● **PERSPECTIVE**

Using organotypic hippocampal slice cultures to gain insight into mechanisms responsible for the neuroprotective effects of meloxicam: a role for gamma aminobutyric and endoplasmic reticulum stress

This perspective aims to put into context the recent article by Landucci et al. (2018) on mechanisms involved in the neuroprotective effects of meloxicam on an organotypic hippocampal slice cultures (OHSCs) model. *In vitro* cell cultures are the main method for studying large quantities of homogeneous cells in an isolated environment. Thus, the use of primary dissociated neuron, astrocyte, oligodendrocyte, microglia, or endothelial cell cultures has become a standard method in many laboratories, contributing substantially to a reduction in the number of *in vivo* assays. Cell cultures allow many different types of assays to be performed in research laboratories, such as survival, proliferation, cell signaling, or studies about the influence of toxic or protective drugs. However, cell cultures do not reproduce the complex cell interactions that occur in the whole organ (Humpel, 2015). Thus, other approaches, such as organotypic cultures, have been developed in recent decades to better align models with *in vivo* situations, with the goal of preserving the original structural and synaptic organization as much as possible. In this regard, the first studies were conducted using hippocampal slices from neonates at 2 to 23 days old. The slices were maintained in culture at the interface between air and a culture medium. They were then placed on a sterile, transparent, and porous membrane and stored in petri dishes in an incubator. This method yielded thin slices that remained one to four cell layers thick and were characterized by a well-preserved organotypic organization (Humpel, 2015).

Donor age is important for organotypic slice cultures, and many assays have been carried out to assess the maturity of the cultured slices. Thus, OHSCs that are prepared from 8-day-old rats and cultured in proper membranes for 12–14 days *in vitro* allow the slices to become mature enough to perform experiments that mimic processes that occur *in vivo*. Two weeks in culture guarantee that slices are not activated by endogenous release of calcium or glutamate, reactive astrogliosis is minimized, and the developing slices have the time to mature and stabilize intrinsic axonal projections. In this regard, slice maturation has been suggested to be associated with the development of new synapses and the stabilization of glutamate transmission (Gerace et al., 2016).

OHSCs have been used as a model for different types of study, such as neurodegeneration, neurotoxicity, and neuroprotection. In particular, OHSCs have been used as a model to study mechanisms of response that occur following cerebral ischemia *in vivo*, as well as the effects elicited by the return of blood flow to the brain after ischemia (known as reperfusion). Ischemia effects are mimicked by exposing OHSCs to oxygen-glucose deprivation (OGD) in a hypoxia chamber for selected periods of time, and reperfusion is mimicked by returning the slices to normoxic conditions, which are called reperfusion-like (RL) conditions. Effects of OGD in the OHSC model have been well characterized, and exposure to OGD for periods ranging from 20 to 40 minutes have shown that, after 24 hours of RL conditions, there is a time-dependent and gradual increase of the injury in the hippocampal CA1 area that involves the selective apoptotic degeneration of pyramidal cells. A period of 30 minutes of OGD allows evaluating the effects of drugs that attenuate CA1 injury, as well as those that produce an aggravation of OGD toxicity (Llorente et al., 2015; Landucci et al., 2018).

Inflammation plays a crucial role in ischemic brain injury. The brain damage induced by ischemia triggers an increase in microglial and astrocyte activity; an increase in the production of cytokines, chemokines, adhesion molecules, and metalloproteinases; and the infiltration of monocytes and leukocytes in damaged brain regions. The role of inflammation in the ischemic process is controversial because a substantial proportion of the inflammatory response seems to aggravate the ischemic lesion, while certain inflammatory responses are beneficial (Kawabori and Yenari, 2015). Therefore, it is necessary to identify harmful and beneficial inflammatory responses to design therapeutic strategies that selectively inhibit harmful responses while improving the beneficial ones.

Cycloxygenases (COX) play a crucial role in the inflammatory response that follows stroke. The role of COX-1 in stroke is unclear and seems to be dependent on the experimental model. While COX-1 deficient mice result in worse outcomes when used in a middle cerebral artery occlusion model of brain ischemia, the inhibition of COX-1 led to an increase in healthy neurons in the hippocampal CA1 region in a model of global cerebral ischemia. These discrepancies may be the result of differences in inflammatory responses between focal and global cerebral ischemia models. COX-2 is induced following brain ischemia. COX-2 inhibition or COX-2 deficient mice result in improved neurological outcome after stroke, and COX-2 overexpression leads to worse outcome (Kawabori and Yenari, 2015). Thus, the use of anti-inflammatory drugs as daily treatments or as treatments for ischemia should be meticulously reviewed.

Meloxicam, a COX-2 preferential non-steroidal anti-inflammatory drug, has been reported to lessen ischemic transcriptional effects in some glutamatergic system genes (see in Landucci et al., 2018), as well as to decrease infarct volume in *in vivo* assays (Jacobsen et al., 2013). Interestingly, the presence of meloxicam in the incubation medium has been reported to decrease cell mortality in the OHSC model after 30 minues of OGD conditions, followed by 24 hours of RL conditions. The presence of meloxicam in the OHSC model also modifies the expression of different glutamatergic genes involved in excitotoxicity induced by OGD (Llorente et al., 2015). Overall, the study of Llorente et al. (2015) shows that meloxicam is able to provide neuroprotection independently of the systemic inflammatory response.

In a recent report, the neuroprotective effects of meloxicam in the OHSC model have been confirmed and are thought to be directly related to gamma aminobutyric A (GABA$_A$) receptors. The authors report that blocking the GABA$_A$ receptor, either with bicuculline (**Figure 1**) or with gabazine, results in loss of the neuroprotective effects of meloxicam (Landucci et al., 2018), giving additional support to the previously described role of the GABAergic system in the effects of non-steroidal anti-inflammatory drugs (Bhattacharya et al., 2014). Landucci et al. (2018) also indicate that the neuroprotective effects of meloxicam rely on the decrease of apoptosis, one of the OGD-induced types of regulated cell death subroutines. Interestingly, the study of Landucci et al. (2018) reveals that the blocking of GABA$_A$ receptors does not significantly increase apoptosis, which indicates that bicuculline elicits other type of cell death subroutines. The effects of meloxicam on apoptosis seem to depend on the cell line where the assays are performed (Fosslien, 2000), and the information of the effects of this agent in the central nervous system are very scarce. Thus, OHCS appears to be an interesting model for the study of the neuroprotective effects of meloxicam, its interactions with GABA$_A$ receptors, and the role of different cell death subroutines linked to OGD in the central nervous system.

Post-ischemic release of GABA, and the subsequent activation of GABA receptors, have been suggested as a contributor to the attenuation of post-ischemic neuronal damage (Cozzi et al., 2002). Different levels of GABA have been reported to play an important role in the responses mediated through GABA$_A$. Thus, high levels of GABA elicit transient inhibitory responses (that is, phasic responses) mediated by the rapid activation of postsynaptic GABA$_A$ receptors. In contrast, continuous low levels of GABA activate extrasynaptic

$GABA_A$ receptors, which results in persistent inhibition of neuronal excitability (tonic response). The peri-infarct area in the cerebral cortex has been reported to present a tonic rather than a phasic neuronal inhibition after stroke (Clarkson et al., 2010). This finding led Landucci et al. (2018) to analyze the pattern of transcription of $GABA_A$ α1, β2, and γ3 subunits in the OHCS model. They found that OGD modifies the pattern of expression of these receptors, which suggests the modification of $GABA_A$ receptor isoforms as a mechanism of response to ischemic damage. The modifications in the $GABA_A$ subunit transcriptional pattern induced by the presence of meloxicam provides additional support to this idea and suggests that the neuroprotective effects of meloxicam are related to changes in $GABA_A$ receptors (Landucci et al., 2018).

In recent years, several reports have described the inflammatory response and its interplay with endoplasmic reticulum stress and its associated unfolded protein response (UPR). The importance of this relationship and the ability of anti-inflammatory agents or endoplasmic reticulum-stress modulators to modify both responses, have been also highlighted after ischemic stroke (Llorente et al., 2013). Endoplasmic reticulum stress is a condition that results from the accumulation of unfolded proteins, and it is one of the major imbalances induced by cerebral ischemia. Endoplasmic reticulum stress leads to the UPR, a complex cellular response that attempts to restore homeostasis by blocking protein synthesis and promoting the expression of proteins, such as chaperones, that help to correctly fold proteins (Schroder and Kaufman, 2005). Meloxicam has been described as being related to the UPR in an *in vivo* global cerebral model (Llorente et al., 2013). Whether meloxicam is able to directly stimulate the UPR or whether it is dependent on the systemic anti-inflammatory response is analyzed in the OHCS model (Landucci et al., 2018). Landucci et al. (2018) evaluate the early UPR response elicited by protein kinase RNA-like ER kinase (PERK) (Schroder and Kaufman, 2005) and report that meloxicam is able to decrease ER stress (**Figure 1**) by maintaining the phosphorylation of eIF2alpha. They also suggest a progressive increase in the levels of ER stress during RL conditions that would be alleviated by meloxicam. Thus, meloxicam seems to be able to directly stimulate UPR independently of its systemic effect.
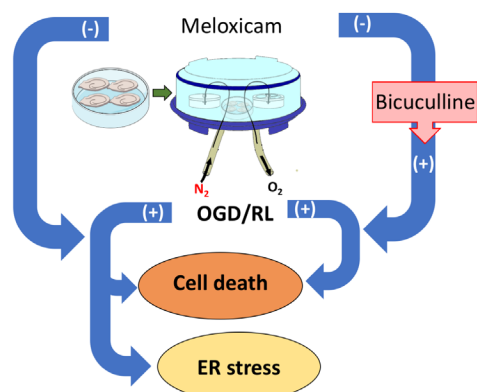
Figure 1 Meloxicam and bicuculline effects on oxygen and glucose deprivation (OGD).
Meloxicam decreases cell death and endoplasmic reticulum stress in the organotypic hippocampal slice culture model after 30 minutes of OGD conditions, followed by 24 hours of reperfusion-like (RL) conditions. Bicuculline, an antagonist of gamma aminobutyric A receptors blocks the neuroprotective effects of meloxicam.

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