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Receptor for Advanced Glycation Endproducts is upregulated in temporal lobe epilepsy and contributes to experimental seizures

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ABSTRACT

Toll-like receptor 4 (TLR4) activation in neuron and astrocytes by High Mobility Group Box 1 (HMGB1) protein is a key mechanism of seizure generation. HMGB1 also activates the Receptor for Advanced Glycation Endproducts (RAGE), but it was unknown whether RAGE activation contributes to seizures or to HMGB1 proictogenic effects.

We found that acute EEG seizures induced by 7 ng intrahippocampal kainic acid (KA) were significantly reduced in *Rage*−/− mice relative to wild type (Wt) mice. The proictogenic effect of HMGB1 was decreased in *Rage*−/− mice, but less so, than in *Tlr4* −/− mice.

In a mouse mesial temporal lobe epilepsy (mTLE) model, status epilepticus induced by 200 ng intrahippocampal KA and the onset of the spontaneous epileptic activity were similar in *Rage*−/−, *Tlr4* −/− and Wt mice. However, the number of hippocampal paroxysmal episodes and their duration were both decreased in epileptic *Rage*−/− and *Tlr4*−/− mice vs Wt mice.

All strains of epileptic mice displayed similar cognitive deficits in the novel object recognition test vs the corresponding control mice.

CA1 neuronal cell loss was increased in epileptic *Rage*−/− vs epileptic Wt mice, while granule cell dispersion and doublecortin (DCX)-positive neurons were similarly affected. Notably, DCX neurons were preserved in epileptic *Tlr4*−/− mice.

We did not find compensatory changes in HMGB1-related inflammatory signaling nor in glutamate receptor subunits in *Rage*−/− and *Tlr4* −/− naïve mice, except for ~20% NR2B subunit reduction in *Rage*−/− mice.

RAGE was induced in neurons, astrocytes and microvessels in human and experimental mTLE hippocampi.

We conclude that RAGE contributes to hyperexcitability underlying acute and chronic seizures, as well as to the proictogenic effects of HMGB1. RAGE and TLR4 play different roles in the neuropathologic sequelae developing after status epilepticus.

These findings reveal new molecular mechanisms underlying seizures, cell loss and neurogenesis which involve inflammatory pathways upregulated in human epilepsy.

Introduction

Investigations of the mechanisms involved in the genesis of seizures led to the identification of key inflammatory pathways activated in the brain in response to ictogenic or epileptogenic injuries (Vezzani et al., 2011). In this regard, we described recently the activation of the High Mobility Group Box 1 (HMGB1)-Toll-like receptor 4 (TLR4) axis in human and experimental epilepsy, and its crucial role in ictogenesis (Maroso et al., 2010).

HMGB1 is a chromatin protein ubiquitously expressed by cells. HMGB1 is also a key trigger of inflammation. Upon its nuclear-to-cytoplasmatic translocation, which occurs during seizures, following tissue injury, or when cells are exposed to specific biological stressors, HMGB1 is extracellularly released and behaves as a proinflammatory cytokine. HMGB1 is one member of a set of endogenous molecules (i.e. the *alarmins* also named *danger associated molecular patterns*, *DAMPs*) released by cells in order to alert the microenvironment of

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ensuing pathological events and start homeostatic programmes for tissue repair (Bianchi, 2007; Bianchi and Manfredi, 2007).

Among the several identified receptors for HMGB1, the two best characterized are TLR4 and Receptor for Advanced Glycation Endproducts (RAGE). Both TLR4 and RAGE are type 1 transmembrane receptors playing key roles in innate immunity activation. Both receptors are constitutively expressed by many cell types, and they can be rapidly upregulated upon interaction with their ligands. Depending on the cellular context and available ligands, the activation of RAGE and TLR4 mediates several cell responses, including inflammation, cell proliferation and migration, and cell differentiation. These receptors are also expressed in CNS where they are endowed of pleiotropic functions. For example, the activation of RAGE by HMGB1, or other endogenous ligands of the S100/calgranulin family, can induce differentiation of immature neurons and has a role in axonal and dendritic elongation (Ding and Keller, 2005; Rauvala and Rouhiainen, 2010). Moreover, these receptors mediate the activation of innate immune mechanisms leading to the onset of inflammatory processes (Bianchi and Manfredi, 2007; Schmidt et al., 2000; Stern et al., 2002).

Both TLR4 and RAGE are induced in neurons and glia following acute and chronic CNS injuries (Gao et al., 2012; Lue et al., 2005; Ma and Nicholson, 2004; Qiu et al., 2008; Sasaki et al., 2001; Zhang et al., 2011). Excessive production and release of "damage-associated molecular pattern" molecules (DAMPs, e.g. HMGB1, S100 proteins, amyloid-β) activating TLR4 and/or RAGE might lead to acute and chronic diseases (Bianchi, 2007). Accordingly, these signaling molecules have been implicated in disease progression in several CNS pathologies (Huttunen et al., 2000; Rauvala and Rouhiainen, 2010; Schmidt et al., 2000; Stern et al., 2002). HMGB1 release from brain cells is involved in the development of ischemic injury and blood–brain barrier (BBB) disruption induced by stroke (Qiu et al., 2008; Zhang et al., 2011), and in neurodegeneration after traumatic brain injury (Gao et al., 2012). Both RAGE and TLR4 mediate these deleterious effects by activation of NF-kB and ERK signaling, leading to tissue inflammation (Qiu et al., 2008; Zhang et al., 2011).

We showed that TLR4 is induced in neurons and astrocytes in the hippocampus of patients with mesial Temporal Lobe Epilepsy (mTLE) (Maroso et al., 2010). In epileptogenic cortical tissue from developmental cortical malformations, TLR4 and RAGE are both induced in dysplastic neuronal cells and reactive astrocytes; RAGE is also increased in balloon cells and giant cells as well as in HLA-DR-positive microglia (Zurolo et al., 2011). The two elective endogenous ligands of these receptors in the CNS, HMGB1 (for both receptors) and S100β (for RAGE) (Bianchi, 2007; Sims et al., 2010) are also induced in human epileptic foci in neuronal and glial cells (HMGB1) (Maroso et al., 2010; Zurolo et al., 2011) or specifically in astrocytes (S100beta) (Griffin et al., 1995), thus supporting the activation of these pathways in human epilepsy. Notably, the activation of the HMGB1-TLR4 axis in animal models contributes to aberrant neuronal excitability underlying seizures (Maroso et al., 2010).

Since HMGB1's effects are often mediated by both TLR4 and RAGE (Mazarati et al., 2011; Rauvala and Rouhiainen, 2010; van Beijnum et al., 2008), we set out to determine the contribution of RAGE to seizures and to HMGB1's proictogenic effects, as compared to TLR4. Moreover, since no information was available yet on the role of innate immune mechanisms in epilepsy development, we studied the role of RAGE and TLR4 in spontaneous epileptic activity induced by status epilepticus in a mouse model of mTLE.

In this study, we used mouse mutants lacking *Rage* or *Tlr4*. A pharmacological approach was precluded since small molecule antagonists of RAGE are under development (Deane et al., 2012) but not yet available. TLR4 antagonists do not cross the intact BBB and, although effective on seizures upon intracerebral injection (Maroso et al., 2010), they display a transient effect (about 2 h) making their use impractical in a progressive model of epilepsy. Our data show that RAGE contributes to seizures as well as to HMGB1 proictogenic effects, and plays a role in neuropathology ensuing after status epilepticus. This evidence, together with the increased expression of RAGE in experimental and human mTLE, identifies a new molecular mechanism potentially involved in human epilepsy.

Material and methods

Animals

We used ~8 week-old wild type (Wt) C57BL6 male mice, strain-, age- and gender-matched to *Rage*−/− and *Tlr4*−/− mice. The generation of the *Rage*−/− mouse line has been described in detail (Andrassy et al., 2008; Liliensiek et al., 2004). Briefly, *Rage*−/− mice are viable and display normal reproductive fitness. No spontaneous disease development was observed in these mice up to the age of 6 months under SPF conditions. No significant difference was reported in the mean body weight between Wt and knock-out (KO) mice, as confirmed in our study (not shown). A series of standard behavioral tests were previously done to identify the phenotype of *Rage*−/− mice (Sakatani et al., 2009). *Rage*−/− mice showed more activity in their home cage during the dark phase but similar exploratory behavior in the open field as compared to Wt mice. *Rage*−/− mice had also an enhanced auditory startle response when exposed to auditory signals. No significant differences were instead observed in the Morris water maze, classical fear conditioning and elevated plus maze (Sakatani et al., 2009). Long-term potentiation was also not affected in *Rage*−/− mice (Origlia et al., 2008). Notably, these mice show an impaired ability to mount an inflammatory response after an innate immune challenge (Liliensiek et al., 2004; Tikellis et al., 2008).

Tlr4−/− mice were developed by The Jackson Laboratories (*Strain Name: C57BL/10ScNJ*). They have a deletion of the *Tlr4* gene resulting in the absence of both mRNA and protein, thus these mice do not respond to lipopolysaccharide (LPS) stimulation. The phenotype characterization of these mice is reported in detail in the vendor web site (C57BL/10ScNJ, Stock Number 003752; http://jaxmice.jax.org/strain/003752.html).

Rage or *Tlr4* deletion, although compromising the innate immune response, does not modify the adaptive immune response both in vitro and in vivo (Liliensiek et al., 2004; Oliveira et al., 2010).

Receptor KO and Wt mice colonies were maintained in SPF facilities at Mario Negri Institute. Mice were housed at a constant room temperature (23 °C) and relative humidity (60 ± 5%) with free access to food and water and a fixed 12 h light/dark cycle. All experimental procedures were conducted in conformity with institutional guidelines that are in compliance with national (D.L. n.116, G.U. Suppl 40, February 18, 1992) and international guidelines and laws (EEC Council Directive 86/609, OJ L 358, 1, December 12, 1987, Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996).

Mouse model of acute seizures

Wt (n = 10), *Rage*−/− and *Tlr4*−/− mice (n = 8 each group) were surgically implanted under general gas anesthesia (1.4% isoflurane in a mixture of 70% N_2O - 30% O_2) and stereotaxic guidance (Balosso et al., 2008; Maroso et al., 2010). Two nichrome-insulated bipolar depth electrodes (60 μm OD) were implanted bilaterally into the dorsal hippocampus (from bregma, *mm*: nose bar 0; anteroposterior −1.8, lateral 1.5 and 2.0 below dura mater; Franklin and Paxinos, 2008). A 23-gauge cannula was unilaterally positioned on top of the dura mater and glued to one of the depth electrodes for the intrahippocampal injection of kainic acid (KA; see later). The electrodes were connected to a multipin socket and, together with the injection cannula secured to the skull by acrylic dental cement. The correct position of the electrodes and injection needle in each mouse was evaluated by post-hoc histological analysis of brain tissue at the end of the experiments (see later).