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Bicuculline Reverts the Neuroprotective Effects of Meloxicam in an Oxygen and Glucose Deprivation (OGD) Model of Organotypic Hippocampal Slice Cultures

Elisa Landucci,[a†] Irene L. Llorente,[b†] Berta Anuncibay-Soto,[c,d] Domenico E. Pellegrini-Giampietro[a] and Arsenio Fernández-López[c*]

[a] *Sezione di Farmacologia Clinica e Oncologia, Dipartimento di Scienze della Salute, Università di Firenze, Viale Pieraccini 6, 50139 Firenze, Italy*

[b] *Neurology Department, David Geffen School of Medicine, University of California, Los Angeles, United States*

[c] *Área de Biología Celular, Instituto de Biomedicina, Universidad de León, 24071 León, Spain*

[d] *Neural Therapies SL, Edificio Institutos de Investigación, Local B14, Universidad de León, 24071 León, Spain*

Abstract—We previously demonstrated that the non-steroidal anti-inflammatory agent meloxicam has neuroprotective effects in an oxygen and glucose deprivation model (OGD) of rat organotypic hippocampal slice cultures. We wondered if GABAergic transmission changed the neuroprotective effects of meloxicam and if meloxicam was able to modulate endoplasmic reticulum stress (ER stress) in this model. Mortality was measured using propidium iodide. Western blot assays were performed to measure levels of cleaved and non-cleaved caspase-3 to quantify apoptosis, while levels of GRP78, GRP94 and phosphorylated eIF2α were used to detect unfolded protein response (UPR). Transcript levels of GRP78, GRP94 and GABAergic receptor α, β, and γ subunits were measured by real-time quantitative polymerase chain reaction (qPCR). In the present study, we show that the presence of meloxicam in a 30 min OGD assay, followed by 24 h of normoxic conditions, presented an antiapoptotic effect. The simultaneous presence of the $GABA_A$ receptor antagonist, bicuculline, in combination with meloxicam blocked the neuroprotective effect provided by the latter. However, in light of its effects on caspase 3 and PARP, bicuculline did not seem to promote the apoptotic pathway. Our results also showed that meloxicam modified the unfolded protein response (UPR), as well as the transcriptional response of different genes, including the $GABA_A$ receptor, alpha1, beta3 and gamma2 subunits. We concluded that meloxicam has a neuroprotective anti-apoptotic action, is able to enhance the UPR independently of the systemic anti-inflammatory response and its neuroprotective effect can be inhibited by blocking $GABA_A$ receptors. © 2018 Published by Elsevier Ltd on behalf of IBRO.

Key words: organotypic hippocampal slices culture, meloxicam, unfolded protein response (UPR), oxygen and glucose deprivation (OGD), GABAergic system.

*Corresponding author. Fax: +34 987 291917.
E-mail addresses: elisa.landucci@unifi.it (E. Landucci), atrida@g.ucla.edu (I. L. Llorente), ba.soto@neuraltherapies.com, banus@unileon.es (B. Anuncibay-Soto), domenico.pellegrini@unifi.it (D. E. Pellegrini-Giampietro), aferl@unileon.es (A. Fernández-López).
† These authors contributed equally to this study.

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ATF4, activating transcription factor 4; BIC, OGD 30 min followed by 24 h of normoxic conditions, both of them in the presence of bicuculline; CA1, Cornu ammonis 1; CCD, charge-coupled device; cDNA, complementary deoxyribonucleic acid; CHOP, CCAAT/enhancer-binding protein (C/EBP) homologous protein or DNA damage-inducible transcript 3; COX2, cyclooxygenase 2; Ct, cycle threshold; DNA, deoxyribonucleic acid; Dnase, deoxyribonuclease; eIF2α, eukaryotic initiation factor 2 (eIF2) alpha; ER stress, endoplasmic reticulum stress; GABA, gamma aminobutyric acid; GRP78, 78 kDa glucose regulated protein or binding immunoglobulin protein (BiP); GRP94, glucose regulated protein 94 or heat shock protein 90 kDA beta; Ig, immunoglobulin; mRNA, messenger ribonucleic acid; Mel, OGD 30 min followed by 24 h of RL conditions, both of them in the presence of 50 μM meloxicam; Mel + Bic, OGD 30 min followed by 24 h of RL conditions, both of them in the presence of meloxicam + bicuculline; Mel + Gab, OGD 30 min followed by 24 h of RL conditions, both of them in the presence of meloxicam + gabazine; MIQE, minimum information for publication of quantitative real-time polymerase chain reaction experiments; NMDA, N-methyl-D-aspartate; Nmx, normoxic conditions; NSAID, non-steroidal anti-inflammatory drug; OD, optical density; OGD, oxygen and glucose deprivation; OHSC, organotypic hippocampal slice culture; p-eIF2α, phosphorylated eIF2α; PARP, poly (ADP-ribose) polymerase; PI, propidium iodide; qPCR, quantitative real-time polymerase chain reaction; RL, reperfusion-like; RLO, 30 min in OGD conditions; RL1, 1 h in normoxic conditions after 30 min in OGD conditions; RL3, 3 h in normoxic conditions after 30 min in OGD conditions; RL24, 24 h in normoxic conditions after 30 min in OGD conditions; RNA, ribonucleic acid; RNS, reactive nitrogen species; ROS, reactive oxygen species; SEM, standard error of the mean; SDS, sodium dodecyl sulfate; STAU, 10 μM staurosporine for 24 h; TBS-T, 0.1% Tween 20 in 20 mM Tris-buffered saline; UPR, unfolded protein response.

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INTRODUCTION

Neuroinflammation is a hallmark of the process that follows cerebral ischemia. Consequently, anti-inflammatory agents, particularly non-steroidal anti-inflammatory drugs (NSAIDs), have been explored as neuroprotective molecules, both in focal and global cerebral ischemia models (Hauss-Wegrzyniak et al., 1999; Bhattacharya et al., 2013; Llorente et al., 2013a; Ugidos et al., 2017). One of these agents, meloxicam, an NSAID that preferentially inhibits cyclooxygenase 2 (COX2) (Fleischmann et al., 2002) has been tested in this study. COX2 produces prostaglandins that inhibit apoptosis and, therefore, selective COX2 inhibitors would reduce prostaglandin synthesis and restore apoptosis (reviewed by Fosslien, 2000). However, different effects on apoptosis can be observed by different selective COX2 inhibitors that can promote or reduce apoptosis depending on the cell line studied. Mechanisms underlying the neuroprotective effects of meloxicam in the brain are largely unknown and rely on neuronal or astroglia cultures, which can reveal no effect on apoptosis (Ramesh et al., 2017) or no antiapoptotic effects in neuroblastoma cultures (Tasaki et al., 2010). In the present study, we demonstrated for the first time the anti-apoptotic effects of meloxicam on organotypic hippocampal culture slices (OHSC).

Excitotoxicity and endoplasmic reticulum (ER) stress are major imbalances in cell homeostasis that result from cerebral ischemia, a consequence of excessive glutamate receptor activation, which is a well-known paradigm of neural cell death (Rothman and Olney, 1986; Choi and Rothman, 1990). Some glutamatergic receptors, such as N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, play a crucial role in stroke (Lai et al., 2014). Meloxicam seems to be involved in preventing excitotoxicity in light of its ability of attenuating the ischemic-induced changes in the transcript levels of different genes of the glutamatergic system, mainly in NMDA and AMPA receptor subunits (Montori et al., 2010a,b,c; Llorente et al., 2013a).

Some reports link the effects of NSAIDs to the GABAergic system (Coyne et al., 2007; Bhattacharya et al., 2014), which plays a critical role in controlling the excitotoxicity elicited by the release of glutamate after oxygen and glucose deprivation (OGD) (Green et al., 2000; Allen et al., 2004; Clarkson et al., 2010). This mechanism is not well understood, but one of the effects that follow ischemia is the release of high levels of gamma aminobutyric acid (GABA) (Cozzi et al., 2002). These levels have been shown to inactivate or reduce the number of cell surface $GABA_A$ receptors in both *in vivo* and *ex vivo* studies (Alicke and Schwartz-Bloom, 1995; Ricci et al., 2011). Chronic exposure to GABA has been reported to decrease the transcription of $GABA_A$ receptor genes in cultured cells (Baumgartner et al., 1994), in *in vivo* studies (Fenelon and Herbison, 1996) and in rat brain slice assays (Llorente et al., 2013b).

We previously reported that meloxicam provides a neuroprotective effect in the OGD model of OHSC by modulating the transcripts of glutamatergic genes (Llorente et al., 2015). Thus, the neuroprotective effects of meloxicam are independent of the systemic anti-inflammatory response and its effects on the blood–brain barrier, and support the notion that meloxicam neuroprotection could prevent excitotoxicity, which raises a first question of this study as to whether $GABA_A$ receptors are playing a role in the inflammatory-independent neuroprotective effects of meloxicam.

ER stress is the accumulation of unfolded proteins due to ATP depletion and Ca^{2+} release into the cytosol elicited by a lack of energy and oxygen, an imbalance that has been reported to occur during cerebral ischemia (Bai and Lyden, 2015) and in OGD-cultured cells (Ibuki et al., 2012; Chiu et al., 2014). ER stress results in increases in reactive oxygen species (ROS), reactive nitrogen species (RNS) and can also lead to cell death and increases in CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP). This transcription factor has been reported to be a marker of unfolded protein response (UPR) activation, together with increases in 78 kDa glucose-regulated protein or binding immunoglobulin protein (BiP) (GRP78) and glucose regulated protein 94 or heat shock protein 90 kDA beta (GRP84) chaperons (Fawcett et al., 1999; Truettner et al., 2009). The involvement of meloxicam in the UPR elicited by ER stress has been reported in a global cerebral model *in vivo* (Llorente et al., 2013a), and raises the second question of this study: can meloxicam directly stimulate the UPR or is this response mediated as a part of the systemic anti-inflammatory response?

In this study, we also demonstrated, for the first time, that the neuroprotective effect of meloxicam involves the $GABA_A$ receptor, as well as how the transcript levels of the most ubiquitous subunits of $GABA_A$ receptors are modified by OGD and meloxicam.

EXPERIMENTAL PROCEDURES

Reagents

Meloxicam sodium salt: 4-hydroxy-2-methyl-N-(5-methyl-2-thiazolyl)-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide sodium hydrate (CAS 71125-39-8) and staurosporine (CAS 62996-74-1) were purchased from Sigma (St Louis, MO, USA). 6-Imino-3-(4-methoxyphenyl)-1(6H)-pyridazinebutanoic acid hydrobromide (gabazine; CAS 104104-50-9) and (-)-bicuculline methiodide (R-(R*,S*)]-5-(6,8-Dihydro-8-oxofuro[3,4-e]-1,3-benzodioxol-6-yl)-5,6,7,8-tetrahydro-6,6-dimethyl-1,3-dioxolo[4,5-g]isoquinolinium iodide (CAS 40709-69-1) were purchased from Tocris (Ellisville, MO, USA). Tissue culture reagents were obtained from Gibco-BRL (San Giuliano Milanese, MI, Italy) and Sigma (St Louis, MO, USA).

Preparation of rat organotypic hippocampal slice cultures

All animal experiments were carried out in accordance with the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines and the Guidelines of the European Union Council Directive (63/2010/EU) for

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