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Enhanced oxidative stress is an early event during development of Alzheimer-like pathologies in presenilin conditional knock-out mice

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ABSTRACT

Conditional double knock-out of presenilin-1 (PS1) and presenilin-2 (PS2) (PS cDKO) in forebrain of mice led to progressive memory dysfunction and forebrain degeneration. These changes in the brain recapitulated most of the neurodegenerative phenotypes of Alzheimer's disease (AD). Oxidative stress in brain tissues is intimately related to AD. In this report, we examined oxidative stress status in cerebral cortex in 2-, 4- and 7-month PS cDKO and the age- and gender-matched control mice (WT). Lipid peroxidation (MDA as the measure) and protein oxidation (protein carbonyl as the measure) were found to be significantly increased in PS cDKO mice over the age points examined, notably in those at 2-month, suggesting that oxidative stress is an early event in response to PS loss-of-function. The oxidative modification of cortical proteins was further confirmed by Oxyblot assay. The investigations into endogenous antioxidant defense (CAT, SOD and GSH-px as measures) revealed a compensatory defense against oxidative stress, particularly at the early age stage, in PS cDKO mice. The expression level of cortical glial fibrillary acidic protein (GFAP) increased in an age-related manner, in particular in 2-month PS cDKO mice, suggesting that the interaction relationship between oxidative stress and inflammatory response may be closely associated with the underlying loss-of-function pathogenesis of AD.

Alzheimer's disease (AD) is the most common cause of dementia among elderly. Mutations in presenilin 1 (PS1) and presenilin 2 (PS2) genes are associated with the early onset of AD [18]. PS1 is an essential component of γ-secretase, which cleaves the C-terminal of amyloid precursor protein (APP) to produce β amyloid (Aβ) that accumulates in senile plaques [9]. Several knock-out animal models have been generated to study the physiological function of PS genes. PS1 knock-out mice display severe cerebral hemorrhage and widespread skeletal abnormalities [32]. PS2 knock-out mice lack any detectable phenotype [12]. While conventional PS1 and PS2 double knock-out mice often resulted in embryonic lethality, precluding the functional analysis of PS in adult [12,17], the forebrain-specific and conditional PS1 and PS2 double knock-out (PS cDKO) mice were indistinguishable from littermate controls during early adulthood, and exhibited age-dependent AD-like neurodegenerative phenotypes although the brain Aβ was decreased [2,3,11,14,30].

Brain is particularly susceptible to oxidative stress because of the high oxygen consumption rate, rich in unsaturated lipids, and a relatively high abundance of redox-capable transition metal ions and a relatively low availability of antioxidant enzymes compared with other organs. Indeed, growing evidence has suggested a pivotal role of oxidative stress in the pathogenesis of AD [16,22,33]. While oxidative damage to neuronal DNAs, proteins and lipids in AD has been described to be brain amyloidosis-origin in nature [5] it has also been evident increasingly in human studies [27] and in AD-like transgenic animals [31,35] that oxidative stress is an early event preceding Aβ deposition in the formation of AD pathologies. Furthermore, previous investigations into oxidative imbalance implicated in underlying AD pathogenic mechanism have been largely based on the AD-like amyloidosis in transgenic animal models. However, data derived from PS cDKO that presents the loss-of-function of pathogenic mechanism of AD with reduced Aβ production are currently scant. The present study aimed primarily to investigate oxidative stress status in the PS cDKO mice with different ages by measuring the levels of lipid peroxidation product and protein oxidation carbonyl in response to the PS inactivation in the brain.

The study was approved by the Animal Ethics Committee and all procedures complied with international standards of humane care in animal experimentation. PS cDKO mice on B6CBA background were generated as described previously [12–14,30]. Age- and gender-matched mice with the same genetic background (B6CBA) were served as wild-type (WT) control. The cerebral cor-

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tex of 2-, 4- and 7-month mice were dissected from each group and homogenized in lysis buffer (6 M urea, 2 M thiourea, 10% glycerol, 50 mM Tris–HCl (pH 7.8–8.2), 2% *n*-OG, 1 mM protease inhibitor) on ice, respectively. The cytosolic fractions were centrifuged at 20,000 × *g* at 15 °C for 15 min. The protein concentration was determined by the Bradford reagent method with BSA standard.

Lipid peroxidation product MDA was determined by photometrical method using the MDA Assay Kit (Nanjing Jiancheng Corp., China). The SOD activity was determined using the SOD Assay Kit-WST (Dojindo Inc., Japan) according to the manufacturer's protocol. CAT and GSH-px activities were measured using the CAT and GSH-px assay kits (Nanjing Jiancheng Corp., China), respectively.

Total protein carbonyls in cortex samples were determined as described before [19]. Briefly, 80 μL of protein samples were mixed with 80 μL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) (in 2 M HCl) and incubated for 1 h at room temperature (RT). Proteins were precipitated with equal volume of 30% TCA, and the pellets were washed three times with 1 mL ethanol–ethyl acetate (1:1, v:v). The pellets were dissolved in 80 μL of 6 M guanidine HCl and kept at 37 °C for 30 min. The carbonyl content was calculated from the absorbance at 366 nm using a molar absorption coefficient of 22,000 M^{-1} cm^{-1}.

DNPH derivatization was carried out on 20 μg of protein for 15 min using Oxyblot Kit (Millipore-Chemicon) following manufacturer's instruction. The derivatives were then resolved by 12% SDS-PAGE and transferred to Hybond-P PVDF membrane (Amersham). After blocking in TBST containing 5% BSA at RT for 60 min, the membranes were washed with TBST and incubated with anti-DNP (1:150) at 4 °C for overnight. The membranes were washed with TBST and incubated with horseradish peroxidase (HRP) conjugated anti-rabbit IgG at RT for 45 min. The membranes were washed again with TBST and the proteins were visualized with the ECL chemiluminescence kit (Amersham) according to manufacturer's protocol. The protein level was normalized to GAPDH.

For Western blot, cortical proteins were prepared as described above. Each protein sample was mixed with loading buffer and heated at 100 °C for 4 min. The samples were then resolved by SDS-PAGE, transferred to Hybond-P PVDF membrane (Amersham) and incubated with antibodies against GFAP (1:2000) (Santa Cruz) and GAPDH (1:20,000) (the internal control).

All data were expressed as the mean ± S.E.M. The differences between the mean values of two groups were determined by Student *t*-test. For comparison of variable between groups with a relative small sample size (i.e., cortical GSH-px in male mice), the non-parametric procedure was used. Statistical significance was set at $p < 0.05$.

Several behavior studies showed that memory and synaptic plasticity deficits in PS cDKO mice appeared at the age of 2-month while severe memory dysfunction started from 6-month old, and these were accompanied by neuronal degeneration in the cerebral cortex in an age-dependent manner [30]. To address the time-course of oxidative stress with the pathological progress of AD, we assessed oxidative status at both lipid and protein levels in the PS cDKO mice with an age spectrum of 2-, 4- and 7-month. Notably, the levels of lipid peroxidation product MDA were significantly elevated in the cortices of PS cDKO mice at the age as early as 2-month (2.8 ± 0.29 vs. 1.96 ± 0.17, $p < 0.03$), and the oxidative stress were even severe and maintained at a higher level during the ages from 4- (4.36 ± 0.57 vs. 2.79 ± 0.17, $p < 0.02$) to 7-month (4.24 ± 0.29 vs. 2.86 ± 0.21, $p < 0.01$), as compared with those of age- and gender-matched WT mice (Fig. 1A). Accordantly, protein oxidative modification levels, as measured as protein carbonyl content by DNPH assay, in the cerebral cortex samples were also significantly higher by 51% (9.19 ± 1.08 vs. 6.11 ± 0.77, $p < 0.04$), 61% (24.93 ± 2.28 vs. 15.43 ± 1.64) and 41% (18.4 ± 2.02 vs. 13.03 ± 0.91) in 2-, 4- and 7-

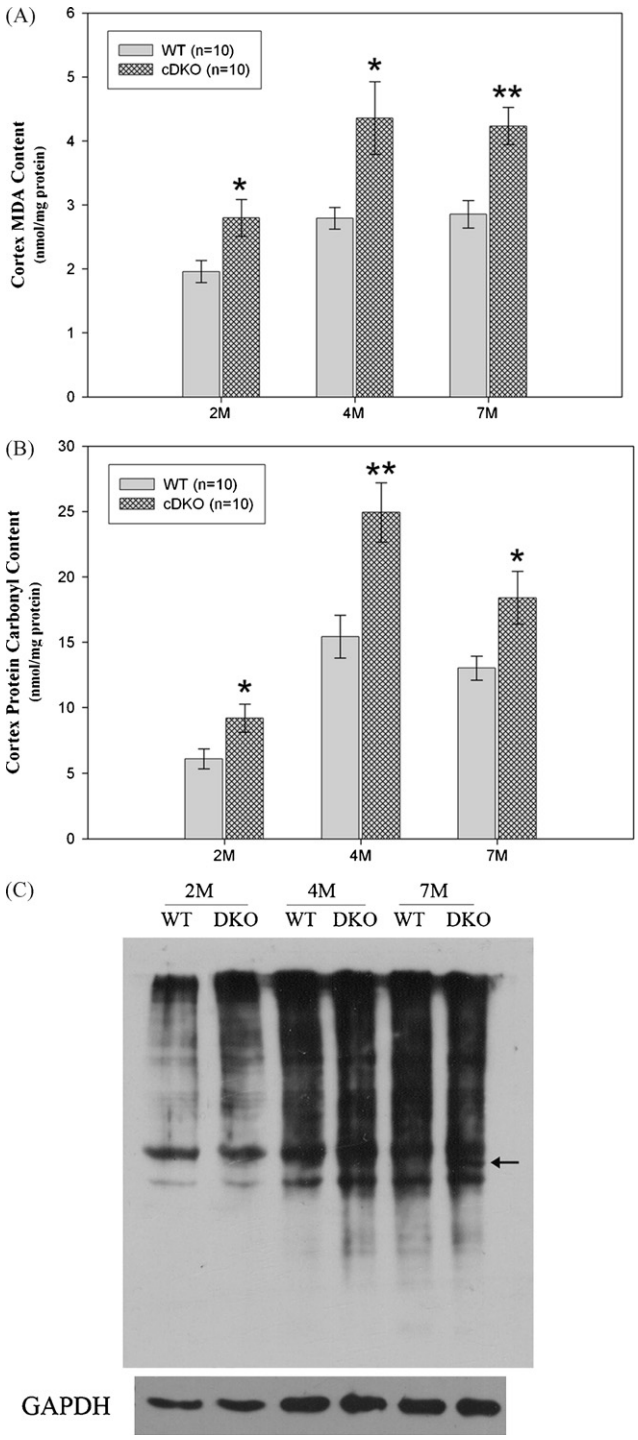

Fig. 1. Enhanced oxidative stress in PS cDKO mice. (A) Cortical MDA contents were significantly increased in PS cDKO mice at the ages of 2-, 4- and 7-month compared to matched WT. (B) Increased cortical carbonylated proteins were found in PS cDKO mice over all age points examined using spectrophotometric DNPH assay. (C) Oxyblot results confirmed the presence of carbonylated proteins in the cortices of PS cDKO mice. The lane total density of PS cDKO was higher compared to that of WT in 2-, 4- and 7-month-old mice. The arrow indicated the band with high degree of oxidative modification in 4- and 7-month PS cDKO mice. GAPDH was used as a loading control. Data were shown as mean ± S.E.M. $^{*}p < 0.05$ and $^{**}p < 0.01$.

month age groups of PS cDKO mice, respectively, as compared with those of WT controls (Fig. 1B). The finding was further confirmed in the mice, especially in those at ages of 4- and 7-month, using Oxyblot immunolabeling of carbonylated proteins (Fig. 1C). DNPH-control-labeled proteins were also applied as a negative control in the assay (data not shown).

To further address oxidative stress status during the pathological development of AD, we assessed endogenous antioxidant defense including CAT, SOD and GSH-px in the cerebral cortices of PS cDKO mice. Cortical CAT activities were unchanged in PS cDKO mice at 2-month age, and were significantly decreased by 58% (0.23 ± 0.05 vs. 0.55 ± 0.11) and 46% (0.12 ± 0.02 vs. 0.22 ± 0.04) in the 4- and 7-month mice, respectively, compared to that of matched controls (Fig. 2A). Contrastingly, cortical SOD activities were markedly higher in 2- and 7-month PS cDKO mice by 12% (5.47 ± 0.19 vs. 4.89 ± 0.16, $p < 0.03$) and 45% (5.74 ± 0.68 vs. 3.94 ± 0.19, $p < 0.03$), respectively, compared to WT controls. SOD activities were also increased in 4-month PS cDKO mice but without statistical significance possibly due to a larger standard deviation (Fig. 2B). There was no significant difference in cortical GSH-px activities between WT and PS cDKO mice over all age points. However, in 2-month male PS cDKO mice the activities of this enzyme appeared to be increased significantly by 19% (43.05 ± 1.92 vs. 36.15 ± 2.17, $p < 0.05$) compared to age- and gender-matched WT (Fig. 2C).

To study the possible source of the enhanced oxidative stress occurred in the brain in response to the loss-of-function of PS, we measured the level of cortical GFAP, a marker of inflammation of cortex, in 2-, 4- and 7-month transgenic and WT mice. Data showed that GFAP expression was increased in the cortices of PS cDKO mice in an age-related manner (Fig. 3), similar to the changes shown in the MDA and protein oxidation results above.

PS play essential roles in the mature cerebral cortex in addition to regulating neuronal differentiation and migration during embryonic development. It involves in proteolytic processing of Notch and Wnt signaling pathways, which are important for neuronal differentiation and cortex development [10]. PSs are also found to reside in the membranes of the endoplasmic reticulum and Golgi apparatus [8]. Previous researches have shown that there are impairments of learning and memory, synaptic plasticity, and neuronal survival in an age-dependent manner in the adult cerebral cortex after inactivation of PSs, and expressions of multiple CREB–CBP target genes, such as c-fos, exon III-containing BDNF transcripts, Egr-1 and synapsin-1, are also reduced in the cerebral cortex [2,30].

On the other hand, increasing evidence has suggested that oxidative damage in brain tissue is intimately related to neurodegeneration including AD. Although it remains elusive as to whether oxidative stress is involved at the onset of AD, it is thought to play a significant role during disease progression, particularly in cellular and tissue damage that occurs throughout AD. The levels of lipid peroxidation products and protein carbonyl formation have been shown to be elevated in the tissue from AD brain [31] and AD transgenic mice, such as Tg2576 mice [35], APP/PS mutant [23], ApoE knock-out [6] and SAMP8 mice [24]. Oxidative characteristics identified so far in AD transgenic mice models appeared to be Aβ-related as Aβ is thought to be a metalloenzyme capable of generating ROS [28]. Nakajima and Shirasawa found PS1-deficient neurons exhibited increased vulnerability to oxidative stress in calcium-dependent manners [26], and it was mediated by nitric oxide (NO) [25]. The present study investigated oxidative response of cerebral cortex to the development of AD pathologies in PS cDKO mice independent of brain Aβ deposition. The results revealed that oxidative stress, expressed as enhanced lipid peroxidation and protein oxidation, was initiated in PS cDKO mice aged as early as 2-month, at least being synchronous with the occurrence of memory and synaptic plasticity deficits in the model [30]. Such

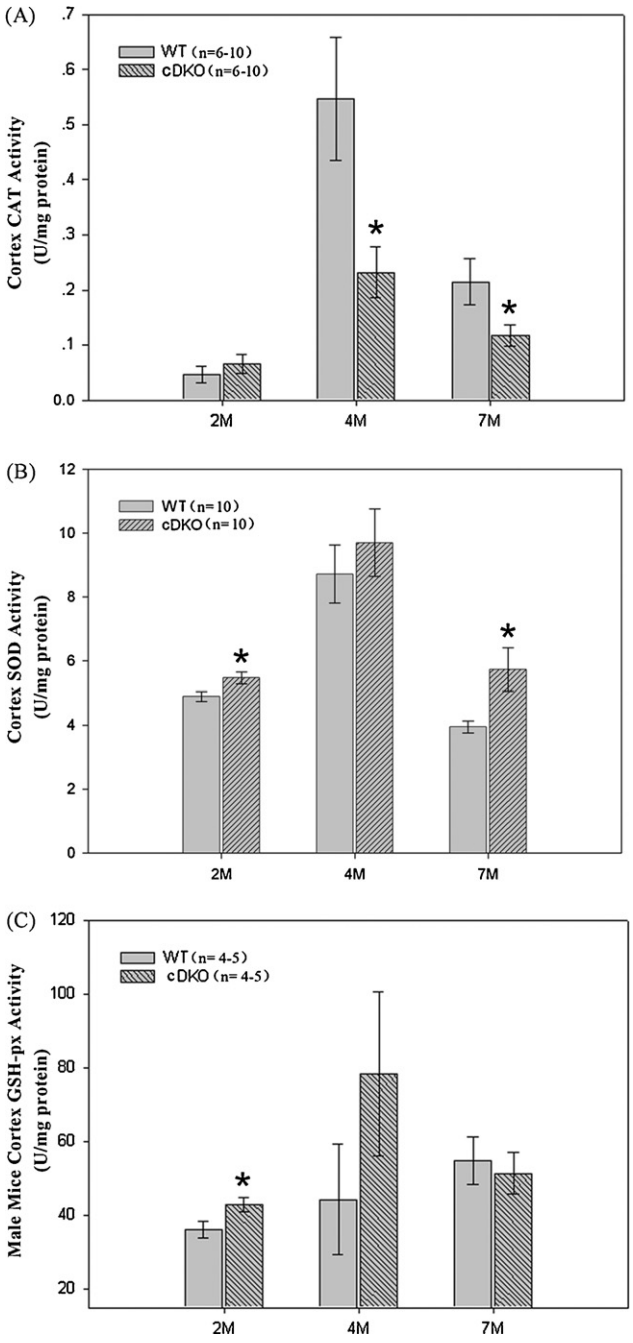

Fig. 2. Alterations in activities of antioxidant enzymes in the cortices of PS cDKO mice. (A) CAT activities were significantly reduced in 4- and 7-month PS cDKO mice relative to WT. (B) SOD activities were elevated significantly in 2- and 7-month PS cDKO mice. (C) GSH activities appeared to be increased significantly in 2-month male PS cDKO mice. Data were shown as mean ± S.E.M. $^*p < 0.05$.

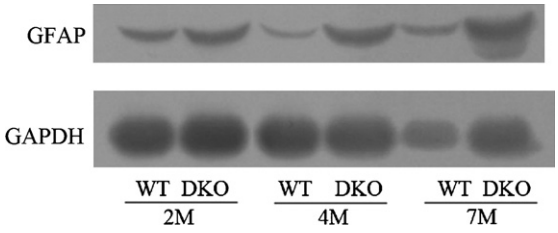

Fig. 3. Western blot analysis of cortical GFAP. The protein levels of GFAP were found to be increased in an age-related manner in PS cDKO mice. GAPDH was used as a loading control.

an enhanced oxidative stress persisted during the age points afterward examined. ROS may damage all types of biological molecules including proteins, lipids and DNA. Lipid peroxidation itself can directly destroy structural integrity of cell membranes and lead to apoptosis and even cell death while the lipid peroxidation product MDA is also neuronal toxic, and in particular may cause protein modification and impair protein functions. Proteins are important molecules because of their functions involved in metabolism and cell structure. Protein carbonylation is an irreversible form of severe or prolonged oxidative damage [7]. Carbonyl groups (aldehydes and ketones) can be directly formed via oxidation of the amino acid residues (e.g., Pro, Arg, Lys, and Thr) on protein side chains or through oxidative cleavage of proteins by either the α-amidation pathway or oxidation of glutamyl side chains [4]. Carbonyl groups may be introduced into proteins by Michael addition reactions of 4-hydroxynonenal, another toxic lipid peroxidation product. Many proteins become more susceptible to degradation by cellular proteases upon carbonylation of one or more residues. Carbonylation would thus lead to impairments of protein structure and function. Our results suggest that remarkable lipid peroxidation and protein oxidative modification in the cerebral cortex is not only an early event in response to the loss-of-function of PS but possibly the culprit responsible for significant development of AD-like pathologies as well in the PS cDKO mice.

On the other hand, it has been reported that some endogenous enzymes that detoxify ROS were increased in the brain of AD [1,20]. SOD, CAT and GSH-px are the three main antioxidant enzymes involved in cellular protection against free radical-induced damages. The activities of several key antioxidant enzymes, particularly CAT, were reduced in parietotemporal cortex of AD patients [15], and it was also reported that the CAT activity in the cerebral cortex of SAMP8 was decreased [29], suggesting that AD brain may be vulnerable to increased ROS production. In the present study, the cortical CAT activity appeared to be unchanged in PS cDKO mice at 2-month as compared with WT controls but significantly reduced in the mice at 4- and 7-month (Fig. 2A). Marcus et al. reported that expression of SOD in various regions of AD brain was much higher than that in non-AD brains, suggesting a compensatory mechanism of CNS against oxidative damage [21]. The over-expression of SOD in neuronal cells of transgenic AD mice could also reduce Aβ toxicity and accumulation [34]. In agreement with these findings, we found an increase in cortical SOD activity in PS cDKO mice over the age points examined, notably in those at 2-month (Fig. 2B). Increased oxidative stress may also be associated with altered glutathione metabolism in AD pathogenesis. Significant elevation of GSH-px activity has also been reported in the hippocampus of AD patients [20]. The cortical mRNA expression of glutathione peroxidase 5 was up-regulated in 6-month PS cDKO mice [3]. In the present study, we did not find significant changes of cortical GSH-px activity in PS cDKO mice. However, an increasing tendency in the activity of this enzyme was observed in 2-month male PS cDKO mice (Fig. 2C). Our observation suggests that in PS cDKO mice there should be also a compensatory defense against oxidative stress, particularly at the early age stage, in response to PS deficiency.

Getting hints from the study of Shen et al., in which in addition to the reduced Aβ production an increase in inflammatory response at both transcriptional and protein levels was also observed in the cortices of 6-month PS cDKO mice [3], we found that the expression of cortical GFAP was up-regulated in a age-related manner over the age points examined in PS cDKO mice (Fig. 3), revealing that the interaction relationship between oxidative stress and inflammatory may be closely associated with the underlying loss-of-function pathogenesis of AD.

In conclusion, enhanced oxidative stress in the cortex of PS cDKO mice is an early event responsible for the loss-of-function of PS towards the AD-like pathologies unrelated with the brain amyloidosis. The cause–effect relationship between oxidative stress and inflammatory response in this PS-deficient AD-like model needs to be further elucidated.

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