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Expression of the Receptor for Advanced Glycation End Products in Oligodendrocytes in Response to Oxidative Stress

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Abstract

Demyelination is a common result of oxidative stress in the nervous system, and we report here that the response of oligodendrocytes to oxidative stress involves the receptor for advanced glycation end products (RAGE). RAGE has not previously been reported in neonatal rat oligodendrocytes (NRO), but, by using primers specific for rat RAGE, we were able to show expression of messenger RNA (mRNA) for RAGE in NRO, and a 55-kDa protein was detected by Western blotting with antibodies to RAGE. Neonatal rat oligodendrocytes stained strongly for RAGE, suggesting membrane localization of RAGE. Addition of low concentrations of hydrogen peroxide (100 μ M) initiated 55-kDa RAGE shedding from the cell membrane and the appearance of “soluble” 45-kDa RAGE in the culture medium, followed by restoration of RAGE expression to normal levels. Increasing hydrogen peroxide concentration (>200 μ M) resulted in no restoration of RAGE, and the cells underwent apoptosis and necrosis. We further confirmed the observation in a human oligodendroglioma-derived (HOG) cell line. Both the antioxidant N-acetyl-L-cysteine and the broad-spectrum metalloproteases inhibitor TAPI0 were able partially to inhibit shedding of RAGE, suggesting involvement of metalloproteases in cleavage to produce soluble RAGE. The level of 55-kDa RAGE in autopsy brain of patients undergoing neurodegeneration with accompanying inflammation [multiple sclerosis and neuronal ceroid-lipofuscinosis (Batten's disease)] was much lower than that in age-matched controls, suggesting that shedding of RAGE might occur as reactive oxygen species accumulate in brain cells and be part of the process of neurodegeneration.

Keywords

oligodendrocytes; reactive oxygen species; hydrogen peroxide; RAGE; shedding proteolysis

Oligodendrocytes have been implicated in the pathogenesis of demyelinating diseases such as multiple sclerosis (MS) in which the active agents are reactive oxygen species (ROS) and the products of inflammation (LeVine, 1992; Smith et al., 1999). Evidence of ROS damage has been reported in demyelinating lesions in the brains of MS patients (Langemann et al., 1992; LeVine and Wetzel, 1998) and in the demyelinating experimental allergic encephalitis (EAE) mouse spinal cord (MacMicking et al., 1992). Lipid peroxidation products and ROS have been identified in cerebrospinal fluid (CSF), plasma, and brain from MS patients (Naidoo and Knapp, 1992; LeVine, 1992; Qin et al., 2007), and this, together with evidence for peroxynitrite modification of proteins (Liu et al., 2001) and mitochondrial damage (Vladimirova et al.,

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1998), strongly supports the view that oxidative damage is important in demyelination. Protein carbonyl content, mainly glutamic semialdehyde (from arginine and proline) and aminoadipic semialdehyde (from lysine oxidation), is a measure of ROS-mediated protein oxidation (Berlett and Stadtman, 1997) and is increased in MS brain (Bizzozero et al., 2005).

RAGE is a 45–55-kDa member of the immunoglobulin superfamily (Rong et al., 2004a,b; Ahmed, 2005). It contains three extracellular domains, a transmembrane domain, and a highly charged intracellular domain that mediates interaction with oxidative stress-related signal transduction molecules such as MAP kinase and nuclear factor- κ B (Lander et al., 1997; Ahmed, 2005). It plays a major role in ligand-receptor cell signaling. In addition to the products of sugar oxidation and the resulting advanced glycation end products (AGE; produced in large amounts in diabetes and progressively in aging), other ligands for RAGE include proinflammatory cytokines such as the S100-calgranulins and amphoterin, as well as fibrillar proteins such as beta-amyloid (Deane et al., 2004; Chou et al., 2004). Engagement of RAGE by AGE triggers the generation of ROS, and the incubation of human endothelial cells with AGE prompted intracellular generation of hydrogen peroxide (Wautier and Schmidt, 2004). RAGE expression levels are much higher in nervous system, such as in the developing embryonic rat brain (Hori et al., 1995; Sakaguchi et al., 2003), than in other tissues in mature animals, except for lung and skin, and a recent study suggests that RAGE may be involved in the regulation of differentiation (Bartling et al., 2005). There is also evidence that normal RAGE is critical for maintaining protein homeostasis in the CNS (e.g., that of the beta-amyloid peptide; Deane et al., 2004) and that altered activity of RAGE may contribute to neuroinflammation, a disconnect between the cerebral blood flow and metabolism, altered synaptic transmission, and neuronal injury.

Although AGEs could play an important role in the pathogenesis of neurodegeneration, Kalousova et al. (2005) did not find any statistically significant differences between patients with MS and controls in terms of the presence of AGE products (Kalousova et al., 2005). However, another ligand of RAGE, S100-calgranulin, was reported to be up-regulated in both MS and an EAE model for MS. Further, induction of EAE in mice caused increased RAGE expression, and injection of soluble RAGE was able to protect against injury in EAE (Yan et al., 2003). In contrast to most reports that RAGE is up-regulated by its ligands (such as AGE), Miura et al. (2004) used quantitative reverse transcription-polymerase chain reaction (RT-QPCR) analysis to show that the monocyte expression of RAGE mRNA was significantly lower in patients with retinopathy than in those without retinopathy and was also significantly down-regulated in patients with nephropathy in comparison with those without nephropathy. In fact, in monocyte-enriched cultures, both RAGE mRNA and protein levels were down-regulated by exposure to glyceraldehyde-derived AGE, the recently identified high-affinity RAGE ligand (Miura et al., 2004). RAGE has also been found to have a protective function in peripheral nerve recovery from injury (Rong et al., 2004a,b). Because there have been several reports of cell membrane receptor protein ectodomain shedding (tumor necrosis factor receptor, betacellulin, and calcineurin) in response to stress (PMA or H_2O_2 ; Zhang et al., 2001; Sanderson et al., 2006; Lee et al., 2007), we investigated the effect of stress on RAGE. Engagement of RAGE by AGE triggers the generation of ROS, and the incubation of human endothelial cells with AGE prompted the intracellular generation of hydrogen peroxide (Wautier and Schmidt, 2004). We report here that RAGE undergoes proteolysis and shedding in response to H_2O_2 and that this might shed light on the role of RAGE in the pathogenesis of demyelinating diseases.

MATERIALS AND METHODS

Chemicals and Antibodies

Polyclonal goat anti-RAGE antibodies against N-terminus (N-16) and C-terminus (C-20) amino acids of RAGE were purchased from Santa Cruz Biotechnology (Santa Cruz, CA; SC-8230 and SC-8229). Both react with RAGE of mouse, rat, and human origin by both Western blot and immunohistochemistry (Yonekura et al., 2003; Sorci et al., 2004). A third monoclonal mouse anti-RAGE antibody was obtained from Chemicon (Temecula, CA) and used as described by Chou et al. (2004). Mouse antimyelin basic protein (MBP) monoclonal antibody was also purchased from Chemicon. N-acetyl-L-cysteine was from Sigma (St. Louis, MO); TAPI0 was purchased from Peptides International (Louisville, KY). The protein assay kit was purchased from Bio-Rad (Hercules, CA). The RT-PCR one step kit was from Qiagen (Valencia, CA), the fluorescein (FITC)-conjugated affinity pure rabbit anti-goat IgG was from Jackson ImmunoResearch (West Grove, PA), and Texas red-labeled goat anti-mouse IgG were purchased from Molecular Probes (Eugene, OR).

Isolation of Primary Oligodendrocyte and Cultures

Neonatal rat oligodendrocytes (NRO) were isolated by the Bottenstein (1986) modification of the method of McCarthy and deVellis (1980) in which the first shake is done at 7 days and then at 1-week intervals, with shakes 2, 3, and 4 giving the highest yield of astrocyte and microglia-free oligodendrocyte (NRO) preparations (Scurlock and Dawson, 1999; Testai et al., 2004). After shaking to dissociate oligodendrocytes from the astrocyte bed layer, they were plated on 60-mm poly-L-lysine-coated dishes and allowed to undergo differentiation; NRO were differentiated for 6 days prior to use (Fig. 1A). Human oligodendrogloma (HOG) cells (Post and Dawson, 1992; Dawson et al., 1993) were grown in monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% gentamicin as described by Scurlock and Dawson (1999). The animal (rat) study protocol was approved by the University of Chicago IACUC, protocol No. 70610.

Human Tissues

Age- and post-mortem-matched control and MS brain samples were obtained from the Human Brain and Spinal Fluid Resource Center (Los Angeles, CA; rmnbbank@ucla.edu). All care was taken to minimize post-mortem autolysis. MS was clinically verified in patients and by the presence of gadolinium-enhancing lesions on brain MRI. The diagnosis of Batten disease (CLN1, -2, and -3) was by a combination of clinical symptoms, post-mortem pathology, and DNA mutation analysis, and samples were obtained through the BDSRA brain bank. The human/animal tissue study protocol was approved by the University of Chicago IACUC, protocol No. 71543.

Reverse Transcriptase-Polymerase Chain Reaction for RAGE mRNA Expression in NRO and HOG

Total RNA was extracted from pure cultured NRO and HOG with the Qiagen total RNA extract kit; RT-PCR was executed with an RT-PCR one-step kit (Qiagen) and primer-pairs specific to a rat RAGE fragment sequence (606 bp) and human RAGE fragment sequence (513 bp). We confirmed the presence of this 606-bp mRNA in rat oligodendrocytes by using upper 5' CCTGAGACGGGACTCTTCACGCTTCGG 3' and lower 5' CTCCTCGTCCTCCTGGCTTTCTGGGGC 3' primers and the presence of this 513-bp RAGE mRNA in HOG by using upper 5' CAGGATGAGGGGATTTCCG 3' and lower 5' AGGGACTTCACAGGTCAGGGTTAC 3'. Briefly, the reaction mixture was prepared in PCR tubes according to the kit menu and placed into a Perkin Elmer GeneAMP PCR System 2400. The programming RT-PCR procedure consisted of reverse transcription (50°C for 30 min),

initial PCR activation (95°C for 15 min), then 40 cycles of 94°C for 30 sec., 65°C for 30 sec for NRO (55°C for HOG PCR annealing temperature), and 72°C for 1 min, followed by a final extension at 72°C for 10 min. The RT-PCR-amplified samples were visualized on 1.5% agarose gels with ethidium bromide.

Western Blotting of Proteins

Protein extracts from cultured NRO differentiated for 6 days and brain samples were subjected to SDS-gel electrophoresis. Proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA), and Western blotting was carried out with the anti-RAGE antibodies according to the manufacturer's instructions. Positive bands were detected with a chemiluminescence kit from Fisher Scientific (Pittsburgh, PA). The Western blot bands were scanned with a Bio-Rad ChemiDoc XRS imager and quantified in Quantity One 4.5.0 software (Bio-Rad).

Staining of NRO and HOG To Show Expression of RAGE

NRO and HOG were grown on four-well tissue culture slides, rinsed twice in PBS, and fixed in freshly prepared 4% paraformaldehyde at room temperature for 15 min, and then double-staining immunohistochemistry was executed. After removal of the fixative and rinsing three times with PBS, the slides were incubated in PBS/1% Triton X-100 for 10 min at room temperature, then with PBS/1% Triton X-100/2% normal goat serum (NGS) for 5 min at room temperature for a total of 15 min. The primary antibody [anti-RAGE (N-16) and anti-MBP] was diluted in PBS/1% Triton X-100/2% NGS and incubated with cells overnight at 4°C. After rinsing six times with PBS for 5 min at room temperature, cells were incubated with secondary antibody (FITC-conjugated rabbit anti-goat IgG for anti-RAGE and Texas red-conjugated anti-mouse IgG for anti-MBP) for 1 hr, then rinsed, and 1 drop of 2% n-propyl gallate in PBS:glycerol (1:1) was added to each well and the slide sealed with nail polish. The immunofluorescence reaction was followed and documented with an Axiovert S100 TV (Carl Zeiss, Inc.).

Isolation of Soluble RAGE From Conditioned Media

Trichloroacetic acid (TCA)/acetone precipitation of soluble RAGE was performed as follows. Equal volumes of condition media were mixed with 20% TCA/80% ice-cold acetone and incubated at -20°C for 1 hr. The protein pellet was precipitated by centrifugation at 12,000 rpm for 15 min at 4°C in a microcentrifuge. The supernatant was discarded, and the protein pellet was washed with 1 ml ice-cold acetone and centrifuged as described above. Acetone was driven off by heating the dry pellet at 95°C for 10 min (Annabi et al., 2005).

Inhibition of RAGE Shedding by Antioxidant and a Metalloproteases Inhibitor

NRO were preincubated with either N-acetyl-L-cysteine (20 and 30 mM) or TAPI0 (50 µM) for 30 min, and then H₂O₂ (100 µM) was added and cells were cultured for a further 1 hr and harvested mechanically, and Western blot assay was carried out as described above.

Statistical Analysis

Results were based on experiments run in triplicate at least twice. Statistical analyses were performed by Student's *t*-test, and results were considered statistically significant at $P < 0.05$.

RESULTS

Evidence for RAGE Synthesis and Protein Expression by NRO and HOG

Six-days-cultured oligodendrocytes (NRO) were maintained in monolayer culture at the degree of confluence and purity indicated by staining for glial fibrillary acidic protein (GFAP) to detect

astrocyte contamination (2% or less) and with OX-42 for microglia contamination (<1%). HOG cells were used at subconfluence.

Analysis of rat brain and isolated NRO total RNA by RT-PCR revealed a fragment of the appropriate size (606 bp) corresponding to RAGE (Fig. 1A). RAGE mRNA expression levels in NRO increased for up to 12 days in Bottenstein differentiating culture conditions. RT-PCR of HOG cell total RNA also showed the expected 513-bp amplified band corresponding to human RAGE (Fig. 1C). The expression of full-length RAGE protein was further confirmed by Western blot of NRO (Fig. 1B) and HOG cell extracts (Fig. 1D) using anti-RAGE antibodies (C-20).

To verify further that RAGE expression was not from the minor astrocyte or microglia contamination, we carried out direct double-immunocytochemistry staining for RAGE with anti-RAGE antibodies (N-16) and anti-MBP antibody. Results showed NRO and HOG cells both to be positive for the cell membrane presence of RAGE (Fig. 2A-H), which is consistent with membrane protein properties of RAGE.

Hydrogen Peroxide Mediates Ectodomain Shedding of RAGE

A detailed study carried out on NRO cells showed (Fig. 3A) that there was actually a very rapid initial decrease (<10 min) in 55-kDa RAGE protein levels in response to H₂O₂ (100 μ M), followed by the recovery of RAGE expression and return to normal levels after 24 hr (Western blot with C-20 anti-RAGE antibody). When the same experiment was carried out on HOG cells, we also observed the initial loss of RAGE caused by H₂O₂ (200 μ M) treatment (Fig. 3B), and the recovery of RAGE to normal by 4–6 hr. A possible explanation for this is that H₂O₂ initiates ectodomain cleavage of RAGE (e.g., producing soluble 45-kDa RAGE), which is released to media and therefore not detected in the cells. During the course of these studies, both H₂O₂ and endothelin-1 were shown to activate probetacellulin (pro-BTC) by proteolytic ectodomain shedding (Sanderson et al., 2006), and, more recently, H₂O₂ has been shown to initiate the rapid proteolytic cleavage and shedding of calcineurin in neurons (Lee et al., 2007).

Higher Peroxide Concentrations Prevent Recovery and Lead to Cell Death

The amount of RAGE expressed by oligodendrocytes (NRO) decreased dramatically as early as 0.1 hr (Fig. 3A) and then increased with time of exposure to 100 μ M hydrogen peroxide (Fig. 3C,E) as measured by Western blot with C-terminal anti-RAGE antibodies. However, increasing the concentration of H₂O₂ to 250, 500, and 1,000 μ M caused an irreversible dose-dependent decrease in RAGE expression (Fig. 3D,F), and cell death and a similar reduction in RAGE expression with increasing concentrations of H₂O₂ were also observed in HOG cells (Fig. 4A). A similar observation of loss of RAGE was made when we used light-activated rose Bengal to induce ROS in NRO (Fig. 4B).

Peroxide Causes Release of Soluble RAGE From the Ectodomain

To isolate soluble RAGE released into the medium by H₂O₂ treatment, we treated cells with H₂O₂ (0, 100, 200 μ M) and collected cells and medium separately. Secreted proteins were precipitated by the TCA method, and analysis by SDS-PAGE and Western blot showed a 45-kDa secreted form of RAGE to occur in the culture medium of cells treated with H₂O₂ (Fig. 5B; Western blot checks RAGE in medium by N-16 anti-RAGE antibody; level of RAGE in cells by C-20 anti-RAGE antibody; Fig. 5A). Because most protein ectodomain shedding is produced by metalloproteases and in some cases metalloprotease activation was verified by oxidative removal of the enzyme's proinhibitory region by a "cysteine-switch mechanism" (Nagase and Woessner, 1999; Roghani et al., 1999), we tested the idea that both anti-oxidants such as NAC and metalloproteinase inhibitors could block H₂O₂-induced RAGE

shedding. NRO were preincubated in N-acetyl-L-cysteine (20 and 30 mM) for 30 min, prior to H₂O₂ (100 μM) addition, and cells were cultured for a further 1 hr and harvested for Western blot assay. NAC partially protects against RAGE cleavage (Fig. 6). We then used a broad-spectrum metalloproteases inhibitor, TAPI0 (50 μM), for 30 min prior to H₂O₂ (100 μM) for 1 hr and showed that TAPI0 also partially reduced the level of RAGE shedding (Fig. 7).

From these studies, we concluded that treatment with H₂O₂ causes the ectodomain shedding of RAGE from the cell surface but that the cell has the capacity to regenerate RAGE unless the H₂O₂ concentration (physiologically ROS) is high enough to cause cell death. These observations suggested an unexpected complexity in the relationship of ROS and RAGE, so we looked at RAGE expression in two neurodegenerative diseases in which inflammatory-related induction of ROS has been implicated in the pathology, namely, MS (Fig. 8A) and Batten's disease (Fig. 8B). In MS brain samples, using plaque from patients who had died of active disease, we observed the significantly reduction of RAGE in all patients (Fig. 8A). A similar reduction in RAGE was observed in Batten's disease brain samples (Fig. 8B), suggesting that the loss of RAGE accompanying neurodegenerative disease might involve free radical-triggered protein shedding. A similar level of RAGE down-regulation has previously been observed in a patient with type 1 retinopathy (Miura et al., 2004) and may be a common pathological response to ROS injury.

DISCUSSION

RAGE has been reported in many tissues and cells such as smooth muscle cells, endothelial cells, astrocytes, and neurons but has not been previously reported in oligodendrocytes. We confirmed the ability of oligodendrocytes to synthesize RAGE by RT-PCR and the presence of RAGE protein by both Western blotting and direct double immunostaining of cultured oligodendrocytes with different anti-RAGE antibodies and anti-MBP antibody. We further confirmed the observation in an HOG cell line in which there was no possibility of astrocyte or microglial contamination. We also confirmed the expression of RAGE on the surface of oligodendrocytes by direct immunostaining. RAGE is a member of the immunoglobulin superfamily (Yonekura et al., 2003, 2005; Chavakis et al., 2004; Ahmed, 2005) with three extracellular domains, a transmembrane domain, and a C-terminal intracellular domain. This latter domain mediates interaction with oxidative stress-related signal transduction molecules such as MAP kinase and nuclear factor-κB (Lander et al., 1997; Deane et al., 2004). The function of RAGE is complicated by the existence of multiple ligands other than AGEs, including proinflammatory cytokines, S100-calgranulins, amphoterin, and fibrillar proteins such as beta-amyloid (Srikrishna et al., 2002; Yonekura et al., 2003, 2005; Deane et al., 2004; Ahmed, 2005). However, there is evidence that RAGE is critical for protein homeostasis in the nervous system (Deane et al., 2004). Thus RAGE has a proposed role in promoting neurite outgrowth (Srikrishna et al., 2002) and may also be involved in the regulation of development and differentiation in the nervous system (Hori et al., 1995; Sakaguchi et al., 2003; Bartling et al., 2005). Our novel finding is that, although peroxide treatment initially removes RAGE from NRO or HOG cells, with treatment with low levels of H₂O₂ (e.g., 100 μM) eventually we see the recovery of RAGE to normal levels in both NRO and HOG cells. However, RAGE expression was not recovered at higher levels of H₂O₂ that lead to increased cell death in both NRO and HOG cells.

We used H₂O₂ to induce oxidative stress, because it has been shown to convert the major membrane lipid, phosphatidylcholine (PC), to OxPC, 4-hydroxy-2-nonenal (HNE), and possibly also malondialdehyde (Chang et al., 2004; Boullier et al., 2005; Qin et al., 2007). Antibodies to OxPC can be detected in MS plaques and CSF (Qin et al., 2007), suggesting that they play an important role in pathogenesis. Oxidative stress (ROS generation) has been implicated in the demyelination and axonal degeneration associated with the acute and chronic

phases of MS but the increased levels of ROS have usually been attributed to infiltrating macrophages and microglia. Protein carbonylation, indicative of ROS-mediated protein oxidation (Berlett and Stadtman, 1997), has been observed in MS gray and white matter (Bizzozero et al., 2005), but there was no significant accumulation of lipid peroxidation products (free MDA or HNE) in either gray matter or white matter from MS brains. This may be because the lipid products rapidly polymerize or form protein adducts. PC degradation products (e.g., POVPC) are extremely reactive and unlikely to occur in the free state, and HNE is also most likely bound to protein (Berlett and Stadtman, 1997). Thus the OxPC detected in MS brain (Qin et al., 2007) most likely exists mainly in the form of lipid polymers (after aldol condensation of OxPC) or OxPC covalently bound to lysyl-15-kDa protein.

The level of RAGE has previously been reported to increase during both diabetes and inflammation as well as in spinal cord tissue from EAE-induced mice, which some consider to be a rodent model for MS (Yan et al., 2003). However, more recent studies suggest that the monocyte expression of RAGE mRNA is significantly lower in type 1 diabetes patients with retinopathy than in those without retinopathy and is also significantly down-regulated in patients with nephropathy in comparison with those without nephropathy. In agreement with this, RAGE mRNA and protein levels were actually down-regulated by exposure to glyceraldehyde-derived AGE (a recently identified high-affinity RAGE ligand) in monocyte-enriched cultures (Miura et al., 2004). We were also unable to confirm the increase in RAGE in the 11 MS brain samples we studied, whereas we did observe (in a separate study) that OxPC formation occurred in these same MS autopsy brain samples (Qin et al., 2007) and that both lipid- and protein-bound OxPC was associated with MS plaques. This suggests that oxidative damage related to the formation of ROS is taking place in active MS plaques and may be instrumental in reducing RAGE, as demonstrated in the oligodendrocyte cell culture studies.

Yan et al. (2003) showed that treatment of EAE mice with a soluble RAGE analog was able to block development of the severe demyelination associated with myelin basic protein-induced EAE in the mice. Yan et al. interpreted their data to mean that increased expression of RAGE in MS or EAE was pathogenic. However, it now seems more likely that full-length RAGE shedding (to produce soluble RAGE under oxidized condition) is a kind of self protective mechanism in neural cells. Rong et al. (2004a,b) concluded that unmodified RAGE functions as a scavenger receptor with a protective, regenerative function and further showed that antagonism of RAGE suppressed peripheral nerve regeneration via recruitment of both inflammatory and axonal outgrowth pathways. Thus RAGE may normally facilitate the repair of minor insults to the CNS, and we interpret our data to mean that the lack of RAGE inhibits repair. This is supported by the observation that continued exposure to moderate levels of H₂O₂ actually led to the induction of RAGE and that RAGE is necessary for repair from oxidative injury.

The proinflammatory and tissue-destructive consequences of RAGE activation have been documented in diabetes (in which carboxymethyllysine adducts of proteins form ligands for RAGE) by showing experimental reversal of symptoms following treatment with anti-RAGE antibody (Hudson et al., 2003). More recently, this has been challenged in diabetes (Miura et al., 2004) and by the work presented here, and it is possible that some of the differences in observations could be explained by the shedding of soluble RAGE. Our results clearly show that RAGE is normally expressed in oligodendrocytes and HOG cells, that it is lost from cells following peroxide or ROS treatment, but that it is then regenerated. The process is believed to involve a calcium-dependent ROS oxidation mechanism (blocked by NAC) in which metalloprotease activation occurs by the oxidative removal of the enzyme's proinhibitory mechanism by a "cysteine switch" (Sanderson et al., 2006). However, because NAC was only a partial blocker, it is also possible that ADAM10-mediated ectodomain shedding as described for betacellulin is also occurring (Sanderson et al., 2006). Blocking with the broad-spectrum

metalloprotease inhibitor TAPI0 was also partial, whereas the H₂O₂-mediated shedding of calcineurin by neurons was insensitive to metalloproteases inhibitors. This suggests that several different mechanisms of shedding are active in brain. RAGE contains the MMP9 cleavage site motif (Pro-XX-Hy-Ser/Thr, where Hy is a hydrophobic amino acid) in the form of a PTAGS sequence at which cleavage would produce the observed 45-kDa soluble RAGE (Hanford et al., 2004). However, in a series of detailed studies, Zhang (2006, unpublished thesis work, University of Mainz) was unable to block cleavage by introducing a mutation (P328E) and suggested that the shedding of RAGE was not sequence specific as far as metalloproteinases were concerned. However, there was a general finding that metalloproteinases rather than lysosomal proteases (as suggested for calcineurin by Lee et al., 2007) are involved in RAGE cleavage and shedding, and this is in agreement with our observations. Thus overexpression of MMP9 increased RAGE shedding, whereas silencing with RNAi decreased shedding (Zhang, 2006). This extends the data supporting the role of MMP9 in ectodermal shedding, and our data are in agreement with its role in a crucial aspect of RAGE regulation and function in the brain.

We therefore propose a model in which oligodendroglial surface RAGE is proteolytically cleaved by ROS-activated metalloproteases. If the ROS are transient or low, the RAGE is regenerated within 24 hr, and the cell suffers no permanent damage. If the ROS persists and reactive species such as OxPC and HNE become abundant, then there is a failure of RAGE resynthesis and ROS can go on to damage membranes and cell processes. There is also the loss of other RAGE signaling functions in the CNS, which could play a role in the progressive degeneration observed in diseases such as MS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

- Ahmed N. Advanced glycation endproducts—role in pathology of diabetic complications. *Diabetes Res Clin Pract* 2005;67:3–21. [PubMed: 15620429]
- Annabi B, Bouzeghrane M, Currie JC, Hawkins R, Dulude H, Daigneault L, Ruiz M, Wisniewski J, Garde S, Rabbani SA, Panchal C, Wu JJ, Béliveau R. A PSP94-derived peptide PCK3145 inhibits MMP-9 secretion and triggers CD44 cell surface shedding: implication in tumor metastasis. *Clin Exp Metastasis* 2005;22:429–439. [PubMed: 16283486]
- Bartling B, Hofmann HS, Weigle B, Silber RE, Simm A. Down-regulation of the receptor for advanced glycation end-products (RAGE) supports non-small cell lung carcinoma. *Carcinogenesis* 2005;26:293–301. [PubMed: 15539404]
- Berlett BS, Stadman ER. Protein oxidation in aging, disease, and oxidative stress. *J Biol Chem* 1997;272:20313–20316. [PubMed: 9252331]
- Bizzozero OA, DeJesus G, Callahan K, Pastuszyn A. Elevated protein carbonylation in the brain white matter and gray matter of patients with multiple sclerosis. *J Neurosci Res* 2005;81:687–695. [PubMed: 16007681]
- Bottenstein JE. Growth requirements in vitro of oligodendrocyte cell lines and neonatal rat brain oligodendrocytes. *Proc Natl Acad Sci U S A* 1986;83:1955–1959. [PubMed: 3456614]
- Boullier A, Friedman P, Harkewicz R, Hartvigsen K, Green SR, Almazan F, Dennis EA, Steinberg D, Witztum JL, Quehenberger O. Phosphocholine as a pattern recognition ligand for CD36. *J Lipid Res* 2005;46:969–976. [PubMed: 15722561]

- Chang MK, Binder CJ, Miller YI, Subbanagounder G, Silverman GJ, Berliner JA, Witztum JL. Apoptotic cells with oxidation-specific epitopes are immunogenic and proinflammatory. *J Exp Med* 2004;200:1359–1370. [PubMed: 15583011]
- Chavakis T, Bierhaus A, Nawroth PP. RAGE (receptor for advanced glycation end products): a central player in the inflammatory response. *Microbes Infect* 2004;6:1219–1225. [PubMed: 15488742]
- Chou DK, Zhang J, Smith FI, McCaffery P, Jungalwala FB. Developmental expression of receptor for advanced glycation end products (RAGE), amphoterin and sulfoglucuronyl (HNK-1) carbohydrate in mouse cerebellum and their role in neurite outgrowth and cell migration. *J Neurochem* 2004;90:1389–1401. [PubMed: 15341523]
- Dawson G, Dawson SA, Post GR. Regulation of phospholipase D activity in a human oligodendroglioma cell line (HOG). *J Neurosci Res* 1993;34:324–330. [PubMed: 8455209]
- Deane R, Wu Z, Zlokovic BV. RAGE (yin) versus LRP (yang) balance regulates alzheimer amyloid beta-peptide clearance through transport across the blood–brain barrier. *Stroke* 2004;35(Suppl 1):2628–2631. [PubMed: 15459432]
- Hanford LE, Enghild JJ, Valnickova Z, Petersen SV, Schaefer LM, Schaefer TM, Reinhart TA, Oury TD. Purification and characterization of mouse soluble receptor for advanced glycation end products (sRAGE). *J Biol Chem* 2004;279:50019–50024. [PubMed: 15381690]
- Hori O, Brett J, Slattery T, Cao R, Zhang J, Chen JX, Nagashima M, Lundh ER, Vijay S, Nitecki D, et al. The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. Mediation of neurite outgrowth and co-expression of rage and amphoterin in the developing nervous system. *J Biol Chem* 1995;270:25752–25761. [PubMed: 7592757]
- Hudson BI, Bucciarelli LG, Wendt T, Sakaguchi T, Lalla E, Qu W, Lu Y, Lee L, Stern DM, Naka Y, Ramasamy R, Yan SD, Yan SF, D'Agati V, Schmidt AM. Blockade of receptor for advanced glycation endproducts: a new target for therapeutic intervention in diabetic complications and inflammatory disorders. *Arch Biochem Biophys* 2003;419:80–88. [PubMed: 14568011]
- Kalousova M, Havrdova E, Mrazova K, Spacek P, Braun M, Uhrova J, Germanova A, Zima T. Advanced glycoxidation end products in patients with multiple sclerosis. *Prague Med Rep* 2005;106:167–174.
- Lander HM, Tauras JM, Ogiste JS, Hori O, Moss RA, Schmidt AM. Activation of the receptor for advanced glycation end products triggers a p21(ras)-dependent mitogen-activated protein kinase pathway regulated by oxidant stress. *J Biol Chem* 1997;272:17810–17814. [PubMed: 9211935]
- Langemann H, Kabiersch A, Newcombe J. Measurement of low-molecular-weight antioxidants, uric acid, tyrosine and tryptophan in plaques and white matter from patients with multiple sclerosis. *Eur Neurol* 1992;32:248–252. [PubMed: 1521544]
- Lee JE, Kim H, Jang H, Cho EJ, Youn HD. Hydrogen peroxide triggers the proteolytic cleavage and the inactivation of calcineurin. *J Neurochem* 2007;100:1703–1712. [PubMed: 17217415]
- LeVine SM. The role of reactive oxygen species in the pathogenesis of multiple sclerosis. *Med Hypoth* 1992;39:271–274.
- LeVine SM, Wetzel DL. Chemical analysis of multiple sclerosis lesions by FT-IR microspectroscopy. *Free Radic Biol Med* 1998;25:33–41. [PubMed: 9655519]
- Liu JS, Zhao ML, Brosnan CF, Lee SC. Expression of inducible nitric oxide synthase and nitrotyrosine in multiple sclerosis lesions. *Am J Pathol* 2001;158:2057–2066. [PubMed: 11395383]
- MacMicking JD, Willenborg DO, Weidemann MJ, Rockett KA, Cowden WB. Elevated secretion of reactive nitrogen and oxygen intermediates by inflammatory leukocytes in hyperacute experimental autoimmune encephalomyelitis: enhancement by the soluble products of encephalitogenic T cells. *J Exp Med* 1992;176:303–307. [PubMed: 1319459]
- McCarthy KD, de Vellis J. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J Cell Biol* 1980;85:890–902. [PubMed: 6248568]
- Miura J, Uchigata Y, Yamamoto Y, Takeuchi M, Sakurai S, Watanabe T, Yonekura H, Yamagishi S, Makita Z, Sato A, Omori Y, Yamamoto H, Iwamoto Y. AGE down-regulation of monocyte RAGE expression and its association with diabetic complications in type 1 diabetes. *J Diabetes Complications* 2004;18:53–59. [PubMed: 15019601]
- Naidoo R, Knapp ML. Studies of lipid peroxidation products in cerebrospinal fluid and serum in multiple sclerosis and other conditions. *Clin Chem* 1992;38:2449–2454. [PubMed: 1458583]

- Nagase H, Woessner JF Jr. Matrix metalloproteinases. *J Biol Chem* 1999;274:21491–21494. [PubMed: 10419448]
- Post GR, Dawson G. Characterization of a cell line derived from a human oligodendroglioma. *Mol Chem Neuropathol* 1992;16:303–317. [PubMed: 1329795]
- Qin J, Goswami R, Balabanov R, Dawson G. Oxidized phosphatidylcholine is a marker for neuroinflammation in multiple sclerosis brain. *J Neurosci Res* 2007;85:977–984. [PubMed: 17304573]
- Roghani M, Becherer JD, Moss ML, Atherton RE, Erdjument-Bromage H, Arribas J, Blackburn RK, Weskamp G, Tempst P, Blobel CP. Metalloprotease-disintegrin MDC9: intracellular maturation and catalytic activity. *J Biol Chem* 1999;274:3531–3540. [PubMed: 9920899]
- Rong LL, Trojaborg W, Qu W, Kostov K, Yan SD, Gooch C, Szabolcs M, Hays AP, Schmidt AM. Antagonism of RAGE suppresses peripheral nerve regeneration. *FASEB J* 2004a;18:1812–1817. [PubMed: 15576484]
- Rong LL, Yan SF, Wendt T, Hans D, Pachydaki S, Bucciarelli LG, Adebayo A, Qu W, Lu Y, Kostov K, Lalla E, Yan SD, Gooch C, Szabolcs M, Trojaborg W, Hays AP, Schmidt AM. RAGE modulates peripheral nerve regeneration via recruitment of both inflammatory and axonal outgrowth pathways. *FASEB J* 2004b;18:1818–1825. [PubMed: 15576485]
- Sakaguchi T, Yan SF, Yan SD, Belov D, Rong LL, Sousa M, Andrassy M, Marso SP, Duda S, Arnold B, Liliensiek B, Nawroth PP, Stern DM, Schmidt AM, Naka Y. Central role of RAGE-dependent neointimal expansion in arterial restenosis. *J Clin Invest* 2003;111:959–972. [PubMed: 12671045]
- Sanderson MP, Abbott CA, Tada H, Seno M, Dempsey PJ, Dunbar AJ. Hydrogen peroxide and endothelin-1 are novel activators of betacellulin ectodomain shedding. *J Cell Biochem* 2006;99:609–623. [PubMed: 16676357]
- Scurlock B, Dawson G. Differential responses of oligodendrocytes to tumor necrosis factor and other pro-apoptotic agents: role of ceramide in apoptosis. *J Neurosci Res* 1999;55:514–522. [PubMed: 10723061]
- Smith KJ, Kapoor R, Felts PA. Demyelination: the role of reactive oxygen and nitrogen species. *Brain Pathol* 1999;9:69–92. [PubMed: 9989453]
- Sorci G, Riuzzi F, Arcuri C, Giambanco I, Donato R. Amphoterin stimulates myogenesis and counteracts the antimyogenic factors basic fibroblast growth factor and S100B via RAGE binding. *Mol Cell Biol* 2004;24:4880–4894. [PubMed: 15143181]
- Srikrishna G, Huttunen HJ, Johansson L, Weigle B, Yamaguchi Y, Rauvala H, Freeze HH. N-glycans on the receptor for advanced glycation end products influence amphoterin binding and neurite outgrowth. *J Neurochem* 2002;80:998–1008. [PubMed: 11953450]
- Testai FD, Landek MA, Dawson G. Regulation of sphingomyelinases in cells of the oligodendrocyte lineage. *J Neurosci Res* 2004;75:66–74. [PubMed: 14689449]
- Vladimirova O, O'Connor J, Cahill A, Alder H, Butunoi C, Kalman B. Oxidative damage to DNA in plaques of MS brains. *Mult Scler* 1998;4:413–418. [PubMed: 9839301]
- Wautier JL, Schmidt AM. Protein glycation: a firm link to endothelial cell dysfunction. *Circ Res* 2004;95:233–238. [PubMed: 15297385]
- Yan SS, Wu ZY, Zhang HP, Furtado G, Chen X, Yan SF, Schmidt AM, Brown C, Stern A, LaFaille J, Chess L, Stern DM, Jiang H. Suppression of experimental autoimmune encephalomyelitis by selective blockade of encephalitogenic T-cell infiltration of the central nervous system. *Nat Med* 2003;9:287–293. [PubMed: 12598893]
- Yonekura H, Yamamoto Y, Sakurai S, Petrova RG, Abedin MJ, Li H, Yasui K, Takeuchi M, Makita Z, Takasawa S, Okamoto H, Watanabe T, Yamamoto H. Novel splice variants of the receptor for advanced glycation end-products expressed in human vascular endothelial cells and pericytes, and their putative roles in diabetes-induced vascular injury. *Biochem J* 2003;370:1097–1109. [PubMed: 12495433]
- Yonekura H, Yamamoto Y, Sakurai S, Watanabe T, Yamamoto H. Roles of the receptor for advanced glycation endproducts in diabetes-induced vascular injury. *J Pharmacol Sci* 2005;97:305–311. [PubMed: 15750291]
- Zhang, L. Unpublished thesis work. University of Mainz; 2006. Proteolysis of the receptor for advanced glycation end products by matrix metalloproteinases..

Zhang Z, Oliver P, Lancaster JJ, Schwarzenberger PO, Joshi MS, Cork J, Kolls JK. Reactive oxygen species mediate tumor necrosis factor alpha-converting, enzyme-dependent ectodomain shedding induced by phorbol myristate acetate. *FASEB J* 2001;15:303–305. [PubMed: 11156944]

Fig. 1.

RAGE expression in NRO and HOG as determined by RT-PCR and Western blot. **A:** RAGE expression in NRO as determined by RT-PCR. Lane 1, DNA ladder; lane 2, 0-day neonatal rat oligodendrocytes; lanes 3 and 4, 5-day and 12-day oligodendrocytes in Bottenstein's differentiation medium; lane 5, adult rat brain extract; lane 6, blank. **B:** Western blot showing RAGE expression in NRO as detected with antibodies to N-terminal (N-16) parts of RAGE. β-Actin is included to show protein loading, and the blots were carried out as described in the text. **C:** RAGE expression in HOG as determined by RT-PCR. Lane 1, DNA ladder; lane 2, blank; lanes 3–5, HOG cell extracts. **D:** Western blot showing RAGE expression in HOG as detected with antibodies to N-terminal (N-16) parts of RAGE. β-Actin is included to show protein loading, and the blots were carried out as described in the text.

Fig. 2. RAGE immunoeexpression in NRO and HOG cells. **A,E:** Phase microscopy of NRO and HOG cells. **B,F:** RAGE immunoeexpression of NRO and HOG cells. **C,G:** NRO and HOG were displayed by anti-MBP antibody. **D,H:** Colocalization for RAGE and MBP demonstrated for NRO and HOG cells. $\times 63$. Scale bars = 20 μm . [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 3.

Time course and concentration course of changes in RAGE levels following NRO and HOG treatment with H₂O₂. **A:** RAGE was detected with anti-RAGE antibodies (C-20), and β-actin was used to show protein loading levels. -, Untreated; +, treated with 100 μM H₂O₂ for the time indicated. Cultured oligodendrocytes (NRO) with or without H₂O₂ (100 μM) treatment were harvested at 0.1 hr, 2 hr, 4 hr, and 24 hr time points; protein extracts were loaded on SDS-PAGE gels; and Western blots were carried out to show that RAGE expression increased with time of H₂O₂ (100 μM) treatment, but, compared with untreated cell protein levels (harvested at same time point), the RAGE protein levels were always lower before 24 hr of 100 μM H₂O₂ treatment. **B:** RAGE was detected with anti-RAGE antibodies (C-20), and β-actin was used to show equal protein loading levels. -, Untreated; +, treated with 200 μM H₂O₂ for the time indicated. HOG cells treated with H₂O₂ (200 μM) were harvested at 0.1 hr, 2 hr, 4 hr, and 6 hr, and protein extracts were loaded on SDS-PAGE gels. The Western blot shows that RAGE expression increases with time of H₂O₂ treatment. Compared with untreated cell protein levels (harvested at same time point), the protein levels were always lower before 4 hr of 200 μM H₂O₂ treatment. **C,E:** RAGE was detected with anti-RAGE antibodies, and β-actin was used to show equal protein loading levels. Cultured oligodendrocytes (NRO) were treated with

H₂O₂ (100 μ M), and cells were harvested at 2 hr (lane 2), 4 hr (lane 3), 8 hr (lane 4), and 24 hr (lane 5). Equal protein extracts were loaded on SDS-PAGE gels and subjected to Western blotting to show that RAGE expression initially decreased and then increased with time of treatment with H₂O₂ (100 μ M), but the control (without H₂O₂ treatment, lane 1) RAGE level was always the highest. **D,F:** Dose response of RAGE levels to high concentration H₂O₂ in NRO detected with anti-RAGE antibody. Lane 1, 0 μ M; lane 2, 250 μ M; lane 3, 500 μ M, lane 4, 1000 μ M H₂O₂ treatment for 24 hr.

Fig. 4. Regulation of RAGE levels by increased H_2O_2 concentration and rose Bengal. **A:** Dose response of RAGE levels to H_2O_2 in HOG detected with anti-RAGE antibody. Lane 1, 0 μ M; lane 2, 250 μ M; lane 3, 500 μ M; lane 4, 1,000 μ M. H_2O_2 treated for 24 hr. **B:** RAGE levels are reduced in NRO by rose Bengal (5 μ M) treatment for 24 hr detected with anti-RAGE antibody. Cultured NRO or HOG were treated with increasing H_2O_2 (up to 1,000 μ M) and oxidation-inducing reagent rose Bengal (5 μ M). After 24 hr, cells were harvested and subjected to Western blot analysis to show that RAGE expression decreases in response to increasing H_2O_2 concentrations and free radical production (rose Bengal).

Fig. 5.

H_2O_2 activated ectodomain shedding of RAGE. **A:** NRO cells were incubated with or without H_2O_2 for 1 hr, cells were harvested, and Western blot was analyzed with C-20 anti-RAGE antibody. Lane 1, control without H_2O_2 treatment; lane 2, 100 μ M H_2O_2 ; lane 3, 200 μ M H_2O_2 . Western blot showing RAGE processed ectodomain shedding by H_2O_2 . **B:** Condition medium was collected, and proteins were precipitated by the TCA method. Western blot was analyzed with N-16 anti-RAGE antibody, and results showed that 45-kDa soluble RAGE was occurred in H_2O_2 -treated condition medium. Lane 1, control without H_2O_2 treatment; lane 2, 100 μ M H_2O_2 ; lane 3, 200 μ M H_2O_2 .

Fig. 6.

H_2O_2 -induced RAGE shedding was partially inhibited by NAC. NRO cells were preincubated in N-acetyl-L-cysteine (20 and 30 mM) for 30 min, and then H_2O_2 (100 μ M) was added to condition media directly, and cells were cultured for a further 1 hr and harvested for Western blot assay with C-20 anti-RAGE antibody. Results showed that NAC just partially protect RAGE cleavage. Lane 1, control; lane 2, control plus 30 mM NAC; lane 3, 100 μ M H_2O_2 ; lane 4, 100 μ M H_2O_2 plus 20 mM NAC; lane 5, 100 μ M H_2O_2 plus 30 mM NAC.

Fig. 7. H₂O₂-induced RAGE shedding was partially inhibited by TAPI0. NRO were preincubated with the broad-spectrum metalloproteases inhibitor TAPI0 (50 μM) for 30 min, then H₂O₂ (100 μM) was added, and cells were cultured for a further 1 hr and harvested for Western blot assay with C-20 anti-RAGE antibody. TAPI0 can partially reduce the level of RAGE shedding. Lane 1, control; lane 2, control plus TAPI0 (50 μM); lane 3, 100 μM H₂O₂; lane 4, 100 μM H₂O₂ plus TAPI0 (50 μM).

Fig. 8.

Comparison of RAGE levels in autopsy brain samples from patients with neuroinflammatory brain disease (MS and NCL) and control brains. **A:** Comparison of RAGE levels in MS and control brain. Lanes 1 and 2, control; lanes 3 and 5, MS plaque region; lane 4 and 6, MS unaffected white matter. C-terminal anti-RAGE antibody were used to show RAGE expression in brain, and β -actin was included to show protein loading levels. **B:** Comparison of RAGE levels in NCL and control brain. Lanes 1–3, controls; lanes 4–6, Batten's disease (CLN1, -2, and -3) brains. C-terminal anti-RAGE antibody was used to show RAGE expression in brain, and β -actin was included to show protein loading levels.