Original Contribution

Oxidative stress resistance in hippocampal cells is associated with altered membrane fluidity and enhanced nonamyloidogenic cleavage of endogenous amyloid precursor protein

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ABSTRACT

Reactive oxygen species (ROS) have important roles as signaling molecules in the regulation of a variety of biological processes. On the other hand, chronic oxidative stress exerted by ROS is widely considered a causative factor in aging. Therefore, cells need to be able to adapt to a chronic oxidative challenge and do so to a certain cell-type-specific extent. Recently, we have shown in oxidative-stress-resistant cell lines, HT22H2O2 and HT22Glu, derived from the neuronal cell line HT22 by chronic exposure to sublethal concentrations of H2O2 and glutamate, that, in addition to the known antioxidant defense mechanisms, e.g., activation of antioxidant enzymes or up-regulation of heat-shock proteins, oxidative stress resistance depends on the composition of cellular membranes. Here, we extend our previous investigations and report increased membrane fluidity in HT22H2O2 and HT22Glu cells compared to the parental HT22WT cells. The increased membrane fluidity correlates with a redistribution of cholesterol, sphingomyelin, and membrane-associated proteins involved in APP processing between detergent-resistant and detergent-soluble membrane subdomains. The altered membrane properties were associated with drastic changes in the metabolism of the Alzheimer disease-associated amyloid precursor protein (APP), particularly toward enhanced production of soluble APPαs, which is a known neuroprotective factor. Thus our -data provide a link between chronic oxidative stress, alterations in membrane fluidity and composition of membrane subdomains, stress adaptation, and APP processing.

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The production of ROS and their detoxification are normal physiological processes, but an imbalance between the production of ROS and their removal may lead to oxidative stress. As inferred from many studies investigating neurodegenerative diseases that are associated with increased generation of oxidative stress, such as Alzheimer disease (AD), Parkinson disease, and amyotrophic lateral sclerosis, the ability to cope with oxidative stress is cell-type specific. In the AD brain, the cerebellum is less affected by neurodegeneration than the hippocampal region or the cortex [1]. Little is known about the physiological mechanisms behind regional sensitivity to oxidative stress. The cell-type-specific equipment of antioxidant defense systems, such as antioxidant molecules and heat-shock proteins, seems to contribute to a distinct cellular response to oxidative stress.

Recently, we have shown that the lipid composition of cellular membranes is an additional important determinant of cell-type-specific stress resistance [2]. We have employed a neuronal cell culture model based on the hippocampal cell line HT22, which was exposed to a mild oxidative stress by chronic exposure to sublethal concentrations of H2O2 or glutamate, resulting in the production of HT22H2O2 and HT22Glu cells, respectively, which are more stress resistant to further exposure to these treatments than the parental HT22WT cells [3]. In HT22 cells, glutamate elicits oxidative stress via its inhibitory action on a cysteine–glutamate antiporter, which results in the depletion of intracellular cysteine and hence the antioxidant molecule glutathione [4]. Both cell lines, HT22H2O2 and HT22Glu, are mutually cross-resistant and show similar lipid profiles [3]. Aside from the increased expression of antioxidants and heat shock proteins, these stress-resistant cells are characterized by a different cholesterol and sphingomyelin metabolism and by an increase in autophagic activity compared to the parental HT22WT cells. Treatment of vulnerable HT22WT cells with a sphingomyelinase inhibitor to increase cellular sphingomyelin levels enhanced stress resistance, confirming the hypothesis that alterations in the cellular lipid balance affect vulnerability to oxidative stress [2]. To deepen our understanding of the reciprocal influence of the

Abbreviations: AD, Alzheimer disease; APP, amyloid precursor protein; DRM, detergent-resistant membrane subdomain; LRP, low-density lipoprotein receptor-related protein; PS-1, presenilin-1; ROS, reactive oxygen species; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluene sulfonate.

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functional network of cellular lipids and oxidative stress, we focused our investigations on differences in membrane fluidity and in the composition of lipid rafts of oxidative-stress-resistant and vulnerable HT22 cells. Lipid rafts are cholesterol- and sphingolipid-rich detergent-resistant membrane subdomains (DRMs). By their dynamic recruitment of transmembrane or membrane-associated proteins, DRMs regulate many physiological processes, such as the activation of signaling pathways or the enzymatic processing of membrane-bound proteins, which may modulate cellular stress resistance. In addition to analyzing the distribution of cholesterol and sphingomyelin between DRMs and non-DRMs, we particularly investigated the localization of proteins involved in the metabolism of APP because APP processing is known to be strongly influenced by alterations in the lipid environment and several studies indicate a role for APP in antioxidant responses [5,6]. It has been demonstrated that higher membrane fluidity favors the nonamyloidogenic γ-secretase cleavage of APP, whereas lower membrane fluidity is associated with preferential β-secretase cleavage, leading to an increase in toxic Aβ fragments [7]. In addition, depending on whether APP is cleaved via the amyloidogenic or the nonamyloidogenic process, APP fragments themselves are known to induce oxidative stress or to act neuroprotective, respectively [8].

Materials and methods

Reagents

The following commercial antibodies were used for Western blotting: polyclonal anti-BACE-1 (Abcam); monoclonal anti-caveolin-1 (Transduction Laboratories); monoclonal A8717 (Sigma) against the C-terminus of APP; monoclonal 22C11 (Chemicon), which is directed against amino acids 66–81 of the amino-terminus of APP; polyclonal anti-nicastrin (Sigma); and monoclonal anti-tubulin and polyclonal anti-actin (Sigma). Polyclonal anti-presenilin-1 antibodies 3108 and 3110 were raised to the loop domain (amino acids 263–407) of presenilin-1 (PS-1), as described previously [9]. Polyclonal antibody 1704, which was raised against the last 15 amino acids of the cytoplasmic domain of human low-density lipoprotein receptor-related protein (LRP), has been described previously [10,11]. Secondary donkey anti-mouse and donkey anti-rabbit horseradish peroxidase-conjugated antibodies were from Dianova. Biotinylated anti-rabbit Ig and the ABC kit (used to enhance the BACE-1 signals) were obtained from Vector Laboratories. All other biochemicals used were obtained from regular commercial sources unless stated otherwise.

Cell culture

The initial isolation and maintenance of HT22WT, HT22H2O2, and HT22C60 cells have been described previously [2,3]. Cells were cultured in DMEM containing 10% fetal calf serum, 1 mM sodium pyruvate, and 1× penicillin/streptomycin from Invitrogen. To maintain the oxidative medium was changed daily to remove residual toxins. Before experiments, cells were cultured for 10 days without toxins and culture medium. 37°C with 1.5% CO2 and 95% air. For inhibition of transmission of differently polarized light. Because of its cationic moiety, TMA-DPH is anchored only in the outer leaflet of the plasma membrane. Because the rate of internalization of TMA-DPH is very slow, the measured anisotropy values represent organization in the cellular plasma membranes [12].

Preparation of DRMs

Cells grown to 70% confluence in a 10-cm dish were washed twice with ice-cold PBS, scraped in 1 ml PBS, collected by centrifugation at 1000 g for 5 min at 4 °C, and resuspended in 300 μl lysis buffer (50 mM Tris–Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 mM CHAPS). After incubation on ice for 30 min the cell lysate was mixed with 300 μl 80% sucrose solution (in lysis buffer without CHAPS) to give a 40% sucrose-containing solution. The 40% sucrose solution was overlaid by a step gradient of 1.3 ml 30% sucrose and 250 μl 5% sucrose. By centrifugation for 120 min at 200,000 g at 4°C, CHAPS-insoluble, lipid-rich, low-density membrane microdomains (floating at the interface between 5 and 30% sucrose) were separated from high-density membrane fractions (collecting at the bottom of the gradient). Ten fractions were collected from the top and 20 μl of each fraction was analyzed by Western blot analysis. For the determination of free unesterified cholesterol and sphingomyelin 50 and 100 μl of each fraction was analyzed using the Amplex red cholesterol assay kit or the sphingomyelinase assay kit, respectively (Invitrogen).

Western blot analysis

Cells from 70% confluent 10-cm dishes were cultured for 24 h. The cells were washed twice and scraped into ice-cold PBS. After centrifugation at 1000 g for 5 min at 4°C the cells were lysed in a buffer containing 62.5 mM Tris, pH 6.8, 2% SDS, 10% sucrose, 5 mM EDTA supplemented with phenylmethylsulfonyl fluoride (5 mg/ml) and aprotinin (1 μg/ml). Extracts were sonicated briefly and denatured for 5 min at 95°C. Protein concentrations of the samples were determined using the BCA kit (Pierce) with bovine serum albumin as a standard. Thirty micrograms of protein/lane was separated by SDS–PAGE on 4–12% Bis–Tris-gradient gels (Invitrogen) and analyzed by Western blotting with the appropriate antibodies. For the determination of soluble (s) APP, gel loading of medium samples was normalized to the corresponding cell extracts. SAPP was detected by the monoclonal antibody 22C11. Western blot signals were quantified by AIDA Image software. Actin or tubulin was used to ensure equal protein loading of cell lysate samples.

Cell surface biotinylation

Cells from 70% confluent cultures were washed twice with ice-cold PBS, chilled on ice for 5 min to block endocytosis, and biotinylated...
with 1 mg EZ-Link Sulfo-NHS-LC-Biotin (Pierce) in 2 ml PBS/culture dish (100-mm diameter) for 1 h at 4°C. After cell surface biotinylation, the cells were washed twice with ice-cold PBS and subsequently incubated on ice with 50 mM glycine/PBS for 15 min to quench the biotinylation reaction. Subsequently, cells were lysed in 500 μl of buffer A (for composition see below) and left on ice for 60 min. After a centrifugation step (10 min, 25,000 g, 4°C) to remove nonsolubilized material, the cell lysates were adjusted to the same protein concentration (120 μg/ml) by addition of buffer A so that the total volume was 500 μl. To each lysate, 60 μl of NeutrAvidin beads (Pierce) was added. After incubation for 1 h at 4°C the supernatant was removed and kept for SDS–PAGE analysis. The beads were washed twice with each buffer, B, C, and D, and then boiled in sample buffer for SDS–PAGE and subjected to Western blot analysis with the 22C11 antibody against APP. The buffer compositions were as follows: buffer A, 50 mM Tris–Cl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1% NP-40, 0.1% sodium deoxycholate; buffer B, 10 mM Tris–Cl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.2% NP-40; buffer C, 10 mM Tris–Cl, pH 7.4, 500 mM NaCl, 2 mM EDTA, 0.2% NP-40; buffer D, 10 mM Tris–Cl, pH 7.4.

**Determination of γ-secretase activity**

Enzymatic activity of γ-secretase in total cell extracts was analyzed using a γ-secretase activity kit (R&D Systems, Wiesbaden-Nordenstadt, Germany) following the manufacturer’s instructions. For the determination of γ-secretase activity, the same amounts of cellular protein were used.

**Statistical analysis**

Statistical significance was determined by the unpaired two-tailed Student t test using the SigmaStat software; significance was set at p ≤ 0.05 and p ≤ 0.005.

**Results**

**Plasma membrane fluidity is increased in HT22H2O2 and HT22Glu cells**

The previously reported differences in lipid composition between oxidative-stress-resistant HT22H2O2/HT22Glu cells and HT22WT cells [2] are probably reflective of differences in membrane fluidity, which will certainly affect the cellular metabolism. To measure plasma membrane fluidity we examined the steady-state fluorescence anisotropy using the dye TMA-DPH. Anisotropy values are inversely proportional to the degree of membrane fluidity. Interestingly, in contrast to the reported decrease in membrane fluidity in cells exposed to acute oxidative stress [13–15], a significant increase in plasma membrane fluidity was found for HT22H2O2 and HT22Glu cells, which were generated by chronic exposure to oxidative stress, compared to HT22WT cells (Fig. 1).

**Cholesterol and proteins contributing to APP processing migrate toward the non-DRM fraction whereas sphingomyelin levels increase in DRMs of stress-resistant cells**

The observed increase in membrane fluidity in HT22H2O2 and HT22Glu cells suggests that the actual localization and/or distribution of lipids and membrane-associated proteins might be altered compared to HT22WT cells. We therefore analyzed the composition of different membrane regions, DRMs and non-DRMs, in all cell lines. Thereby we concentrated on the localization of cholesterol, sphingomyelin, and membrane-associated proteins involved in APP processing. Cholesterol is a major component of DRMs, in which amyloidogenic APP processing takes place after APP has been endocytosed, whereas processing of APP to the nonamyloidogenic form by α-secretase has been reported to take place in non-DRMs of the plasma membrane [16–18]. To investigate whether cholesterol distribution between the DRM and the non-DRM fractions is changed in HT22H2O2 and HT22Glu versus HT22WT cells, we determined the relative amount of cholesterol in DRMs of these cell lines. DRMs were isolated by extracting cells with 20 mM CHAPS and subsequent sucrose density gradient ultracentrifugation. The cholesterol content in DRMs of HT22H2O2 and HT22Glu cells was significantly reduced whereas it was increased in the non-DRM fraction compared to HT22WT cells (Fig. 2A). In contrast, the relative proportion of sphingomyelin was increased in DRMs and decreased in non-DRMs of the stress-resistant cells (Fig. 2B). Furthermore, we studied the localization of caveolin-1, as a typical DRM marker; of APP; and of...
several proteins that play important roles in APP processing and clearance, such as nicastrin, PS-1, BACE-1, and LRP. In HT22WT cells we found a substantial fraction of caveolin-1, nicastrin, LRP, and PS-1 in DRMs (at the interface between 5 and 30% sucrose, Fig. 3, left). A majority of cellular proteins were detected in the non-DRM (40% sucrose) fractions at the bottom of the gradient as shown by Coomassie staining of the proteins after gel electrophoresis (Fig. 3). This clearly demonstrated that caveolin-1, nicastrin, LRP, and PS-1 are truly enriched in the DRM fraction. In comparison to the above-mentioned proteins, only a minor population of BACE-1 and full-length APP resided in DRMs in all investigated cell lines (Fig. 3). In all cells, BACE-1 occurred mainly as a dimer. Dimerization of BACE-1 has been reported recently for several tissues and cell lines and seems to facilitate substrate binding and cleavage [19,20].

Importantly, in HT22H2O2 and HT22Glu cells the relative amounts of all analyzed proteins in DRMs were reduced compared to those in HT22WT cells, suggesting a general disintegration of CHAPS-resistant DRMs in stress-resistant cells (Fig. 3). In all cells, BACE-1 occurred mainly as a dimer. Dimerization of BACE-1 has been reported recently for several tissues and cell lines and seems to facilitate substrate binding and cleavage [19,20].

**APP processing is shifted toward the nonamyloidogenic pathway in HT22H2O2 and HT22Glu cells**

The increased plasma membrane fluidity and the observed shift in cholesterol, APP, and other AD-associated APP-processing proteins to non-DRMs led us to hypothesize that α-secretase activity should be altered in oxidative-stress-resistant cells compared to stress-sensitive cells. Western blot analysis using antibodies that are directed against the C-terminus of APP showed the three isoforms of full-length APP (APPfl) in neuronal cells: the mature, N- and O-glycosylated, and the immature forms of APP695, APP751, and APP770 (Figs. 3 and 4A, second row from top). Comparison of APPfl protein in total lysates of stress-resistant and vulnerable cells showed clearly a reduction in the highest glycosylated APPfl protein in HT22H2O2 and HT22Glu cells, reasoned from the reduced appearance of the highest of the three bands compared to HT22WT cells (Fig. 4A, second row from top). Relative to the APPfl protein, levels of sAPP released into the medium by HT22H2O2 and HT22Glu cells increased compared to HT22WT cells (detected by N-terminal APP antibody 22C11, Fig. 4A, top row). Because Western blot analysis by antibodies directed against the C-terminus of APP gave a strong signal for CTFα, but did not detect any CTFβ in these cell lines (Fig. 4A, third row from top), the increase in sAPP in the medium of HT22H2O2 and HT22Glu cells reflects an increase in α-secretase-cleaved APP, sAPPα.

APP shedding by α-secretase occurs mainly at the cell surface [17]. The observed increase in sAPPα prompted us to examine whether stress-resistant cells have more APP localized at the plasma membrane than the vulnerable parental cells. Indeed, we observed a strong increase in cell surface-biotinylated APP in HT22H2O2 cells compared to HT22WT cells (Fig. 4B). This is in line with the observed enhancement in α-secretase cleavage.

![Fig. 3.](image-url) Proteins contributing to APP processing show reduced levels in DRMs of HT22H2O2 and HT22Glu cells compared to HT22WT cells. DRMs from HT22WT, HT22H2O2, and HT22Glu cells were isolated by extraction of cells with 20 mM Chaps (30 min, 4 °C) and subsequent sucrose density gradient centrifugation. Coomassie staining after gel electrophoresis showed that in all cell types the majority of proteins were localized in the non-DRM 40% sucrose fractions (20 μl of each fraction was loaded per lane). Western blot analysis showed relative enrichment of caveolin-1, a DRM marker, in the DRM fractions (at the interface between 5 and 30% sucrose, left). Partial migration into the DRM fractions was also found for full-length APP (APPfl). Note that the APP711 and APP770 bands migrate together in the chosen gel system. Nicastrin, LRP, BACE-1, and PS-1 were also detected in the DRM fraction in HT22WT cells. In HT22H2O2 and HT22Glu cells the amounts of all analyzed proteins in the DRM fractions were reduced compared to HT22WT cells (right). For each protein one representative blot of three independent experiments is shown.
Oxidative stress is generally acknowledged to be associated with AD pathology and has been described as a possible mediator of cell damage in AD. Although the entire brain is subject to an oxidative challenge, certain brain areas are more vulnerable to an oxidative attack than others. Here we searched for the molecular basis of possible endogenous defense mechanisms that distinguish oxidative-stress-resistant cells from cells with a high sensitivity for oxidative stress. For this study we used three neuronal cell lines that differ in their vulnerability to oxidative stress. Recently, we reported that changes in the lipid composition of these cell lines might be one important factor that determines the differential vulnerability to oxidative stress [2]. Now we report that the observed differences in lipid composition result in increased membrane fluidity in stress-resistant cells and correlate with changes in APP processing, shifting APP processing toward an increased release of the neuroprotective sAPPx fragment.

The unique localization of cholesterol enriched in the outer leaflet of the plasma membrane in DRMs suggests that alterations in cellular lipid composition and trafficking due to oxidative stress will affect the activity of membrane-bound proteins involved in APP metabolism. It is important to stress that modulation of total cholesterol by just 10% is sufficient to visibly change DRM composition and membrane fluidity [21]. We have previously shown that cholesterol accumulated in oxidative-stress-resistant cells mainly in lysosomes and not in the plasma membrane [2]. Hence, despite the observed increase in total cholesterol of about 13% in HT22H2O2 cells [2], the shift of cholesterol out of the DRM fraction to the non-DRM fraction led us to expect an increase in membrane fluidity. In addition, the observed accumulation of the cholesterol precursors lanosterol and desmosterol [2], which exert weaker condensation effects on lipid acyl chains than cholesterol [22–24], would also suggest a shift to a more disordered plasma membrane. Indeed, plasma membrane fluidity was significantly enhanced in both stress-resistant cell lines compared to HT22WT cells. Increased membrane fluidity might protect cells against oxidative stress, which is known to rigidify membranes [25–27]. This hypothesis is supported by a study showing that the fluidity of synaptic plasma membranes isolated from different rat brain regions proved to be higher in the cerebellum, a brain region that is relatively resistant to oxidative stress, than in the hippocampus or cortex, two relatively vulnerable brain regions [28]. Interestingly, the most fluid synaptic plasma membrane of the cerebellum showed the lowest cholesterol level of the three investigated brain regions.

The observed increase in membrane fluidity is also important with regard to APP processing because higher plasma membrane fluidity due to methyl-β-cyclodextrin-induced cholesterol depletion has been shown to promote α-secretase cleavage of APP and, therefore, the nonamyloidogenic form [29]. Interestingly, in that study Kojro and colleagues also document that replacement of cholesterol by its precursor lanosterol strongly enhances α-secretase activity [29], which is in agreement with our data showing increased lanosterol levels and enhanced α-secretase activity in oxidative-stress-resistant cells. In contrast, cholesterol accumulation in the plasma membrane has been reported to increase the rigidity of the plasma membrane and to decrease α-secretase processing of APP [7]. The observed changes in HT22H2O2 and HT22Glu cells toward nonamyloidogenic APP processing to sAPPx will probably contribute to their increased stress resistance. sAPPx is known to act as a neuroprotective cytokine by blocking activation of the proapoptotic c-Jun N-terminal kinase signaling cascade [30,31]. In summary, alterations in the lipid composition of cellular membranes and in membrane fluidity, induced by a permanently increased oxidative microenvironment, are prone to change many different metabolic

Levels of the C-terminal fragment of APP, which results from α-secretase cleavage, were not increased as we would have expected from enhanced α-secretase cleavage, but markedly reduced in HT22H2O2 and HT22Glu cells (Fig. 4A, third row). This indicates increased processing of APPCTFα by γ-secretase. Measurement of γ-secretase activity showed indeed increased activity in HT22H2O2 cells (Fig. 4C). In concert with this, treatment for 30 h with the γ-secretase inhibitor L685,458 (1.5 μM) reduced APPCTFα levels and further inhibition does not visibly increase the amount of APPCTFα above the already high levels. Direct measurement of the γ-secretase-generated intracellular APP fragment (AICD) were not possible given that the endogenous levels of AICD in HT22 cells are difficult to detect.
processes taking place in distinct membrane compartments (here shown for APP processing), which then may influence the vulnerability of cells to oxidative stressors.

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