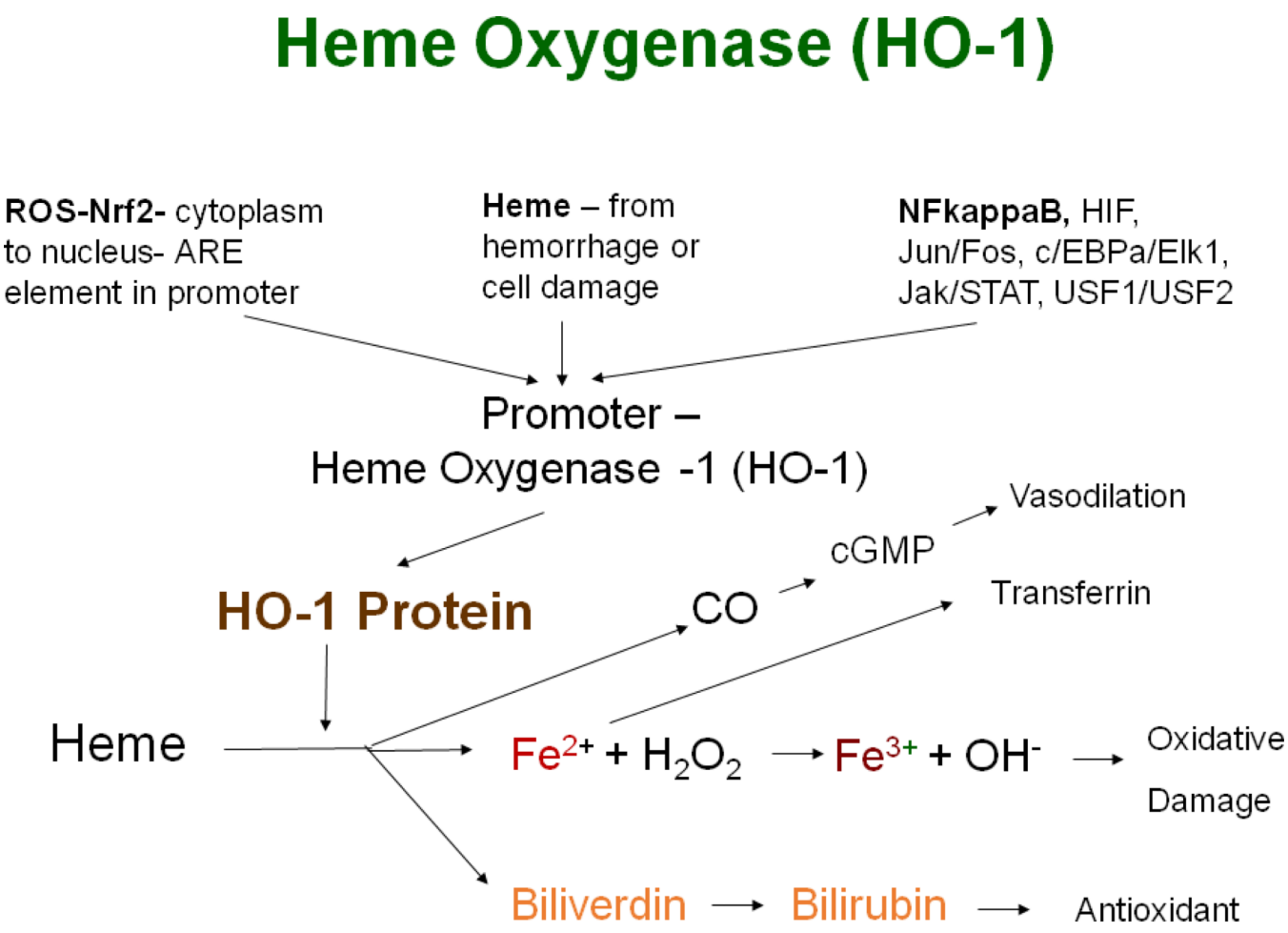

Figure 6.

Heme Oxygenase (HO-1) metabolizes heme. The HO-1 promoter has elements for many transcription factors including Nrf2 and NFkappaB, and also responds to heme. These induce HO-1 protein which then metabolizes heme to CO, ferrous ion and biliverdin. CO mediates vasodilation via cGMP. Ferrous iron can be bound by transferrin and other iron binding proteins to prevent oxidative stress; or be acted upon by hydrogen peroxide to produce hydroxyl ions to increase oxidative stress. Biliverdin is metabolized by biliverdin reductase to bilirubin which serves as an anti-oxidant.

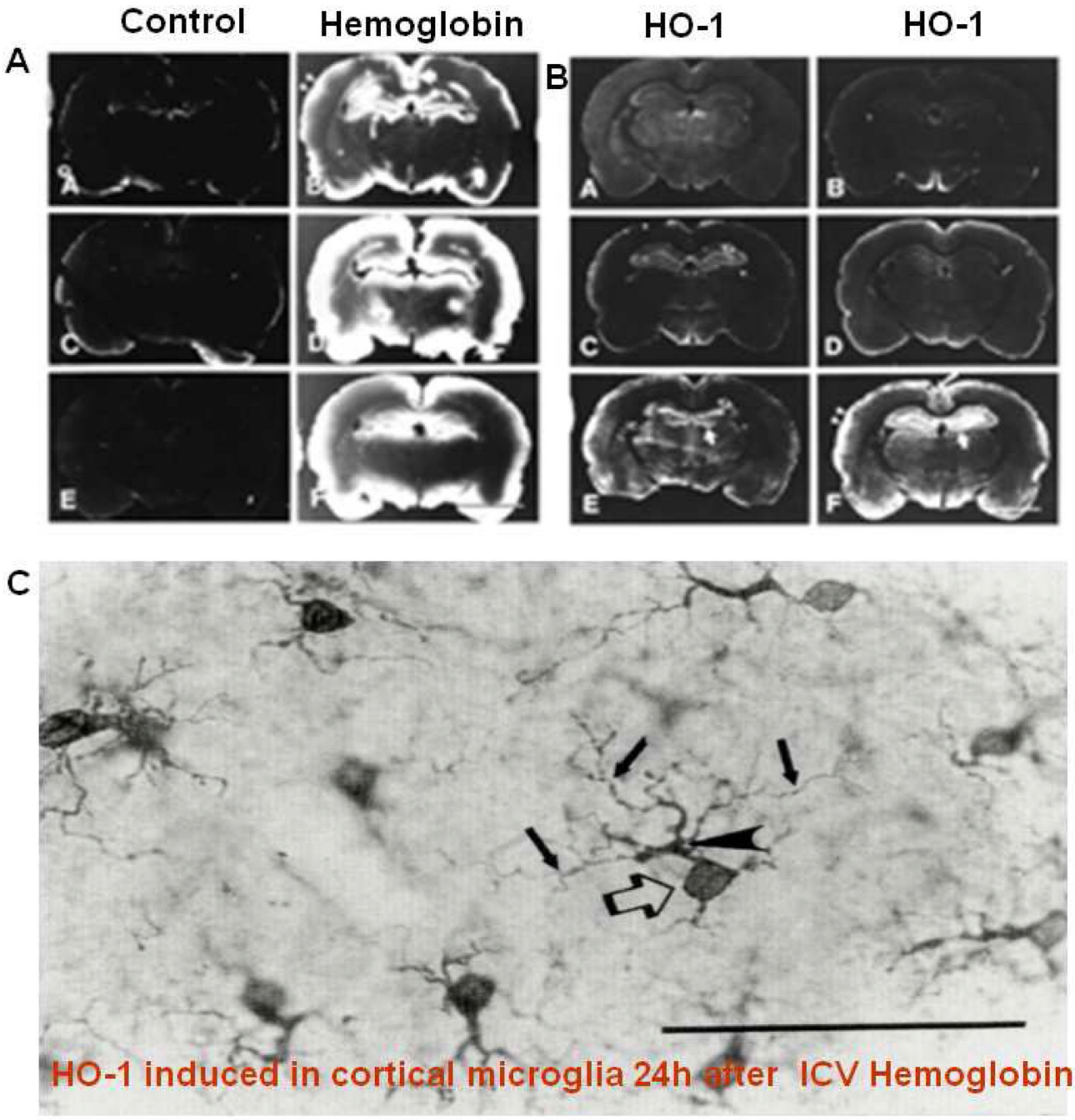

Figure 7.

Induction of HO-1 protein in microglia throughout brain following cisternal injection of hemoglobin. Hemoglobin injected into the cisterna magna slowly enters brain over 24 hours (Panel A – B,D,F). Hemoglobin injected into the cisterna magna induces the HO-1 protein throughout brain over a period of 24 hours (Panel B). The hemoglobin injected into the cisterna magna (intracerebro-ventricular) induces HO-1 protein in microglia throughout cortex 24 hours later (C). This figure is adapted from Turner et al [3] [33].