HYPOXIA INDUCIBLE FACTOR-1 MEDIATES NEURONAL EXPRESSION OF THE RECEPTOR FOR ADVANCED GLYCATION ENDPRODUCTS FOLLOWING HYPOXIA/ISCHEMIA

Paola Pichiule1, Juan Carlos Chavez3,4, Ann Marie Schmidt2, and Susan J. Vannucci1

1Department of Pediatrics and 2Surgery, Columbia University, New York, NY 10032; 3Burke Medical Research Institute, White Plains, New York, NY 10605, and 4Department of Neurology and Neuroscience, Weill Medical College of Cornell University, New York, NY 10021.

Activation of the receptor for advanced glycation endproducts (RAGE) by its multiple ligands can trigger diverse signaling pathways with injurious or pro-survival consequences. In this study, we show that RAGE mRNA and protein levels were stimulated in the mouse brain after experimental stroke and systemic hypoxia. In both cases, RAGE expression was primarily associated with neurons. Activation of RAGE-dependent pathway(s) post-ischemia appear to have a neuroprotective role since mice genetically deficient for RAGE exhibited increased infarct size 24 hours after injury. Upregulation of RAGE expression was also observed in primary neurons subjected to hypoxia or oxygen-glucose deprivation (OGD), an in-vitro model of ischemia. Treatment of neurons with low concentrations of S100B decreased neuronal death after OGD, and this effect was abolished by a neutralizing antibody against RAGE. Conversely, high concentration of exogenous S100B had a cytotoxic effect that seems to be RAGE-independent. As an important novel finding, we demonstrate that hypoxic stimulation of RAGE expression is mediated by the transcription factor hypoxia-inducible factor-1. This conclusion is supported by the finding that HIF-1α down-regulation by Cre-mediated excision drastically decreased RAGE induction by hypoxia or desferrioxamine. In addition, we showed that the mouse RAGE promoter region contains at least one functional HIF-1-binding site decreased hypoxia-dependent promoter activation. Specific binding of HIF-1 to this putative HRE in hypoxic cells was detected by chromatin immunoprecipitation assay.

The receptor for advanced glycation end products (RAGE) is a member of the immunoglobin superfamily of cell surface molecules. It was originally identified by its capacity to bind advanced glycation endproducts (AGEs), adducts that accumulate during natural aging and are produced at an augmented rate during diabetes (1). Subsequently, several other ligands for this receptor have been reported including amyloid-β peptide, high-mobility group box 1 (HMGB1), some members of the S100/calgranulin family and Mac-1 (2-5). Multiple studies indicate that RAGE signaling has profound stimulatory effects on gene expression of inflammatory mediators, a mechanism that has been implicated in the pathogenesis of diabetic complications in the periphery (for review see (6). In the central nervous system, the expression of RAGE has been described in several cell types. During the embryonic and early postnatal period, RAGE is highly expressed by hippocampal, cortical and cerebellar neurons (4). Expression of this receptor is limited in the normal adult brain, but enhanced in pathological conditions like Alzheimer’s disease (5;7). In this pathology, RAGE expression was observed not only in neurons but also in endothelial cells, and microglia (5;8). Several RAGE ligands are produced in the brain under normal and pathological conditions; interactions of these ligands with RAGE can lead to multiple molecular and cellular consequences, depending
on the cell type involved and the nature and concentration of the ligand. S100B is one such ligand. In addition to its intracellular function, when secreted S100B can exert autocrine and paracrine effects that have trophic or toxic impact depending on its concentration. It has been proposed that activation of RAGE can mediate both sets of effects (9). In vitro studies using primary neurons or neuroblastoma cells showed that stimulation of RAGE by low levels of S100B lead to neurite outgrowth, and activation of pro-survival pathways during stress conditions like trophic factor deprivation, glutamate, N-methyl-D-aspartate or amyloid-β toxicity (9-12). On the other hand, treatment of primary neurons, astrocytes or microglia with relatively high doses of S100B leads to oxidative stress, NF-κB activation, expression of pro-inflammatory mediators and cytotoxicity (9;11;13;14). Little is known about the in-vivo synergistic or inhibitory effects of S100B and the other RAGE ligands, and for some of these proteins (i.e. HMGB1, S100B and amyloid-β) other cell surface interaction sites besides RAGE have been postulated (15;16). Recently, it was reported that expression of RAGE is enhanced by ischemia both in the brain and heart (17;18). In addition, it is well established that the production of the glial-derived protein S100B is substantially enhanced upon activation in response to cerebral ischemia (19;20). These reports support the possibility of an effective activation of RAGE-mediated signaling in the ischemic brain, the consequences of which have not been explored. Regulation of many cellular responses to ischemia requires the concerted activation of various transcription factors including hypoxia inducible factor 1(HIF-1). HIF-1 is a heterodimer composed of HIF-1α and HIF-1β subunits; HIF-1α is the regulatory component of this complex and its expression is exquisitely regulated by an oxygen-dependent post-translational modification that targets HIF-1α for proteosomal degradation (for review see (21)). During hypoxia, HIF-1α is stabilized, translocates into the nucleus where it binds HIF-1β and forms the active HIF-1 complex. Interaction of HIF-1 with its consensus DNA-binding site is required for the hypoxia-induced expression of a vast array of target genes, which are involved in various cellular and systemic adaptive responses to hypoxia, including erythropoiesis, angiogenesis, vasomotor regulation, cell proliferation and survival, cell death, and matrix metabolism, among others (22;23). HIF-1α is upregulated in the hypoxic-ischemic brain (24-26), however potential interactions with S100B or RAGE have not been investigated. The purpose of this study was to investigate this potential signaling pathway in hypoxia-ischemia. We confirm earlier findings of neuronal RAGE upregulation after cerebral hypoxia-ischemia insult (HI) in the mouse and further demonstrate that RAGE activation in the ischemic brain may have a neuroprotective role since RAGE-null mice displayed exacerbated brain damage when subjected to HI. In addition, by using a loss-of-function approach, we show that HIF-1 is required for RAGE expression during hypoxia. Chromatin immunoprecipitation experiments demonstrate that hypoxia induces the association of endogenous HIF-1α to the RAGE promoter. Finally, we show that activation of RAGE in primary neurons by low concentrations of S100B improves neuronal survival after oxygen-glucose deprivation. Conversely, high concentrations of S100B have RAGE-independent cytotoxic effect. Together, our results indicate that HIF-1 mediates the transcriptional activation of RAGE expression in neurons, and activation of this receptor can be viewed as part of the glia-neuron communication system that might be important for the neuronal response to ischemic insults, and explain the basis for the bi-phasic effects of S100B in the brain.

**Experimental Procedures**

**Animals**-The generation of RAGE-null mice has been described in detail elsewhere (27;28). The RAGE-null animals used for the present study were provided by Dr. A.M. Schmidt; these animals have been backcrossed into the C57Bl/6J strain for >10 generations at Columbia University animal facility. Age-matched wild type C57Bl/6J mice were used as controls (The Jackson Laboratories, Bar Harbor, ME). RAGE-null mice are viable, display normal development and reproductive capacity. We found no differences in pre-ischemic body weight and blood glucose levels among RAGE-null and control animals. Toth et.al recently described normal brain weight and no white matter abnormalities in RAGE-null mice (29). Mice carrying conditional HIF-1α floxed
alleles (HIF-1α<sup>F/F</sup>) were generously provided by Dr. Randal Johnson (University of California, San Diego). These animals (C57Bl/6 genetic background) were generated by engineering loxP sites flanking exon 2 of the HIF-1α gene as described previously (30).

**Induction of unilateral cerebral hypoxia-ischemia damage-** Hypoxia-ischemia was induced in male RAGE-null or wild-type mice (12-15 weeks) as described previously (31-32). Animals were anesthetized with isoflurane (2.5% induction, 1.5% maintenance), and the right common carotid artery was isolated and double ligated with 4-0 surgical silk. The incision was sutured, and animals were allowed to recover with access to food and water for 2 hours. Then, animals were exposed to hypoxia (30 min) in custom-designed plexiglass chambers individually equipped with oxygen and temperature sensors (OxyCycler A-series, BioSpherix, Redfield, NY). Oxygen levels during hypoxic exposure were monitored and controlled at a constant concentration of 8% O2 balanced with nitrogen. Chamber temperature was maintained at 35°C; preliminary experiments showed that at this temperature, animals' rectal temperature was 37°C. After hypoxia, animals were allowed to recover to normoxia for 30 min in the chambers, and then returned to their cages. Animals were euthanized at 6 hours to 5 days of recovery. The duration of systemic hypoxia was chosen to assure consistent injury in 80% of the animals and low mortality rate by 48 h of recovery. Animals with right common carotid artery ligation without hypoxia were used as sham-operated controls.

**Systemic hypoxia-C57Bl6J male mice (12 weeks old) were exposed to normobaric hypoxia (10% O2 balanced with nitrogen) for 24 and 48 hours (BioSpherix) with access to food and water. Littermate controls were kept under normoxia in a similar chamber for the duration of the experiment. Animals were sacrificed immediately after hypoxia.

**Infarct volume and immunohistochemistry-** Brains were quickly removed from skull and frozen in dry-ice chilled isopentane (-30°C). Coronal sections (25 μm) were cut serially in a Leica cryostat and mounted on superfrost slides (VWR). The extent of brain infarction was identified using cresyl violet staining; infarcted area was determined indirectly by subtracting the area of healthy tissue in the ipsilateral hemisphere from the area of the contralateral hemisphere. Infarction volume was calculated by integration of infarct areas measured in 12 equidistant sections (at 250 μm intervals) encompassing the entire lesion. For immunohistochemistry, sections were fixed with ice-cold methanol (-80°C), incubated with PBS containing 0.4% (v/v) Triton X-100 and 10% (w/v) normal serum for 2 hours. Subsequently, sections were incubated overnight at 4°C with rat polyclonal antibody against RAGE (1:200; Vector Laboratories, Burlingame, CA) and Cy3-conjugated streptavidin (1:500, Molecular Probes). Sections were subsequently double-stained for the neuronal specific nuclear protein NeuN (1:200, Chemicon) using the Vector M.O.M Immunodetection kit. An additional group of sections were double-stained with the astrocyte marker glial fibrillary acidic protein (GFAP), using a polyclonal rabbit anti-GFAP antibody (1:400; Dako) and a secondary anti-rabbit antibody conjugated with fluorescein (1:100; Invitrogen). Staining was analyzed and documented using an Axiovert 200M microscope equipped with AxioVision software. The specificity of RAGE immunohistochemistry was confirmed by showing negligible staining in the ischemic RAGE-null mouse brain (not shown).

**Cell culture and treatments-** Primary neuronal cultures were prepared from cerebral cortices of wild type C57Bl/6J or homozygote conditional floxed HIF-1α (HIF-1α<sup>F/F</sup>) mouse embryos (E15), according to the protocol described by Chavez et al (33). Briefly, dissected cortices were dissociated in Earl’s balance salt solution (EBSS) containing papain (50 U/ml) and DNase I (100 U/ml). Cells were seeded in poly-D-lysine coated plates under serum-free conditions using Neurobasal medium supplemented with B27, glutamine (2mM), glutamate (25 μM) and β-mercaptoethanol (25 mM) (Invitrogen, Carlsbad, CA). On the fourth day of plating, one-half of the medium was replaced with glutamate-free B27/Neurobasal medium, and subsequently cultures were fed every 4 days with glutamate-free medium. These cultures contain >90% neurons as determined by microtubule associated protein-2 cytochemistry. Experiments were performed in neurons at day in vitro 10-15 (D.I.V) unless otherwise indicated.
3T3 NIH cells (ATCC) were grown in DMEM medium supplemented with 10% bovine fetal serum. Primary neurons were stimulated with bovine brain S100B protein (Calbiochem), desferrioxamine (Sigma) or dimethyloxalglycine (Sigma) freshly dissolved in PBS at the indicated concentrations. RAGE-blocking antibody (Ab-RAGE) was provided by Dr. A.M. Schmidt (Columbia University, NY).

*Hypoxia and Oxygen Glucose deprivation*- A custom-made temperature controlled hypoxic/anaerobic glove-box system was used (Coy Laboratories, Michigan). This system is equipped with an inverted microscope that allows visual inspection of cell viability before terminating each experiment. For hypoxia treatments, the system was set at 37°C with an atmosphere of 0.5%O₂, 5%CO₂ and 94%N₂, and all solutions were pre-equilibrated for at least 12 hours before each experiment. Cells were transferred into the chamber, washed with PBS, and incubated with fresh media for up to 24 hours in a humidified internal incubator. At the end of hypoxia, cell harvesting and lysis were performed within the chamber. In agreement with a previous report (33), this degree of hypoxia did not produce cell death (data not shown). For oxygen-glucose deprivation (OGD), the glove-box system was set at 37°C with an atmosphere of 5% CO₂, 5% H₂, 90% N₂ (anaerobic). Neurons were transferred into the chamber, washed with PBS and incubated with a pre-equilibrated glucose-free balance salt solution for up to 60 minutes. At the end of the procedure, cells were removed from the chamber, fresh Neurobasal media was added (reperfusion) and cultures were returned to a regular incubator. At different periods of reperfusion, neurons were harvested for immunoblot analysis, and cell death was determined using MTT assay following the manufacturer's protocol (CellTiter 96® Assay, Promega, Madison, WI). For experimental controls, cultures were subjected to the same procedures but maintained at normoxia with glucose-containing media in a standard cell-culture incubator.

*Adenoviral vector construction and transduction*- A nonreplicative adenovirus in which the Cre recombinase gene is under the regulation of the CMV promoter was obtained from Vector Biolabs. Reporter adenovirus encoding green fluorescence protein (AdGFP) was used as control. For adenoviral transduction, cortical neurons derived from homozygous HIF-1α/F/F mice were prepared as described above. At D.I.V 6, cells were infected with AdCre or AdGFP at a multiplicity of infection of 100. Significant deletion of floxed HIF-1α alleles by AdCre infection was accomplished at 7 days post-infection as determined by Western blot. Adenoviral infection with either AdCre or AdGFP did not affect neuronal viability (data not shown).

*Preparation of tissue and whole-cell lysates*- Right and left hemispheres were dissected from ischemic brains and frozen in liquid nitrogen. Samples were homogenized with a Polytron homogenizer using ice-cold lysis RIPA buffer (1x PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (Complete, Roche). Homogenates were centrifuged at 10,000g for 10 min (4°C), and supernatants were collected for immunoblots. For the preparation of whole-cell lysates, cells were harvested, washed with PBS, and centrifuged (2000 g x 10 min). The resulting cell pellet was subsequently processed as described above. In all cases, protein concentrations were determined by Bradford protein assay with bovine serum albumin as standard (Bio-Rad, Hercules, CA).

*Western blot analysis*- Samples were electrophoresed on SDS-PAGE under reducing conditions and transferred to nitrocellulose membrane (Bio-Rad) by standard procedures. Membranes were blocked with 10% nonfat dry milk and incubated with the following primary antibodies: rabbit anti-RAGE (Santa Cruz), anti-β-actin (Santa Cruz), anti-HIF-1α (R&D) and anti-HIF-1β (Novus Biologicals Littleton, CO). After washing, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies. Antigen-antibody complexes were visualized by enhanced chemiluminescence detection (ECL, Amersham, Piscataway, NJ). Results were visualized and quantified with Kodak Image Station 4000. Membranes were stripped and reprobed as needed.

*Real time PCR analysis*- Total RNA from cell cultures or brain samples was extracted using RNgents® Total RNA isolation System (Promega). Complementary DNA was synthesized from 2.5 μg total RNA using the Superscript III system with oligo-dT (18) primer (Invitrogen, CA). Real time PCR analysis was performed using
0.5 μl of the final cDNA synthesis mix and mouse specific Taq-Man based gene expression assays (Applied Biosystems). The following assays were employed: Advanced glycation endproducts (Ager) (Mm00545815_m1), vascular endothelial growth factor (Vegf) (Mm00437304_m1), Erythropoietin (Epo) (Mm00433126_m1), and β-actin (Mn00607939_s1). The PCR reaction was carried out in an ABI 7500 real time PCR thermocycler (Applied Biosystems, CA). All reactions were performed in duplicate and independently repeated at least three times. Results were normalized to β-actin, and expressed as fold-increase relative to control.

**DNA electrophoretic mobility shift assay (EMSA)**-Crude nuclear extracts were prepared as described previously (33) and used for EMSA to detect HIF-1 binding activity. All probes were commercially generated (Invitrogen), annealed and radiolabeled with [γ-P32]-ATP using T4 polynucleotide kinase. After incubation of probes with nuclear extracts (5 μg of proteins), DNA:protein complexes were resolved in 5% polyacrylamide gels, and the signal was visualized using a phosphorimager (FUJIFILM). Supershift experiments were performed by pre-incubating nuclear extract with 1 μg of monoclonal HIF-1α antibody on ice for 30 min prior to the addition of labeled probe. The sequences of the sense strands of the oligonucleotides were as follows: 5’gcctggacacagtgttcttagcct 3’ (RAGE-Wt, –1071/-1052, contains a putative HIF-1 binding site), 5’gcctggacaaaagggtttcttagcct 3’(RAGE-Mut), 5’ gcctcaacgtgtgtcctagctca 3’(Epo-Wt), and 5’ gcctaaaagctgtctca 3’(Epo-Mut).

Generation of promoter constructs, transient transfection and reporter gene assays- A fragment containing the 5’-flanking region (~2000 bp) of the mouse RAGE gene was generated from mouse genomic DNA by PCR using the following primers: forward 5’attgctagccagatgacagtaacagtc3’ and reverse 5’attaagcttccatctcccatctgttgc3’. This product was cloned into the Nhe1 and HindIII site of the pGL3-basic vector (Promega), and the generated plasmid was designated pRAGE-luc1. Three additional RAGE promoter constructs (pRAGE-luc2, pRAGE-luc3 and pRAGE-luc4) were generated using the same downstream primer as for pRAGE-luc1 and the following Nhe1 site-containing upstream primers: F2(attgctagccagatgacagtaacagtc3’), F3(attgctagccagatgacagtaacagtc3’) and F4(attgctagccagatgacagtaacagtc3’). In the pRAGE-mutHRE construct, the putative HRE of pRAGE-luc1 was replaced from 5’-ACGTG-3’ to 5’-AAAAG-3’ using the QuikChange site-directed mutagenesis kit (Stratagene). All constructs were verified by DNA sequencing. 3T3 NIH cells at about 90% confluence in 24-well plates were transiently transfected with reporter plasmid (0.5 μg) using Lipofectamin 2000 (Invitrogen) according to the manufacturer’s directions. To correct for variable transfection efficiency, cells were cotransfected with the pRL-SV40 vector (0.05 μg) encoding the Renilla luciferase gene. Transfected cells were allowed to recover for 24 h in fresh medium, and then treated with DFO (100 μM), DMOG (300 μM) or subjected to 0.5%O2. Cells were lysed and luciferase activity was determined with a multiwell luminescence reader (Molecular Devices), by using the Dual-Luciferase Reporter Assay System (Promega).

**Chromatin immunoprecipitation- ChIP assays** were performed as described previously (33). HIF-1α+/F neurons and AdCre-infected neurons (referred to as HIF-1α−/−) were exposed to normoxia or hypoxia (0.5% O2 for 1 to 8 hours) and immediately fixed with 1% formaldehyde/PBS. Immunoprecipitation was performed using 2.5 μg of anti-HIF-1α antibody (R&D) or rabbit IgG as negative control. Immunoprecipitated DNA complex was amplified by PCR using primers encompassing the HRE-containing region of the RAGE promoter: forward 5’tgtgtcttaaataaacatgctgatttatttt’3 and reverse 5’tttaaactatttgcagagtggaaaagatt’3. PCR products were separated by electrophoresis through 1.5% agarose gels and visualized by ethidium bromide staining.

**Statistical analysis**- Data are presented as mean ± standard deviation. Statistical comparisons among groups were made using a one-way ANOVA test with Tukey correction. A p<0.05 was considered statistically significant.

**RESULTS**

**Induction of RAGE expression after cerebral ischemia.** The effect of brain ischemia on RAGE expression was examined using a model of unilateral hypoxic-ischemic (HI) brain damage in...
the adult mouse (31;32). This methodology consists of a permanent unilateral ligation of the right common carotid artery followed by a short period of systemic hypoxia (8% oxygen, 30 min), which causes a reduction of blood flow in affected brain areas by 50-60% (31). Reperfusion of the ipsilateral hemisphere commences with the return to normoxia. As previously described, this model produces ischemic brain damage that is restricted to the hemisphere ipsilateral to the ligation (Fig 1A). There is no obvious neuropathology on the contralateral side of the brain, or after unilateral carotid occlusion alone (sham-operated animals).

At various intervals of reperfusion after HI, RAGE mRNA and protein levels were evaluated in the ipsilateral and contralateral cerebral hemispheres. Real time RT-PCR showed a significant increase of RAGE mRNA levels in the ischemic but not in the contralateral hemisphere, as compared to the sham-operated animals (Fig 1B). This upregulation was evident at 12 hours of recovery (~70% increase), and continued at 2 and 5 days of recovery (~150% increase). Similarly, immunoblot analysis showed a transient increase of RAGE protein levels in the ischemic hemisphere, with maximal induction observed at 24 and 48 hours of recovery (Fig 1C). The polyclonal RAGE antibody used in this assay recognized a major immunoreactive band at approximately 50 kD and additional closely migrating bands that may represent spliced or less glycosylated forms reported previously (34-36). Immunostaining performed at 24 hours after HI revealed that induction of RAGE occurred mainly in neurons surrounding the infarction, as indicated by double staining with the neuronal marker NeuN (Fig 1A). None of the RAGE-positive cells were positive for GFAP (Fig 1A). Conversely, very little staining was evident in the non-damaged hemisphere (Fig 1A). To ascertain whether RAGE-dependent signaling contributes to ischemic brain damage, we subjected wild-type and RAGE-null mice to HI. At 12 hours of reperfusion, there was no significant difference in infarct volume between wild-type and RAGE-null mice. However, RAGE-null mice showed larger infarct volumes at 24 hours of recovery (~24% increase, Fig 1D) suggesting progressive injury, whereas the infarct volume in the wild-type was not different between 12 and 24 hours.

Systemic hypoxia increases RAGE expression in the brain. We then asked whether RAGE expression in the brain can also be modulated by prolonged systemic hypoxia, a stress that unlike HI does not produce significant neuronal death. For this purpose, animals were exposed to 10% O₂ for 24 and 48 hours, and RAGE mRNA levels evaluated in cortex by real-time RT-PCR. Our results showed a significant induction of RAGE mRNA levels in cortex after hypoxia (~80% and 140 % by 24 and 48 hours of hypoxia, respectively) (Fig 2A). The kinetics of RAGE mRNA stimulation closely followed that of VEGF and Epo, which have been previously reported to be hypoxia-inducible genes in the brain (25;37)(Fig 2A). Immunohistochemistry performed at 48 hours of hypoxia showed that RAGE staining co-localized primarily with NeuN-positive neurons and was detected throughout the forebrain (Fig 2B). No significant levels of RAGE protein were detected in the normoxic brains.

HIF-1-dependent regulation of hypoxic expression of RAGE in primary neurons. The in vivo upregulation of RAGE expression in the brain after prolonged hypoxia or ischemia led us to explore the possibility that this gene might be regulated by the conserved HIF-1-dependent pathway. For this purpose, we first evaluated the response of RAGE in cortical primary neurons to desferrioxamine (DFO, 100 μM, 24h) or dimethyloxalglycine treatment (DMOG, 300 μM, 24h), two well-characterized pharmacological HIF-1 inducers under normoxic conditions (38;39). Similar to hypoxia, DFO and DMOG led to an increase in HIF-1α accumulation, while HIF-1β levels were not affected. Interestingly, RAGE mRNA levels were induced not only by hypoxia (0.5% O₂, 24h) but also by DFO and DMOG treatment (~2.3-fold increase, p<0.05). These results resembled those obtained for VEGF mRNA levels (Fig 3), an established HIF-1 target.

To further study the role of HIF-1 in the hypoxic induction of RAGE, the expression of HIF-1α was downregulated in primary neurons using Cre-mediated deletion. For this approach, primary neurons were isolated from homozygous mice harboring lox-P sites flanking exon 2 of the HIF-1α gene (referred to as HIF-1α F/F). These HIF-1α F/F neurons were then transduced with adenovirus encoding Cre-recombinase (AdCre) to generate HIF-1-deficient neurons (referred as HIF-1∆/∆).
Untreated cells or cells infected with adenovirus encoding GFP (AdGFP) were used as controls. Using this approach, previous reports have shown that infection of HIF-1α/F/F neurons or astrocytes with AdCre results in efficient excision of floxed HIF-1α allele (33). Accordingly, immunoblot analysis of AdCre-infected neurons subjected to hypoxia (0.5% O2, 24h) confirmed a substantial decrease of hypoxic HIF-1α protein levels starting at 4 days after the infection, while HIF-1β was not affected (Fig 4A). Conversely, AdGFP-infected neurons showed normal HIF-1α induction after hypoxia. Based on these results, subsequent experiments with HIF-1α/F/F cells were performed at 7 days post-infection. Interestingly, exposure of non-infected or AdGFP-infected neurons to hypoxia (0.5% O2, 24 hours) or DFO led to upregulation of RAGE mRNA and protein levels, whereas HIF-1Δ/Δ neurons presented abrogated RAGE induction (Fig 4B). As expected, HIF-1Δ/Δ neurons also showed a significant attenuation of the induction of VEGF after hypoxia or DFO (Fig 4B and C).

**Hypoxia increases RAGE promoter activity.** The transcription factor HIF-1α binds to a conserved hypoxia-response element (HRE) of target genes containing a HIF DNA binding site referred to as the core HRE (23). Sequence analysis of the 5′-flanking region of the mouse RAGE gene revealed the presence of a putative binding site for HIF with the characteristic motif, 5′-RCGTG-3′, located at position -1063/-1059 upstream of the transcription start site. To determine whether this promoter region mediates RAGE activation in response to hypoxia, we constructed a series of deletion reporter plasmids in which fragments of RAGE 5′-flanking sequences were fused to the firefly luciferase gene (Fig 5A). For this purpose, a ~2 kbp RAGE promoter construct (pRAGELuc1, -2168/-59) was used as a template for the generation of shorter plasmids with truncated 5′-ends: pRAGELuc2 (-1650/-59), pRAGELuc3 (-1175/-59) and pRAGELuc4 (-950/-59). In addition, a mutant plasmid was generated from pRAGELuc1 by introducing a 4bp substitution through site-directed mutagenesis at the putative HIF binding site (pmutRAGE-luc). Due to the low transfection efficacy of primary neurons, 3T3 NIH cells were used for these experiments. 3T3 NIH cells were transiently transfected with these constructs and exposed to normoxia, hypoxia (0.5% O2), DFO (100 μM) or DMOG (300 μM) for 24 hours. Our results showed that luciferase activity of pRAGELuc1, pRAGELuc2 and pRAGELuc3 was stimulated by hypoxia, DFO and DMOG by ~2-fold relative to normoxic controls (Fig 5A). However, pRAGELuc4 or pmutRAGE-Luc constructs were insensitive to hypoxia, DFO or DMOG (Fig 5A). These experiments indicate that a hypoxia-sensitive region extending between -1175 bp and -950 bp in the RAGE promoter harbors regulatory elements including a potential HRE that was sufficient for transcriptional stimulation of the reporter gene.

**HIF-1 binding to the HRE present in the RAGE promoter.** To assess the ability of HIF-1α to interact with the HRE consensus sequence identified in the RAGE promoter, we performed electrophoretic mobility shift assays (EMSA) and chromatin immunoprecipitation analysis (ChIP). EMSA was performed using radiolabeled oligonucleotides harboring the core HRE and flanking sequence of the RAGE promoter (wtRAGE). Fig 5B shows that this oligomer bound constitutive factors present in nuclear extracts of both normoxic and hypoxic neurons. Importantly, an induced binding activity was present in the hypoxic but not normoxic extracts. This hypoxia-induced complex was supershifted by antibodies raised against HIF-1α, suggesting that HIF-1α was a component of this complex (Fig 5B). Disruption of the HIF-1 recognition sequence in the probe (mutRAGE) resulted in loss of binding to nuclear extracts of hypoxic neurons. Similar results were obtained when the assay was performed with an oligonucleotide corresponding to the HRE of the 3′enhancer of Epo gene, an established HIF-1 target (Fig 5B). For ChIP assays, HIF-1α/F/F and HIF-1α/Δ/Δ primary neurons were exposed to normoxia or hypoxia (0.5% O2, 1 to 8 hours). Following cross-linking, an antibody directed against HIF-1α was used to immunoprecipitate the crosslinked DNA-protein complex. PCR analysis of isolated DNA fragments indicates that hypoxia causes HIF-1α recruitment to the endogenous HRE-containing RAGE promoter region. Confirming the specificity of these results, decreased association of HIF-1α with the RAGE promoter was observed in hypoxic HIF-1α/Δ/Δ neurons. In neurons maintained under normoxic conditions, HIF-1α was not significantly associated with the RAGE promoter (Fig 5C).
Effect of RAGE activation by S100B on oxygen-glucose deprivation induced neuronal death. The production and release of the glia-derived protein S100B is greatly augmented by cerebral ischemia (19;20). We next proceeded to study the cellular effects of RAGE activation by S100B in primary neurons subjected to oxygen-glucose deprivation (OGD), an in vitro model of ischemia-reperfusion injury. Exposure of cortical neurons to OGD (60 min) followed by recovery resulted in a progressive decrease of cell viability; by 24 hours there was a ~70 % reduction of cell survival compared to control (Fig 6A). Western blot analysis of RAGE after OGD showed stimulation of RAGE at 3-12 hours of recovery (Fig 6B). Treatment of neurons with S100B (24 h), at a concentration range of 0.1 nM to 200 nM, under normal oxygen/glucose conditions did not have any effect on cell viability (Fig 6C). Additional experiments with higher doses of S100B (250 nM and 500 nM) showed mild toxicity, as cell viability decrease by 20%. Treatment of neurons with S100B (100 nM) for 1 to 24 hours prior to OGD did not have any protective effect against this stress (Fig 6D). In contrast, treatment of neurons with S100B immediately after OGD (0.1 nM to 200 nM, 24 h) led to protection (Fig 6E). Pro-survival effects were noted with 10 nM, and maximal effects were observed with 50-100 nM S100B (~40% increase of cell survival). Higher S100B concentrations (250 or 500 nM) failed to exert any protective effect against OGD. To determine whether the neuroprotective effects of S100B require the RAGE receptor, neurons were co-treated with a specific RAGE neutralizing antibody (50 μg/ml) and S100B immediately after OGD. This led to a complete blockade of the pro-survival effect of S100B confirming that this protective outcome requires RAGE signaling. In contrast, the toxic effect of relatively high doses of S100B (250 or 500 nM) was not affected by this antibody (Fig 6C).

DISCUSSION
Injuries to the mammalian brain, such as hypoxia and ischemia, can induce both neurodegenerative and neuroregenerative responses. An understanding of the specific pathways involved is essential in order to design therapeutic interventions that inhibit neurodegeneration/death pathways while promoting neuroregenerative/plasticity pathways. In the present study, we employed in vivo and in vitro models of hypoxia and ischemia to investigate the response of RAGE to cerebral injury. We show that RAGE is expressed at very low levels under normal conditions in mouse brain, but is significantly upregulated in response to prolonged, mild hypoxia or hypoxia-ischemia; in both conditions, RAGE expression colocalizes primarily with neurons. We also demonstrate that this response is mediated by the interaction of the transcription factor HIF-1 with an HRE sequence on the RAGE promoter, and that HIF-1 mediated stimulation of neuronal RAGE following hypoxia-ischemia is part of the neuroprotective/neuroregenerative response that also involves the glial-derived RAGE ligand S100B. To the best of our knowledge this may represent a direct ligand-independent activation of the RAGE promoter by HIF-1. It is also possible that hypoxia can affect the release of specific RAGE ligands which can subsequently modulate RAGE expression (e.i. HMGB1).

The biology of RAGE has been predominantly studied in the context of diabetic complications in the periphery, and Alzheimer’s disease in the brain. In these situations, evidence indicates that RAGE-mediated signaling leads to pro-inflammatory gene expression, a mechanism believed to be involved in cellular dysfunction (5;6;40). Very limited reports have explored the function of RAGE in acute injury such as cerebral ischemia, a stress that also leads to extensive release of glia-derived S100B and HMGB1, suggesting an effective stimulation of RAGE in this scenario(18-20;41). Excessive accumulation of other RAGE ligands such as AGEs and amyloid β-peptide are not likely in the ischemic adult brain; however, as a consequence of senescence or diabetes, AGEs levels do increase and can potentially elicit RAGE-dependent responses (42). In the present study, we found a sustained induction of RAGE mRNA and protein levels after cerebral hypoxia/ischemia. This response was confined to the ischemic hemisphere while the contralateral hemisphere showed unchanged RAGE expression. Additionally, our results showed increased RAGE immunolabeling in neurons located in the ischemic hemisphere at 24 and 48 hours of recovery, which is in agreement with a previous study demonstrating increased
RAGE mRNA by \textit{in situ} hybridization in ischemic neurons at 72 hours of recovery from brain hypoxia-ischemia in rats (18). However, the possibility that other cell types express this receptor at different stages during the evolution of the infarct can not be ruled out. In this regard, in contrast to our findings, Toth et al recently reported RAGE immunolabeling not only in neurons but also in endothelial and glial cells in diabetic and normal mouse brain(29). The reasons for this discrepancy are currently unknown but may be related to differences in primary antibodies and/or staining protocols that could lead to variations in labeling effectiveness. In our study, specificity of RAGE staining was confirmed by showing negligible staining in ischemic RAGE-null mice.

Insight into the potential role of RAGE in the pathophysiology of stroke was obtained using mice genetically deficient for RAGE. Our results showed that during early reperfusion (12 hours), RAGE-null animals displayed comparable infarct size to wild-type mice. At 24 hours of recovery, there was no increase in infarct size in wild-type; however, RAGE-null animals developed larger infarct volume. Brain damage was not evaluated at later intervals and the possibility exists of a further evolution of the infarction in both RAGE-null and wild-type animals. Nevertheless, these initial results indicate that activation of RAGE-dependent processes in wild-type animals may prevent ongoing cell death in the peri-infarct area halting spread of the infarction. Our data do not exclude the possibility that germline deletion of RAGE affects systemic parameters like vascular tone that might indirectly account for the greater ischemic brain injury.

In stark contrast to our results in brain ischemia, in a rodent model of myocardial ischemic-reperfusion injury, Bucciarelli et al found that pharmacological blockade or genetic ablation of RAGE resulted in attenuation of heart ischemic damage and dysfunction, suggesting that in this context activation of RAGE-dependent signaling has a deleterious effect (17). Further reflecting the complexity of RAGE function, in a paradigm of peripheral nerve injury, augmented RAGE expression was found associated with axons and infiltrating macrophages, and in this context, inhibition of RAGE resulted in deficient nerve regeneration (43;44). However, in experimental diabetic neuropathy, genetic ablation of RAGE led to partial protection from diabetes-induced nerve functional deficits (45). Therefore, these studies suggest that that the outcome of RAGE activation \textit{in-vivo} varies depending on the spatio-temporal expression of this receptor and the extent of ligand availability.

The diverse biological effects of ligand-RAGE interaction have also been uncovered in cell-culture studies. While evidence exists that at least in some cultured cells, activation of RAGE induces the expression of pro-inflammatory genes (e.i COX-2 and TNF-1α), RAGE can also regulate potentially neuroprotective genes such as bcl-2 and Creb (9;13;46-48). Our \textit{in-vitro} experiments involving oxygen-glucose deprivation showed a dual effect of exogenous S100B on neuronal survival, being neuroprotective at low doses and neurotoxic at high doses. These results are in accordance with previous studies showing survival effects of S100B at relatively low concentrations against serum deprivation, glutamate, NMDA or amyloid-β peptide neurotoxicity (9;11;12). The requirement of RAGE for S100B protective effects was demonstrated in some of these paradigms (9;11).Consistent with these findings, we found that the neuroprotective effect of low concentrations of S100B against OGD was abrogated by RAGE-blocking antibodies. However, discrepancies exist regarding the involvement of RAGE in S100B toxicity. Our results using RAGE neutralizing antibodies indicated no involvement of RAGE in S100B-induced cell death in primary neurons; however, Huttunen el al reported contrary results using neuroblastoma cells overexpressing signal-deficient RAGE (9). An important consideration regarding the nanomolar range of action reported for S100B by the present and previous reports (11;12;49) is the apparent discrepancy with other studies which indicate a moderate binding affinity (micro-molar range) of RAGE for this ligand (50;51). This raises the uncertainty of whether low concentrations of S100B can effectively bind to RAGE, and leads to the consideration that low concentrations of S100B might mediate neuroprotection by pathways not involving direct RAGE-signaling but still requiring RAGE indirectly. However, because these binding studies were performed using sRAGE immobilized on a sensor chip surface, it is possible that the kinetics...
of RAGE-S100B interaction are quite different at
the physiological conditions of the plasma
membrane.
The novel contribution of our studies regarding the
underlying mechanism of hypoxia-induced RAGE
expression is that, like many hypoxia inducible
genes, this receptor is regulated by the
transcriptional activator hypoxia inducible factor 1
(HIF-1). We showed that RAGE mRNA levels
were induced by hypoxia and pharmacologic
activators of HIF-1, namely DFO and DMOG. In
neurons with decreased HIF-1 activity generated
by a Cre-lox based approach, the upregulation of
RAGE by hypoxia or DFO was abolished, and a
similar effect was observed with VEGF, a well
characterized HIF-1 regulated gene. Analysis of
the proximal promoter region (~2.5 kb) of the
mouse RAGE gene showed at least one putative
HIF-1-binding sites with the consensus sequence
‘5-ACGTG-3’. When this region was cloned into a
luciferase reporter vector and transfected into 3T3 fibroblast, luciferase expression was induced by
hypoxia, DFO or DMOG. Deletion analysis
revealed a unique functional hypoxia response
element located ~1000 bp upstream of the
proposed transcription start site. Mutation of this
potential HIF-1 binding site abrogated hypoxia-
induced luciferase activation. Moreover, DNA
binding activity of HIF-1 to this putative HRE was
demonstrated by EMSA analysis and chromatin
immunoprecipitation assay in hypoxic neurons.
Although these data conclusively establish that
HIF-1 regulates RAGE expression during hypoxia,
a single HRE might not be sufficient to convey
efficient hypoxia sensitivity to this promoter.
Thus, it is conceivable that in-vivo other
transcription factors are involved by acting
synergistically with HIF-1 to regulate
hypoxic/ischemic RAGE expression. For instance,
functional NFκ-B binding sites have been
identified in the human RAGE promoter that can
be also important for the transcriptional regulation
of this gene especially in ischemic situations (52).
The link between HIF-1 and RAGE described here
may have ramifications for other aspects of
hypoxia, such as tumor development, in which
RAGE-mediated signaling events have been
implicated (53). As many tumors show elevated
expression of HIF-1α caused by hypoxia inherent
to growing tumors and/or genetic mutations (54),
it will be interesting to investigate whether the
elevated levels of HIF-1 underlie the increased
RAGE expression found in some tumors.
In conclusion, the results of this study indicate that
activation of the HIF-1/RAGE/S100B pathway in
neurons results in a neuroprotective response to
hypoxic/ischemic stress and could be a target for
strategies promoting post-ischemic survival.

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FOOTNOTES
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The abbreviations used are: RAGE, Receptor for advanced glycation endproducts; HIF-1, Hypoxia-inducible factor-1; HRE, hypoxia-response element; HI, hypoxia-ischemia; OGD, oxygen-glucose deprivation.

FIGURE LEGENDS

Figure 1. RAGE expression in the mouse brain: effect of hypoxia-ischemia. A). Boxed area in cresyl violet-stained section indicates the area where representative photomicrographs were taken (a). Double immunostaining of RAGE (green) with NeuN (red) at 24 hours of recovery from HI is shown in panel b.
and c. Higher magnification view of NeuN-positive neurons expressing RAGE (yellow) is shown in merged image (d). Double-staining with GFAP (red) showed no colocalization with RAGE (d). Expression of RAGE was only detected in the ipsilateral (b) but not in the contralateral hemisphere (e). Real time RT-PCR of RAGE in the ipsilateral (ischemic) and contralateral cerebral hemispheres of mice subjected to HI and different durations of recovery/reperfusion (6 hours to 5 days). Data was normalized to β-actin and expressed relative to the contralateral hemisphere of sham-control (C) that was arbitrarily defined as 1. Data are expressed as mean ± SD from 4 animals per group *p<0.05 vs sham. C) Representative immunoblot of RAGE in the ipsilateral and contralateral hemispheres of animals subjected to HI followed by recovery. C, Sham-operated animal control; +, mouse lung lysate positive control (+). Co-detection of β-actin was performed to assess equal loading. D) Evaluation of infarct volume at 12 and 24 hours after the onset of ischemia. Data is presented as mean ± SD (n=5-6) *p<0.05 compared to wild-type.

Figure 2. Induction of RAGE by systemic hypoxia. A) Real-time RT-PCR analysis of RAGE in the brain cortex of mice subjected to systemic hypoxia (H, 10% O₂ for 24 or 48h). Data was normalized to β-actin and expressed relative to normoxic-control (N) that was arbitrarily defined as 1. Data are expressed as mean ± SD from 4 animals per group *p<0.05 vs normoxia. B) Immunohistochemical analysis of RAGE (green) in the mouse brain at 48 hours of hypoxia (scale bar, 40 μm). Colocalization of RAGE with NeuN-positive neurons (red) is shown in merged image.

Figure 3. Expression of RAGE in neurons subjected to hypoxia and hypoxia-mimetic agents. Levels of RAGE mRNA were assessed by real-time RT-PCR analysis in primary neurons exposed for 24 hours to hypoxia (H, 0.5% O₂), desferrioxamine (DFO, 100 μM) or dimethyloxalglycine (DMOG, 300 μM). Results were normalized to β-actin and expressed as fold-induction compared with normoxia (N) (mean ±SD, n=6, *p<0.05). Bottom panel is a representative immunoblot demonstrating increased HIF-1α but not HIF-1β levels after hypoxia, DFO or DMOG treatment.

Figure 4. Cre recombinase-mediated deletion of HIF-1α affects hypoxia-induced RAGE expression. A) Primary neurons derived from HIF-1αF/F transgenic mice were infected with AdGFP or AdCre. AdCre-infected cells were exposed to hypoxia at several days post-infection (1 to 7 days). Representative immunoblot analysis demonstrating Cre recombinase-mediated reduction of HIF-1α but not HIF-1β levels in hypoxic AdCre-infected cells. Conversely, AdGFP-infected cells exposed to normoxia (N) or hypoxia (H, 0.5% O₂, 24h) at 7 days post-infection showed normal HIF-1α response. B and C) RT-PCR analysis of RAGE and VEGF transcripts performed in AdGFP or AdCre-infected neurons exposed to normoxia (N), hypoxia (H), vehicle (C) or DFO (100 μM). Non-infected wild-type (not shown) and non-infected HIF-1αF/F neurons were used for comparison. All RT-PCR results were normalized to β-actin and expressed as fold-induction compared to wild-type cells treated with vehicle or kept at normoxia (n=6) *p<0.05 compared with normoxic or vehicle-treated cells.

Figure 5. RAGE is a target gene of HIF-1α. A) Schematic representation of the 5′-deleted RAGE promoter-luciferase reporter constructs. Potential transcription factors binding sites identified in this area are represented by boxes. Numbering refers to position relative to the transcription initiation site (+1). NIH/3T3 cells were co-transfected with the indicated RAGE-luciferase reporter constructs (pGL3-basic vectors) together with pRL-SV40 as a transfection control. pRAGE-mutHRE is the mutant form of pRAGE-luc1, with point mutations at the putative core HRE. After transfection, cells were exposed to normoxia (N), 1% O₂ (H), DFO (100 μM) or DMOG (300 μM) for 24h. Promoter activity was expressed as a ratio of firefly/renilla luciferase activities (RLU=relative luciferase units, mean±SD, n=4,*p<0.05 vs normoxia). B) EMSA performed with nuclear extracts from primary neurons exposed to normoxia or 0.5%O₂ and 32P-labeled probes containing either wild-type (Wt) or mutated (Mut) HRE from RAGE or Epo gene. For supershift assay, binding reactions contained antibody against HIF-1α. The positions of the putative HIF, constitutive(C), and supershift (SS) complexes are indicated by arrow heads. C) Chromatin
immunoprecipitation carried out with HIF-1α^FF and HIF-1α^ΔΔ neurons. Cells were harvested at 0, 1, 4, and 8 hours of hypoxia (0.5% O₂). Immunoprecipitation (IP) was performed with monoclonal anti-HIF-1α antibody. Coprecipitated genomic DNA fragments were evaluated by PCR using primers flanking the HRE-containing region of the RAGE promoter. HIF-1α^ΔΔ neurons were generated by AdCre infection as described in Materials and Methods.

Figure 6. Effect of S100B on neuronal survival to oxygen-glucose deprivation (OGD). A) Cell viability was assessed in primary cortical neurons subjected to OGD (60 min) followed by recovery (0 to 24 hours). B) Representative RAGE immunoblot in neurons subjected to OGD followed by reperfusion (0 to 12 hours). C) Primary neurons were incubated for 24 hours with the indicated concentrations of S100B in the presence of anti-RAGE antibody or non-immune IgG under normal glucose-oxygen conditions. *p <0.05 vs untreated group. D) Neurons were pretreated with S100B (100 nM) in the presence of anti-RAGE antibody and subjected to OGD (60 min) followed by reperfusion (24h). E) Neurons were exposed to OGD (60 min) followed by 24 hours of reperfusion. Treatment with S100B (0-500 nM) and anti-RAGE antibody was performed at the onset of reperfusion. *p<0.05 vs OGD. In all cases, cell death was determined using MTT assay, and data expressed as percentage of cell survival (mean±SD, n=4-6). C and N= normal glucose/oxygen conditions, H= 0.5% O₂ for 24 hours.
Figure 1

A

B

C

D

Relative RAGE mRNA levels normalized to β-actin

Contralateral

Ipsilateral

C

Ipsilateral

Contralateral

β-actin

RAGE (~50 kD)

Infarct Volume (mm²)

12h

24h

*
Figure 2

A

![Bar graph showing relative mRNA levels normalized to ß-actin. Legend includes RAGE, VERF, and Epo.](image)

B

![Immunofluorescence images of cells under 21% and 10% O2 conditions.](image)
Figure 3

![Graph showing relative mRNA levels normalized to β-actin for different conditions: N, H, DFO, DMOG. The graph compares RAGE and VEGF conditions. Bar graphs indicate increased expression in some conditions.](http://www.jbc.org/Downloaded from)
Figure 5

A

| Construct                | -2168/-59 | -1650/-59 | -1175/-59 | -950/-59 |
|--------------------------|-----------|-----------|-----------|-----------|
| pRAGE-luc1               |           |           |           |           |
| pRAGE-luc2               |           |           |           |           |
| pRAGE-luc3               |           |           |           |           |
| pRAGE-luc4               |           |           |           |           |
| pRAGE-mutHRE             |           |           |           |           |

B

![DNA gel electrophoresis](image)

C

| Condition | 0 | 1 | 4 | 8 |
|-----------|---|---|---|---|
| RAGE      |   |   |   |   |
| IP: Anti-HIF1α |   |   |   |   |

Legend:
- N: Normal
- H: Hypoxic
- DF0: Deferoxamine
- DMOG: Dimethyl sulfoxide

Note: Antioxidant treatments (N, H, DF0, DMOG) and HIF-1α conditions (F/F, Δ/Δ) are indicated, along with relative luciferase units (RLU).
Hypoxia inducible factor-1 mediates neuronal expression of the receptor for advanced glycation endproducts following hypoxia/Ischemia

Paola Pichiule, Juan C. Chavez, Ann Marie Schmidt and Susan J. Vannucci

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