PERSPECTIVE

Combining anti-inflammatory and unfolding protein responses to fight stroke

This perspective shows an attempt to obtain synergy effects to fight ischemic stroke by combining agents acting on two different homeostatic mechanisms: inflammation and unfolded protein response (Anuncibay-Soto et al., 2018). Different pharmacological approaches have been assayed to alleviate cerebrovascular accident (stroke), which represents one of the most devastating diseases in the elderly. There are two main types of cerebrovascular accidents: ischemic and hemorrhagic strokes. The former, reaching up to 87% of incidences, represents the most relevant form of cerebrovascular accident. The deprivation of glucose and oxygen due to ischemia results in a lack of energy in cells that leads them to activate mechanisms to recover homeostasis or to death if they cannot overcome the damage. In this regard, after the blood flow in a specific area of the brain is blocked (focal ischemia), two areas can be detected in the injured tissue: the ischemic core, where blood flow is reduced to less than 7 mL/100 g per minute and the penumbra area where some blood flow (7–17 mL/100 g per minute) remains. The ischemic core is characterized by the presence of necrosis, an immediate, irreversible and non-regulated type of cell death. The penumbra surrounds the ischemic core, and presents regulated cell death subroutines such as apoptosis or necroptosis. The delayed cell death in the penumbra area provides some opportunities to prevent the neuronal demise and a considerable effort in research has been focused on limiting the damage in this area. The global cerebral ischemia model where blood flow is blocked to the whole brain for a short time does not present an ischemic core and can be considered as a penumbra model for the whole brain. Thus, this model is very useful for comparing the responses of different areas of the brain to ischemia and in pharmacological research aimed to look for therapies that rescue the damaged cells in the penumbra area (Nour et al., 2013).

Most of the cell components suffer the effects of the loss of energy, including ionic imbalance and swelling of the cell due to depletion of adenosine triphosphate (ATP) needed to maintain the activity of Na^+/K^+ ATPase pumps. This induces a massive release of glutamate, which induces the opening of both voltage-dependent and glutamate-regulated calcium channels in selectively vulnerable neurons. The resulting accumulation of Ca^{2+} in the cytosol activates several calcium-dependent proteases and lipolysis, which causes the formation of reactive oxygen species, leading to extensive damage by oxidization of cellular molecules (Liang et al., 2007). One of the results of the lack of energy is the accumulation of unfolded/misfolded proteins in the cell, a condition known as endoplasmic reticulum stress. To overcome endoplasmic reticulum stress, the cell ignites the unfolded protein response. This response is activated by three different sensors located in endoplasmic reticulum membrane: protein kinase RNA-like endoplasmic reticulum kinase, inositol-requiring protein 1 alpha, and activating transcription factor 6. If the endoplasmic reticulum stress cannot be alleviated by the unfolded protein response, the cell will initiate the apoptosis process (Kumar et al., 2003). The neuroprotective effect of an enhancer of the protein kinase RNA-like endoplasmic reticulum kinase-unfolded protein response pathway, salubrinal, administered prior to ischemia has been described in a middle cerebral artery occlusion model. The post-ischemic administration of salubrinal has been recently reported to provide neuroprotection in the CA1 hippocampal area, a well-known ischemia-vulnerable area (Anuncibay-Soto et al., 2016).

Ischemic brain areas can recover normal blood flow either by recanalization due to direct clot lysis/retrieval of the thrombus that caused the ischemia or by augmentation of downstream collaterals (Nour et al., 2013). This process, called reperfusion, restores glucose and oxygen to the area. Reperfusion is required for survival of the tissue but the restoration of blood flow results in overproduction of reactive oxygen species in mitochondria, rapidly reducing the endogenous antioxidant capacity of the cell. The excessive reactive oxygen species levels damage DNA, nucleic acids and lipids resulting in mitochondrial swelling, cell injury and death, which enhances the inflammatory responses. The initial inflammatory response plays a neuroprotective role; however, excessive inflammation (sterile inflammation) results in more damage and worsens the stroke outcome (Bai and Lyden, 2015). One of the key responses to the enhancement of the inflammatory response is the impairment of the neurovascular unit, formed by astrocites, microglia, endothelial cells and neurons (Fiebich et al., 2014). Swelling of the end-feet of the astrocites and the degradation of the basal lamina by matrix metalloproteinases (are a part of the typical inflammatory response in the brain, allowing blood components to enter into the brain parenchyma. During post-ischemic reperfusion, microglia are activated to phagocytose cell debris, but they also release pro-inflammatory molecules such as interleukin-1, tumor necrosis factor-alpha and interleukin-6 that enhance the inflammatory response and reactive oxygen species production (Bai and Lyden, 2015). In fact, inflammation represents one of the most relevant effects elicited by stroke, which has led to consideration of anti-inflammatory agents as possible therapies against stroke. Different non-steroidal anti-inflammatory drugs have been assayed. The peripheral side effects of anti-cyclooxygenase (COX)-1 non-steroidal anti-inflammatory drugs have led to development of more selective anti-COX-2 agents but this has not necessarily improved the protective effects against stroke. In fact, a number of members of the "coxibs" family, the more recent and selective anti-COX-2 non-steroidal anti-inflammatory drugs, have been withdrawn from the market because they increase the risk of stroke or cardiovascular disease (Cairns, 2007).

The different sources of damage and their onset at different times after ischemia support a logical search for synergistic mechanisms to improve therapies against stroke. In this regard, the crosslink between the unfolded protein response and the inflammatory response is widely described (Hasnain et al., 2010). A recent report compares treatments with salubrinal alone, robenacoxib alone or salubrinal + robenacoxib looking for synergistic effects against stroke (Anuncibay-Soto et al., 2018). Robenacoxib was chosen as one of the more recent members of the coxib family that presents very high selectivity for COX-2 and whose effects on stroke were not previously tested.

Surprisingly, robenacoxib accelerates the neuronal loss and is detrimental to the CA1 region, being unable to prevent neuronal demise 7 days after ischemia, but in combination with salubrinal it prevents this deleterious effect (Anuncibay-Soto et al., 2018). The authors hypothesized that this effect could be the result of vasoconstriction as a consequence of blocking COX-2 in endothelial cells. Agents that are too selective against COX-2 would not prevent platelet activation, which requires COX-1 cross-reactivity (Cairns, 2007; Patrono, 2016). Since inflammation-induced platelet activation is one of the most common thromboembolic complications in patients with cerebrovascular disease, the use of these selective agents could result in worse outcomes. Thus, the use of robenacoxib seems to present a detrimental effect in the CA1 region after ischemia and could increase the damage after a cerebrovascular accident (Anuncibay-Soto et al., 2018).

In this regard, the strong microglial activation in the CA1 region to an anti-inflammatory agent such as robenacoxib is another surprising result in the report of Anuncibay-Soto et al. (2018). Again, this effect can be reversed by salubrinal. The authors suggest that the selective binding of robenacoxib to COX-2 introduces an unbalance in the COX-1 and COX-2 functions that could account for its deleterious effect. Selective functions of COX-1 and COX-2 have been described to play different, indispensable roles that cannot be substituted and would play different functions in the two-hit hypothesis of neuroinflammation. In this model, the first hit is the injury itself, for example hypoxia-ischemia, which leads to the activation of microglia cells inducing the activation of COX-2 and prostaglandin E2 release (pre-neuroinflammation). The second hit occurs when damaged neurons release cytosolic ATP into the extracellular medium, which activates neuronal and glial purinergic receptors enhancing microglial prostaglandin E2 release (neuroinflammation) (Fiebich et al., 2014). In light of this model, robenacoxib would be unable to stop neuroinflammation mediated by COX-1, which would explain the microglial activity and neuronal damage. The combination of salubrinal and robenacoxib would decrease the activity of both isoforms of COX and would explain the higher decrease in the microglial activation observed after this combined treatment (Anuncibay-Soto et al., 2018).

The detrimental effects of some highly selective anti-COX-2 non-steroidal anti-inflammatory drugs, including robenacoxib, suggest that use of anti-inflammatory agents that present activity against COX-1 and COX-2 should be considered in stroke treatments, providing proper controls to prevent peripheral side effects of COX-1 activity (Anuncibay-Soto et al., 2018). Both COX proteins are involved in the stroke response and it has been proposed that progressive steps in the ischemic damage started by COX-1 activation and followed by COX-2 are mediators in a global cerebral ischemia model (Candelario-Jalil et al., 2003). Furthermore, traditional non-steroidal anti-inflammatory drugs with activities against COX-1 and COX-2 present less risk for stroke and cardiovascular pathologies (Roumie et al., 2008).

The effects of robenacoxib or its combination with salubrinal on the endothelial cells is also analyzed in the hippocampal CA1 area in the study of Anuncibay-Soto et al. (2018). The study shows different effects of robenacoxib on the endothelial cells in the CA1 region. Thus, while treatment with salubrinal ameliorated the ischemia-induced damage on the tight junctions of the endothelial cells, robenacoxib increased the damage. In this case the damage cannot be recovered by the combination of salubrinal and robenacoxib. In this regard, robenacoxib cannot reduce the ischemic-induced increase in the transcriptional levels of matrix metalloproteinase-9, while salubrinal, alone or in combination with robenacoxib, is able to reduce this increase (Anuncibay-Soto et al., 2018). In contrast, the intrinsic COX-2 activity of endothelial vascular cells (Patrono, 2016) could account for the robenacoxib-dependent decrease of vascular cell adhesion molecule 1 and intercellular adhesion molecule 1 transcription, which would prevent the impairment of neurovascular unit at the endothelial level. The authors conclude that reducing the endoplasmic reticulum stress seems to be more relevant than inhibiting COX-2 to prevent basal lamina degradation.

In summary, treatment with the highly selective anti-COX-2 agent robenacoxib does not result in neuroprotection, in fact, it makes neuronal loss appear several days sooner. This effect can be prevented by combining the effect with salubrinal. It must be highlighted that the neuroprotective effects of acute treatment with salubrinal can be observed for at least 7 days before the ischemic insult. Robenacoxib increases the glial activation induced by ischemia, while salubrinal does not, but the combination seems able to revert it. The effects of the combination of salubrinal and robenacoxib suggest that an adequate combination of neuroprotective agents can be stronger than their individual neuroprotective effects but the times and agents to use have to be chosen very carefully (Anuncibay-Soto et al., 2018) (**Figure 1**).

This work was supported by the Spanish Ministerio de Economia y Competitividad (MINECO) and co-financed with FEDER funds "una manera de hacer Europa" (reference RTC-2015-4094-1), Junta de Castilla y León (reference LE025P17) and Neural Therapies SL (reference NT-Dev01).

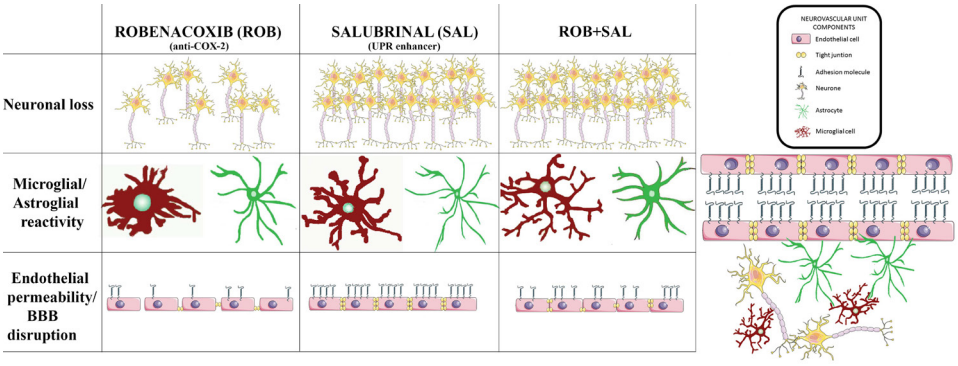

Figure 1 Isolated and combined action of robenacoxib and salubrinal on the the neurovascular unit.
The image represents the main effects of the treatment with salubrinal (SAL), robenacoxib (ROB) and the combined treatment with rob + sal on the neurovascular unit components. First row shows the treatment effects on the neuronal loss. Second row indicates the effects on the glial (microglial and astroglial) reactivity. Third row represents the effects on the endothelial permeability (cell adhesion molecules expression) and on the blood-brain barrier (BBB) disruption. COX-2: Cyclooxygenase-2.

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***Received:** October 8, 2018*
***Accepted:** November 2, 2018*

***doi:** 10.4103/1673-5374.245468*
***Copyright license agreement:** The Copyright License Agreement has been signed by all authors before publication.*
***Plagiarism check:** Checked twice by iThenticate.*
***Peer review:** Externally peer reviewed.*

***Open peer reviewer:** Masaaki Hori, Juntendo University School of Medicine, Japan.*
***Additional file:** Open peer review report 1.*

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P-Reviewer: Hori M; C-Editors: Zhao M, Yu J; T-Editor: Liu XL