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Mitochondrial Enzymes and Endoplasmic Reticulum Calcium Stores as Targets of Oxidative Stress in Neurodegenerative Diseases

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Considerable evidence indicates that oxidative stress accompanies age-related neurodegenerative diseases. Specific mechanisms by which oxidative stress leads to neurodegeneration are unknown. Two targets of oxidative stress that are known to change in neurodegenerative diseases are the mitochondrial enzyme α-ketoglutarate dehydrogenase complex (KGDHC) and endoplasmic reticulum calcium stores. KGDHC activities are diminished in all common neurodegenerative diseases and the changes are particularly well documented in Alzheimer's disease (AD). A second change that occurs in cells from AD patients is an exaggerated endoplasmic reticulum calcium store [i.e., bombesin-releasable calcium stores (BRCS)]. H_2O_2, a general oxidant, changes both variables in the same direction as occurs in disease. Other oxidants selectively alter these variables. Various antioxidants were used to help define the critical oxidant species that modifies these responses. All of the antioxidants diminish the oxidant-induced carboxy-dichlorofluorescein (cDCF) detectable reactive oxygen species (ROS), but have diverse actions on these cellular processes. For example, α-keto-β-methyl-*n*-valeric acid (KMV) diminishes the H_2O_2 effects on BRCS, while trolox and DMSO exaggerate the response. Acute trolox treatment does not alter H_2O_2-induced changes in KGDHC, whereas chronic treatment with trolox increases KGDHC almost threefold. The results suggest that KGDHC and BRCS provide targets by which oxidative stress may induce neurodegeneration and a useful tool for selecting antioxidants for reversing age-related neurodegeneration.

KEY WORDS: Reactive oxygen species; KGDHC; bombesin-releasable calcium stores; Alzheimer's disease; oxidants; antioxidants.

INTRODUCTION

Overwhelming evidence indicates that damage from reactive oxygen species (ROS) occurs in Alzheimer's disease (AD) brain (Markesbery *et al*., 1999; Smith *et al*., 2000). For example, the reactive aldehyde acrolein is present throughout the AD brain. Many or most tangles contain acrolein; however, acrolein is also present in brain areas that do not have tangles, suggesting that oxidative stress is more pervasive than tangles (Calingasan *et al*., 1999). The presence of hydroxynonenal, another reactive aldehyde (Markesbery *et al*., 1999), also indicates that reactive aldehydes and lipid damage is extensive in the brain. Protein oxidation (Aksenov *et al*., 2001; Lyras *et al*., 1997) and nitration (Smith *et al*., 1997) indicate damage to protein. ROS-induced damage to all four DNA bases and to RNA (8-hydroxyguanosine) occurs in brains from AD patients (Lyras *et al*., 1997). The presence of ferritin, hemoxygenase, and reactive iron are indicators of ongoing oxidative stress (Rottkamp *et al*., 2001). A recent study of autopsy brains suggests that measures of oxidative stress are greatest early in the disease and then decline (Nunomura *et al*., 2001). Although the inability to make temporal measurements in autopsy brain makes mechanistic approaches equivocal, results in transgenic mice also indicate that oxidative stress is an early change. In these mice, β-amyloid (Aβ) is deposited in brain because of overexpression of a human amyloid

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precursor protein transgene with a double mutation found in a Swedish family with early-onset AD. Measures of oxidative stress (isoprostane levels) in these mice precede the surge of Aß production and plaque formation by more than a month (Pratico *et al.*, 2001). Whether a similar, early increase in isoprostanes occurs in human brain is unknown. The specific targets of this oxidative stress and how the changes might be reversed is the focus of this review.

THE α-KETOGLUTARATE DEHYDROGENASE ENZYME COMPLEX (KGDHC) AND NEURODEGENERATIVE DISEASE

Brain metabolism is diminished in AD, and a possible underlying cause of the decline is a reduction in the activity of the KGDHC. KGDHC consists of three proteins: E1k, E2k, and E3. KGDHC is a key and arguably rate-limiting enzyme of the tricarboxylic acid cycle. KGDHC is thiamine-dependent, and is diminished in the brains of individuals with thiamine deficiency (i.e., Wernicke-Korsakoff patients), who have severe memory deficits (Butterworth *et al.*, 1993). Several groups report that KGDHC is diminished in brains from patients with AD, and no contravening reports exist (Gibson *et al.*, 2000). The AD-related reduction in KGDHC activity occurs in genetic and nongenetic forms of AD. Diminished activities occur in brain regions with severe pathology, as well as in areas that show minimal pathology. In the non-genetic forms of the AD, the immunoreactivity of these three components is not altered (Mastrogiacomo *et al.*, 1993), whereas in genetic forms of AD, protein levels of E1k and E2k, but not E3 decline (Gibson *et al.*, 1997). This suggests that the enzyme can be inactivated by multiple mechanisms (Fig. 1). The relation of the decline in KGDHC to the pathophysiology of AD varies with apolipoprotein geno-type. In patients with one apolipoprotein E4 allele, the correlation of KGDHC activities to the clinical dementia rating is very high ($r = 0.7$). In this same subpopulation, the correlation with plaques ($r = 0.11$) and tangles ($r = 0.32$) is very low (Gibson *et al.*, 2000). The decline in KGDHC also occurs in brain regions away from the regions of pathology, indicating that the reduction is not just secondary to neurodegeneration. Further, the decline does not appear to be a general loss in mitochondrial function because another mitochondrial enzyme, glutamate dehydrogenase, is unchanged. Thus, brain KGDHC activities decline with AD, and the changes appear to be pathophysiologically important.

KGDHC is diminished in several neurodegenerative disorders. These include progressive supranuclear palsy

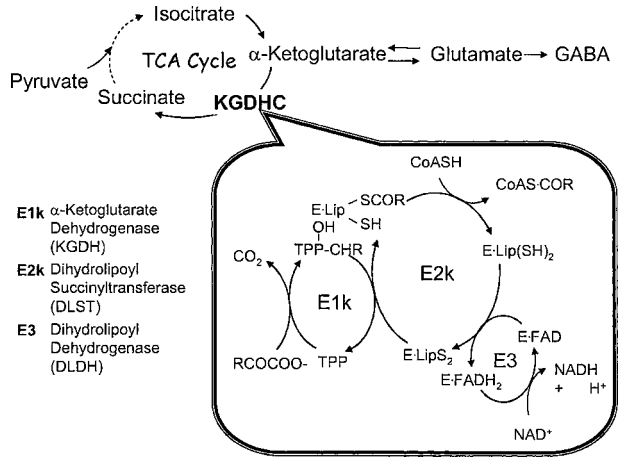

Fig. 1. KGDHC and metabolism.

(Park *et al.*, 2001), Parkinson's disease (Gibson *et al.*, 2003), and Huntington's disease (Klivenyi *et al.*, 2004). As in AD, the decline occurs in both areas of neurodegeneration and in other brain areas. Further, the results are consistent with KGDHC being more sensitive to the disease process (e.g., some undetectable form of ROS than other common measures of oxidative stress). For example, a decline in cerebellar KGDHC in PSP patients occurs although there is not an increase in malondialdehyde, protein carbonyl formation, or protein nitration (Park *et al.*, 2001).

CONSEQUENCES OF DIMINISHED KGDHC ACTIVITIES

Diminishing KGDHC activities has profound consequences on cell and brain function. In cells, inhibition of KGDHC correlates with release of cytochrome C and activation of caspase pathways, and these events precede alterations in the mitochondrial membrane potential (Huang *et al.*, 2003). In brain slices, inhibition of acetylcholine synthesis is particularly sensitive to KGDHC inhibition (Gibson and Blass, 1976). Two experimental approaches suggest that diminished KGDHC activities do not induce the neurodegeneration, but predispose to damage by other means. The first approach is thiamine deficiency, which leads to selective neurodegeneration. A thiamine derivative is a cofactor for KGDHC, and a major consequence of thiamine deficiency is a decline in the activity of KGDHC. However, neither the distribution of KGDHC nor the response of KGDHC to neurodegeneration suggests that KGDHC is directly responsible for neuron death. Instead, the results suggest that the decline in KGDHC predisposes to other insults that promote the neuronal death

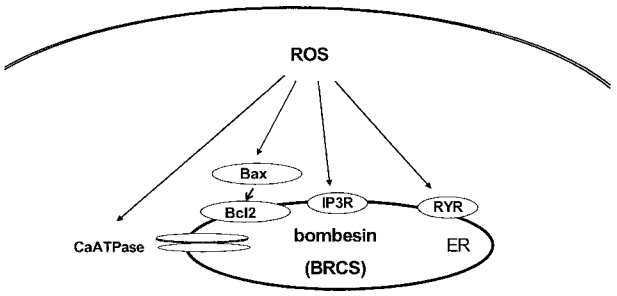

Fig. 2. Bombesin-releasable calcium stores (BRCS).

(Sheu *et al.*, 1998). A second approach to test the consequences of a diminished KGDHC activity is to use transgenic mice that have reduced levels of the E3 component of KGDHC, and thus diminished KGDHC activities. These mice did not show any pathological changes. However, lesions induced by MPTP and 3-NP were much larger in these mice (Klivenyi *et al.*, 2004).

EXAGGERATED ENDOPLASMIC RETICULUM STORES OF CALCIUM OCCUR IN AD AND IN ANIMAL MODELS OF AD

A second change that accompanies AD is an exaggeration in the internal pools of calcium. Both mitochondrial (Gibson *et al.*, 1997) and endoplasmic reticulum (Gibson *et al.*, 1996; Ito *et al.*, 1994) stores differ between cells from AD patients and controls. The increase in $[Ca^{2+}]_i$ after the addition of bombesin or bradykinin in the absence of calcium is used to assess endoplasmic reticulum stores of calcium. Experimentally, these are defined as bombesin or bradykinin releasable calcium stores (BRCS). The BRCS are exaggerated in fibroblasts from AD patients (Ito *et al.*, 1994). The increases in these pools appear related to the changes in capacitative calcium entry (Leissring *et al.*, 2000; Yoo *et al.*, 2000) and to the reduced flux of calcium into the cells (Peterson *et al.*, 1985). BRCS has been examined in fibroblasts from multiple individuals with AD, including non-genetic forms of AD, and in those bearing PS1 (Ito *et al.*, 1994) and APP (Gibson *et al.*, 1997) mutations. Increases in BRCS also occur in cells that have been transfected with mutant PS-1 (Guo *et al.*, 1996) and in both fibroblasts and neurons from transgenic mice bearing a presenilin-1 mutation (Leissring *et al.*, 2000). Thus, many approaches indicate that this calcium store is altered in cells from AD patients. The possible actions of ROS on the BRCS are shown in Fig. 2.

KGDHC AS A TARGET OF OXIDATIVE STRESS AND FOR REVERSAL WITH ANTIOXIDANTS

Oxidants produce inactivation of KGDHC just as seen in AD. KGDHC is inactivated by a variety of oxidants including peroxynitrite, NO (Park *et al.*, 1999), hydroxylnonenal (Humphries *et al.*, 1998), H_2O_2 (in mM concentrations), chloroamine (μM concentrations), and sodium hypochlorite (in nM concentrations). H_2O_2 diminishes KGDHC activity in synaptosomes (Chinopoulos *et al.*, 1999), fibroblasts (Gibson *et al.*, 2002), and N2a cells. Although all forms of AD have reduced brain KGDHC activities, protein levels as determined by immunoreactivity decline in some forms of AD but not others. Even related oxidants can produce a dichotomy similar to that which occurs in AD brain. Both NO and peroxynitrite diminish KGDHC activities. However, peroxynitrite, but not NO, diminishes immunoreactivity of E1k and E2k. The first pattern is similar to that observed in AD patients bearing the APP670/671 mutation (Table I).

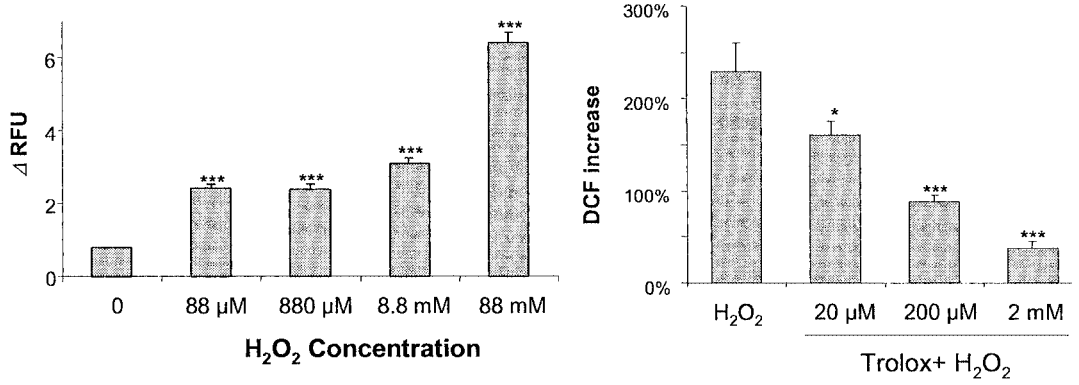

Fig. 3. H_2O_2-induced increase in ROS is sensitive to Trolox (1-h. treatment). Asterisks indicate difference from the control groups: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Table I. Selective Changes in KGDHC Immunoreactivity with Oxidative Stress

	E1k	E2k	E3
NO(SNP)	↔	↔	↔
Peroxynitrite	↓	↓	↔

The second pattern is similar to that seen in AD patients with no known genetic basis (Mastrogiacomo *et al.*, 1993).

KDGHC activity is reduced in animal and cell models with increased oxidative stress. Transgenic superoxide dismutase (SOD2) knockout mice have reduced KGDHC activities in their brains (Hinerfeld *et al.*, 2004). KGDHC is diminished. In cells that overexpress monoamine oxidase (MAO), and increased substrate (i.e., more ROS) exaggerates the reduction in KGDHC (Kumar *et al.*, 2003).

The sensitivity of KGDHC to ROS suggests that antioxidants should protect KGDHC. The interactions with KGDHC have been studied with H_2O_2. H_2O_2 produces a dose-dependent increase in reactive oxygen species as detected with cDCF. Trolox reduces the ROS production in a dose-dependent manner (Gibson *et al.*, 2002) (Fig. 3). However, under these same conditions, trolox does not protect KGDHC against H_2O_2 in acute condition (Fig. 4). This was also true of the antioxidants *N*-acetylcysteine and DMSO. On the other hand, if cells are incubated with trolox for 5 days, there was a dose-dependent increase in KGDHC. Following H_2O_2 treatment, all trolox-treated cells had higher KGDHC activities than control cells or H_2O_2 alone (Fig. 4). The intermediate concentration of trolox diminishes the H_2O_2-induced reduction in KGDHC activities by 50% (Gibson *et al.*, 2002). These results suggest that under some conditions (e.g. acute or chronic trolox treatment with some antioxidants), it is possible to protect KGDHC.

Table II. Select Changes in BRCS with Various Oxidants

	DCF-ROS	Change in BRCS
H_2O_2	++	+
SIN-1	+++++++	0
t-BHP	+++	++
HX/XO	+++++++	−
SNAP	++++	++
SNP	++	0

BRCS AS A TARGET OF OXIDATIVE STRESS AND REVERSAL WITH ANTIOXIDANTS

BRCS are selectively altered by various oxidants (Table II). BRCS is increased by *tert*-butyl-hydroxyperoxide (*t*-BHP), H_2O_2, and *S*-nitroso-*N*-acetylpenicillamine (SNAP), is unaffected by 3-morpholinosyndnonimine (SIN-1) or sodium nitroprusside (SNP), and is diminished by hypoxanthine/xanthine oxidase (HX/XO). These oxidants produce ROS that can be distinguished with other fluorescent indicators, including DAF and amplex red (data not shown).

The interactions of the antioxidant trolox with H_2O_2-induced changes in BRCS are not predictable (Fig. 5; Gibson *et al.*, 2002). Under the conditions that reduce H_2O_2-induced cDCF-detectable ROS, trolox exaggerates H_2O_2-induced (88 μM) increases in BRCS. However, chronic treatment with trolox has no effect on BRCS (i.e., nearly the opposite effects that are observed with KGDHC). Acute treatment with di-methylsulfoxide (DMSO) also exaggerates the BRCS, while NAC has no effect (data not shown). An understanding of the interaction of various oxidant species with various antioxidants will possibly reveal which species lead to the AD-like changes in BRCS.

Further experiments tested the ability of α-keto-β-methyl-*n*-valeric acid (KMV) (20 mM) to reduce

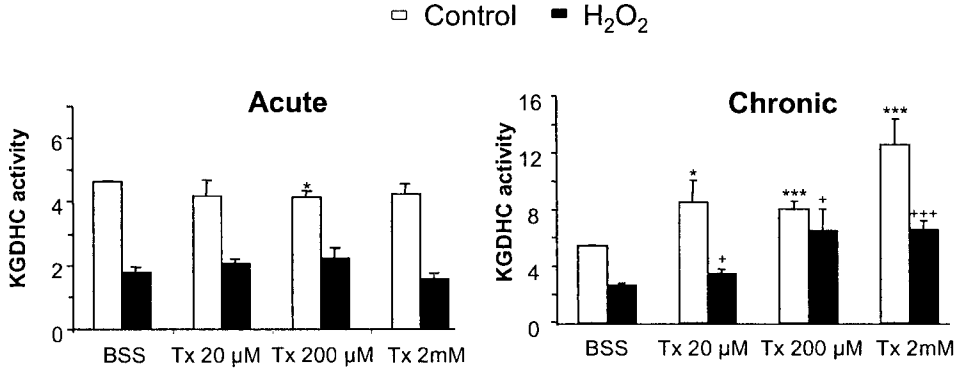

Fig. 4. Trolox provides limited protection of KGDHC from H_2O_2. Asterisks indicate difference from the control groups: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

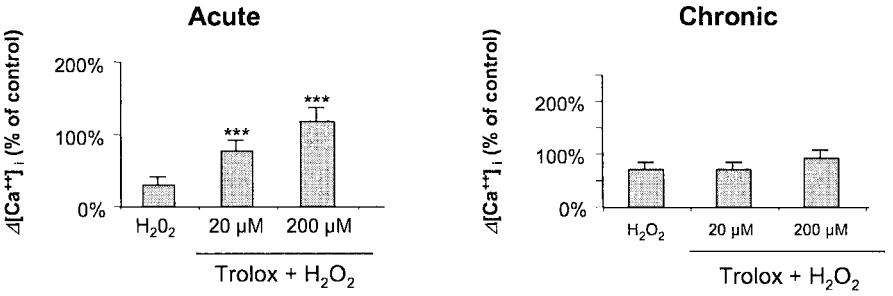

Fig. 5. Trolox exaggerates the effects of H_2O_2 on BRCS. H_2O_2 (final 88 μM) was added in both acute and chronic trolox treatments. Asterisks indicate difference from the control groups: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

c-DCF-detectable ROS and interact with BRCS (Fig. 6). KMV appears to be a unique antioxidant. KMV diminishes c-DCF-detectable ROS that are induced by H_2O_2, hypoxia, and SIN-1, but does not neutralize 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF)-detectable NO induced by SIN-1. KMV reduces both BRCS and the H_2O_2-induced change in BRCS. Thus, KMV is better than trolox at protecting against H_2O_2 when BRCS is regarded as the target. The results suggest that the same H_2O_2-induced ROS that reacts with KMV may also underlie the changes in BRCS related to AD.

CONCLUSIONS

Oxidative stress is a common feature of neurodegenerative diseases. This may lead to oxidation of key components of BRCS and of KGDHC. The disease-related changes in these processes in disease can be patterned by select oxidants. The results suggest these changes may be important in normal signaling of the molecules as well in neurodegeneration. Therefore, reversal of the changes in these processes by select antioxidants may be beneficial.

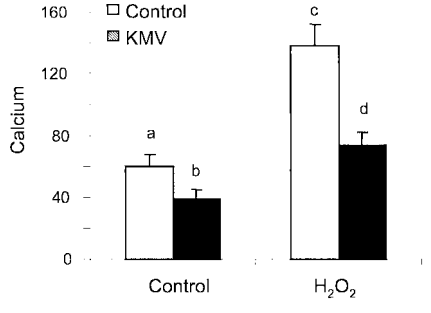

Fig. 6. KMV protects BRCS against H_2O_2. H_2O_2 (88 μM) was added after 1 min basal $[Ca^{2+}]_i$ measurements in the presence of KMV (20 mM). Data are means ± SEM. Values with different letters denote significance ($P < 0.05$).

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