The Role of Caveolin-1 Phosphorylation in AQP4 Membrane Expression in a Model of Oxidative Stress in Primary Astrocyte Cultures

by

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ABSTRACT

Astrocytes play an important role in a wide variety of physiological processes and in disease states such as ischemia. Ischemic damage in the brain involves apoptotic cell death of neurons as well as astrocytes and it has been suggested that reactive oxygen species (ROS) generated as a consequence of ischemia are a major factor in triggering cell death. The water-permeable channel, aquaporin 4 (AQP4), which is expressed at high concentrations in astrocytes, is an important determinant of mortality and morbidity in mice subjected to ischemia, however the effects of ROS on AQP4 expression and the underlying mechanisms are still obscure. In the present study, we used primary astrocyte cultures to examine the expression of AQP4 under oxidative stress using hydrogen peroxide. First, we showed that H_2O_2 induces a significant increase of both AQP4 mRNA and protein levels and that this effect is inhibited by the anti-oxidant N-acetylcysteine. Second, we demonstrated using cell surface biotinylation that H_2O_2 increases AQP4 plasma membrane expression independently of the newly synthesized pool of AQP4. In parallel, we found that caveolin-1 undergoes a dose- and time-dependent phosphorylation in astrocytes treated with H_2O_2 and that this effect is inhibited by the *src* kinase inhibitor PP2. More importantly, PP2 inhibits the H_2O_2-induced increase in AQP4 cell surface expression, suggesting that the phosphorylation of caveolin-1 and possibly other proteins may play a role in this process. To investigate this further, we used MDA-435 cells expressing Y14F and Y14D caveolin-1 mutants transfected with AQP4 and found that these cells exhibit a decrease and an increase in AQP4 membrane expression, respectively. Furthermore, caveolin-1 knock down in astrocytes inhibits H_2O_2-induced increase in AQP4 cell surface expression. Together these findings show that the phosphorylation of caveolin-1 Y14 is a key regulator of AQP4 cell surface expression in oxidative stress possibly by altering AQP4 internalization and trafficking resulting in its redistribution within different compartments of the cell.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AA	arachidonic acid
ADAMTS	adamlysins
AJ	adhesion junctions
AMF	autocrine motility factor
AMPA	a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
Apaf-1	apoptotic protease activating factor 1
M1-AQP4	aquaporin-4 isoform M1
M23-AQP4	aquaporin-4 isoform M23
AQPs	aquaporins
ATP	adenosine-5'-triphosphate
AVD	apoptotic volume decrease
Bad	B-cell lymphoma 2 (Bcl-2)-associated death
BBB	blood-brain barrier
Bcl-2	B-cell lymphoma 2
Ca^{2+}	calcium ion
CaMKII	$Ca^{2+/}$calmodulin-dependent protein kinase II
CBD	caveolin binding domain
Cl^-	chloride ion
CNS	central nervous system
CSD	caveolin scaffolding domain
CSF	cerebro-spinal fluid
DAP	dystrophin-associated protein
DAPC	dystrophin associated protein complex
DEP-1	density enhanced phosphatase
DG	dystroglycan
DHPG	3,5-dihydro-oxyphenylglycine
EC	endothelial cell
ECF	extracellular fluid
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
eNOS	endothelial nitric oxide synthases
ER	endoplasmic reticulum
FAK	focal adhesion kinase
FFEM	freeze fracture electron microscopy
FRAP	fluorescence recovery after photobleaching
GLAST	Na^+-coupled L-glutamate/L-aspartate transporter
GLT-1	glutamate transporter-1
GM1	monosialotetrahexosylganglioside
GM-CSF	granulocyte macrophage colony-stimulating factor
GSH	glutathione
GSHPx	glutathione peroxidase
GTP	guanosine triphosphate
H^+	hydrogen ion

H_2O_2	hydrogen peroxide
HDL	high density lipoprotein
HIF-1α	hypoxia inducible factor-1α
HMG CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HNE	4-hydroxynonenal
ICAM-1	inter-cellular adhesion molecule 1
ICH	intracerebral hemorrhage
IEG	immediate early genes
IL	interleukin
ILM	inner limiting membrane
iNOS	inductible Nitric Oxide Synthase
K^+	potassium ion
Kir4.1	inward rectifier K^+ channel 4.1
LDL	low density lipoprotein
LTP	long-term potentiation
MAPK	mitogen activated protein kinase
MCAO	middle cerebral artery occlusion
MD	muscular dystrophy
mGluR	metabotropic glutamate
MIP	major intrinsic protein
MMP	matrix metalloproteinases
MRI	magnetic resonance imaging
MTLE	mesial temporal lobe epilepsy
Na^+	sodium ion
NAD(P)H	nicotinamide adenine dinucleotide phosphate
NMDA	N-methyl D-aspartate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
NOSs	specific nitric oxide synthases
NPA	Asparagine-Proline-Alanin
O_2^-	superoxide
OAPs	orthogonal array particles
OH	hydroxyl
$ONOO^-$	peroxynitrite
ONOOH	peroxynitrous acid
ONS	oxidative/nitrosative stress
PDBu	phorbol 12, 13-dibutyrate
PDZ	PSD-95, Dlg and Zo-1
PECAM-1	platelet endothelial cell adhesion molecule
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PTPμ	protein-tyrosine phosphatase
RNS	reactive nitrogen species
ROS	reactive oxygen species

RVD	regulatory volume decrease
RVI	regulatory volume increase
SH2	domain-containing phosphatases
SOD	superoxide dismutase
SPT	single-particle tracking
SV40	simian virus 40
TIRFM	total internal reflection fluorescence microscopy
TLE	temporal lobe epilepsy
TNFα	tumor necrosis factor alpha
tPA	tissue plasminogen activator
VEGF	vascular endothelial growth factor
VE-PTP	vascular endothelial tyrosine phosphatase
XO	xanthine oxidase

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1 INTRODUCTION

1.1 Cerebral edema

Cerebral edema is a fairly common pathophysiological entity encountered in many clinical conditions. By definition cerebral edema is due to a progressive accumulation of water content in the intracellular and/or extracellular spaces of the brain that results in pathological swelling [1]. Under physiological conditions, the extracellular fluid and intracellular fluid have the same osmotic pressure, therefore maintaining a water balance in the brain. However, the extracellular or intracellular osmolarity is subjected to changes in pathophysiological conditions at a complex cellular level, which generates osmotic gradients that lead to excessive water flow resulting ultimately in brain edema [2]. Edema in the brain may be topographically classified into focal or global edema. Focal edema is generated from an osmotic pressure gradient with adjacent regions and may result in tissue shift and herniation. Examples of focal edema can be found around tumors, hematomas and infarctions. Global edema widely affects the whole brain and it may cause intracranial hypertension, compromise perfusion, lead to generalized ischemia and in some cases to death [3, 4]. Cardiopulmonary arrest, severe traumatic injury, and liver failure are common causes of global cerebral edema. Based on different pathophysiological mechanisms responsible for the production of edemas [1], cerebral edema is generally categorized into four types:

1) Cytotoxic edema, in this type of edema the blood brain barrier (BBB) remains intact. This edema is due to the derangement in cellular mechanisms resulting in the inadequate functioning of the sodium and potassium pump in the glia cell membrane. The Na^+/K^+-ATPase pump spends the energy from ATP hydrolysis and maintains cellular homeostasis. The cellular retention of Na^+ caused by the malfunction of the pump due to the deprivation of ATP leads to rapid water uptake [5]. Astrocytes in the gray and white matters are swollen in this type of edema, especially in their peri-capillary foot processes. Cytotoxic edema is seen in severe hypothermia, early ischemia, stroke, and hypoxia as well as cardiac arrest.

2) Vasogenic edema is characterized by intra-vascular fluid and protein penetration into the extracellular space of the brain parenchyma when the BBB is altered due to the breakdown

of tight endothelial junctions. Once plasma constituents cross the BBB, the edema spreads quickly to other brain areas. This type of edema is seen in response to tumors, focal inflammation, trauma and late stages of cerebral ischemia [5]. Some pathophysiological mechanisms contributing to BBB dysfunction are: physical disruption by arterial hypertension or trauma, tumor facilitated release of vasoactive and endothelial destructive compounds (e.g. arachidonic acid, histamine, excitatory neurotransmitters and free radicals) [6].

3) Osmotic edema, normally cerebral-spinal fluid (CSF) and extracellular fluid (ECF) osmolarities of the brain are slightly lower than that of the plasma. When the plasma is diluted by excessive water intake in pathophysiological conditions, the brain osmolality exceeds the serum osmolarity creating an abnormal pressure gradient which goes down and results in water flow into the brain causing edema. The physical disruption of BBB and infiltration of the plasma into the parenchyma after a surgery or intracerebral haemorrhage can cause this type of brain edema [7].

4) Interstitial edema is seen in hydrocephalus when outflow of CSF is obstructed and intraventricular pressure increased. The result is the movement of Na^+ and water across the ventricular wall into the paraventricular space. Interstitial edema is different from vasogenic edema because unlike the plasma, CSF contains almost no proteins [8].

1.1.1 Cytotoxic edema

Cytotoxic edema, also called cellular edema, oncotic cell swelling, or oncosis, is characterized by the swelling of all the cellular elements of the brain. It is initiated as soon as the onset of acute cerebral ischemia. Neurons, glia, and endothelial cells swell within minutes of hypoxia, due to extracellular cations entering the cells and accumulating intracellularly drawing water into the cells. Under physiological condition, the membrane Na^+/K^+-ATPase pumps three Na^+ out of the cell for every two K^+ pumped in. In cytotoxic edema, extracellular Na^+ and other cations enter into neurons and astrocytes and accumulate intracellularly, in part due to failure of energy-dependent mechanisms of extrusion. Unchecked influx of cations occurs largely through cation channels. Cation influx in turn drives influx of anions which maintains electrical neutrality. In combination these

phenomena generate concentration gradients between intracellular and extracellular spaces that drive influx of water, resulting in osmotic expansion of the cell and giving rise to cytotoxic edema [9, 10]. The cascade begins with the deprivation of energetic molecules such as adenosine triphosphate (ATP) in cells due to the abrogation of oxidative phosphorylation responses to hypoxia [11]. The cells quickly use up the reserve of ATP and unless oxygen/glucose is restored the deranged cellular machinery loses its ability to pump Na^+ and K^+ via the Na^+/K^+ ATPase pump that requires continuous expenditure of ATP necessary to maintain homeostasis. When ATP is absolutely depleted, it causes higher intracellular cation accumulation and enhances osmotic overload that in turn promotes water influx. In cytotoxic edema, the BBB is intact and impermeable to ions and fluids and is not accompanied by an increase in the net volume of the brain. For an actual increase in brain volume to occur, additional fluid must be added to the brain's extracellular space. The movement of ions and water into cells in cytotoxic edema results in the depletion of these constituents from the extracellular space. Newly established gradients for Na^+ and other osmotically active solutes between the intravascular space and the extracellular space are the driving forces for the transendothelial movement of edema fluid across the BBB. However, the stored potential energy of these ionic gradients cannot manifest into solute and water movement until the permeability of cerebral endothelial cells of the BBB is altered. Cytotoxic edema (cellular swelling) begins within 30 minutes following middle cerebral artery occlusion (MCAO) and persists for up to 24 hours after ischemic reperfusion [12, 13]. Swelling is more prominent in astrocytes than in neurons as they are involved in the clearance of anions and glutamate [14-16] which are the major sources that cause osmotic overload that in turn promotes water flow.

Glutamate is the major excitatory neurotransmitter in the mammalian CNS. It has been implicated in the pathogenesis of stroke and other neurological diseases [17]. Clearance of glutamate from the extracellular space is accomplished primarily by transporter-mediated uptake. Glutamate transporters are expressed by many cell types in the CNS, including astrocytes, neurons, oligodendrocytes, microglia and endothelia [18]. Of these, uptake by astrocytes is most important for maintaining normal extracellular glutamate concentration. The extracellular concentration of glutamate is usually in the low micromolar range, compared to that in the millimolar range inside the cell [19]. GLT-1 and GLAST achieve glutamate uptake against this concentration gradient by coupling transport of Na^+ and K^+

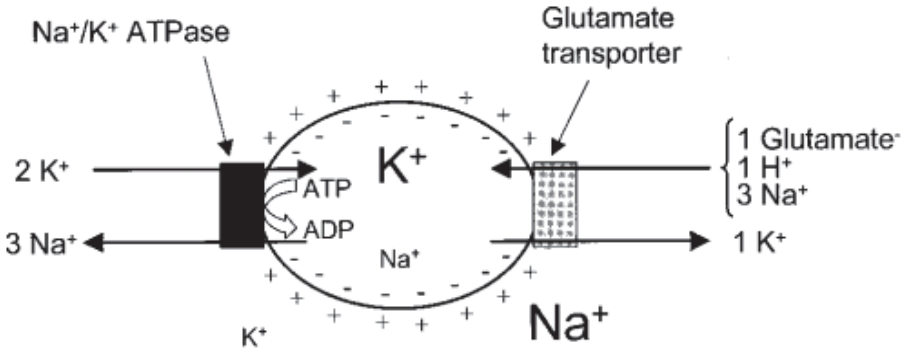


Figure 1.1 Schematic representation of glutamate uptake. Uptake of glutamate and H^+ is driven by the coupled transport of Na^+ and K^+ down their respective concentration gradients. The cell membrane potential also contributes to the uptake driving force because there is a net inward movement of positive charges with each glutamate transported. Anderson et al., Glia 32:1–14 (2000).

down their respective concentration gradients, with inward movement of glutamate and H^+. The cell membrane potential also contributes to the driving force because of the net inward movement of positive charges with each glutamate molecule transported into the cell (glutamate carries negative charge). It was suggested that the stoichiometry of the Na^+-dependent transporters is inward cotransport of 3 Na^+ ions and 1 H^+ ion and an outward counter-transport of 1 K^+ ion with the uptake of each glutamate anion into the cells (Fig. 1.1) [20]. The failure of Na^+- K^+ channel in response to cerebral ischemia/hypoxia prevents K^+ from being pumped into the cell resulting in the increase of its extracellular concentration and a decrease of its intracellular concentration. Concomitantly, the extracellular Na^+ concentration falls and its intracellular concentration increases leading to membrane depolarization due to the failure of Na^+/K^+-ATPase pump. These changes in Na^+ and K^+ concentration gradients impact the glutamate uptake [21]. Consequently glutamate transporters are expected to reverse and pump glutamate out of cells and release it in the extracellular space. Detailed calculations [22] predict that the ion concentration changes occurring in ischemia will result automatically in reversed operation of glutamate transporters occuring until the extracellular glutamate concentration ($[Glu]_o$) rises to ~60 μmol/L, a level known to trigger neuronal cell death. The excessive accumulation of glutamate further enhances the abnormal influx of Na^+ and Ca^{2+} into the cells via N-methyl-D-aspartate (NMDA) and the metabotropic glutamate (mGluR) receptors resulting in excessive water influx [23].

The swelling of astrocytes may compress the microcirculation in ischemic areas as early as 30 minutes after MCAO by narrowing down the capillary lumen, blocking further oxygen supply and inducing prolonged ischemia, excitotoxicity, cerebral herniation and ultimately mortality [24] [13]. In the brain, the defense of cell against swelling is crucial due to spacial constraints imposed by the skull.

1.1.2 Vasogenic edema

Vasogenic edema develops after 4-6 hours of ischemia, reaches a maximum at 36-72 hours and lasts 7-14 days [25]. The key feature of vasogenic edema is the disruption of the BBB and subsequent leakage of the intravascular fluid into the extracellular space of the

brain parenchyma [26]. The BBB integrity is essential for maintaining the normal function of the brain. It provides a highly selective barrier between vascular space and the brain tissue that enables the maintenance of the microenvironment that is crucial for overall homeostasis of the brain [27]. The anatomical components of the BBB are the capillary endothelium and the astrocytic foot processes that surround the cerebral capillaries. The astrocytes surround the endothelial cells of the BBB, providing biochemical support to those cells [28, 29]. Vasogenic edema occurs usually later than cytotoxic edema [30]. It has been reported that the components of perivascular extracellular matrix (ECM) including laminin, collagen, agrin, and fibronectin are attenuated in the ischemic areas between 3 and 24 hours after MCAO or in brain tumors and subrachnoid haemorrhage that are the major causes of vasogenic edema [31-35]. The mechanism of BBB disruption involves endothelial cell alteration and basal lamina degradation by matrix metalloproteinases (MMP) or adamlysins (ADAMTS) that are responsible of digesting almost all ECM molecules [36]. As a result of BBB disruption, albumin and other high molecular weight molecules enter the cerebral interstitial space from the plasma, which creates an osmotic environment allowing water influx into the brain [37]. This phenomenon peaks at 12 hours after permanent MCAO [38] or 1h after a transient MCAO [39].

Proinflammatory cytokines produced and secreted by brain microvascular endothelial cells during cerebral ischemia include interleukin (IL)-α and β, IL-6, and GM-CSF [40-42]. In many cases, these cytokines are involved in initiating changes in adhesion molecules on endothelial cells such as PECAM-1, E-selectin, and ICAM-1 [43]. This allows immune cells to cross the broken down BBB and infiltrate the central nervous system (CNS).

In vasogenic edema, the BBB disruption is accompanied by the up-regulation of vascular endothelial growth factor VEGF-A and VEGF-B, a major inducer of angiogenesis, secreted by macrophage, astroglia and neurons [44-47]. VEGF-A is known to produce structural alterations in the endothelium, including an alteration of the structure of tight junctions. VEGF-B, unlike VEGF-A, may be an essential factor for the maintenance of endothelial homeostasis [42]. In stroke, VEGF accelerates the development of edema during the sub-acute stage. However, it may improve recovery by inducing new vessel formation in the subsequent stages [48].

It is generally believed that cytotoxic edema is an initial first step that precedes

vasogenic edema [49].

1.1.3 Cerebral edema in stroke

Stroke can be categorized as hemorrhagic or ischemic based on their pathophysiological mechanisms and account for 20% and 80% of stroke cases, respectively. Pathways leading to edema formation in hemorrhagic stroke differ from those in ischemic stroke.

1.1.3.1 Cerebral edema in hemorrhagic stroke

In hemorrhagic stroke, following the initial physical trauma from the hydrostatic effect of intracerebral hemorrhage (ICH), perifocal edema formation is initiated by clot derived proteins and vasoactive substances impairing the BBB integrity. Thrombin, plasminogen activator and urokinase contribute to brain edema formation [50-52]. The activation of thrombin was shown to cause inflammatory cell infiltration and scar formation [53], as well as direct disruption of the BBB by inducing endothelial cells retraction [54]. The presence of plasminogen activator and urokinase has been found to enhance this effect by competing for thrombin inhibitors. The opening of the BBB leads to the formation of a proteinacious ultrafiltrate causing vasogenic edema peaking at 10-20 days in humans following ICH [53]. In later phases, thrombin, hemoglobin degradation products, and inflammatory mediators together facilitate the development of vasogenic edema.

1.1.3.2 Cerebral edema in ischemic stroke

Ischemic stroke is the most common and serious form of ischemic brain injury. It causes a loss of neural function resulting from a critical reduction in cerebral blood flow. Brain metabolism rapidly declines as the supply of oxygen and glucose delivered by blood is decreased. It initiates a sequence of different edema mechanisms in a stepwise fashion that contributes to its high morbidity and mortality. The disruption of cerebral blood flow in ischemia leads to the impairment of ATP synthesis, causes insufficient Na^+/K^+ ATPase inducing net accumulation of intracellular Na^+, which draws water into the cell creating cytotoxic edema. This edema occurs in the first minutes of ischemia. In addition, increased

H^+ ions due to ischemic acidosis are expelled out of the cells in exchange for Na^+ ions which worsen cytotoxic edema. Further cellular damage is caused by ischemia resulting in BBB disruption through a number of proposed mechanisms such as disrupted Ca^{2+} signaling [55] and the involvement of other molecules including VEGF [45] and MMPs [56]. The disruption of the BBB causes water accumulation in the extracellular space leading to the formation of vasogenic edema. Depending on the degree of ischemia, the BBB may lose its entire physical integrity leading to hemorrhagic conversion.

1.2 Cerebral ischemia

Oxygen is valuable because it is important in many ATP-producing cycles occurring throughout the body such as, the Krebs cycle, and the electron transport chain, which maintain cellular metabolism. During cerebral ischemia, due to the reduced oxygen and glucose supply to brain that causes the failure of ATP production, the reserve of ATP is depleted within minutes resulting in a failure of energy production. One of the most important consequences of the loss of available ATP is the failure of ATP-dependent ion pumps and the subsequent dissipation of the resting membrane potential of neurons. As discussed previously, the maintenance of the resting membrane potential is ultimately dependent on Na^+-K^+-ATPase, which exports Na^+ and imports K^+ against their concentration gradients at the cost of ATP. As this pump is electrogenic, it normally produces hyperpolarizing currents. Decreased activity, therefore, leads to depolarization because of the loss of hyperpolarizing currents. Accordingly, many CNS neurons depolarize rapidly after ATP depletion. Depolarization of the presynaptic neuron releases neurotransmitters like glutamate and dopamine, which are excitatory in nature [57]. The dysfunction of the ionic pumps also generates edema and swelling of neurons. Loss of Na^+-K^+-ATPase activity has other consequences in addition to depolarization. Many ion pumps, including the Na^+-Ca^{2+} exchanger, Na^+-H^+ exchanger, are dependent on the transmembrane Na^+ gradient for their activity. As this gradient declines, so does the activity of these exchange systems which leads to further disruption of ionic equilibrium. The early damage is reversible to some extent, however continuation of the ischemic condition for prolonged periods of time results in the irreversible damage to neurons and glia.

Despite the enormous scientific efforts that have been put to clarify the pathophysiology of cerebral ischemic damage, the cellular mechanisms responsible for neuronal cell death after cerebral ischemia remain unclear [58]. Current understanding of the molecular mechanisms such as excitotoxicity, ionic homeostasis, free radicals generation, mitogen activated protein kinase (MAPK) activation, and alteration in gene expression (Fig. 1.2) involved in cerebral ischemic reperfusion injury will be discussed in section 2.3 and 2.4.

1.2.1 Glutamate excitotoxicity

The accumulation of glutamate and increase in intracellular calcium [Ca^{2+}] are major contributors to neuronal damage during ischemic insult [59]. Ischemia triggers excessive glutamate release from presynaptic nerve terminals and astrocytes into the extracellular space andsubsequent activation of NMDA [60], amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) [61], Kainate [62], and G-protein coupled metabotropic glutamate receptors [63]. NMDA receptors are ion channels that allow passage of Ca^{2+} and Na^{+} ions. When activated by glutamate, the NMDA receptor allows Ca^{2+} to enter the cell. Large influxes of Ca^{2+} into neurons stimulate Ca^{2+} dependent enzymes including proteases, kinases, phospholipases, and nitric oxide synthase (NOS) [64]. If these processes are not properly regulated, they can eventually lead to cell death accompanied by the breakdown of the cytoskeleton, free radical formation, alterations in gene expression and protein synthesis and membrane dysfunction [65]. In the cases of kainite and AMPA receptor activation, the likely mode of injury is sensitization of the CA1 pyramidal cells during ischemia causing lethal cell injury from abnormal Ca^{2+} regulation in these cells or other metabolic derangement not yet understood [66-68].

Glutamate-induced swelling of astrocytes in various CNS regions has been observed *in vivo* and in *vitro* [13, 76] This type of swelling could be associated with the general class of Na^{+} coupled co-transporters that transport glutamate in astrocytes [69]. In astrocytes, the various processes of glutamate uptake and receptor activation of ion channels may work in parallel to affect glial swelling.

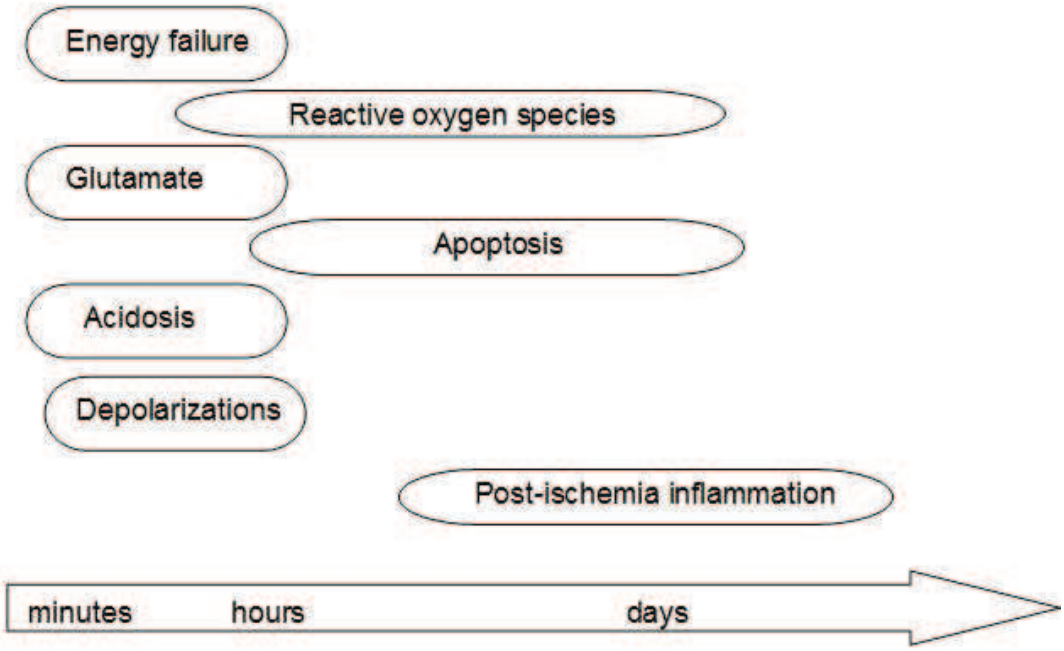


Figure 1.2 Temporal sequence of the mechanisms mediating focal cerebral ischemic damage. Schematic diagram from Contemporary Neuroscience™ Cerebral ischemia: Molecular and Cellular Pathophysiology. Edited by Wolfgang Walz. ISBN: 0-89603-540-9.

1.2.2 Calcium overload

Normally, the extracellular concentration of Ca^{2+} is about 10,000 times greater than the intracellular concentration. This difference is maintained by at least four mechanisms: 1) active extrusion of Ca^{2+} from the cell by an ATP-driven membrane pump, 2) exchange of Ca^{2+} for Na^{+} at the cell membrane driven by the intracellular to extracellular concentration gradient of Na^{+} as a result of the Na^{+}/K^{+} pump, 3) sequestration of intracellular Ca^{2+} in the endoplasmic reticulum (ER) by an ATP-driven process, and 4) accumulation of intracellular Ca^{2+} by oxidation-dependent Ca^{2+} sequestration inside the mitochondria.

The derangement of Na^{+}/K^{+} pump during ischemia virtually eliminates three of the four mechanisms of cellular Ca^{2+} homeostasis [70]. This causes a massive and rapid accumulation of Ca^{2+} in the cell. The diminished capacity of oxidative phosphorylation chains in mitochondria by oxygen deprivation causes an overload of Ca^{2+} too, as Ca^{2+} pumping across mitochondrial membrane consume energy as well. Elevated intracellular Ca^{2+} activates membrane phospholipases and protein kinases, which trigger the disruption of the plasma membrane further reducing the efficiency of the Ca^{2+} pump leading to further Ca^{2+} overload and a failure to regulate intracellular Ca^{2+} levels [71].

1.2.3 Free radicals

The generation of free radicals is associated with brain injury. The most common free radicals studied are superoxide (O_2^{-}), hydrogen peroxide (H_2O_2), hydroxyl (OH), and nitric oxide (NO) [72]. Free radicals are molecules possessing an unpaired electron in the outer orbit making them highly reactive. Free iron is a key catalyst of free radical-mediated injury and is readily available in injured brain tissue [72]. Free radicals damage endothelial cells leading to the disruption of the BBB. Free radical scavengers such as vitamin E, ascorbic acid, superoxide dismutase protect the cells against free radical-induced damage [73]. Free radicals contribute not only to the mitochondrial ultrastrutural changes seen after cerebral ischemia, but also to biochemical alterations such as the decrease of oxidative phosphorylation [74].

1.2.4 Gene activation

Accumulation of glutamate in the extracellular space and calcium overload in neurons activates many downstream processes including gene transcription. More than 100 genes which are denoted as immediate early genes (IEG) have been reported to be expressed within 15 min of the insult [75]. These include many proteins such as cytokines, enzymes and transcription factors such as *c-fos, fos-B, c-jun,* and *junD* which are involved in the transcriptional activation of other genes [76]. In parallel, heat shock genes are activated continuously or immediately following the IEG expression to stabilize the stressed cells (Fig. 1.3).

1.2.5 Potential therapies

In the synthesis of cholesterol in the liver, HMG CoA is converted to mevalonic acid by the enzyme HMG CoA reductase. Normally this enzyme is inhibited by a high dietary intake of cholesterol and conversely a reduction of dietary cholesterol may increase HMG CoA reductase activity. The HMG-CoA reductase inhibitors, commonly called statins are structural analogues of HMG CoA that competitively inhibit the enzyme, preventing cholesterol synthesis [77]. A decline in intracellular cholesterol levels promotes increased expression of cell surface low density lipoprotein (LDL) receptors and uptake of circulating LDL. HMG-CoA reductase inhibitors such as atorvastatin, fluvastatin, lovastatin, pravastatin and simvastatin have been reported to reduce plasma concentrations of LDL cholesterol by 20-60%, increase high density lipoprotein (HDL) cholesterol by 5-15% and reduce triglyceride by 10-45% [78].

Statins have been shown to reduce morbidity from coronary heart disease but also have an anti-oxidant effect that is neuroprotective during cerebral hypoxia, ischemia, and reperfusion by inhibiting isoprenylated proteins that are cholesterol-lowering independent. They also reduce leucocyte-induced LDL oxidation, increase α-tocopherol/total cholesterol ratio and maintain the enzymatic activity of HDL-peraoxonase that is consumed during HDL oxidation. Their effects are extended to preservation of superoxide dismutase (SOD) activity and the inhibition of cytokine-mediated up-regulation of iNOS, promoting an inflammatory

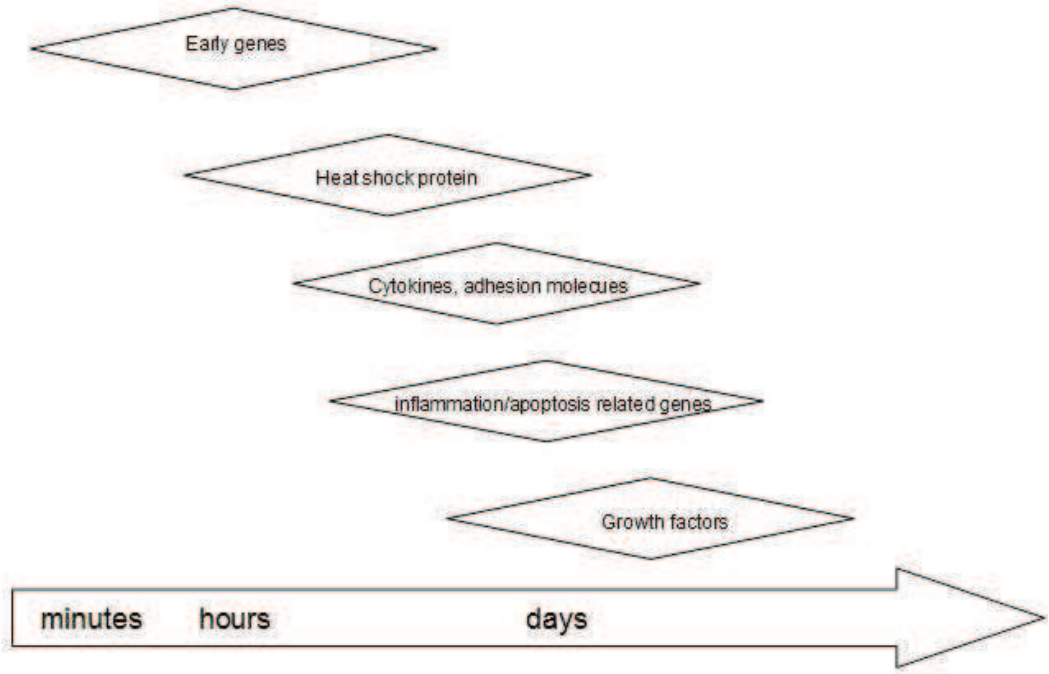


Figure 1.3 Temporal sequence of gene expression following focal cerebral ischemia. Schematic diagram from Contemporary Neuroscience™ Cerebral ischemia: Molecular and Cellular Pathophysiology. Edited by Wolfgang Walz. ISBN: 0-89603-540-9.

response and oxidative damage through the increased production of $ONOO^-$ [79].

Erythropoietin (EPO) is a glycoprotein hormone that controls red cell production and has a neuroproductive effect via its ability to deplete iron, decrease glutamate toxicity,activate apoptosis as well as its antiflammatory and antioxidative properties [80]. Clinical trials using erythropoietin have reported a reduction in infarct volumes and a better outcome in patients 30 days after stroke [81].

Neuroinflammation is a key event in postischemic injury and a number of anti-inflammatory drugs have been used to prevent neuroinflammation in stroke [82]. Indeed, the anti-inflammatory drug minocycline combined with the tissue plasminogen activator (tPA) has been shown to reduce the infarction volume and MMP-9 actvity in blood levels [35].

1.3 Mechanisms involved in cell volume regulation

Under physiological conditions, extracellular and intracellular osmolarities are subject to fluctuations due to changes in ionic content. Such fluctuations generate osmotic gradients that can induce severe damage to leading to cell death [83]. Therefore, preventing or minimizing excessive cell volume alteration is critical and is the mechanism for cells to successfully adapt to their microenvironment. Cells possess two primary mechanisms to regulate physiological volume stability when they encounter stress and both mechanisms function by altering ionic content within the cell ultimately driving water to its respective compartment.

To restore cellular volume in front of hypertonic challenges, cells undergo regulatory volume increase (RVI) [84], whereby several plasma membrane ion channels act alone or in concert to recruit Na^+ or Cl^- into the cell, creating an osmotic gradient responsible for drawing water out of the extracellular fluid and restoring cellular volume [85, 86]. Conversely, the regulatory volume decrease (RVD) reestablishes cellular volume when cells are exposed to hypotonic conditions by mechanisms designed to expel K^+ and Cl^- [87, 88]. *In vivo* and under physiological conditions, the cell size does change dramatically during the process of apoptosis. Cells undergoing apoptosis lose water and shrink in a process that is known as the apoptotic volume decrease (AVD). The AVD adopts a similar mechanism as RVI and RVD to make use of ionic fluxes across the plasma membrane and create the

osmotic gradients necessary to bring about volume change.

Much work has been done on the various ionic channels constituting these volume regulatory mechanisms, however little attention was given to the pathway(s) through which water crosses the plasma membrane. The water channel proteins termed aquaporins (AQPs) are critical in regulating this water movement in a large number of physiological systems and defects of AQP function have been implicated in numerous diseases and pathological conditions such as stroke [89], loss of vision and cataracts [30, 58, 90], obesity [91]. The importance of these channels in neural tissue function [92] and their role in the onset of brain edema in response to hypoxia and ischemia have been widely investigated [93, 94].

1.4 Aquaporins

Simple diffusion through the lipid bilayer is not the only way water crosses the plasma membrane. A water channel family, the aquaporins, facilitates water flux through the plasma membrane of many cell types, providing a higher water permeablility than simple diffusion. Aquaporins are a subset of the Major Intrinsic Protein (MIP) family of proteins [95]. To date, 13 AQPs (designated AQP0-AQP12) have been identified and cloned in mammals. Their molecular weight is approximately 30 kD and varies due to glycosylation on the extracellular domains [96, 97]. Evidence based on AQP-1, 3, 4, 9 knockout mice revealed various function of AQPs ranging from reduced tumor angiogenesis and cellular migration to providing energy substrates such as glycerol to epidermis and fat tissues for skin hydration and fat metabolism [98, 99].

Monomeric AQP polypeptides exhibit six transmembrane helix domains with intracellular carboxyl and amino termini. The channel functions as a heterotetramer when at the plasma membrane. Each monomer surrounds a water pore that forms a transporter allowing water transport across the membrane in both directions [100]. Mammalian AQPs can be divided into two groups (orthodox AQPs and aqualyceroporins) based on their genomic organization and ability to exclusively transport water (AQP0, AQP1, AQP2, AQP4, AQP5) or both water and small nonionic molecules such as glycerol and urea (AQP3, AQP7, AQP9, AQP10) [101]. Other AQPs including AQP6, AQP8, AQP11 and AQP12 have a unique function and genomic organization (with the exception of AQP6) and remain

unclassified [102]. AQPs not only act as a water channel proteins as they are abundantly expressed in fluid-transporting tissues such as kidney, liver, and epithelia, but are also expressed in neurons and astroglia indicating that these channels play fundamental roles in normal cellular function of the brain [103]. Generally, water permeability results from the combination of simple water diffusion through the lipid bilayer and movement through AQPs. Nevertheless, in many cell types the water transport through AQPs constitutes the major permeability pathway and AQPs act as a rate-limiting step in this process [104, 105].

1.4.1 Aquaporins in the brain

Several AQP isoforms are expressed in brain tissue. AQP1, for example, is restricted to the choroid plexus of the lateral ventricles where it appears to be responsible for providing water to the cerebral spinal fluid [105, 114]. It is also expressed in primary sensory neurons responsible for nociception. Interestingly, no phenotypic differences in nociception were detected in the AQP1$^{-/-}$ mouse [106], suggesting that redundant mechanisms may be present. AQP1 has recently been reported to be localized to astrocytes [107] and AQP1 has been linked to migrating ability of malignant astrocytes [108]. Indeed reintroducing AQP1 into glioma cell lines shows that AQP1 is not only sufficient to restore water permeability but also enhances cell growth and migration, typical properties of gliomas.

AQP4 is by far the most predominant water channel in the brain and is found in ependyma and pial surfaces in contact with the CSF as well. It is expressed in periventricular areas, and astrocytic endfeet adjacent to blood vessels [109-111]. This distinct expression pattern of AQP4 allows water exchange between blood and brain, a crucial interface for homeostatic volume regulation. The function of AQP4 has been widely explored and will be discussed in the following section.

AQP9 protein is strongly expressed in brain as well as liver, testes, epididymis and spleen [112-115]. This AQP channel is permeable to water and several solutes including carbamides, polyols, purines, and pyrimidines [116]. Its expression in the CNS is primarily restricted to catecholaminergic neurons, which are not thought to be directly involved in osmoregulation. In this context, it is believed that brain AQP9 may function as a metabolic transporter of glycerol and lactate. Indeed, it has been suggested that AQP9 might participate

in the *lactate shuttle* model [117]. Under pathological conditions such as hypoxia/ischemia, AQP9 may participate in the clearance of excess lactate from the extracellular environment.

In 1994, AQP4 mRNA was first detected in the rat brain by in situ hybridyzation [111] and AQP4 structure was revealed using crystallography [118-120]. A polypeptide chain of AQP4 contains six α-helix transmembrane domains, they forms a pore that allows water movement by surrounding the two shorter helices containing two NPA motifs (Asparagine-Proline-Alanin). Due to the absence of cysteine in position 189, AQP4 is not sensitive to mercury (Fig. 1.4A) [111].

Monomers of AQP4 physically bind together and form homotetramers. The assembly of AQP4 at the plasma membrane forms large clusters (100 nm) called orthogonal array particles (OAPs). Indeed, evidence from the literature has shown that the AQP4-null mouse lacks OAPs, whereas oocytes transfected with AQP4 cDNA present with OAPs that are visible through freeze fracture electron microscopy (FFEM) (Fig. 1.4B) [51, 121]. It is not clear why AQP4 forms OAPs, whereas other AQPs do not. Sequence alignments of OAP-forming vs. non-OAP-forming AQPs have not been informative in identifying contact surfaces that could energetically stabilize OAP assembly [122]. It was recently postulated that the colocalization of OAPs comprising AQP4 and the inward rectifier K^+ channel 4.1 (Kir4.1) in retinal Muller cells might facilitate local water flux associated with K^+ siphoning [123].

Recent studies reveal that AQP4 has six splice variants, AQP4a-f [124]. Two isoforms of AQP4 result from two different mRNAs with different translation initiation sites either at the first methionine residue, M1 (AQP4a), or at the second methionine, M23 (AQP4c) (Fig. 1.4A). Therefore, the amino acid sequence of M1-AQP4 is longer than M23-AQP4 by 22 residues at its N-terminus [83, 124, 125]. It is subjected to palmitoylation on cysteines 13 and 17 and these can interact with the N-terminal residues and the first NPA motif [126, 127]. Visualizing fluorescently labeled AQP4 mutants in cell membranes using quantum-dot single-particle tracking (SPT) and total internal reflection fluorescence microscopy (TIRFM) revealed that M1-AQP4 and M23-AQP4 have different mobilities at the plasma membrane [128]. M23-AQP4 is nearly immobile in the membrane and tends to promote the formation of large OAPs at astrocyte endfeet, whereas M1-AQP4 diffuses freely in the membrane, resulting in the disruption of OAP formation. It has been shown that M1-AQP4

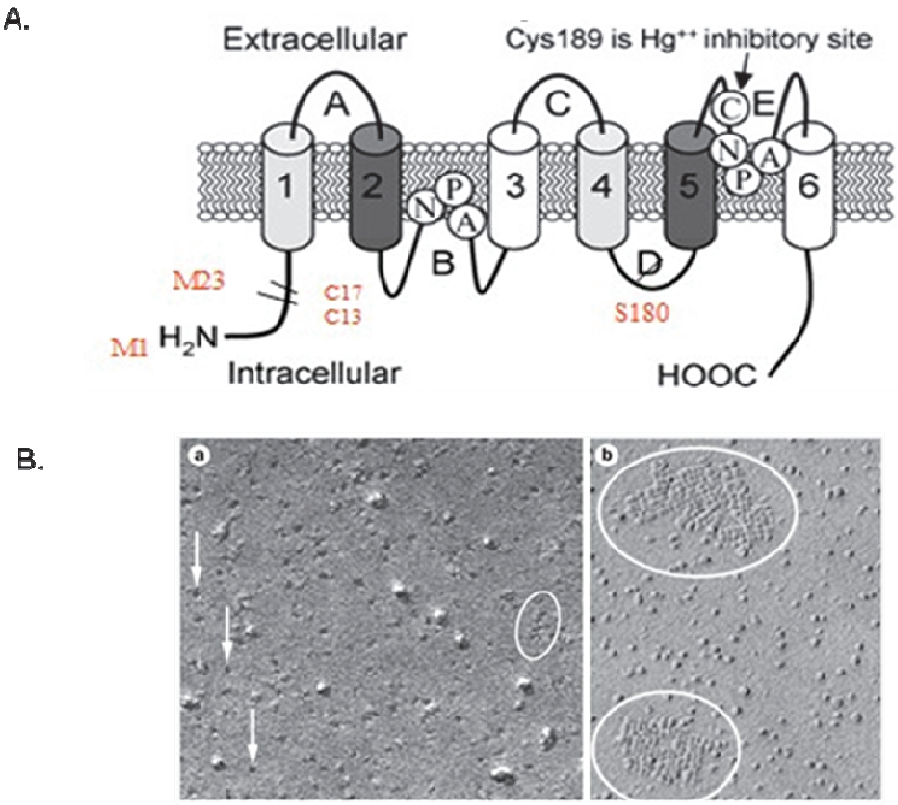


Figure 1.4 Schematic representation of AQP4 structure and M1/M23-dependent formation of orthogonal array of particles (OAPs). A. AQP4 monomer consists of six transmembrane domains surrounding a water pore and two conserved NPA motifs (Asn-Pro-Ala) that allow water but not small solutes or protons to pass across the pore. AQP4 contains a PDZ-binding motif localized at the C-terminus and residues that can undergo palmitoylation (C13 and C17) or phosphorylation (S180). **B.** In CHO cells transfected with M1-AQP4, most tetramers are present as singlets (left panel), only very few (<5%) are organized into small OAPs. In CHO cells transfected with M23-AQP4, large OAPs are detectable at the plasma membrane (right panel). From Jarius and Wildemann. *Nature Reviews Neurology* 6: 383-392 (2010).

palmitoylation is partially involved in this effect [129, 130]. The relative amount of M1-AQP4 versus M23-AQP4 at the membrane determines the size of OAPs, which are made of hundreds of tetramers. The ratio between M23-AQP4 and M1-AQP4 is approximately 3:1 in brain [124, 127, 131-133]. OAP formation by M23-AQP4 is stabilized by hydrophobic intermolecular interaction involving the N-terminal residues. Two lines of evidence suggested that AQP4 PDZ-binding domain deletion does not prevent OAP assembly. First, the observed diffusion of C-terminal deletion mutant of M23-AQP4 (M23Δ6) is lower than AQP4-M1. Second, short-range SPT indicated that diffusion of individual M23Δ6 tetramers is identical to that of full-length M23-AQP4. Therefore, it was concluded that the C-terminal PDZ-binding domain of AQP4 is not involved in OAP assembly but does provide an anchor to assembled OAPs, probably by interacting with structures on the cytoplasmic side of the membrane [130].

1.4.2 Phosphorylation of aquaporin 4

AQP4 has many phosphorylation sites (Fig. 1.4A) that are substrates for protein kinase C (PKC), G and A or Ca^{2+}/calmodulin-dependent protein kinase II [93, 134-138]. Serine 180 is a substrate for PKC [148, 150, 151]. Activators of PKC such as phorbol 12-myristate 13-acetate (PMA) and phorbol 12, 13-dibutyrate (PDBu) used either 60 min before or 30 min following focal ischemia prevent brain edema [139]. In addition, these two agents were reported to decrease the water permeability of AQP4 when expressed in oocytes [140].

In LLCPK1 cells expressing AQP4, activation of PKC with phorbol 12 13-dibutyrate (PDBu) and dopamine decrease the osmotic water permeability of the membrane. This effect was abolished by treatment with the PKC selective inhibitor Ro-31-8220 [93, 134]. AQP4 bearing the Ser180Ala mutation in cytosolic loop D is not affected by such a treatment, suggesting that Ser180 in loop D is a specific target residue for PKC-mediated phosphorylation of AQP4 and is involved in mediating water permeability. On the other hand, the mutation of Ser180 does not affect the formation of OAPs, indicating that OAP formation is independent of Ser180 phosphorylation induced by PKC [93]. The phosphorylation of Ser111 in loop B, which has been shown as a potential phosphorylation site for CaMKII increases the water permeability of AQP4 and the evidence shows that the membrane water

permeablity of astrocyte cell line expressing exogenous AQP4 was increased by lead treatment, which potentiates CaMKII activation [141]. The water permeability of AQP4 bearing the mutation Ser111Ala was not affected by lead treatment, suggesting that AQP4-Ser111, which is specifically phosphorylated by CaMKII, involved in the regulation of water permeability. Glutamate or 3,5-dihydr-oxyphenylglycine (DHPG), mGluR 1/5 agonist induce an increase in water permeability of astrocyte primary cultures or an astrocyte cell lines expressing AQP4. These effects are mediated by intracellular Ca^{2+} signaling that is prevented by the neuronal nitric oxide synthase (nNOS) inhibitor 7- nitroindazole. However, it does not affect water permeability in AQP4-Ser111Ala expressing cells. These results suggest that mGluR-CaMKII-NO might act upstream of PKC mediated-phosphorylation of Ser111 [141].

1.4.3 Aquaporin 4 function

AQP4 is localized to astrocyte processes adjacent to blood vessels as well as at ependymal and pial surfaces in contact with CSF (Fig. 1.5A) Similarly, in retina, AQP4 is enriched in Müller cell endfeet abutting blood vessels and the inner limiting membrane (ILM) [142]. This distribution pattern suggests a role of AQP4 in water movement in and out of the central nervous system [38].

AQP4 has been shown to play a role in water accumulation in the early stage of brain edema (Fig 1.5B), but also in the removal of excess water involved in brain swelling [143]. A growing body of evidence based on human diseases and animal models of diseases suggest that AQP4 plays a major role in water transport [100, 144-147]. In human, three studies reported six mutations in AQP4 that alter water transport [144-146]. Multiple mouse models have been employed to explore the role of AQP4 in the pathogenesis of cerebral edema. The main approaches have been either to knockout AQP4 expression completely or to disrupt the polarized subcellular expression of AQP4 [100, 147]. A key potential mechanism for altered subcellular distribution of AQP4 is the disruption of the dystrophin associated protein (DAP) complex, which has an essential role in anchoring AQP4 at the perivascular astrocyte membrane. First, in dystrophin-null mice, the expression of AQP4 is dramatically reduced in astroglial endfeet surrounding capillaries and at the glia limitans (CSF-brain interface) despite the lack of alteration in total AQP4 protein levels [100]. Secondly, mice deficient in

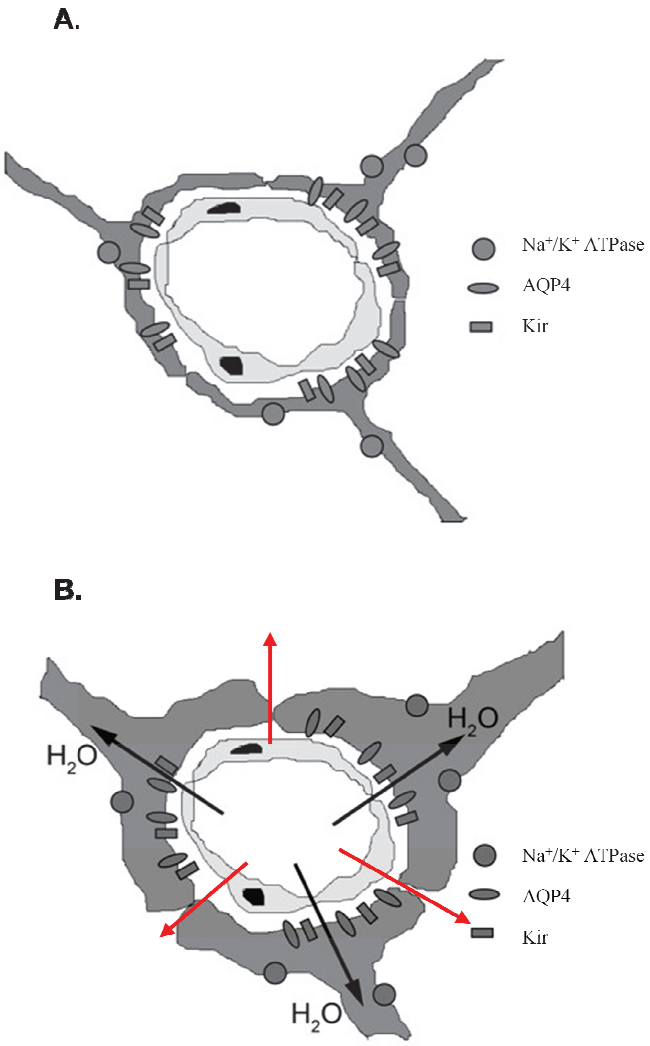


Figure 1.5 Distribution of AQP4 at astrocyte endfeet and its role in cytotoxic edema. .A. Interaction of glial cell endfeet with the vascular endothelial cells and positioning of AQP4, Na^+-K^+-ATPase, and Kir potassium channels. **B.** In cytotoxic edema, glial cells swell due to AQP4-mediated water flow from the blood into the brain. From Ayus et al., Am J Physiol Renal Physiol 295: 619-624 (2008).

α-syntrophin, an adapter protein associated with dystrophin, show marked loss of AQP4 from perivascular and subpial membranes as judged by quantitative immunogold electron microscopy [42, 147]. These studies indicate that alterations in components of the DAP complex affect the subcellular targeting and function of AQP4. In this regard, α-syntrophin-dificient mice also demonstrated a deficit in the extracellular K^+ clearance following evoked neuronal activity [147].

Phenotypic analysis of the AQP4 null mouse has provided new insights into the mechanisms of water transport during the development of cerebral edema [148]. The loss of AQP4 has a significant impact on pathological responses of the CNS. The deletion of AQP4 results in a reduction of brain swelling and the infarct size and volume in cytotoxic brain edema models induced by water intoxication or traumatic brain injury [149]. These studies have also shown that AQP4 null mice have improved survival and neurological outcome following water intoxication, focal cerebral ischemia and bacterial meningitis [143, 150]. On the contrary, these mice have more brain swelling in models of vasogenic edema where excess fluid accumulates in the extracellular space, due to impairment of the AQP4-dependent water clearance (Table. 1.1) [148]. Based on these findings it was concluded that AQP4 facilitates the clearance of water in vasogenic cerebral.

1.4.4 Expression of aquaporin 4

The expression of AQP4 during brain edema has been widely investigated and the controversial results obtained in different models suggest that redundant mechanisms occur during brain edema [151-156]. Two peaks of hemispheric swelling have been shown to coincide with two peaks of AQP4 up-regulation at 30 minutes and 40 hours after transient focal brain ischemia [154, 156]. Nevertheless, many studies have shown that AQP4 expression decreases from 1 to 24 hrs post ischemia [153, 156-158]. Several groups have shown that hypoxia/ischemia insult evoked a marked decrease in astrocytic AQP4 mRNA and protein expression [93, 153, 159, 160]. Furthermore, it has been shown that the hypoxia inducible factor-1α (HIF-1α) regulates expression of AQP4 in hypoxic conditions [161]. On the other side, several studies have shown that in the models of vasogenic edema,

Brain edema types	BBB integrity	Caused by	AQP4 effect
Cytotoxic	Intact	Water intoxication	Increased cell swelling, increased edema
		Acute hyponatremia	
		Focal cerebral ischemia	
Vesogenic	Leaky	Brain tumor	Decreased edema
		Cortical freeze injury	
		Intraparenchyma fluid infusion	
		Brain abcess	

Table 1.1 Summary of AQP4 effects in different types of brain edema. Modified from Eric Beitz et al., Aquaporins, Handbook of Experimental Pharmacology 190. Springer. (2009).

including traumatic brain injury [58, 155, 162-164], brain abscess [150, 165]or subarchnoid hemorrhage [166, 167], the expression of AQP4 increases after 24 hours and subsequently drops at 48 hours [151, 152]. Interestingly, the distribution of such upregulated AQP4 is mainly localized at the BBB, the ependyma and the glia limitans where edema fluid clearance occurs [168]. These variations of AQP4 expression have been correlated with the resolution of edema via the use of magnetic resonance imaging (MRI) [169]. It should be noted, however that each of these studies used various hypoxia/ischemia-inducing methodologies to stimulate edema (cytotoxic vs. vasogenic) in various brain regions (focal vs. global, for example) which vary in their hypoxia/ischemia vulnerabilities. The well established role of AQP4 in the bidirectional transport of water is consistent with the down-regulation of AQP4 during hypoxia/ischemia as a protective factor against water influx into astrocytes at early stages of edema, and its up-regulation at later stages assisting therefore water clearance from astrocytes [170]. It is widely agreeable that excess fluid leaves the brain parenchyma through three different routes: across the BBB, the ependyma into the ventricles and across the glia limitans into the CSF (Fig. 1.6) [171]. The localization of AQP4 at those specific sites is crucial for water efflux as shown in the syntrophin null mouse with mislocalized of AQP4 [42, 172]. Together these observations suggest that the up-regulation of AQP4 is beneficial in cases of vasogenic edema. [151, 166].

An abmormal accumulation of extracellular K^+ in the brain has been implicated in the generation of seizures in patients with mesial temporal lobe epilepsy (MTLE) and hippocampal sclerosis [173]. Several studies revealed a spatial overlap of Kir4.1 and AQP4 in astrocyte endfeet contacting capillaries and suggested that buffering of K^+ via Kir4.1 depends on concomitant transmembrane flux of water in the same cell [53, 174, 175]. These parallel water influxes are thought to be necessary to dissipate osmotic imbalances due to K^+ redistribution. In agreement with this hypothesis, clearance of extracellular K^+ is compromised upon reduction of the perivascular pool of astroglial AQP4 and impaired K^+ buffering in concert with prolonged seizure duration is observed in $AQP4^{-/-}$ mice [174]. In the sclerotic hippocampus of TLE patients dislocation of water channels in concert with reduced expression of Kir channel in astrocytes probably underlies the impaired K^+ buffering leading to increased seizure propensity.

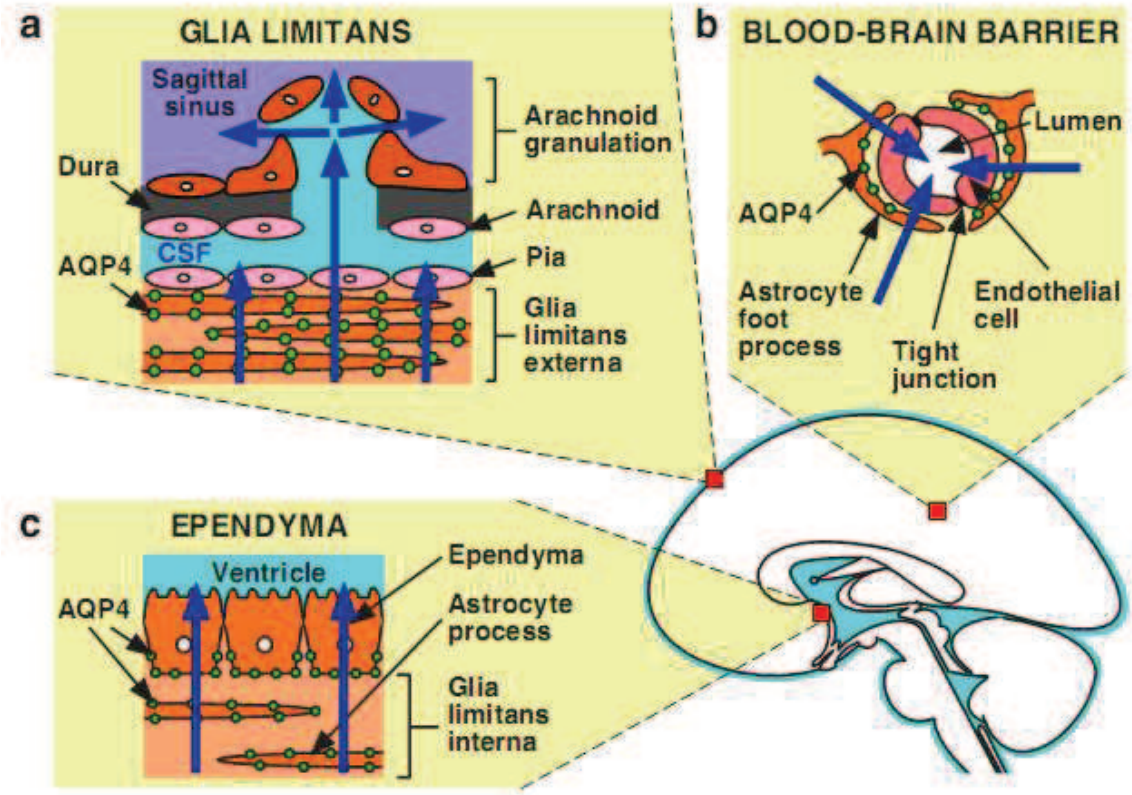


Figure 1.6 Routes of water exit in brain edema. In both cytotoxic and vasogenic brain edema, excess fluid is eliminated through aquaporin-4 (AQP4) rich barriers from **a.** the glia limitans externa into the subarachnoid space; **b.** the blood-brain barrier into the bloodstream; **c.** the glia limitans interna and ependyma into the ventricles. From Papadopoulos and Verkman. Pediatr Nephrol 22: 778–784 (2007).

1.4.5 Role of the dystroglycan-associated protein complex in aquaporin 4 polarized distribution

Muscular dystrophy (MD) is characterized by progressive muscle weakness and degeneration. Central to a large number of MDs is a defect in the DAP complex [176]. Mutations in different components of this complex, defects in their post-translational modifications and interaction with the ECM have been shown to cause MDs as well as brain abnormalities [177, 178]. The DAP complex includes numerous intracellular proteins such as syntrophin, dystroberevin and dystrophin, which are downstream of dystroglycan. The dystroglycan (DG) gene encodes a precursor protein that is post-translationally cleaved into α-DG and β-DG. β-DG is a transmembrane protein, whereas α-DG represents the extracellular component of the DAP complex [179, 180]. α-DG binds the extracellular matrix proteins laminin, agrin and perlecan and neurexin [179, 181-187]. Via its C-terminal domain β-DG binds directly to dystrophin, which in turn binds F-actin, syntrophin and dystrobrevin (Fig. 1.7) [188]. The link between the actin cytoskeleton and the ECM through the DAP complex enables the maintenance of muscle fibres during contraction-relaxation cycles [182, 187, 189-191].

The DAP complex is also present in the CNS where DG is referred to as cranin [179, 186]. Indeed, several forms of MDs also present a large spectrum of disabilities, including learning disability, and mental retartation and epilespy.

Immunolocalization studies show that dystrophin, utrophin, dystrobrevin, syntrophin, and DG are localized and concentrated postsynaptically in Purkinje cells in cerebellum, pyramidal neurons in hippocampus and cerebral cortex, and at astrocyte endfeet in the brain as well as Müller cells endfeet in the retina [192]. In these cell domains, A growing body of evidence suggests a role for the DAP complex in the subcellular distribution of various channels [193-195]. This prompted the investigation of expression level of AQP4 in mice deficient in various components of the dystroglycan complex.

Co-immunoprecipitation studies show that AQP4 binds to the dystrophin complex through its interaction with α-syntrophin and Dp71. α-syntrophin deficient mice lack polarized expression of AQP4 in astrocyte endfeet. Immunogold labeling demonstrates an ~eightfold reduction of AQP4 reactivity at the perivascular astrocyte endfeet of α-syntrophin

deficient mice compared to wild-type. Such mislocalization was near equivalent to the phenotype of AQP4 knockout mice [147]. The development of cytotoxic edema also significantly retards in α-syntrophin deficient mice following acute hyponatremia and transient cerebral ischemia [100]. A similar impact has been shown in the dystrophin mutant *mdx* mouse [175]. Interestingly, these mutant mice have similar AQP4 expression levels as the wild-type controls, indicating the mere mislocalization of AQP4 is sufficient to impair channel function to a significant extent. Recent data has demonstrated that marked reduction of AQP4 in glial cells of the retina (Müller cells) isolated from Dp71-deficient mice [175]. Electrophysiological studies have shown reduced potassium currents in relation to the reduced AQP4 expression further fortifying the concept of a Kir4.1-AQP4 K^+-water trafficking complex.

The abolishment of AQP4 polarization by modulating the components of the DAP complex is a more attractive strategy than manipulating the AQP4 expression directly because it avoids changing AQP4 expression in other tissues. A recent study demonstrated that AQP4 is organized in the plasma membrane into several distinct pools that have different sizes that range from >>1MDa to ~500kDa [37]. Despite the fact that the dystrophin-dependent perivascular AQP4 pool is dramatically decreased, a smaller AQP4 pool expressed at the inner limiting membrane in the retina, the subpial endfoot layer and the ependymal cells are not altered, indicating that the dystrophin complex is not necessary for the proper localization of all AQP4 pools. Likewise, α-syntrophin deficiency does not impair AQP4 localization in the glia limitans, confirming the finding that the AQP4 pool at the glia limitans in the brain is not dependent on α-syntrophin [37].

1.5 Reactive oxygen species

Reactive oxygen species (ROS) are a natural by-product of oxygen metabolism and include oxygen ions, free radicals, and peroxides. They are highly reactive small molecules due to the presence of unpaired electrons. When biological stress occurs, the level of ROS increases, resulting in cellular damage [196]. In contrast, in certain physiological processes, ROS is beneficial and plays a role in the induction of mitogenic responses, immune defence, apoptosis, and breakdown of toxic compounds [197].

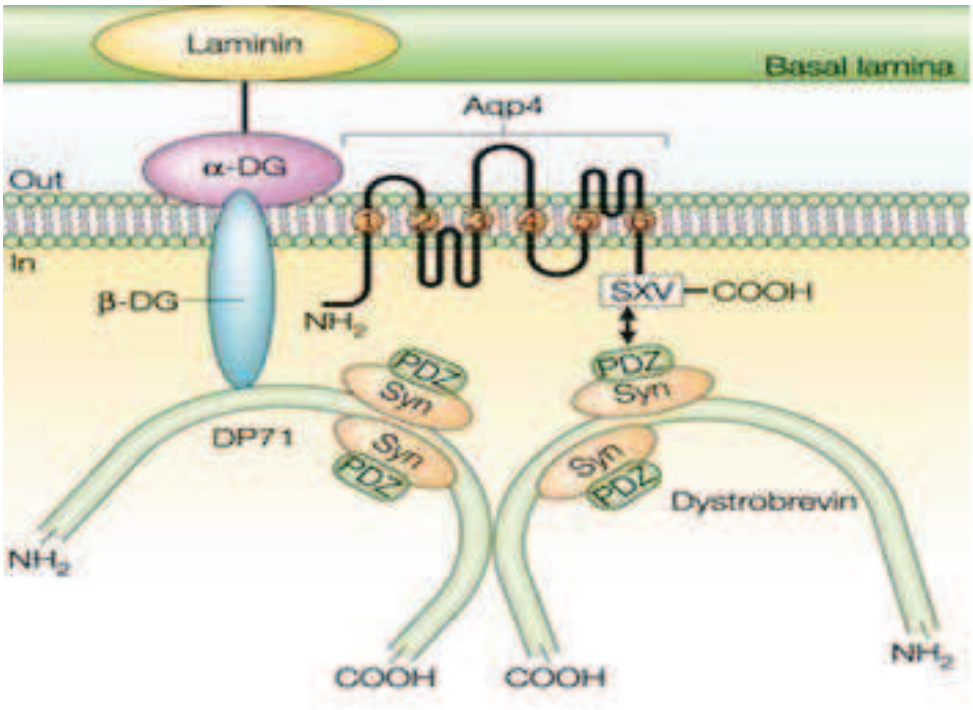


Figure 1.7 Schematic representation of the molecular interactions between the DAP complex, the extracellular matrix and AQP4 at perivascular astrocyte endfeet. β-DG is a transmembrane protein that binds to extracellular α-DG. α-DG binds to laminin in the extracellular matrix. Dystrophin binds to syntrophin which interacts with AQP4 via its PDZ domain. β-DG acts as a bridge linking cytoskeletal actin to the extracellular matrix. Amiry-Moghaddam and Ottersen. Nature Reviews Neuroscience 4: 991-1001 (2003).

O_2^- is the primary ROS and generates H_2O_2 by dismutation. O_2^- is produced in cells via a number of enzymatic reactions and common cellular sources of O_2^- include auto-oxidation of small molecules, such as hemoglobin, mitochondrial components and oxidative enzymes, e.g. xanthine oxidase (XO), nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase, cyclooxygenases, and oxidation of unsaturated fatty acids [197]. The rate of O_2^- generation is dependent on the concentration of oxidisable substrates, the availability of oxygen, and activity of antioxidant enzymes. OH^- is generated from H_2O_2 in the presence of ferrous iron that has been reduced by O_2^- [197]. A variety of ROS is generated by the reaction of oxygen radical with other components, e.g. O_2^- with nitric oxide (NO) produces peroxynitrous acid (ONOOH) that spontaneously decomposes to produce OH^- [197].

Additionally, reactive nitrogen species (RNS) are also generated in normal physiological and pathological conditions. The nitric oxide radical (NO) is generated by specific nitric oxide synthases (NOSs), neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) that metabolise L-arginine to L-citrullin [30]. NO plays a role in regluating neural signaling, blood pressure, smooth muscle relaxation and immune surveillance. Nitrosative stress is the term describing the increased production of RNS. O_2^- and NO can react to produce the peroxynitrite aniaon ($ONOO^-$), which can cause DNA fragmentation and lipid peroxidation [198].

1.5.1 Role of reactive oxygen species in normal physiological processes

It has been well established that ROS have a central role to play in the induction of apoptosis. Aside from their role in apoptosis, ROS have now also been shown to play an important role in cell survival signalling and redox regulation of stress-induced signalling pathways. Lower levels of ROS have been demonstrated to prevent apoptosis [199, 200]. There is substantial evidence showing that ROS can cause direct modifications such as disulphide bond formation and glutathionylation of signalling proteins, altering therefore the activity of certain proteins and alter their sensitivity to apoptosis [201].

Many studies have shown that ROS enhance cell survival by triggering the activation of certain signalling pathways that protect against cell death. These have included activation of the MAPK pathway [202], ROS-induced activation of phospholipase C-γ 1 [203], epidermal

growth factor receptor (EGFR)-dependent activation of Akt [204], activation of VEGFR-3 signalling in response to H_2O_2 [205], activation of NF-κB [203] and PI3K/Akt [58].

H_2O_2 has been shown to inhibit the activity of PTEN through oxidation of its active site cysteine residue, resulting in activation of the PI3K/Akt survival pathway and thus promoting cellular proliferation and preventing apoptosis [199]. It has been demonstrated that addition of exogenous H_2O_2 stimulated the phosphorylation of Akt [206]. ROS have also been shown to be responsible for the activation of *src* leading to phosphorylation of EGFR and subsequent activation of extracellular signal-regulated protein kinase and Akt signalling pathways, ultimately providing protection from anoikis via the degradation of Bim [207].

1.5.2 Role of reactive oxygen species in normal brain physiology

ROS is crucial for neuronal signaling in both the central and the peripheral nervous system. In the peripheral nervous system, ROS inhibit acetylcholine release from motor nerve endings via ATP [208] and may enhance or depress synaptic transmission in the CNS [209]. Indeed, ROS enhance the effect of PKC promoting increased excitatory postsynaptic potentials in the hippocampus [210]. H_2O_2 has been shown to inhibit dopamine release modulate vesicular neurotransmitter release [211].

Microglia and astrocytes are major sources of ROS, which modulate synaptic transmission and non-synaptic communication between neurons and glia. O_2^-, H_2O_2, and NO all participate in neuron-glia communication in the hippocampus [212]. When neuronal activity increases, ROS and RNS diffuse to the myelin sheath of oligodendrocytes, activating PKC and post-translationally modifying myelin basic protein, a key structural component of myelin [212].

ROS has been shown to induce synaptic long-term potentiation (LTP) required for memory formation [213, 214]. In the aged transgenic mouse overexpressing extracellular SOD prolonged hippocampus LTP, improved cerebellum-dependent learning and hippocampus-dependent spacial learning were observed [159]. The level of in EC-SOD mouse strain, O_2^- levels are lower than in the wild type mouse, suggesting that there is an age-dependent alteration in O_2^- and its consequent modulation of synaptic plasticity, learning and memory [214].

1.5.3 Cellular effects of reactive oxygen species in ischemia stroke

Lipid peroxidation is one of the major consequences of ROS-mediated injury in brain. This leads to the production of conjugated dienic hydroperoxides that decompose and are converted into aldehydes, dienals or alkanes. In stroke, the hydrolysis of membrane phospholipids and release of free fatty acids, e.g. arachidonic acid (AA) are increased due to enhanced activity of phospholipase A2 [215]. ROS are a byproduct of AA metabolism and react with lipid to produce lipid peroxides that are further degraded into aldehydes such as 4-hydroxynonenal (HNE), which is toxic to neurons and white matter [215].

ROS are involved in oxidative modification and endonuclease-mediated DNA fragmentation [216, 217]. DNA oxidation may activate repair enzymes, the reaction involves these enzymes inducing a rapid depletion of intracellular energy.

Evidence from the literature has shown that ROS act via redox sensitive signal transduction pathways [3]. In primary mouse endothelial cells, ischemia has been shown to activate the Erk1/2 pathway by inducing VEGF through its receptor, Flk-1 [218]. ROS is also implicated in p38-MAPK and HSF-1 pathways [219].

ROS induces the release of cytochrome c from mitochondria that binds apoptotic protease activating factor 1 (Apaf-1) and deoxyadenosine triphosphate, forming apoptosomes that activate caspase-9 [220]. ROS is also involved in the induction of the phosphorylation and translocation of the B-cell lymphoma 2 (Bcl-2)-associated death promoter (Bad) from the cytosol to the mitochondria in models of brain ischemia [221]. These pro-apoptotic factors subsequently activate caspase 3 and finally lead to apoptotic cell death.

1.5.4 Role of reactive oxygen species in endothelial cell permeability

Numerous studies from *in vitro* and *in vivo* have shown that ROS can increase endothelial permeability, and this effect can be reversed by antioxidant and free raical scavengers. Rac1 is a major component of the vascular NADPH oxidase complex [222]. Expression of constitutively active Rac1 results in ROS production concomitant with disruption of VE-cadherin at cell junctions, tyrosine phosphorylation of α-catenin, and ultimately increase permeability [223]. The mechanism involved has been clarified, Rac-

mediated ROS production leads to activation of the tyrosine kinase Pyk2, which subsequently phosphorylates β-catenin and thus destabilizes the adhesion junctions (AJ) [224]. ROS generation is considered as a downstream of a variety of vasoactive factors, such as VEGF. It has been shown that VEGF-induced junctional disruption and ROS production similarly involves Rac1 activation [222].

ROS alter vascular permeability involves regulation of junctional protein phosphorylation, which is associated with increase permeability. ROS can strongly inactivate protein phosphatase by oxidation of active cysteine residue in the catalytic site [225]. The prevalence of phosphatases (VE-PTP, DEP-1, PTPμ, SHP-2) at endothelial cell (EC) junctions suggests that they are critical for maintaining the low basal phosphorylation levels conductive to junctional integrity [226]. Localized production of ROS and inactivation of phosphatases at these sites contribute to elevation of phosphorylation and junctional disruption. ROS may also regulate junctional permeability by affecting the organization of the actin cytoskeleton [227], via the activation of focal adhesion kinase (FAK) [228].

1.5.5 Generation and clearance of reactive oxygen species

It has been demonstrated that approximately 2% to 5% of the electron flow in isolated brain mitochondria produces superoxide anion radicals (O_2^-) and hydrogen peroxide (H_2O_2) [83]. These constantly produced ROS are scavenged by SOD, glutathione peroxidase (GSHPx), and catalase [83]. Other small molecular antioxidants, including glutathione (GSH), ascorbic acid, are involved in detoxification of free radicals too [229, 230]. The conjunction of anoxic depolarization and decreased glutamate transport by GLT1 and GLAST1 during ischemia lead to a dramatic increase of extracellular glutamate concentration from 0.6 to 320 μM [231]. Correspondingly, decreased intracellular glutamate concentration in astrocytes limits the available amount of glutamate, which is necessary for the production of glutathione, the most abundant antioxidant in the CNS [232]. The limited generation of glutathione therefore leads to increased ROS levels generated by mitochondrial free radical production. Ischemic damage can paradoxically be amplified by reperfusion that corresponds to the restoration of oxygenated blood flow to the ischemic tissue. This is mainly due to the generation of ROS, which may result from the reactivation of respiratory

complexes blocked in a reduced state without functional coordination (inefficient transfer of electrons generating superoxide anions). ROS have direct effects on the respiratory chain components such as complex I, resulting in decreased efficiency of oxidative phosphorylation. Changes in complexes III and IV were also observed, but this would occur later during the deleterious process [233]. They also cause inhibition of the enzymes of the Krebs cycle (i.e. aconitase). ROS cause non-specific damages to lipids, proteins and mitochondrial DNA and induce peroxidation of cardiolipin, a major constituent of the inner membrane, which increases the inhibition of oxidative phosphorylation. It was demonstrated that permanent MCAO was accompanied by a steady increase of ROS production for at least 3 hours and reperfusion after 1 hour of transient MCAO produced a burst in ROS formation. Increased ROS is involved in regulating many cerebral ischemia-related genes. For example, it can directly activate MMP9, accelerating therefore MMP9-mediated breakdown of the BBB. MMP9 can also activate interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNFα), leading to worsening this phenotype [234].

1.5.6 Reactive oxygen species and gene expression

Emerging evidence indicates that ROS, especially superoxide and hydrogen peroxide, are important signaling molecules that serve as second messengers to activate multiple intracellular proteins and enzymes in brain ischemia, including growth factor receptor [235], *c-src* [236], p38 mitogen-activated protein kinase [202], Ras [19], and Akt/protein kinase [199]. Activation of these signaling cascades and redox-sensitive transcription factors leads to the induction of many genes that play important functional roles in the physiology and pathophysiology in CNS [237]. One of the transcription factors that has been investigated during the past few years is NF- B [212]. Hydroxyl radicals (OH^-) and superoxide anion (O_2^-) participate in the activation of NF- B and antioxidant that scavenge these free radicals inhibit NF- B activation [238].

1.5.7 Reactive oxygen species and aquaporin 4 expression

In brain, manganese was shown to induce cell swelling in cultured astrocytes, and

oxidative/nitrosative stress (ONS) has been shown to mediate such swelling. Treatment of cultures with manganese has been shown to increase AQP4 protein in the plasma membrane but not the total cellular AQP4 protein and mRNA levels, suggesting that increased AQP4 levels in cultured is due to the increased stability and/or increased trafficking where increased level of oxidative stress might involved [239]. They also demonstrated that the MAPKs and MAPK activation were implicated in this effect. Nontheless, other groups previously reported that both mRNA and protein level of AQP4 were increased by hydrogen peroxide and p38-MAPKs was involved in mediating this effect [240]. Those contrary results might be due to different concentrations of ROS used by different group.

1.6 Raft-dependent endocytosis

Lipid rafts are plasma membrane microdomains enriched in cholesterol and sphingolipids that are involved in the endocytosis of receptor molecules at the cell surface via a process called raft dependent endocytosis. The invaginations of plasma membrane caveolae are a subdomain of lipid rafts constitutes of caveolin-1, -2 and -3 [241, 242].

1.6.1 Caveolae

Caveolae belongs to the category of membrane microdomains referred to as lipid rafts that are enriched in lipids including cholesterol and sphingolipids (GM1, sphingomyelin and ceramide) this specific composition results in a higher degree of organization of the lipid constituents in these membrane microdomains [243]. Unlike lipid rafts, representing planar, morphologically indistinguishable regions of the plasma membrane, caveolae are coated by a unique family of proteins, termed caveolins that oligomerize to generate large macromolecular complexes [244]. Caveolins define caveolae architecture and morphology [245-248]. Caveolin concentration is high in caveolae (100-200 molecules per caveolae) [249] and they form the typical 60-80 nm invaginations of the plasma membrane [250].

1.6.2 Caveolins

The caveolin is composed of the three isoforms in mammalian cells, caveolin-1, -2 and -3 [251-253]. Caveolin-1 and-2 is abundantly expressed in a variety of tissues. Caveolin-3 is more restricted in muscle and in glial cells [33, 254]. Caveolin-1 forms homo-dimers, and heterodimers with caveolin-2. They aggregate into oligomeric structures containing 14-16 molecules in Golgi and then coalesce into large macromolecular complexes that define caveolae architecture at the plasma membrane [250]. A large body of evidence in the literature shows that either caveolin-1 or caveolin-3 is required to form caveolae. Caveolin-2 appears less important in this respect, although the presence of this isoform does modulate efficiency of caveolae formation [89].

Caveolin-1 has two isoforms, caveolin-1α (residues 1-178) and shorter splice variant caveolin-1β (residues 32-178), both of them generated from same full-length mRNA with a different initiation site [67, 255, 256]. Interesting, caveolin-1β is found mainly in shallow caveolae and at the leading edge of migrating cells [256].

1.6.3 Caveolin-1

A hydrophobic central domain between residues 102 and 134 is inserted in the inner leaflet of the plasma membrane. This region adopts a hairpin-like conformation, thereby exposing both $COOH^-$ and NH_2^- terminus to the cytoplasm [250, 257]. Immediately adjacent to the hydrophobic domain in the N terminal region (residue 82-101), a modular sequence called the 'caveolin scaffolding domain' (CSD) is required for caveolin dimerization, as well as interactions between caveolin-1 and numerous signalling proteins that contain a 'caveolin binding domain' (CBD). In addition, a WW-like domain represent in caveolin-1 (residues 98-132) [258] and play a role in caveolin-1-mediated degradation of the inducible isoform of nitric oxide synthase (iNOS) via the proteasome pathway. A combination of components is required for directing caveolin-1 to the Golgi (amino acids 66-70), oligomerization (amino acids 91-100 and 135-140) and transport to the cell surface (amino acids 71-80) [259, 260]. The COOH-terminal region contains three palmitoylated cysteine residues that are required for oligomerization [261, 262], as well as two separate regions implicated in cross-linking

caveolin dimmers [263]. A minimal sequence of 10 amino acids (46-55) in N-terminus of caveolin-1 is required for localization of the protein to the rear of migrationg cells and formation of caveolae [264].

1.6.4 Caveolae/raft-dependent endocytosis

It has been shown that caveolae and raft mediate the internalization of toxins, viruses, growth hormone, and membrane receptors [265-269]. It was well known that the GTPase activity of dynamin is requred for the pinching off caveolae from the plasma membranes. The expression of a dominant-negative dynamin K44A mutant (dynK44A) which is deficient in GTP hydrolysis, prevents caveolae budding and further reduces raft-mediated internalization of various molecules [58, 203, 268, 270-273]. FRAP studies indicate that caveolin-1 GFP is highly immobile at the plasma membrane [268, 274, 275]. The evidence in the literature shows that low expression levels of caveolin-1 in *ras* and *abl*-transformed NIH-3T3 cells is associated with the increased endocytosis of AMF to the ER, and this effect was reversed by the reintroduction of caveolin-1, indicating caveolin-1 as a negative regulator of caveolae/raft-dependent endocytosis [273]. Caveolin-1 therefore appears to be stabilized the anchoring invaginated rafts to the plasma membrane and retarding their dynamin-dependent budding and detachment rather than inducing raft invagination.

1.6.5 Role of caveolin-1 tyrosine phosphorylation in raft-dependent endocytosis

Caveolae endocytosis is blocked by treatment of cells with tyrosine kinase inhibitor while addition of the phosphatase inhibitor okadaic acid triggers endocytosis [268, 276-278]. Caveolin-1 is phosphorylated by Scr kinase at Tyrosine 14 [279]. Activation of *v-src* in Rat-1 cells is responsible for caveolin-1 phosphorylation and is associated with loss of plasma membrane caveolae [280]. The phosphorylation of caveolin-1 has been shown to be required for lipid raft marker GM1 internalization [281]. However, its role in other molecule receptors anchoring to the plasma membrane and traffiking remains unknown. In addition, caveolin-1 phosphorylation on tyrosine 14 is involved in flatternning, aggregation and fusion of caveolae vesicles [282]. In pancreatic cancer cells, EGF stimulation of *src*-mediated

caveolin-1 phosphorylation leads to a marked increase in the number of assembled caveolae at the cell surface [283]. Transcytosis of albumin across the endothelial cell monolayer mediated by *Src* kinase is associated with Cav1 phosphorylation [278]. It was demonstrated that *Src* kinase activity is also crucial for the stimulation of caveolae internalization by glycosphingolipids and cholesterol [276].

Simian virus 40 (SV40) recruitment to caveolae triggers local tyrosine phosphorylation [268]. However, inhibiting tyrosine phosphorylation does not prevent SV40 recruitment to caveolae but prevents the recruitment of dynamin to caveolae, indicating that tyrosine phosphorylation is essential for dynamin-dependent caveolae budding [268]. Similarly, the Src-dependent internalization of albumin via a Gi-coupled pathway requires interaction of its receptor gp60 with caveolin-1 and dominant-negative *src* reduces phosphorylation of dynamin-2 and its association with caveolin-1, resulting in reduced albumin uptake [284]. This suggests that tyrosine phosphorylation regulates caveolar budding by controlling dynamin recruitment to caveolae [249]. However, the requirement of tyrosine kinases in the raft-dependent uptake of AMF in cancer cells expressing low levels of caveolin-1 and in dynamin-independent raft uptake of SV40 in caveolin-$1^{-/-}$ cells [249] point to a complex role of tyrosine phosphorylation in raft-dependent endocytosis.

1.7 Project outline and hypotheses

It has been 25 years since Demopoulos [285] proposed that free radicals contribute to brain lesions in stroke. Current knowledge of the pathophysiology of ischemia damage and of the cellular and molecular mechanisms involved suggests that amelioration of ischemic lesions could be achieved by agents reducing cellular acidosis, or by those that abort the production of or scavenge free radicals. The poor therapy available for cerebral ischemia that develops after stroke contributes significantly to the morbidity and mortality. The discovery of AQP4 in brain provided new insights into water routes in and out of brain. AQP4 represents a new therapeutic target in the treatment of cerebral ischemia and cerebral edema formation. At the molecular level, understanding the basic physiological processes and mechanisms regulating AQP4 functional expression is very important.

Interestingly, some studies indicated that the expression of AQP4 is regulated by p38

MAPK pathway and the transcription factor NFκb [240, 286, 287]. As these factors have been proposed to be secondary messengers for ROS implicated in the development of brain ischemia, it would be interesting to investigate the expression of AQP4 in increased ROS.

My thesis further investigates the involvement of ROS and determines the role of tyrosine phosphorylation of caveolin-1 in cell surface expression of AQP4 in oxidative stress. My working hypotheses are **1)** ROS regulate the expression of AQP4 in primary astrocytes cultures, **2)** ROS might affect AQP4 membrane expression, and this effect is independent of the *de novo* synthesis of AQP4, **3)** The phosphorylation of caveolin-1 regulates H_2O_2-induced increase in AQP4 membrane expression, and **4)** Changes in AQP4 expression due to oxidative stress is implicated in astrocyte apoptosis.

2 REGULATION OF AQUAPORIN 4 MEMBRANE EXPRESSION BY CAVEOLIN-1 PHOSPHORYLATION IN A MODEL OF OXIDATIVE STRESS

2.1 Introduction

The membrane water-permeable channel aquaporin-4 (AQP4) is highly expressed in brain [110, 126, 288]. The peculiar distribution pattern of AQP4 at astrocytes' endfeet facing the BBB and those forming the glia limitans *in vivo*, suggests that AQP4 is involved in brain volume homeostasis. Indeed, a growing body of evidence based on transgenic mouse models has shown that AQP4 plays a key role in astrocyte swelling that underlies cytotoxic brain edema [143, 150, 289]. Furthermore, its has been demonstrated that AQP4 is also involved in water accumulation within the ECS of the brain parenchyma in models of vasogenic edema [162]. These findings indicate that AQP4 is a potential therapeutic target in the treatment of cerebral edema in response to various CNS pathologies including stroke.

AQP4 forms heterotetramers at the plasma membrane that aggregate to form supramolecular assemblies called orthogonal arrays of particles (OAPs). Although the physiological relevance of OAPs remains disputable, it has been suggested that they play a role in water permeability [290]. AQP4 has two isoforms, the longer isoform (M1) initiates translation at Met-1 and a shorter isoform (M23) initiates translation at Met-23, which lacks 23 amino acids at the N-terminal compared to M1 [83, 87, 111, 291]. It has been shown that M23-AQP4 alone forms large size OAPs, whereas M1-AQP4 forms only heteretetramers [132]. The concentration ratio of M1 to M23 is a crucial determinant of the size of OAPs as greater amounts of the M23 isoform contribute to the formation of a larger pool of OAPs [128]. Interestingly, cerebral ischemia leads to the dissociation or reduction of OAPs [129, 292, 293]. However, the expression pattern of M1 and M23 has not yet been evaluated in such condition.

Reactive oxygen species (ROS) are generated as a result of cerebral ischemia and their role in brain injury has been substantiated in various models [239, 294-296]. These oxidants can oxidize DNA, lipids, proteins and can also lead to apoptotic or necrotic cell death [297-301]. They have been shown to activate over 100 genes [302] and many signaling pathways

are activated by oxidative stress including the p38-MAPK and NFκB pathways [170, 273, 303]. Of particular interest, ROS have been reported to regulate AQP4 expression [239, 240]. It was demonstrated that AQP4 expression could be induced by direct exposure to ROS in human neuroblastoma cells and that the expression level of AQP4 in brain was altered in cerebral ischemia which is an important contributor to cell swelling [139, 304]. A previous study reported that manganese, an oxidative stress inducer, enhances APQ4 membrane expression [239]. However, the mechanism underlying the manganese-induced AQP4 membrane overexpression is still elusive and the mechanism in alterating expression of AQP4 or protein stability in oxidative stress is still unclear.

By far the best characterized mechanisms for membrane proteins trafficking were categorized into clathrin dependent and clathrin independent pathway. Recent data have also shed light on the role of lipid rafts and caveolae on trafficking of raft-associated proteins and lipids, defined by its clathrin independence, dynamin dependence, and sensitivity to cholesterol depletion [58]. In a previous study, we have shown that AQP4 codistributes with caveolin-1 in lipid raft enriched-fractions [305]. Interestingly, residue Y14 of caveolin-1 undergoes phosphorylation by *Src* kinase in oxidative stress [306]. Moreover, tyrosine phosphorylation of caveolin-1 alters the trafficking of molecules such as GM1 and several membrane receptors [112, 307].

In the current study, we used primary astrocyte cultures to examine the expression of AQP4 under oxidative stress using hydrogen peroxide. First, we showed that H_2O_2 induces a significant increase in the expression levels of both AQP4 mRNA and protein, and that this effect is inhibited by the anti-oxidant N-acetylcysteine (NAC). Second, we demonstrated, using cell surface biotinylation, that H_2O_2 increases AQP4 plasma membrane expression and that this change is independent of the *de novo* AQP4 synthesis. Finally, we found that the phosphorylation of caveolin-1 Y14 residue is a regulator of H_2O_2-induced increase in cell surface expression. To our knowledge, these findings are the first to show that caveolin-1 phosphorylation plays a key role in the regulation of AQP4 cell surface expression in oxidative stress possibly by altering AQP4 internalization and trafficking resulting in its redistribution in different compartments of the cell.

2.2 Materials and methods

2.2.1 Antibodies and reagents

The following antibodies were used in the present study: rabbit anti-AQP4 against rat GST-AQP4 corresponding to residues 249-323 (Alomone Laboratories, Jerusalem, Israel), rabbit anti-caveolin-1 against the N-terminus of human caveolin-1 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-caveolin-1-Y14 (BD Biosciences, USA) and mouse anti β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). The antioxidant, N-acetylcysteine (NAC) was purchased from Sigma-Aldrich; the inhibitor of protein biosynthesis, cycloheximide, and the kinase inhibitor, PP2, were purchased from Calbiochem.

2.2.2 Cell culture

Primary astrocyte cultures were prepared from the hippocampus of neonatal day 1 rat pups (Spague-Dawley, Charles River). Hippocampi were dissected, and meninges and choroid plexus were removed. Then they were cut into small pieces and incubated for 15 min with 0.05% trypsin (3.0 mg/ml; Gibco, Burlington, Canada). Dissociated hippocampi were then plated in culture flasks and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 1 mM L-glutamine (Gibco) for 2-3 weeks. The culture medium was changed every 3 days. To remove microglia and oligodendrocyte progenitors, the flasks were shaken the day following the plating. Astrocytes were transferred to 12 well plates for 48 h and treated with increasing concentrations of H_2O_2 for 2 h.

Three MDA-435 cell lines expressing Caveolin1-mRFP, Caveolin-1(Y14F)-mRFP, and Caveolin-1(Y14D)-mRFP (generous gift from Drs B Joshi and IR Nabi, UBC, Canada) were grown to confluence on 18 mm coverslips for 3-5 days in RPMI 1640 supplemented with 10% fetal bovine serum, 1% Penicillin-Streptomycin (PS) (Gibco), 1% Glutamate (Gibco) either on 12 well plates for western blotting or on 18 mm coverslips for immunoflurescence. The immunofluorescence was conducted 48 h following the transfection of MDA-435 wild type and mutant cells with 4 μg of VSV-AQP4 (generous gift from Jean Merot; INSERM U533,

Faculté de Médecine, Nantes, France) using Lipofectamine-2000 (Invitrogen, USA).

2.2.3 Reverse transcription (RT)-PCR

Total RNA was isolated using Rneasy Mini Kit (Qiagen) and cDNAs were genetated from 1 μg of total RNA using Superscript II RNase H^- reverse transcriptase (Invitrogen) primed with oligo$(dT)_{18}$ (Invitrogen). Alterations of the AQP4 transcript was assessed on 2 μl of synthesized cDNA using a set of primers previously described ([240]; Forward: 5'-TTGGACCAATCATAGGCGC-3'; Reverse: 5'-GGTCAATGTCGATCACATGC-3') and Paltinum-Taq DNA polymerase (invitrogen). PCRs were performed by co-amplifying β-actin as the internal standard using the following primers: 5'-GACCTGACTGACTACCTCAT-3' (Forward), 5'-TCGTCATACTCCTGCTTGCT-3' (Reverse).

2.2.4 Cell surface biotinylation

Confluent astrocyte and MDA-435 cultures were treated with H_2O_2 for 1 hours, washed with cold DPBS (Gibco) three times for 5 min each and then labeled for 30 min at 4^oC using 0.5 mg/ml EZ-Link Sulfo-NHS-LC-Biotin (Pierce Biotechnology, Rockford, IL, USA). The biotin solution was removed and the reaction was quenched with 50 mM NH_4Cl in DPBS for 10 min. The cells were extensively washed with DPBS prior to being scraped off the flask and centrifuged at 5000 rpm for 5 min. They cell pellet was then incubated for 1 h with extraction buffer (25 mM Tris pH 7.4, 25 mM glycine, 150 mM NaCl and 5 mM EDTA) containing 1% Triton X-100 and 1 x complete protease inhibitor cocktail (Roche, Laval, QC, Canada). The biotinylated proteins were precipitated using streptavidin covalently attached to agarose beads (Pierce Biotechnology, Rockford, USA) and subjected to wester blotting.

2.2.5 Immunofluorescence

The MDA-435 cell lines expressing Caveolin1-mRFP, Caveolin-1(Y14F)-mRFP, and Caveolin-1(Y14D)-mRFP were rinsed with warm PBS and fixed with 4% (w/v) paraformaldehyde prepared in 0.1M phosphate buffer for 20 min. They were then rinsed 3 x

15 min with PBS and incubated for 1 h at room temperature (20-22^{o}C) in a solution containing 2% bovine serum albumin (Sigma) and 0.25% Triton X-100. The immunolabelling was performed by incubating the cells at room temperature for 1hr in the presence of the antibody to AQP4 (1:200). Subsequently, the cells were rinsed 3 x 15 min with PBS and incubated with Alexa Fluor 488 goat anti-rabbit IgG for 1hr (1/200; Moleculer Probes, USA). After several washes with PBS, the coverslips were mounted on glass slides using Prolong Gold Antifade reagent (Invitrogen, Burlington, ON, Canada). To confirm the specificity of the labelling, control cells were treated equivalently in the absence of the antibody to VSV. Fluorescent labeling of cultured cells was visualized using a confocal microscope (Fluoview 1000; Olympus) and an Uplan Apochromat 1.35 NA 60x objective (Olympus).

2.2.6 siRNA transfection

To silence caveolin-1 expression, astrocytes were transfected in suspension before plating with 100 nM *Cav-1* siRNA (siGENOME and ON-TARGETplus SMARTpool siRNA reagents; Dharmacon) using Lipofectamine 2000 (Invitrogen) following the manufacturers' protocol. Scrambled *Cav-1* siRNA was used as a control (ON-TARGETplus siCONTROL nontargeting siRNA; Dharmacon). Two days after plating, astrocytes were treated with 200 μM H_2O_2 for 1hr before being subjected to cell surface biotinylation as described below.

2.2.7 Immunoblotting

The cells were harvested and lysed on ice for 20 min in extraction buffer (25mM Tris pH7.4, 25mM glycine and 150 mM NaCl) containing 1% Triton X-100, 1 x complete protease inhibitor cocktail and 5 mM EDTA. Nuclei and cellular debris were removed from the suspension by centrifugation at 13,200 rpm for 10 min. Extracted proteins were denatured by boiling for 1 min in reducing sample buffer and then loaded on a 12% sodium dodecyl sulfate-polyacrylamide electrophoresis gels. The gels were electrotransferred to nitrocellulose membranes (Bio-Rad, Mississauga, ON, Canada). The blots were probed with antibodies to AQP4 (1/1000), Cav-1 (1/1000), pCav1 (1/1000) and β-actin (1/10000). Bound

antibodies were detected using horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1/2000; Jackson ImmunoResearch, USA). Signals were visualized on Bioflex econo films (Interscience, Markham, ON, Canada) using chemiluminescence (Amersham Biosciences, Buckinghamshire, UK).

2.2.8 Quantitative and statistical analysis

The densitometric analysis of the immunoblot signals was determined using Image Gauge 4.21 software (Fujifilm) and the signal intensity for total expression levels of AQP4 was normalized to that of β-actin. In the case of the cell surface biotinylation, we first normalized the signal intensity of cell surface AQP4 against that in the AQP4 input. These ratios were then normalized against those found in the control untreated cells. All statistical analyses were performed using Prism 4.00 software.

2.3 Results

2.3.1 Hydrogen peroxide induces an increase in both AQP4 protein and mRNA levels

In order to determine the effect of ROS on AQP4 expression, we analyzed primary astrocyte cultures treated with increasing concentrations of H_2O_2 (50 μM to 1 mM) both by immunoblot an RT-PCR. The data show that H_2O_2 induces an increase in AQP4 protein expression level (Fig 2.1A and B). Likewise, AQP4 transcript levels increase significantly in H_2O_2-treated astrocytes (Fig 2.1C and D). While 200 μM of H_2O_2 does not increase significantly AQP4 protein levels, this concentration is sufficient to induce a significant increase of AQP4 transcript level by 64% ± 28.

2.3.2 The free radical scavenger, N-acetyl-cysteine (NAC), reverses the effect of hydrogen peroxide on AQP4 expression

To evaluate the effect of the anti-oxidant, N-acetylcysteine (NAC), astrocytes were first incubated in the presence of 5 mM NAC for 2h prior to the treatment with increasing

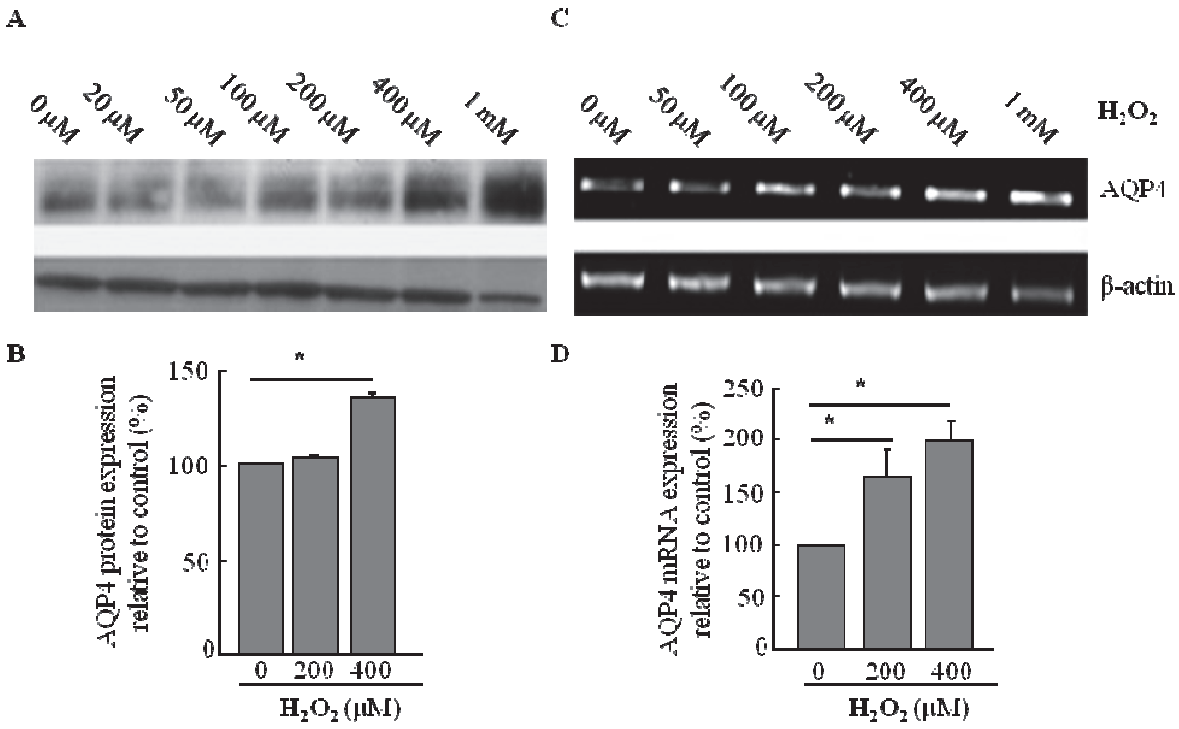


Figure 2.1 Hydrogen Peroxide increases AQP4 protein and mRNA expression levels in astrocytes. Representative immunoblots and RT-PCR of AQP4 protein and mRNA expression levels from astrocytes treated with increasing concentrations of H_2O_2 (**A**, **C**). Histograms of the densitometric analysis of the immunoblots and RT-PCR represent the mean pixel intensity relative to control ±SD of three experiments (**B**, **D**). The asterisks represent statistically significant changes of AQP4 expression in astrocytes as determined by the two-tailed Student's t-test (*$p<0.05$). β-actin was used a loading control for both the immunoblots and RT-PCR (**A**, **C**).

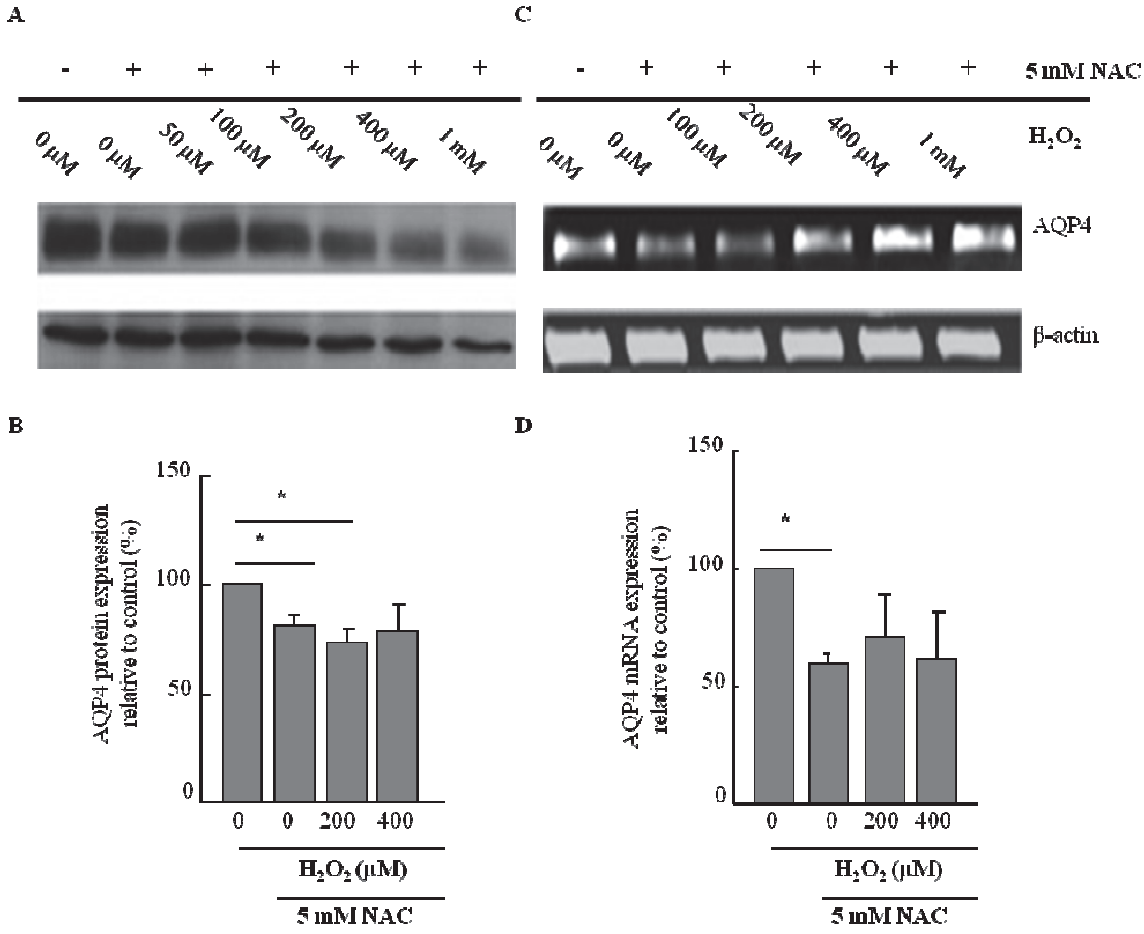


Figure 2.2 The free radical scavenger, N-acetyl-cysteine (NAC), reverses the effect of hydrogen peroxide on AQP4 expression. Representative immunoblots and RT-PCR of AQP4 protein and mRNA expression levels from astrocytes treated with 5 mM of N-acetylcysteine (NAC) for 2 hours prior to their treatment with increasing concentrations of H_2O_2 (**A**, **C**). Histograms of the densitometric analysis of the immunoblots and RT-PCR represent the mean pixel intensity relative to control ±SD of three experiments (**B**, **D**). The asterisks represent statistically significant changes of AQP4 expression in astrocytes as determined by the two-tailed Student's t-test (*$p<0.05$). β-actin was used a loading control for both the immunoblots and RT-PCR (**A**, **C**).

concentrations of H_2O_2. Interestingly, we found that the basal expression levels of AQP4 in the absence of exogenous H_2O_2 are decreased by 20% ± 5 in NAC-treated compared to untreated astrocytes (Fig 2.2A and B). Furthermore, NAC inihibits the H_2O_2-mediated increase in AQP4 protein levels (compare Fig.1A and B to Fig. 2A and B). Likewise, basal expression levels of AQP4 mRNA are significantly decreased (41% ± 4) in NAC-treated versus untreated astrocytes (Fig. 2.2C and D). In addition, NAC effectively inhibits the H_2O_2-induced increase of AQP4 mRNA expression levels (compare Fig 2.1C and D to Fig 2.2C and D).

2.3.3 Hydrogen peroxide increases AQP4 cell surface expression independently of its *de novo* synthesis

Using cell surface biotinylation, we found that membrane expression of AQP4 is increased by almost two fold in astrocytes treated with 200 and 400 μM, respectively (Fig 2.3A and B). To verify whether this increase was due to enhanced synthesis of AQP4, we used the inhibitor of protein biosynthesis, cycloheximide (CHX). First, we treated astrocytes with 100 μg/ml of CHX for 2, 4, 6 and 8 hours and found that efficient inhibition of AQP4 synthesis occurred as early as 2 hours following the treatment (Fig 2.4A). Second, we performed cell surface biotinylation on cells treated with 100 μg/ml cycloheximide for 2 hours and 200 or 400 μM H_2O_2 for 1 hour and found that inhibition of protein synthesis did not alter the H_2O_2-induced increase of AQP4 membrane expression (Fig 2.4B). This result indicates that H_2O_2 enhances AQP4 membrane expression independently of its *de novo* synthesis.

2.3.4 Hydrogen peroxide induces caveolin-1 phosphorylation via the *src*-family kinases

Previous studies have demonstrated that acute oxidative stress induces the phosphorylation of residue Y14 of caveolin-1 in endothelial cells [239]. Here, we found similar results in astrocytes showing that caveolin-1 is phosphorylated on Y14 by H_2O_2 in dose- and time-dependent manners (Fig. 2.5A and B). Indeed, the level of caveolin-1 phosphorylation increased gradually and reached a maximum when astrocytes were treated

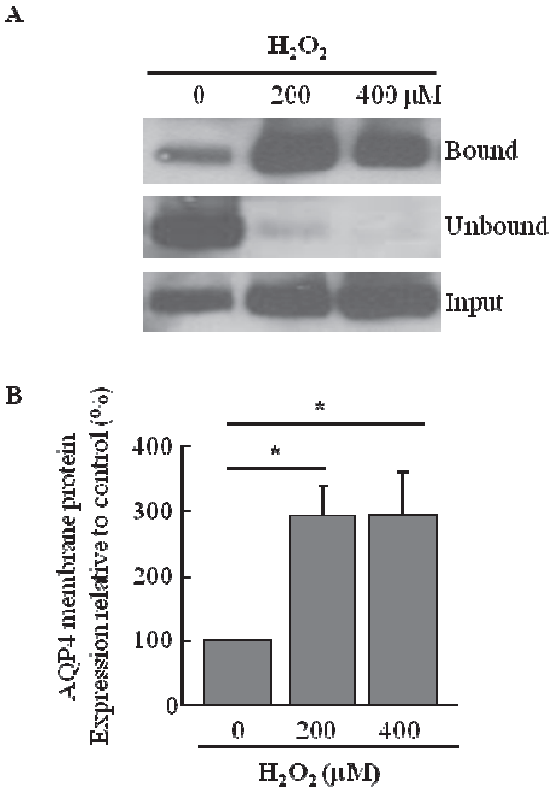


Figure 2.3 Hydrogen peroxide-induced increase in AQP4 cell surface expression is independent of its de novo synthesis. Biotinylated cell surface fractions from untreated astrocytes and astrocytes treated with H_2O_2 were immunoblotted for AQP4 (Bound; **A**). Histograms of the densitometric analysis represent the mean pixel intensity relative to control ±SD of three experiments (**B**). The asterisks represent statistically significant increases of AQP4 cell surface expression compared with control untreated astrocytes as determined by the two-tailed Student's t-test (*$p<0.05$).

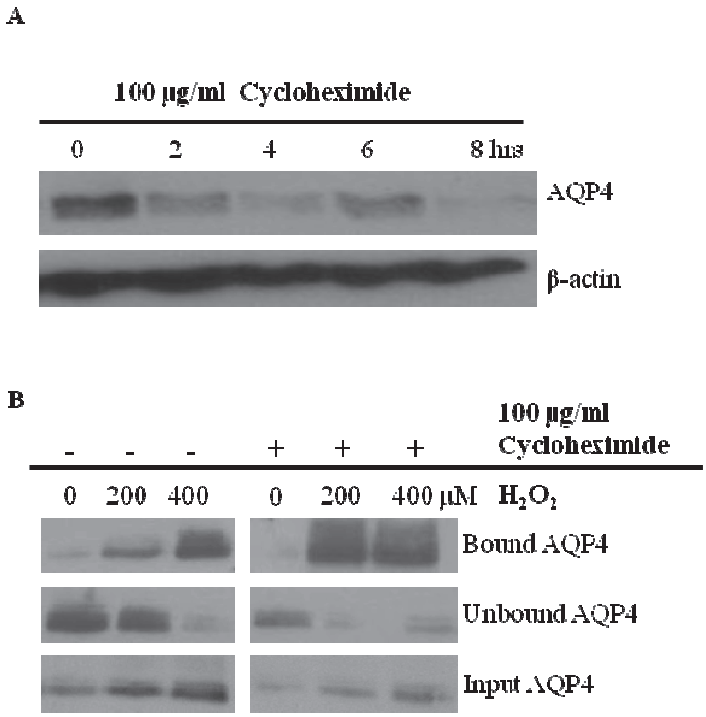


Figure 2.4 Hydrogen peroxide-induced increase in AQP4 cell surface expression is independent of its de novo synthesis. Whole cell extracts from astrocytes treated with 100 µg/ml of cycloheximide for 2, 4, 6 and 8 hours were immunoblotted for AQP4 (**A**). Biotinylated cell surface fractions from astrocytes treated with H_2O_2 alone or with H_2O_2 plus 100 µg/ml of cycloheximide were immunoblotted for AQP4 (Bound AQP4; **B**). Note that protein synthesis inhibition with cycloheximide does not alter H_2O_2-induced increase of AQP4 cell surface expression.

with 400 μM (Fig. 2.5A). This phosphorylation takes place as early as 5 min after H_2O_2 treatment and increases gradually with increasing durations of H_2O_2 treatment (Fig. 2.5B).

Since caveolin-1 is known to be phosphorylated by the *src* tyrosine kinase in other cell systems [308], we used the *Src* inhibitor, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo [3,4-*d*] pyrimidine (PP2), to see whether it prevents caveolin-1 Y14 phosphorylation induced by H_2O_2. We found that astrocytes incubated in the presence of 100 μM PP2 for 1 hour prior to their treatment with 200 μM H_2O_2 present a concentration-dependent decrease of caveolin-1 Y14 phosphorylation when PP2 is used at 10, 20 and 40 μM (Fig. 2.5C). Moreover, this phosphorylation is completely abolished with 100 μM PP2 (Fig. 2.5C).

To investigate the potential role of caveolin-1 phosphorylation in increased AQP4 cell surface expression induced by H_2O_2, astrocytes were treated with 100 μM PP2 for 1 hour prior to being incubated in the presence of H_2O_2 for 1 hour. We found that the inhibition of the *src* tyrosine kinase with PP2 prevented the H_2O_2-induced overexpression of AQP4 membrane expression (Fig. 2.5D and E). This result suggests that phosphorylation of caveolin-1 and other downstream effectors may be involved in the regulation of AQP4 membrane expression in oxidative stress.

2.3.5 Tyrosine phosphorylation of caveolin-1 regulates H_2O_2-induced increase in AQP4 cell surface expression

To determine whether caveolin-1 phosphorylation is required for H_2O_2-induced overexpression of AQP4 at the cell surface, we used MDA435 breast cancer cells expressing caveolin-1, Y14F loss of function mutant caveolin-1 or Y14D constitutively phosphorylated mutant caveolin-1. These cells were transfected with the VSV-AQP4 [138] and were assayed for AQP4 expression both by immunoblot and immunofluoresnce. We found a high efficiency of transfection in the wild type MDA435 cells as well as in those expressing the wild type caveolin-1 and either one of the caveolin-1 mutants (Y14F and Y14D; Fig. 2.6A). The immunofluorescence data show that AQP4 is expressed in mRFP-positive cells expressing caveolin-1, Y14F or Y14D caveolin-1 (Fig. 2.6B-J). Next, we assessed the effect of tyrosine phosphorylation of caveolin-1 on the expression level of AQP4 at plasma membrane by cell surface biotinylation. We found that in basal conditions, the

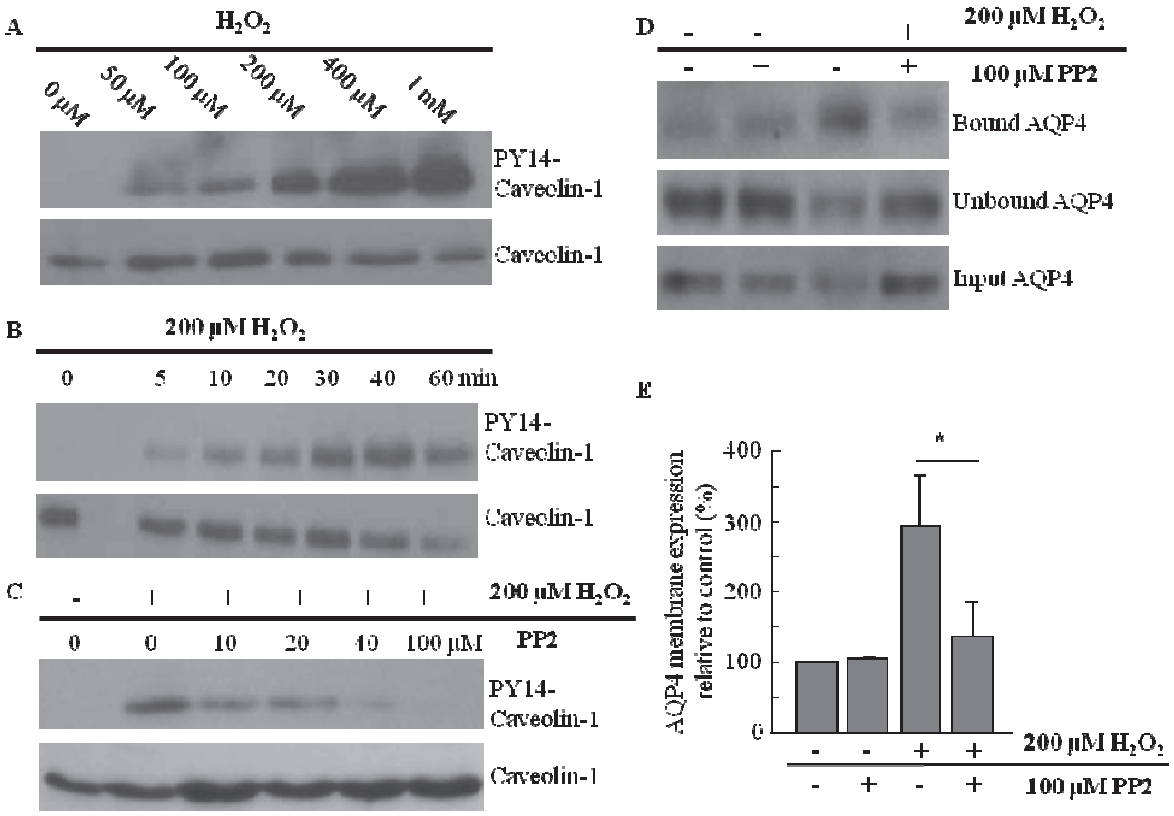
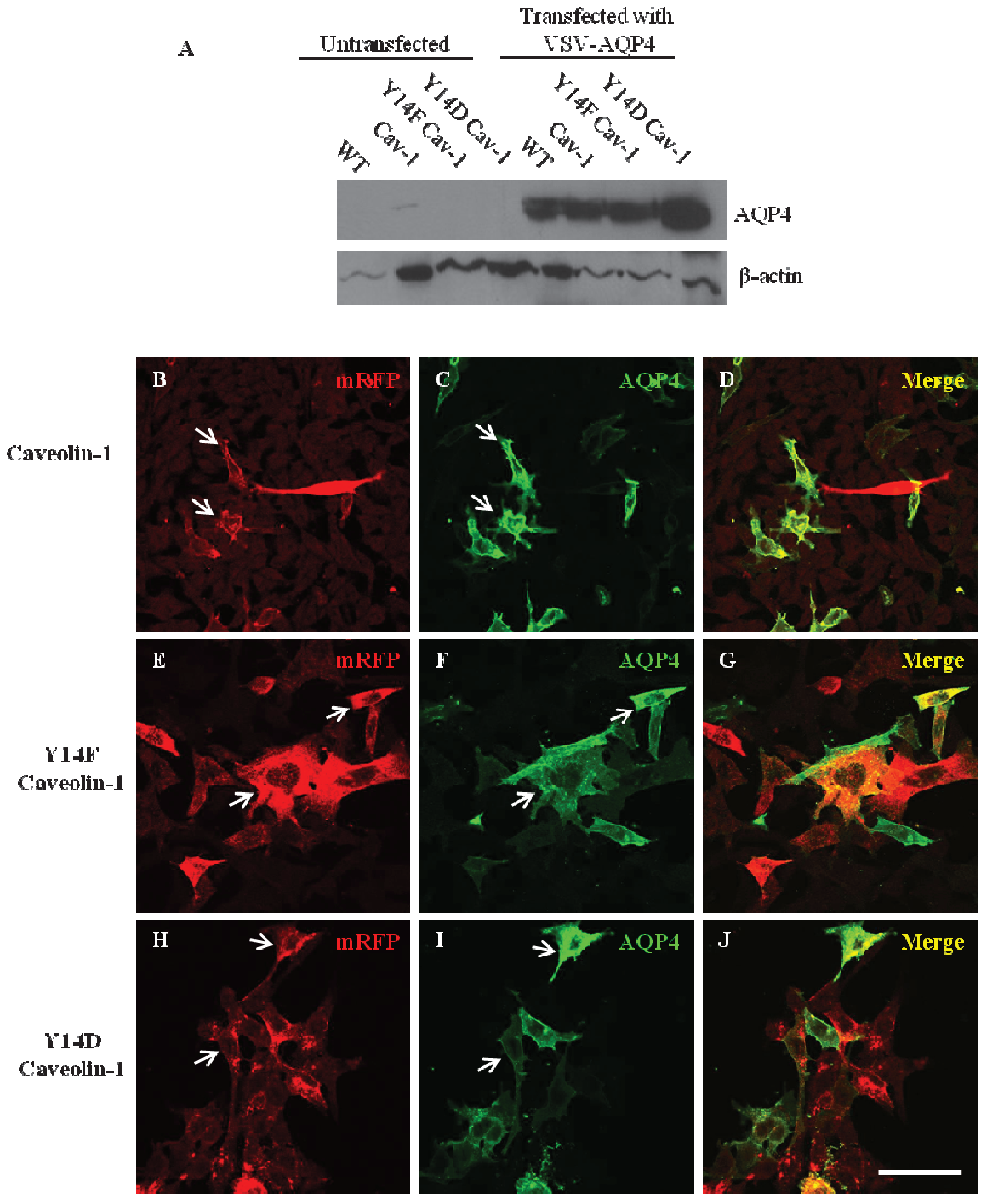


Figure 2.5 The Src kinase inhibitor, PP2, inhibits the H2O2-induced increase of caveolin-1 phosphorylation. Representative immunoblots of phosphorylated caveolin-1 Y14 and caveolin-1 from astrocytes treated with increasing concentrations of H_2O_2 (**A**) or for increasing durations of time (**B**) as well as from astrocytes incubated with PP2 for 2 h prior to their treatment with H_2O_2 (**C**). Note that PP2 inhibits significantly the caveolin-1 Y14 phosphorylation. Biotinylated cell surface fractions from astrocytes treated with either 200 µM H_2O_2 or 100 µM PP2 alone, or with both H_2O_2 and PP2, were immunoblotted for AQP4 (Bound AQP4; **D**). Note that PP2 prevents AQP4 increased cell surface expression induced by H_2O_2. Histograms of the densitometric analysis represent the mean pixel intensity relative to control untreated astrocytes ±SD of three experiments (**E**). The asterisk represents a statistically significant decrease of AQP4 cell surface expression in astrocytes treated with both H_2O_2 and PP2 compared to astrocytes treated with H_2O_2 alone as determined by the two-tailed Student's t-test (*$p<0.05$).

Figure 2.6 Effective transfection of MDA-435 cells expressing wild type caveolin-1, Y14F or Y14D caveolin-1 with the VSV-AQP4 transgene. A. Representative immunoblot from wild type MDA-435 as well as caveolin-1-, Y14F and Y14D caveolin-1-expressing MDA-435 cells transfected with VSV-AQP4-M1 and probed for AQP4 and actin (**A**). Note the high level of AQP4 expression in the transfected compared to the untransfected cells. Selected fields containing MDA435 cells co-expressing caveolin-1 (**B**)**,** Y14F (**E**) or Y14D-caveolin-1 (mRFP-positive cells; **H**) and VSV-AQP4 are represented (**C**, **F** and **I**). Merged images are represented in **D, G** and **J**. Scale bar = 30μm.


A
Untransfected
Transfected with VSV-AQP4
WT
Cav-1
Y14F Cav-1
Y14D Cav-1
AQP4
β-actin
Caveolin-1
Y14F Caveolin-1
Y14D Caveolin-1
B
mRFP
C
AQP4
D
Merge
E
F
G
H
I
J

phosphomimetic Y14D caveolin-1-expressing cells exhibit a significant increase (68% ± 9) in AQP4 cell surface expression (Fig. 2.7A and B), indicating that the phosphorylation of Y14 residue of caveolin-1 regulates AQP4 plasma membrane expression. Conversely, the cells expressing the Y14F loss of function mutant caveolin-1, present a significant decrease in AQP4 membrane expression (31% ± 1; Fig 2.7A and B). Caveolin-1-expressing cells treated with 200 μM H_2O_2 present a significant increase (45% ± 17) in membrane expression of AQP4 compared to untreated cells (Fig 2.7A and B), whereas those expressing the loss of function Y14F caveolin-1 do not show an increase (Fig 2.7A and B). As for the phosphomimetic Y14D caveolin-1-expressing cells, H_2O_2 induces a similar increase as in untreated cells (Fig 2.7A and B). Together these data demonstrate that the phosphorylation of residue Y14 of caveolin-1 is involved in the regulation of cell surface expression of AQP4 in oxidative stress.

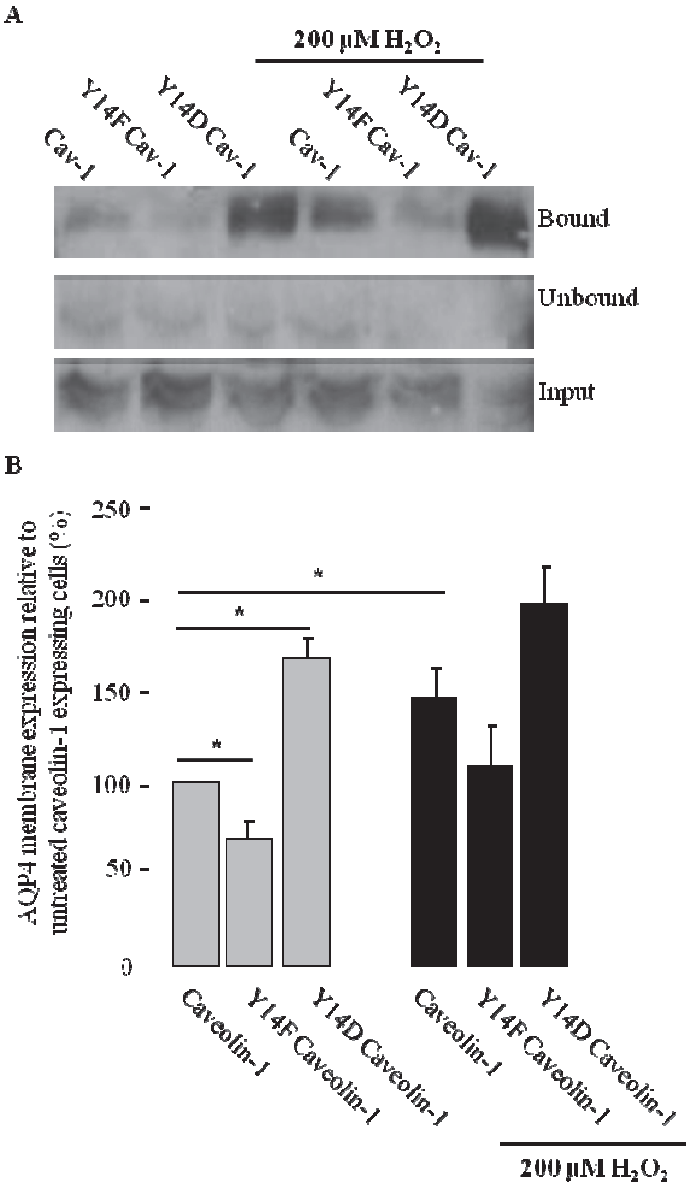
In order to evaluate the role of caveolin-1 in H_2O_2-induced increase of AQP4 membrane expression in astrocytes, caveolin-1 expression was knocked down using double stranded siRNA targeting rat caveolin-1 (si*Cav-1*). We first determined that the knockdown efficiency was approximately 41.81% ± 6.25 (Fig 2.8C). We then evaluated the impact of caveolin-1 deficiency on H_2O_2-induced overexpression of AQP4 cell surface expression and found that it is significantly reduced by almost 2 folds when compared to *siCtl* H_2O_2-treated astrocytes (Fig 2.8A and B). Interestingly, the amount of membrane-bound caveolin-1 is increased in *siCtl* H_2O_2-treated astrocytes compared to untreated astrocytes (data not shown), suggesting that ROS redistributes caveolin-1 within cell compartments enabling its regulation of AQP4 cell surface expression.

2.4 Discussion

2.4.1 Reactive oxygen species increase AQP4 expression levels and promote it targeting to the plasma membrane

Several recent studies have provided evidence supporting the role of the water permeable channel, aquaporin 4 (AQP4), in water transport in brain. AQP4 is the most abundant water channel in brain and is mainly concentrated in the perivascular astrocyte

Figure 2.7 Constitutive tyrosine phosphorylation of caveolin-1 on residue Y14 enhances AQP4 cell surface expression. Biotinylated cell surface fractions from H_2O_2-treated or untreated control caveolin-1, Y14F and Y14D caveolin-1-expressing MDA-435 cells were immunoblotted for AQP4 (Bound, **A**). Note that the Y14F caveolin-1 MDA-435 cells express less AQP4, whereas the Y14D caveolin-1 MDA-435 cells express more AQP4 at the cell surface than caveolin-1 MDA-435 cells both in H_2O_2-treated and untreated cells (**A**). Histograms of the densitometric analysis represent the mean pixel intensity relative to untreated caveolin-1 MDA-435 cells ±SD of three experiments (**B**). The asterisks represent statistically significant changes of AQP4 cell surface expression compared with caveolin-1-expressing MDA-435 cells as determined by the two-tailed Student's t-test (*$p<0.05$).


A
200 μM H_2O_2
Cav-1
Y14F Cav-1
Y14D Cav-1
Cav-1
Y14F Cav-1
Y14D Cav-1
Bound
Unbound
Input
B
AQP4 membrane expression relative to untreated caveolin-1 expressing cells (%)
250
200
150
100
50
0
*
*
*
Caveolin-1
Y14F Caveolin-1
Y14D Caveolin-1
Caveolin-1
Y14F Caveolin-1
Y14D Caveolin-1
200 μM H_2O_2

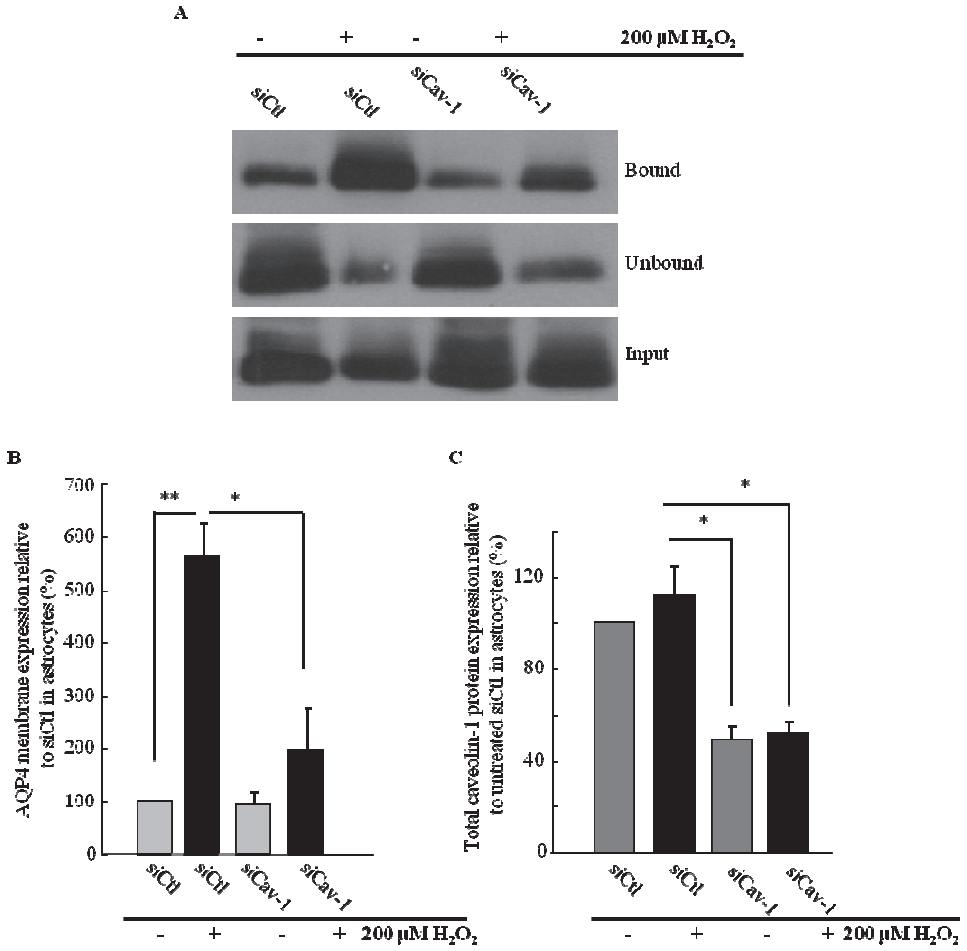


Figure 2.8 Caveolin-1 deficiency inhibits the H2O2-induced increase in AQP4 cell surface expression in astrocytes. Biotinylated cell surface fractions from astrocytes transfected either with siRNA targeting caveolin-1 (si*Cav-1*) or with scambled siRNA (si*Ctl*) and treated or not with 200 µM H_2O_2 were immunoblotted for AQP4 (Bound, **A**). Note that caveolin-1 knock down inhibits the H_2O_2-induced increase in AQP4 cell surface expression. Histograms of the densitometric analysis of bound AQP4 (**B**) and caveolin-1 total expression (**C**) represent the mean pixel intensity relative to untreated siCtl astrocytes ±SD of four experiments. The asterisks represent statistically significant changes of AQP4 cell surface expression and caveolin-1 total expression levels in H_2O_2-treated siCav-1-transfected astrocytes compared with untreated siCtl-transfected astrocytes as determined by the two-tailed Student's t-test (*$p<0.05$).

endfeet. The specific distribution of AQP4 to these specialized cell membrane domains is thought to be of major importance and several studies have suggested that AQP4 facilitates the bi-directional water transport across the plasma membrane in response to osmotic gradients, thereby maintaining water homeostasis in the brain. Studies in astrocyte cultures from AQP4-null mice have proved that AQP4 is indeed the principal channel for water transport in these cells. By reducing water transport from the bloodstream to the intracellular space, the deletion of AQP4 in mice reduces water accumulation in brain following water intoxication and focal cerebral ischemia [143]. On the contrary, the AQP4 null mouse has more brain swelling in models of vasogenic edema where excess fluid accumulates in the extracellular space, due to impairment of the AQP4-dependent water clearance [11]. Therefore, AQP4 plays a key role in water transport and in the development of cerebral edema. Brain edema is invariably associated with stroke and a considerable body of evidence suggests that the production of high levels of reactive oxygen species (ROS) is involved in brain damage in stroke and reperfusion after stroke. A number of studies have reported alterations in AQP4 expression and localization *in vivo* in animal models of ischemic stroke as well as *in vitro* [157, 240]. However, the underlying mechanism for such changes remains largely unknown. In the present study we investigated the effects of the ROS, hydrogen peroxide (H_2O_2) on AQP4 expression as well as the mechanism involved in such effects in primary astrocyte cultures. We showed that H_2O_2 induces the overexpression of both AQP4 mRNA and protein levels in astrocytes. Interestingly, we found that this is accompanied by an increase in AQP4 cell surface expression. These data indicate that ROS do not only alter AQP4 transcription and translation but modulate its stability and potentially targeting to plasma membrane domains as well. Indeed, similar findings have been reported by [240] and [309] in models of hyperosmolar stress induced by mannitol and oxidative stress induced by manganese, respectively. However, in the latter report manganese increases AQP4 cell surface expression without affecting its total protein and mRNA expression levels. Moreover, in accordance with the studies of [240], [309] and [310], inhibition of protein synthesis using cycloheximide did not prevent the H_2O_2-induced increase of AQP4 cell surface expression, indicating that this it is independent of the newly synthesized pool of APQ4.

A previous study has shown that the free radical scavenger, edavarone attenuates cerebral ischemic injury by suppressing AQP4 [311]. More recently, free radical scavengers

including vitamin E and catalase have been reported to inhibit AQP4 upregulation seen in an *in vitro* model of fluid percussion in astrocyte cultures [310]. In light of these data, we wanted to determine the effect of the free radical scavenger, NAC on H_2O_2-induced increase of AQP4 protein levels and mRNA. We found that NAC inhibits efficiently both AQP4 mRNA and protein overexpression (Fig 2.1E-H). Inhibition of oxidative stress may therefore represent a potential strategy for counteracting complications associated with AQP4 in cerebral ischemia. Interestingly, NAC reduces significantly the basal levels of AQP4 mRNA in the absence of exogenous H_2O_2 (Fig 2.1H). Together, these data suggest the presence of an Antioxidant Response Elements (ARE) located in the AQP4 promoter that may participate in the regulation of AQP4 transcriptional activity via its affinity to specific transcription factors in physiological and pathophysiological conditions in response to endogenous ROS and excessive ROS production, respectively. Indeed, the promoters of both exon 0 and exon 1 of AQP4 contain activator protein 1 (AP1) elements [125]. AP1 is a heteromeric transcription factor composed of proteins that belong to the c-Fos, c-Jun, ATF and JDP families. Interestingly, transient expression of c-Fos and c-Jun, members of the leucine zipper family of AP1, has been reported in astrocytes after focal cerebral ischemia induced by MCAO [312] making these transcription factors potential candidates involved in the changes of AQP4 expression in oxidative stress.

2.4.2 Caveolin-1 phosphorylation is responsible for the H_2O_2-induced targeting of AQP4 to the plasma membrane

Several studies have shown that a tyrosine residue (Y14) located at the NH2-terminus of caveolin-1 can be rapidly phosphorylated in response to a number of cellular stresses, including oxidative stress [313-316]. Here, we show that H_2O_2 induces the Y14 phosphorylation in a dose- and time dependent-manner. Since Y14 of caveolin-1 is a target of Src-kinase phosphorylation [313] and H_2O_2 causes a significant phosphorylation of caveolin-1 at Y14 in A549 human carcinoma cells [317], we asked whether Src kinase inhibition using PP2 prevents the H2O2-induced phosphorylation of caveolin-1 in astrocytes. Our data show that inhibition of the Src kinase family prevents effectively caveolin-1 Y14 phosphorylation supporting the hypothesis that caveolin-1 is a substrate of Src-kinase phosphorylation in

astrocytes as well. There is an extensive body of literature implicating caveolin-1 phosphorylation and caveolae in the trafficking of a variety of receptor-tyrosine kinases and serpentine G-protein-coupled receptors [318-320]. This led us to investigate the possible role of caveolin-1 in AQP4 targeting to plasma membrane domains in oxidative stress. We therefore used MDA-435 cells expressing either the constitutively phosphorylated Y14D caveolin-1 or the loss of function Y14F caveolin-1 and found an increase of AQP4 cell surface expression in the Y14D caveolin-1 expressing cells and a lack of H_2O_2-induced increase in the Y14F caveolin-1 expressing cells. These data support the hypothesis of a role of caveolin-1 phophorylation on residue Y14 in the targeting of AQP4 to specific cell domains i.e. the plasma membrane. This is further substantiated by data showing that the Src-mediated phophorylation of caveolin-1 is required for caveolar trafficking of EGFR under oxidative stress [317]. Furthermore, the knock down of caveolin-1 reduces dramatically the AQP4 cell surface expression levels in H_2O_2-treated astrocytes. Together, these data indicate that AQP4 is targeted to plasma membrane domains via a caveolae-mediated pathway. We hypothesize that the phosphorylated form of caveolin-1 presents preferential binding to specific proteins thereby influencing their targeting to different lipid membrane microdomains of the plasma membrane. In support of this hypothesis, we have shown that caveolin-1 and AQP4 codistribute in detergent resistant membranes in astrocytes [305], however it remains to be determined whether oxidative stress enhances the co-distribution of phosphorylated caveolin-1 and AQP4. To our knowledge this is the first demonstration that Y14 caveolin-1 phosphorylation dependent on Src-mediated phosphorylation plays a role in AQP4 targeting at the plasma membrane in oxidative stress.

3 GENERAL DISCUSSION

3.1 Potential role of aquaporin 4 in astrocytes' viability

Brain ischemia results from cardiac arrest, stroke and trauma [321]. These conditions can cause severe brain damage and are a leading cause of death and long-term disability. As supporting cells to neurons and maintaining neuronal survival, astrocytes are less susceptible to ischemia damage. Although astrocytes are more resistant than neurons to most stress conditions *in vitro*, certain astrocyte subtypes, such as the glial fibrillary acidic protein (GFAP)-negative protoplasmic astrocytes that predominate in the gray matter, may be equally or more sensitive than neurons to ischemia *in vivo* [322]. Cell culture studies have shown that astrocytes can undergo apoptosis by many factors associated to ischemia, including acidosis, oxidative stress, substrate deprivation and cytokines [322]. AQP4 which is abundantly expressed in astrocytes has been widely investigated as a water channel that mediates water flow in and out of the brain, and is well known to be implicated in brain edema that follows ischemic damage. However, its role in astrocytes viability and apoptotic cell death remains elusive.

The decrease in cell volume during apoptosis is a highly conserved phenotype and many studies have highlighted the importance of ion efflux, particularly K^+, in this process [248, 323, 324]. The loss of intracellular K^+ is not only concomitant to AVD, but occurs also prior to cytochrome *c* release, caspase-3 activation and DNA degradation [214]. K^+ loss establishes an osmotic gradient that draws water out of the cell and forces cells to shrink.

A reduction in $[K^+]_i$ is essential for activation of apoptotic enzymes [325-328] but a loss of ions does not necessarily imply a reduction in K^+ concentration if the loss is matched by an efflux of water due to a new osmotic gradient established by extracellular and intracellular K^+concentrations. In order to reach a reduction of intracellular K^+ concentration, the water should not be transported out of the cell concomitantly to K^+. Indeed, it has been shown that cells that are succeptible to AVD have a very low water permeability, whereas the non-apoptotic cells have normal water permeability [329]. This change in water permeability during the AVD would thus allow the loss of K^+ to be unbalanced from the loss of water and

facilitate a decrease of $[K^+]_i$ implying a role of water channel in the reduction of K^+ concentration during AVD. In parallel, several studies have demonstrated that the AQP4-null mouse has a better survival outcome compared to wild type in cerebral edema caused by focal brain ischemia or hypoxia [150, 330]. These studies have shown that the lack of AQP4 prevents the water influx from the blood to astrocytes in models of cytotoxic edema [150, 330]. However, the role of AQP4 in astrocyte viability and the effect of AQP4 on intracellular K^+ concentration during astrocyte apoptosis in brain ischemia have not been evaluated yet. In the present study, we first addressed the effect of oxidative stress, which is implicated in the development of brain ischemia, on astrocyte viability. Our data show that astrocytes with the AQP4 gene knocked down have a better survival outcome than wild type cells (9% less apoptosis than siCTL and wild type astrocytes; 20% less apoptosis plus necrosis than siCTL and wild type). We also show that the overexpression of AQP4 in astrocytes increases the levels of both apoptotic and necrotic cell death, suggesting a role for AQP4 in cell death cascades which may be mediated by AVD.

Several lines of evidence have suggested that alterations in the transmembrane gradients of K^+ play an important role in apoptosis [331, 332]. Reduction of the K^+ electrochemical gradient by increasing extracellular K^+ inhibits apoptosis induced by different apoptotic inducers [327, 328, 333]**.** Pharmacological blockade of voltage gated K^+ channels by 4-aminopyridine, Ca^{2+} activated K^+ channels by tetraethylammonium, quinine or rectifer type K^+ channels by clofilium prevent cell death induced by different stimuli in several cellular systems [204, 334-336]. The efflux of K^+ and the concomitant decrease of intracellular K^+ activate key events in the apoptosis cascade, such as caspase cleavage, cytochrome c-dependent formation of the apoptosome, and activation of endonucleases. The intracellular concentration of K^+ is thus far known to be partially regulated by the concentration of water, which is in turn regulated by water permeability. Electrophysiological studies have shown reduced K^+ currents in relation to the reduced AQP4 expression further fortifying the concept of a Kir4.1/AQP4 potassium-water trafficking complex. The role of AQP4 in apoptotic pathways in astrocytes during oxidative stress and its regulation of the intracellular K^+ concentration will be further evaluated.

3.2 Regulation of aquaporin 4 mRNA expression

In our study, we demonstrated that within 1 hr of hydrogen peroxide treatment, a common reagent increasing ROS, an increase of both mRNA and protein expression levels of AQP4 can be observed coinciding with *in vivo* results that AQP4 expression is significantly increased 1 hr after cerebral ischemic onset. Recent studies revealed that MAPK induces AQP4 expression in cultured astrocytes and since ROS activate MAPK [239, 273, 309, 337], we hypothesize that hydrogen peroxide-induced increase in AQP4 expression in astrocytes might be mediated by the p38-MAPK pathway.

Interestingly, we found that the H_2O_2-induced increase in AQP4 expression levels is abolished by the anti-oxidant reagent NAC, indicating the presence of a potential Antioxidant Response Element (ARE) in the AQP4 promoter. This *cis*-acting enhancer sequence may mediate transcriptional activation of a group of specific genes in cells exposed to oxidative stress. From TESS, we identified an ARE-containing the 5'-TGAC-3' tetranucleotide within AQP4 core sequence that resembles the half-site recognized by members of the AP-1 (consensus: 5'-TGACTCA-3') and the ATF/CREB (concensus: 5'-TGACGTCA-3') families of transcription factors. To date, three major signal transduction pathways have been implicated in the regulation of the ARE and these include the MAPK, PI3K and PKC pathways. Interestingly, these pathways have also been shown to be involved in the regulation the AQP4 expression in a broad spectrum of conditions [139, 240, 338].

3.3 Regulation of aquaporin 4 cell surface expression in astrocytes

AQP4 is the predominant water channel in brain and is targeted to specific membrane domains of astrocytes. Previous studies have demonstrated that the interaction between the dystrophin-glycoprotein complex and the ECM are responsible for targeting AQP4 at the glial endfeet [100, 147]. α-syntrophin plays an important role in AQP4 targeting at the perivascular astrocyte membrane [147]. It has been demonstrated that there are two peaks of maximal hemispheric swelling at 1hr and 48 hrs after brain ischemia, coinciding with two peaks of AQP4 expression in astrocyte endfeet [339]. However, the molecular mechanism

involved in its targeting and trafficking during brain ischemia remains largely unknown. We found that H_2O_2 increases significantly the expression of both AQP4 mRNA and protein levels. We then looked at the effect of H_2O_2 on AQP4 cell surface expression. By treating astrocytes with H_2O_2 to mimic increased ROS during the brain ischemia, we found an increase of AQP4 expression at the plasma membrane. This is corroborated by the findings *in vivo* showing that AQP4 expression and more specifically the M1 isoform increases in brain ischemia [168]. The ratio between AQP4 isoforms M1 and M23 influences the size of OAPs, which determines the water permeability [131]. Early disorganization of OAPs in the astrocyte endfeet has been previously observed by freeze fracture after global cerebral ischemia and was shown to precede astrocyte swelling [293]. Recently, it has been shown that agrin plays a crucial role in the formation of OAPs at astrocyte endfeet, indicating that the ECM regulates the ratio between the M1- and M23-AQP4 isoforms [38]. The clustering of AQP4 at the astrocyte endfoot membrane depends on the expression of components of the ECM such as agrin and laminin {Guadagno, 2004 #1431;Warth, 2004 #1433} as well as on that of members of the DAP complex such as syntrophin and dystrophin [42, 147]. Modifications of the level of expression of M1- and M23-AQP4 may affect water movement and possibly the properties of the BBB [38].

An early induction of AQP4 correlating with the absence of edema formation 1h after ischemia onset with thrombin preconditioning (TPC), has been reported [168]. On the other hand, increased AQP4 expression has been shown to enhance water permeability in oocytes and it is very likely that increased AQP4 expression in astrocyte endfeet contributes to exacerbate cytotoxic edema after cerebral ischemia [69, 132]. Our cell surface biotinylation assay shows a shift of both AQP4-M1 and AQP4-M23 isoforms to the plasma membrane *in vitro* by oxidative stress, suggesting a change in OAP size during cerebral ischemia which might be due to the effect of increased ROS generation on both isoforms [131]. Our furture studies will focus on the effect of H_2O_2 on the expression of M1- and M23-AQP4 isoforms at the plasma membrane. Furthermore, the role of different residues of M1- and M23-AQP4 on AQP4 expression the plasma membrane in response to H_2O_2 will be determined (the phosphorylation site S111, S180, the palmitoylation site C13, C17) by transfecting different AQP4 mutants in CHO cells.

3.4 Trafficking of aquaporin 4 in brain ischemia

Our present study shows that H_2O_2-induced overexpression of AQP4 at the plasma membrane is not due to its *de novo* synthesis since inhibition of protein synthesis with cycloheximide did not prevent this effect. Other groups as well as our previous studies have demonstrated that the the interaction of the DAP complex with the ECM plays a key role in AQP4 targeting to perivascular astrocyte enfeet. However, the mechanisms regulating AQP4 trafficking to the plasma membrane in astrocytes have not been investigated yet. When brain ischemia occurs, large quantities of ROS are generated and activate the MMP-9 that degrades major ECM components such as collagen IV [56]. Other studies have reported a decrease in the expression of adhesion molecules such as laminin in the perivascular extracellular space in the ischemic brain [340]. In addition, an *in vitro* study showed a correlation between endothelial cell death and ECM disruption by MMP activation. Together these data imply that ROS-mediated ECM degradation may be involved in altering the AQP4 expression at the cell surface, however neither the mechanism nor the molecules involved have been characterized. In the present study, we began to unravel the mechanisms involved in the regulation of AQP4 cell surface expression and we demonstrate that caveolin-1, a major component of caveolae in lipid rafts, which cofractionates with AQP4 in lipid enriched domains, regulates H_2O_2-induced increase of AQP4 surface expression in astrocytes.

3.5 Relevance and future directions

The relevance of these studies needs to be considered in terms of both therapeutic and pathological implications in brain. Understanding the mechanisms underlying the physiological, pathophysiological and pharmacological effects that either impair or enhance astrocyte volume may help in the design of therapies that alleviate CNS manifestations in brain edema.

In order to identify the AQP4 promoter sequence that regulates the response to oxidative stress, we generated 5 different luciferase reporters by inserting 5 different promoter sequences of AQP4 into pLuc3. By aligning these promoter sequences to transcription factor

binding domains (Transcription Element Search Software-TESS- database), we found many transcription factor binding sites in the AQP4 promoter. Some sequences correspond to *c-jun- and c-fos*-binding domains, which are components of activator protein-1 (AP-1), formed by their hoterodimers. AP-1 is important in NF-AT-mediated cytokine gene activation in many cases [341]. The signal transduction pathway responsible for AP-1 activation involves *c-jun* and p38 kinase-activated protein kinase, which is activated by oxidative stress.

A Chip assay will be used to find transcription factors that bind to the AQP4 promoter and the experiments will focus on their function in the regulation of AQP4 expression in response to oxidative stress *in vitro* and in brain ischemia *in vivo*. These experiments [56] will be carried out both on astrocyte cultures subjected to oxidative stress and brain tissue subjected to MCAO. Using both *in vitro* and *in vivo* models of oxidative stress in brain may enable the identification of novel signaling pathways involved in the regulation of AQP4 expression in pathological conditions.

Several mechanisms of oxidative damage in ischemia and post-ischemic brain have been outlined, leaving little doubt that oxidative stress is a major contributor to ischemic brain injury [342-344]. The advent of transgenic mutants and relatively selective pharmacological antioxidants has allowed improved definition of the varied mechanisms of oxidative stress and potential targets for therapeutic interventions. Our study revealed that AQP4 is a new therapeutic target of antioxidants since these are able to counteract the effect of ROS on AQP4 expression which expression is enhanced in oxidative stress. By evaluating the effect of antioxidants on AQP4 expression in primary atrocyte cultures subjected to oxidative stress using a high throughput screening, we may identify components that could effectively attenuate cytotoxic edema. In our future studies, *in vivo* experiments will be conducted using MCAO, which has been shown to cause cytotoxic edema and in parallel increases endogenous ROS level, to determine the changes in M1- and M23-AQP4 expression. Subsequent experiments will address whether the alteration in the M23/M1-AQP4 ratio impacts OAPs formation using FFEM.

The findings in this thesis demonstrate that the phosphorylation of tyrosine 14 of caveolin-1 regulates the H_2O_2-induced AQP4 membrane expression. This effect is specific to AQP4 since β-DG membrane expression remains unchanged despite the phosphorylation of caveolin-1. In addition, the interaction between β-DG and caveolin-1 is not altered in the

presence of H_2O_2 (data not shown). In our previous studies, we showed that AQP4 is enriched in lipid raft-containing fractions where it co-distributes with caveolin-1, whereas β-DG co-distributes with the TfR and α-tubulin in non lipid raft-containing fractions [305]. These data may explain the selective effect of caveolin-1 phosphorylation on AQP4 but not β-DG cell surface expression in the presence of H_2O_2.

A growing body of evidence suggests that caveolae are directly involved in the internalization of membrane components, extracellular ligands like cholera toxin, folic acid and serum albumin [277, 345-348]. The binding of a ligand to its receptor triggers phosphorylation of tyrosine residues in proteins associated with the caveolae coat, indicading that the pinching off of caveolae can be regulated by synchronized function of kinases and phosphatases. The exact mechanism of this regulation, however, is not entirely known. The integrin-signaling cascade, which controls focal adhesion assembly and turnover, is specifically required to activate or maintain caveolae/raft-mediated endocytosis [349]. Ca^{2+}/calmodulin-dependent signaling and actin turnover orchestrated by Cdc42/Rac1 are two interconnected systems that exert opposite effects on the endocytic routes, suppressing caveolae/raft-mediated endocytosis. A more complex regulatory activity is observed for the stress-induced and mitogenic signalling cascades (JNK/MAPK), which can exert positive as well as negative effects on integrin-signaling-and Ca^{2+}/calmodulin-dependent endocytic routes. In our future studies, we will focus on investigating signaling pathways involved in the caveolin1-mediated AQP4 endocytosis in oxidative stress.

To date, there is little evidence showing a correlation between caveolin-1 and brain injury and the conclusions are controversial based on different models. Indeed, it has been reported that increased expression of endothelial caveolin-1 precedes decreased expression of tight junction proteins occludin and claudin-5 following a lesion, as the expression of tight junction proteins are crucial for the maintenance of the BBB integrity [350]. This evidence suggests that caveolae and caveolin-1 have an important role in early BBB breakdown and could be potential therapeutic targets in the prevention of early brain edema. In an experimental intracerebral hemorrhage (ICH) model, it has been shown that caveolin-1 knockout mice have smaller injury volumes, milder neurologic deficits, less brain edema and neuronal death compared to wild-type mice [239]. The protective mechanism in caveolin-1 knockout mice was associated with a marked reduction in leukocyte infiltration, a decrease in

the expression of inflammatory mediators and reduced matrix metalloproteinase-activity. Our study reveals a novel role of caveolin-1 Y14 phosphorylation in the regulation of AQP4 increased membrane expression in response to oxidative stress in astrocytes, suggesting that the phosphorylation of caveolin-1 may be implicated in brain edema and act as an intermediate between increased ROS levels and AQP4 cell surface overexpression. Our future studies will focus on **1)** the phosphorylation level of caveolin-1 and the AQP4 expression at astrocyte endfeet in rats subjected to MCAO, **2)** the role of cavoelin-1 in altering M1 and M23-AQP4 isoforms' expression at the cell surface, **4)** the impact of oxidative stress on the association between the GTPase dynamin-2 and caveolin-1. Subsequently, the implication of caveolin-1 phosphorylation in the formation of OAPs will be examined in the normal brain as well as the ischemic brain.

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