Celeboxib-mediated neuroprotection in focal cerebral ischemia: an interplay between unfolded protein response and inflammation

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Ischemic stroke results from the temporary or permanent lack of blood supply in the brain due to the occlusion of a brain blood vessel. Around 85% of patients with cerebrovascular accidents suffer from ischemic strokes. Although cerebrovascular accidents represent the major cause of death and permanent disability worldwide, thus far, only processes addressed at eliminating the vessel obstruction (chemical or mechanical) have been successfully developed. Many neuroprotective strategies have been tested in preclinical studies, but clinical trials have, so far, failed to result in beneficial effects. These issues may be due to the very complex pathophysiology of ischemic stroke, which involves the integration of multiple signaling pathways ultimately resulting in neuronal loss.

Endoplasmic reticulum-stress and UPR in cerebral ischemia: Some of the consequences of cerebral ischemia are depletion of adenosine 5′-triphosphate (ATP) levels and an imbalance in cellular Ca^{2+} homeostasis. These two events impair proteostasis and compromise proper endoplasmic reticulum (ER) function, leading to the accumulation and aggregation of misfolded/unfolded proteins in the ER lumen, a condition known as ER stress. To counteract this harmful effect, cells activate a mechanism called the unfolded protein response (UPR). The UPR is ignited by three ER transmembrane protein sensors: inositol-requiring enzyme 1 (IRE1), double-stranded RNA-activated protein kinase-like ER kinase (PERK) and activation transcription factor 6 (ATF6), each of which activates a different signaling pathway, thus orchestrating a complex and finely-tuned cellular response aimed at: (1) shutting down translation of most proteins to reduce the unfolded protein load in the ER lumen, (2) increasing protein folding capability by inducing ER chaperones and (3) activating degradation pathways of misfolded/unfolded proteins. However, the UPR is a double-edged sword; if the stress is too severe or persistent, the UPR pathway switches from pro-survival to a proapoptotic response through three different pathways: CHOP/GADD153 (C/EBP [CCAAT/enhancer binding protein] homologous protein), c-Jun N-terminal kinases and caspase 12 (Xin et al., 2014).

A recent report (Santos-Galdiano et al., 2020), which utilized a rat model of 1-hour transient middle cerebral artery occlusion (tMCAO) followed by 12 and 48 hours of reperfusion, showed increases in the levels of glucose-regulated protein 78 (GRP78) and in the amount of polyubiquitinated proteins, which are considered the major hallmarks of ER stress. This study also reported that the increased levels of CHOP and caspase 12 proteins correlate with the progressive increase in infarct volume observed across the reperfusion times, suggesting a key role of ER stress-induced apoptosis in the neuronal damage that follows cerebral ischemia. Data reported by Santos Galdiano et al. (2020) are consistent with a previous report (Nakka et al., 2010), which indicates an uneven activation of the different UPR arms: quick onset of activation of the PERK pathway, prior to 12 hours of reperfusion, and very low or no activation of the IRE1 pathway.

The ability of ER stress/UPR modulators to reduce brain damage in different stroke models evidences the relevance of these pathways in the neuronal damage that follows cerebral ischemia. Thus, the administration of salubrinal, an enhancer of the early PERK-UPR pathway, decreased the infarct volume and reduced ER stress in a tMCAO model (Nakka et al., 2010). This neuroprotective effect of salubrinal has also been reported in a two-vessel occlusion/hypotension rat model of global cerebral ischemia, which revealed a reduction in overall neuronal loss (Anuncibay-Soto et al., 2016) and necroptotic activity (Font-Belmonte et al., 2019).

Crosslink between UPR and inflammation: When activated, the UPR participates in upregulating inflammatory processes. The three UPR sensors (PERK, IRE1 and ATF6) elicit the expression of proinflammatory cytokines and enzymes involved in immunomodulation, such as cyclooxygenase 2 (COX-2). This response is mainly mediated by the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), as well as the proteins of the mitogen activated protein kinase (MAPK) family, c-Jun N-terminal kinases and p38. However, the relationship between ER stress and inflammation in different disease-specific

contexts is still poorly understood and novel mechanisms integrating ER stress and inflammation in neurons, astroglia and microglia continue to emerge; for a detailed review see Sprenkle et al. (2017). Overall, ER stress-induced inflammation aims to control the tissue damage and contribute to tissue repair. In fact, the inflammatory response is beneficial as the first line of defence against ischemic insult. However, the sustained and excessive inflammatory response causes a feed forward loop that results in neural tissue damage and aggravates the ischemic lesion.

Many current pharmacological strategies focus on reducing post-ischemic inflammation in order to control damage progression. Several studies have shown that the use of traditional non-steroidal anti-inflammatory drugs improves neurological outcomes following stroke. However, these agents present different efficiencies and important harmful side effects. To counteract these detrimental effects, pharmaceutical companies have developed selective COX-2 inhibitors as anti-inflammatory drugs, known as the "coxib" family. However, several members of the coxib family actually increase the risk of suffering an ischemic stroke, with some even being withdrawn from the market (rofecoxib and valdecoxib). Moreover, a more recent coxib, robenacoxib, has been reported to accelerate neuronal loss after transient global cerebral ischemia (Anuncibay-Soto et al., 2018).

Nevertheless, a member of the coxib family, celecoxib, has been described as a safer anti-inflammatory agent that presents no or a very low correlation with increased risk of stroke. Celecoxib has been reported to attenuate cell death, both in oxygen and glucose deprivation assays performed on brain slices (Lopez-Villodres et al., 2012) as well as in an *in vivo* intracerebral hemorrhage model (Sinn et al., 2007). Additionally, these neuroprotective effects have been observed in the tMCAO model, in which celecoxib reduced the infarct volume and improved neurological outcomes when administered 1 and 24 hours after the onset of reperfusion (Santos-Galdiano et al., 2018).

Celecoxib-dependent neuroprotection involves ER stress reduction: Celecoxib seems to play additional roles besides the inhibition of COX-2. In this regard, celecoxib has been reported as an anti-tumoral agent with pro-apoptotic effects in cultured cells (glioblastoma cell lines). These effects have been associated with celecoxib-dependent increases in ER stress related to the PERK-UPR pathway in a COX-2-independent manner (Pyrko et al., 2008). However, these "*in vitro*" effects contrast with those observed *in vivo*, where treatment with celecoxib after 1 hour of tMCAO was shown to reduce protein levels of the chaperone

GRP78 and the amount of polyubiquitinated proteins, thus indicating an overall reduction in ER stress (Santos-Galdiano et al., 2020). Further support for celecoxib-dependent ER stress reduction, and its neuroprotective effect, relies on the celecoxib-dependent decreases observed in the protein levels of CHOP and caspase 12, which are widely used markers of the initial stages of ER stress-induced apoptosis, both at 12 and 48 hours of reperfusion. These results fit the neuroprotective effect previously reported in the same model (Santos-Galdiano et al., 2018). These opposing effects of celecoxib on ER stress and apoptosis seen between the "*in vitro*" and "*in vivo*" models has been hypothesized to be related to the systemic inflammatory response mediated by celecoxib in the "*in vivo*" treatment (Santos-Galdiano et al., 2020).

In contrast to the effects of celecoxib in glioblastoma cell lines, post-ischemic celecoxib treatment in the tMCAO model does not modify the PERK-UPR pathway, although it does activate the IRE1-UPR pathway. Thus, celecoxib treatment significantly increases mRNA levels of spliced X box-binding protein 1 (XBP1s), the hallmark of IRE1-UPR pathway activation, at both 12 and 48 hours of reperfusion. Treatment with celecoxib prevents the neuronal density reduction at 12 hours of reperfusion, however, is unable to reduce microglia activation at this time (Santos-Galdiano et al., 2018), suggesting that the early neuroprotection from celecoxib relies more heavily on decreasing ER stress and activating the IRE1-UPR pathway than on its anti-inflammatory properties. The strong activation of the IRE1-UPR pathway by celecoxib could explain the observed reduction in ER stress-induced apoptosis, and agrees with the previously reported effect of increased XBP1s levels in reducing cell death after ischemic insult (Ibuki et al., 2012). The effect of celecoxib on the IRE1-UPR pathway highlights the potential of increasing this UPR arm as a therapeutic strategy in stroke.

Santos-Galdiano et al. (2020) also analyzed the two possible mechanisms underlying the unfolded protein clearance promoted by celecoxib treatment: the ubiquitin proteasome system and autophagy. Their report shows that celecoxib administration increases mRNA levels of proteasome catalytic subunits (β1, β2 and β5) and decreases levels of the proteasomal substrate ubiquitin-binding protein p62/sequestosome-1 (p62/sqstm1), without changing the microtubule-associated protein 1 light chain 3B (LC3B) II/I ratio. As increases in the ratio LC3B II/I mirror increases in autophagosome formation, we inferred that celecoxib treatment does not modify autophagy degradation. Thus, these data support that celecoxib treatment promotes the ubiquitin proteasome system, rather than autophagy, as the main degradation pathway for reducing the unfolded protein overload in the ER lumen and reducing ER stress. The correlation between the celecoxib-dependent increases in proteasome catalytic subunits and XBP1s supports a therapeutic potential of the IRE1-UPR pathway through boosting ER-associated degradation when proteostasis is impaired.

In conclusion, the strong neuroprotective effect of celecoxib reported after 1 hour of middle cerebral artery occlusion includes a celecoxib-dependent IRE1-UPR pathway activation that reduces the ER stress and, consequently, the ER stress-induced apoptosis. These effects correlate with activation of the ubiquitin proteasome system. Contributions to the ER stress response by the anti-inflammatory effect of celecoxib remain unknown, although data reported in the Santos-Galdiano et al. (2020) manuscript highlight the IRE1-UPR pathway as a promising therapeutic target following stroke.

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