A free radical-generating system regulates APP metabolism/processing

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ABSTRACT

Oxidative stress, a risk factor in the pathophysiology of Alzheimer’s disease, is intimately associated with aging. We previously reported that the X-XOD free radical generating system acts as a modulator of lipid metabolism and a mild inducer of apoptotic death. Using the same cell model, the present study examines the metabolism/processing of the amyloid precursor protein (APP). Prior to inducing cell death, X-XOD promoted the secretion of β-secretase-cleaved soluble APP (sAPPβ) and increased the level of APP carboxy-terminal fragments produced by α and γ secretase (αCTF and γCTF/AICD). In contrast, it reduced the activity of β-secretase and the level of secreted Aβ. The present results indicate that mild oxidative stress maintained throughout culturing regulates APP metabolism/processing in SK-N-MC human neuroblastoma cells.

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1. Introduction

Alzheimer’s disease (AD) is an age-related disorder characterized by progressive memory loss and cholinergic neurodegeneration [1]. The disease is associated with a variety of pathological features in the brain, such as extracellular senile plaques and intracellular neurofibrillary tangles. The main component of these senile plaques is β-amyloid peptide (Aβ), which is derived from amyloid precursor protein (APP), a type I transmembrane protein.

APP is cleaved by three proteases: α-, β-, and γ-secretase. Cleavage by α-secretase occurs within the Aβ domain, preventing the formation of Aβ and resulting in the production of a secreted ectodomain of APP (sAPPα) plus a shorter COOH-terminal fragment of APP (αCTF). The latter is further cleaved by the γ-secretase complex, resulting in γCTF/AICD (the APP intracellular domain) [2]. It is reported that sAPPα is neurotrophic [3,4]. Aβ is generated from APP through proteolytic cleavage by β-secretase at the β-site, followed by γ-secretase cleavage at the position of downstream amino acid residues 40 or 42; this generates Aβ1–40 or Aβ1–42, respectively.

Oxidative stress (OS) increases with age [5]. Many studies have recorded OS in the brains and peripheral tissues of patients with AD, as well as in animal models of the disease [6]. The xanthine/xanthine oxidase (X-XOD) system is a free radical-generating system that induces cell death in cerebellar granule neuronal cultures via reactive oxygen species (ROS) [20]. In earlier work we adapted this system to generate a human neuronal cell model of mild OS that allowed the analysis of free radical-induced events preceding cell death [21]. Using this model, the present study investigates in vitro the consequence of OS on APP metabolism/processing. The results show that, in the SK-N-MC human neuroblastoma cell line, the X-XOD system increases the intracellular level of non-amyloidogenic CTF (γCTF) and the secretion of sAPPα prior to cell death. Further, it reduces the secretion of Aβ (Aβ1–40 and Aβ1–42) leading to the intraneuronal accumulation of APP-derived fragments: CTFs (γCTF, and later βCTF) and sAPPα. The data provided support a model for the regulation of APP metabolism/processing as an early response to OS.

2. Materials and methods

2.1. Materials

The cells used in this work were SK-N-MC human neuroblastoma cells obtained from the American Type Culture Collection (HTB-10; ATCC), and SK-N-MC cells overexpressing APP 695 (the C2 cell line) [8]. Xanthine (X) and catalase were purchased from Sigma, xanthine oxidase (XOD) from Roche, and trolox from Calbiochem. Culture medium components were purchased from Gibco Laboratories. Other chemicals were purchased from Merck or Sigma.
2.2. Cell culture and exposure to oxidative stress

Cells were cultured in minimal essential medium (MEM) supplemented with 10% foetal bovine serum, 100 μM penicillin G and 100 μg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO₂. One day before stimulation with X-XOD, exponentially growing cells at 80–90% confluence were placed in culture dishes with six multiwells (M-6). On the day of stimulation, the cells were placed in fresh medium in the presence or absence of an antioxidant product (trolox or catalase; see below) 1 h before X-XOD treatment. This was performed as previously described [21].

2.3. Analysis of cell injury

The extent of cell injury after exposure to OS was evaluated using the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [7] with minor modifications [21].

2.4. Preparation of the culture medium for analysis following X-XOD treatment

After X-XOD treatment, samples of culture medium were collected, centrifuged and concentrated 10-fold by lyophilization in the presence of a protease inhibitor cocktail (2.5 mM EDTA, 10 μM leupeptin, 1 μM pepstatin, 1 mM phenylmethylsulphonyl fluoride). Samples were then stored at −70°C until their use in Western blotting analysis or sandwich ELISA.

2.5. APP specific antibodies

Anti-APP antibody MAB348/22C11 (Chemicon International, Inc.), or monoclonal antibody 6E10 (Covance), or the anti-C terminal fragment (antibody C1, a kind gift of Paul Mathews, or alternatively rabbit anti-amyloid precursor protein C-terminal fragment from Sigma) were used for the immunodetection of APP and its proteolytic fragments. The ability of each antibody to recognize the different fragments, according to their epitope location in APP, is summarized below (X indicates recognition). Antibodies specific for βAPP1–40 or βAPP1–42 (Biosource International) were used where indicated.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>holoAPP</th>
<th>sAPPα</th>
<th>sAPPβ</th>
<th>αCTF</th>
<th>βCTF</th>
<th>γCTF/C</th>
<th>C. Fluorescence was read using a Fluoroskan II Neonat (Flow Laboratories) (excitation and emission wavelengths 320 and 405 nm, respectively).</th>
</tr>
</thead>
<tbody>
<tr>
<td>22C11</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>6E10</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
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<tr>
<td>Anti-Ct</td>
<td>X</td>
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<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>

2.6. Aβ detection by sandwich ELISA

Monoclonal antibody 6E10 was used as the capture antibody, and antibodies specific for βAPP1–40 or βAPP1–42 as detection antibodies (Biosource International). The assays were performed as described previously [8].

2.7. Western blotting

The concentrations of APP, or alternatively of CTFs, were evaluated by Western blotting. Cells were washed in cold phosphate buffer saline (PBS) and lysed in Laemmli’s SDS sample buffer before loading onto 8 or 10–16.5% SDS–polyacrylamide gels. Cell-number-equivalent samples were subjected to Western blotting. Protein bands were transferred to nitrocellulose membranes (BioRad). These membranes were further processed by incubating with the 22C11, 6E10 or anti-Ct antibodies for 2 h at room temperature, respectively. This was followed by incubation with secondary antibody (anti-mouse/horse [Vector Laboratories] horseradish peroxidase conjugate or anti-rabbit/goat [Nordic] horseradish peroxidase conjugate) and visualization of the bands using the enhanced chemiluminescence Western-blotting analysis system (Pharmacia Biotech). As an internal control, α-tubulin levels were examined (via the reaction with anti-α-tubulin [Sigma]) in the same blots.

2.8. Immunofluorescence confocal microscopy

Cells grown on coverslips and incubated with X-XOD were fixed with 4% paraformaldehyde in PBS at room temperature for 20 min and permeabilized with PBS containing 0.2% Triton X-100 and 2% horse serum at 4°C overnight. To examine intraneuronal APP fragments, double labelling with the monoclonal antibody 6E10 and the polyclonal antibody directed against the C-terminus of APP was performed. Cells were incubated at room temperature for 2 h with primary antibodies and following rinses were incubated at room temperature for 1 h with Alexa 488 anti-mouse immunoglobulin G (green) (Invitrogen) and Alexa 555 anti-rabbit immunoglobulin G (red) (Invitrogen). For staining of the nucleus, coverslips were incubated in PBS containing 5 μg/ml DAPI (Sigma) at room temperature for 15 min. Cells were examined using a Zeiss LSM510 META confocal microscope or a Zeiss Axiovert 200 fluorescence microscope equipped with a 63× oil-immersion objective. Pictures were taken with a digital camera Spot RT slider (Diagnostic) using the MetaMorph™ imaging system software package. Images were processed using Adobe Photoshop CS3.

2.9. β-Secretase activity

β-Secretase activity was determined using fluorogenic peptide substrate IV (R&D Systems). The cells were lysed using 100 μl of cell extraction buffer (0.5% sodium deoxycholate, 1% NP-40 in PBS, pH 7.1), centrifuged at 10 000×g for 20 min at 4°C, and the supernatant removed. Protein quantifications were made using the Bradford protein assay (BioRad). The supernatants were transferred (100 μl/well; 10 μg total protein) to 96-well microplates with 100 μl of 2× reaction buffer (40 mM MES, 20% glycerol, 4 mM DTT, pH 5.0) and 1.5 μl of substrate in each well; the wells were then made up to 200 μl with Milli-Q water. Plates were incubated in the dark for 18 h at 37°C. Fluorescence was read using a Fluoroskan II Neonat (Flow Laboratories) (excitation and emission wavelengths 320 and 405 nm, respectively).

2.10. Reactive oxygen species (ROS) assay

The ROS assay was modified from previously described techniques [22]. Briefly, M-96 plates were seeded at 40 000 cells/well and after exposure to different treatments were rinsed twice in HKRB (20 mM HEPES, 103 mM NaCl, 4.77 mM KCl, 0.5 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 15 mM glucose, pH 7.3). They were then incubated in a 30 μM solution of 2,7-dichlorodihydrofluorescein diacetate, H₂DCFDA (Molecular Probes) for 3 h, shielded from the light. They were then washed twice with HKRB and incubated in 200 μl of HKRB at 37°C for 60 min. The H₂DCFDA fluorescence was recorded using an Infinite²⁰⁰ microplate reader (Tecan Trading AG) at excitation/emission wavelengths of 485/535 nm.

2.11. Statistics

Results were expressed as means ± standard errors. Statistical differences were determined using the Student t test. Significance was set at P < 0.05.
3. Results

3.1. X-XOD increased sAPPα secretion in SK-N-MC cells prior to inducing cell injury

The effects of OS on sAPP secretion were examined by Western blot analysis using the 6E10 antibody, which recognizes secreted APP derived from non-amyloidogenic α-cleavage (sAPPα). The results (Fig. 1A) showed a fivefold increase in sAPPα in the culture medium of cells treated for 24 h with X-XOD compared to control cells; at later culture times (48 h), sAPPα levels in the treated cells were higher but the difference with respect to control cells was not significant. Analysis of the same culture media with the antibody 22C11, which recognizes all the secreted forms of APP (total sAPP), returned results similar to those above (double those at 24 h of X-XOD treatment, not shown), suggesting that sAPPα is the major form of sAPP induced by X-XOD at early treatment times.

To determine whether the promotion of sAPPα release by X-XOD was the result of an increase in the quantity of cellular APP, the APP protein was sought in cellular lysates by Western blot analysis using the 22C11 antibody, which recognizes full-length APP (holoAPP; hAPP) and the APP fragments containing the N-terminal (sAPP) (see Section 2). The results (Fig. 1B) showed that treatment with X-XOD for 24 h had no significant effect on cellular APP levels. Similarly, in a previous study using whole genome expression microarrays, no changes in mRNA after 24 h of X-XOD treatment were detected [21]. At longer exposure times (48 h), an increase in 22C11-immunoreactivity became evident, representing approximately three times the total APP with respect to control cells (Fig. 1B). The two protein bands recognized by 22C11 corresponded to hAPP (upper band) and sAPP (lower band) according to their apparent molecular weight and because the upper band was also recognized by a C-terminal specific antibody, whereas the lower band was not (Fig. 1C).

The cell damage induced by X-XOD was assessed. Cell viability was measured using the MTT assay (Fig. 2) at 24 and 48 h of treatment. While at 48 h the loss of viability was significant, at 24 h no adverse effects were seen. These results agree with previous data showing minimal apoptotic signals at this treatment time [21].

Together, the above results indicate that, at a time preceding the transmission of cell death signals, X-XOD promoted sAPPα release into the culture medium without significantly affecting APP expression. It is therefore concluded that, at early X-XOD treatment times, the promotion of sAPPα secretion might be due to increased activity of the α-secretase processing pathway. The situation was more complex at prolonged treatment times, when X-XOD also modulated APP expression.

3.2. The X-XOD system increased CTF levels in the SK-N-MC cells

The carboxy-terminal fragments of APP – αCTF, βCTF and γCTF/AICD – that remained associated with the cells were monitored by Western blotting using the C-terminal specific antibodies. X-XOD treatment induced a strong increase in the levels of αCTF and γCTF/AICD (Fig. 3A). As occurred with secreted sAPPα (Fig. 1A), the intracellular increase in αCTF was highest and most significant at 24 h, confirming the activation of the α-secretase processing pathway at this time. The 6E10 antibody was used to specifically...
measure βCTF (Fig. 3B), and a slight reduction was detected at 24 h, followed by an increase at 48 h; this time course could be due to significantly reduced β-secretase activity at 24 h, which recovers at 48 h (Fig. 3C).

The accumulation of αCTF and γCTF/AICD might be a normal cellular response to mild OS since, when other human neuroblastoma cells (SH-SY5Y) were treated for 24 h with X-XOD, αCTF and γCTF/AICD accumulation was also detected (not shown).

These results suggest that mild OS activates the α-secretase proteolysis pathway, which is probably a survival response. The activation of β-secretase and the accumulation of APP fragments derived from this processing are later events induced in parallel with death pathways.

### 3.3. Antioxidants modulate the accumulation of α-secretase-derived APP fragments induced by X-XOD

The participation of ROS in the regulation of APP metabolism induced by the X-XOD system was assessed using ROS scavengers. Using the ROS-sensitive probe H2DCFDA, investigations were made into whether X-XOD actually induces an increase in ROS, and whether the antioxidants catalase and trolox [23,24] modulate these ROS concentrations in the present cell model. X-XOD induced an increase in the levels of ROS that was inhibited by the...
antioxidants in a dose-dependent manner (Supplementary Fig. 1). Further, catalase and trolox induced a significant recovery of the loss of cellular viability caused by X-XOD, indicating that the effect of X-XOD on cellular viability was indeed mediated by ROS (not shown).

Interestingly, the doses of antioxidants partially inhibiting the increase in ROS levels induced by X-XOD further enhanced the increase in intracellular levels of αCTF (Fig. 4A and B) without affecting APP levels (not shown). This enhancement was particularly evident when the relative quantities of α and βCTF were measured (Fig. 4C).

Together, these data suggest that non-amyloidogenic α-cleavage of APP is elicited as a cellular response to OS, and that this response is activated more strongly when the OS is mild.

3.4. The X-XOD system reduced Aβ secretion and led to the intraneuronal accumulation of the APP-derived fragments CTFs and sAPPα

Since the quantity of Aβ peptide produced by the SK-N-MC cells was undetectable, SK-N-MC cells that stably overexpress APP 695 – the C2 cell line [8] – were employed to monitor changes in Aβ. In the X-XOD-treated C2 cells, increases in αCTF and γCTF/AICD were seen without significant changes in APP expression, as was recorded in the parental SK-N-MC cell line. Cell viability loss at 24 h and 48 h was also similar to that seen for the parental cell line (data not shown). This suggests that X-XOD-induced APP processing via α-secretase is independent of the basal APP expression level.

The two main species of amyloid peptide, Aβ1-40 and Aβ1-42, were quantified using sandwich ELISA. Fig. 5A shows that the levels of secreted Aβ1-40 or Aβ1-42 were significantly reduced at 24 h of X-XOD treatment, followed by a partial recovery of control levels at 48 h.

Attempts were made to confirm and extend the findings made by Western blotting by performing immunofluorescence analyses aimed at determining the cellular localization of the APP fragments. The C2 line was used since βCTF (and probably Aβ) represent a relatively small proportion of the total APP fragments and are therefore particularly difficult to monitor in the parental line (see Fig. 4B). Double immunofluorescence labelling of C2 cells exposed to X-XOD for 24 h was undertaken, using the 6E10 antibody (green) and the C-terminal specific antibody (red) to generate confocal images (Fig. 5B). The 6E10-immunoreactive protein species is probably composed of hAPP, sAPPα, βCTF and a small amount of Aβ peptide. The C-terminal–positive staining might correspond to hAPP and CTFs. However, the absence of staining in the control cells by the anti-Ct antibody indicates that, at least in these immunofluorescence experiments, anti-Ct positive signals are due only to CTFs.

In the control cells, the 6E10-positive structures were small vesicles distributed throughout the cytoplasm. The X-XOD treatment led to the accumulation of three types of structure according to their location and staining: (i) 6E10-positive and C-terminal...
negative (green-only) clusters concentrated in the cytoplasm and near the plasma membrane; given their immunoreactive profile, these clusters are probably vesicular structures containing sAPPα, similar to those described in cell cultures of several lineages including primary hippocampal neurons exposed to Aβ peptide [19] (which also induces OS); (ii) C-terminal-positive and 6E10-negative (red-only) structures, mainly located in cytoplasmic vesicles (Golgi?) (well known subcellular sites of APP cleavage and subsequent production of CTFs and sAPP) which would be composed of a CTF, and (iii) structures positive for both the C-terminal and 6E10 antibodies (yellow); these could be of two types, containing sAPPα and CTFs together or βCTF exclusively. The Western blot data suggest that the majority of the yellow signals correspond to vesicles containing CTFs and sAPPα. The early accumulation of βCTF with small amounts of Aβ has been associated with pre-plaque amyloid-associated neuropathology [25].

4. Discussion

The cause of neuronal loss in AD seems to be associated with increased OS caused by the peroxidation of lipids [9], as well other oxidative changes in proteins and DNA [10] – processes that could be attenuated by antioxidants [11]. Indeed, free radical production has been implicated in the progression of several neurodegenerative diseases and in the aging process [12].

The present results indicate that OS leads to increased levels of the APP non-amyloidogenic carboxy terminal fragment (αCTF) at the time of minimal oxidation-induced neuronal death. After just 24 h of X-XOD treatment, when the viability of the SK-N-MC cells remained unaffected, increased sAPPα was seen in the culture medium and no significant change in the cellular expression of hAPP was noticed. SAPPα has trophic effects on cerebral neurons in culture, stimulates neurite outgrowth, and regulates synaptogenesis.
Previous work has shown that sAPPα exerts neuroprotective effects, such as the rescue of neurons from the neurotoxic insult induced by Aβ, and the increased expression of certain survival genes [13].

However, at 48 h, the time of cell death, an increase in intracellular sAPPα was seen, although its secretion to the culture medium was not significant. An increase in HAP levels was also recorded in agreement with the increase in APP mRNA detected from 36–48 h (data not shown). This increase in intracellular sAPPα has also been described in cell cultures of a variety of lineages (including primary neurons) exogenously exposed to Aβ [19]. The Aβ peptide has been shown to induce oxidative stress and the expression of APP [26].

Interestingly, the βCTF levels were slightly reduced at 24 h of treatment. This, together with the increase in secreted sAPPα, corroborates that the non-amyloidogenic APP processing pathway is favoured before neuronal death signals become dominant. Catalase and trolax, which prevented X-XOD-induced cell death, further enhanced the increase in βCTF and secreted sAPPα, suggesting a protective role of α-secretase APP processing in response to OS. Similar results have recently been described in hippocampal cells in which the resistance response to OS involves drastic changes in APP metabolism, in particular αCTF fragment accumulation and an increase in sAPPα production [27]. At the time of neuronal death (48 h), the levels of αCTF decreased and those of βCTF increased with respect to their values at 24 h. This must reflect a change in the APP processing/metabolism towards the amyloidogenic pathway, illustrating that the balance between the α- and β-secretase pathways is of physiological importance [28,29]. The recovery of β-secretase activity at 48 h is consistent with the increase in βCTF fragments. βCTF fragments expressed in cells, or of exogenous origin, cause even more neurotoxicity than Aβ [30].

Although the precise functions of CTFs are unknown, they alter membrane currents and calcium homeostasis [14], stimulate mitogen-activated protein kinase pathways, activate the transcription factor NF-κB, and elevate iNOS [15]. They accumulate in the nucleus and bind to Fe65 [16] and CP2 to alter transcription [15]. The translocation of amyloidogenic CTF to the nucleus has been implicated in neuronal death in vivo [17].

Recently, the activation of α-secretase and the suppression of Aβ40 secretion by the lipid compound 1,3-carypyloyl-2-arachidonyl glycerol has been reported [18,31]. In previous work we showed that the X-XOD system induces the overexpression of genes related to cholesterol and lipid metabolism [21], and the results of the present work show this system reduces the secretion of Aβ40–42 and Aβ1–42 suggesting a link between OS, lipid metabolism and APP metabolism. This reduced secretion may be explained by the reduction (although not complete stoppage) of β-secretase activity. This would leave α-secretase to cleave the APP molecules not processed by β-secretase, reflected as an increase in sAPPα.

APP proteolytic processing and trafficking are closely related; indeed, processing can occur in different cell organelles during intracellular transport [32,33]. Immunofluorescence analysis involving confocal microscopy showed that, before neuronal death (i.e., X-XOD treatment for 24 h), amino- (sAPPα) and carboxy-terminal (αCTF rather than βCTF) APP fragments are accumulated. These appeared in vesicular structures concentrated in the cytoplasm, as seen in the production of sAPPα and CTFS [19].

It seems increasingly apparent that OS is a primary progenitor of AD and not merely an epiphenomenon [34]. The present results outline a dynamic cell model in which APP proteolytic processing, and perhaps the catabolism and trafficking of derived fragments, are modulated by the X-XOD system. These findings provide strong evidence for a substantial role for APP and its cleaved products in selective OS-induced neuronal loss. Monitoring the changes in the α/β-secretase activity ratio might be a practical way of following end-stage alterations in the APP processing pathway in response to different antioxidant treatments. More studies on the novel mechanisms by which α-secretase activity is regulated by OS might lead to a new therapeutic approach in the treatment of AD.

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Appendix A. Supplementary data


References
