Oxidative stress increases internal calcium stores and reduces a key mitochondrial enzyme

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Abstract

Fibroblasts from patients with genetic and non-genetic forms of Alzheimer’s disease (AD) show many abnormalities including increased bombesin-releasable calcium stores (BRCS), diminished activities of the mitochondrial α-ketoglutarate dehydrogenase complex (KGDHC), and an altered ability to handle oxidative stress. The link between genetic mutations (and the unknown primary event in non-genetic forms) and these other cellular abnormalities is unknown. To determine whether oxidative stress could be a convergence point that produces the other AD-related changes, these experiments tested in fibroblasts the effects of H2O2, in the presence or absence of select antioxidants, on BRCS and KGDHC. H2O2 concentrations that elevated carboxy-dichlorofluorescein (c-H2DCF)-detectable ROS increased BRCS and decreased KGDHC activity. These changes are in the same direction as those in fibroblasts from AD patients. Acute treatments with the antioxidants Trolox, or DMSO decreased c-H2DCF-detectable ROS by about 90%, but exaggerated the H2O2-induced increases in BRCS by about 4-fold and did not alter the reduction in KGDHC. Chronic pretreatments with Trolox more than doubled the BRCS, tripled KGDHC activities, and reduced the effects of H2O2. Pretreatment with DMSO or N-acetyl cysteine diminished the BRCS and either had no effect, or exaggerated the H2O2-induced changes in these variables. The results demonstrate that BRCS and KGDHC are more sensitive to H2O2 derived species than c-H2DCF, and that oxidized derivatives of the antioxidants exaggerate the actions of H2O2. The findings support the hypothesis that select abnormalities in oxidative processes are a critical part of a cascade that leads to the cellular abnormalities in cells from AD patients. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Alzheimer’s disease; Calcium; Reactive oxygen species; Fibroblast; α-Ketoglutarate dehydrogenase complex; Antioxidant

1. Introduction

Overwhelming evidence indicates that oxidative stress occurs in brains of patients with Alzheimer’s disease (AD) [1–4]. Although reports suggest that oxidative damage is the earliest event in AD [5], studies of autopsy brain cannot distinguish if the markers of oxidative stress result from, or cause the neurodegeneration. Oxidative stress also occurs in brains of mice bearing genetic mutations that reproduce some phenotypic features of AD [6,7]. Oxi-
dative stress precedes plaque formation in mice with mutations that lead to AD-like plaques, [8]. Previous studies in humans indicate that metabolic diseases also cause plaques [9] and tangles [10]. Oxidative stress in cells promotes production of amyloidogenic forms of amyloid precursor protein [11,12] and tangle formation [13,14]. Amyloid-β-peptide impairs metabolism [15], causes oxidative stress [1], and can directly produce H₂O₂ through metal ion reduction [16]. Antioxidants protect against amyloid-β-peptide toxicity [1]. Cells from AD patients [17], cells bearing mutations that cause AD [18] and cybrids [19] from AD patients have abnormalities in the ability to handle oxidative stress. Calcium uptake by mitochondria isolated from fibroblasts of AD patients is sensitive to selective oxidative stressors and to antioxidants [20]. The persistence of abnormalities in the ability to handle oxidative stress in cultured fibroblasts from AD patients suggests that this is an inherent property of AD cells [17,20,21]. Together these findings suggest that oxidative stress is a critical part of a cascade of events leading to AD.

Cellular signaling systems and mitochondrial processes are abnormal in cultured fibroblasts from patients with AD and in transfected cells bearing AD-causing mutations. Fibroblasts from AD patients have abnormalities in amyloid-β-peptide production, calcium dynamics, energy metabolism, β-adrenergic-induced formation of cyclic AMP, G protein interactions, and the phosphatidyl inositol cascade (see reviews [22,23]). An exaggeration of the bombesin-releasable calcium store (BRCS), or IP₃-releasable calcium pool, is one of the most replicable findings [24], and is related to reported changes in capacitative calcium entry [5]. BRCS has been examined in fibroblasts from multiple individuals with AD including non-genetic forms of AD, and in those bearing PS1 [25] or APP mutations [26], and appears to be diagnostic in some populations [27]. BRCS is also altered in brain and fibroblasts from PS-1 transgenic mice [5]. The mitochondrial enzyme α-ketoglutarate dehydrogenase complex (KGDHC) is reduced in brains from AD patients [28,29], and fibroblasts from familial [30,31] and non-familial AD patients [30,31]. Furthermore, KGDHC activity in fibroblasts bearing AD-causing presenilin-1 mutations is more sensitive to oxidative stress [32]. Thus, the occurrence of multiple AD-related changes in fibroblasts is well documented. Fibroblasts can be used to examine the interactions of these abnormalities and to determine the cascade of events leading to multiple changes.

The goal of the current studies was to determine whether oxidative stress in fibroblasts could lead to the changes in calcium and KGDHC that occur in cells from patients with AD. A fibroblast line from a young individual was used because the interpretation would be complicated if other unknown factors (e.g., age-related changes in membranes) had already compromised the cell line. The current experiments lay the foundation for future studies that will be done on cells from multiple AD patients in which antioxidants can be tested in cells with the same genetic background of the patients. Oxidative stress was induced in cells with a general oxidant, H₂O₂, which produces numerous reactive oxygen species (ROS). Specific antioxidants were tested with and without H₂O₂ to better define the nature of the ROS that leads to the changes. The antioxidants that were used included Trolox (Tx, a water soluble analogue of vitamin E which is a general antioxidant that acts on membranes and in the cytosol), N-acetyl cysteine (NAC, a precursor of glutathione) and dimethylsulfoxide (DMSO, a hydroxyl radical scavenger) [33]. Comparisons of the ability of the antioxidants to alter KGDHC or BRCS, to diminish H₂O₂-induced ROS and to reverse H₂O₂-induced changes in KGDHC and BRCS were used to test the role of particular ROS.

2. Materials and methods

2.1. Reagents

Fura-2 AM and 6-carboxy-dichlorodihydrofluorescein diacetate acetylomethyl ester (c-H₂DCF-AM) were purchased from Molecular Probes (Eugene, OR, USA). The fluorescent dyes were made into stocks in dry DMSO and stored under nitrogen at −80°C. The final DMSO concentration while loading the dyes into the cells was 0.2%. Tx was from Calbiochem (San Diego, CA, USA). Bombesin, DMSO and all other chemicals were from Sigma Chemical (St. Louis, MO, USA).
2.2. Fibroblasts

A fibroblast line from a young control donor GM8399 was purchased from Coriell Cell Repository (Camden, NJ, USA) and maintained by our established procedures [34]. Viability was determined with Erythrosin-B [34]. Cells were grown in Dulbecco’s modified Eagle media with 1 g/l glucose (Gibco, Grand Island, NY, USA) and supplemented with 10% fetal bovine serum (Sigma). Cells were sub-cultured weekly by trypsinizing and reseeding at 10^4/cm^2. For imaging experiments, fibroblasts were seeded at 500/cm^2 on MatTek glass bottom Petri dishes (Ashland, MA, USA) 5–6 days before the experiments.

2.3. Fluorescence imaging of intracellular calcium ([Ca^{2+}]_i) [25,26]

Fibroblasts were loaded with 2 μM-Fura-2 AM in balanced salt solution (BSS; in mM: NaCl 140, KCl 5, MgCl_2 1.5, glucose 5, HEPES 10, CaCl_2 2.5; pH 7.4) for 1 h at room temperature (23°C) in the dark. Cells were then rinsed and kept in calcium free BSS buffer for measurement of [Ca^{2+}]_i on the microscope stage. The calcium free BSS was the same as BSS except that added CaCl_2 was reduced to 0.1 mM and EGTA was added to 1 mM. The calculated free [Ca^{2+}] in the buffer was 7 nM (MaxChelator v 6.63 by Chris Patton, Stanford University, Hopkins Marine Station, Pacific Grove, CA 93950-3094, USA). Measurements of [Ca^{2+}]_i were started exactly 3 min after the cells were changed into calcium free BSS. In all the treatment groups, basal [Ca^{2+}]_i was measured for 1 min before any manipulations. Measurements were made with an ImageMaster system from PTI (Photon Technology International, Lawrenceville, NJ, USA). Excitation wavelengths of 350 and 378 nm (band passes 2–4 nm) were selected with two monochrometers and interchanged with chopper mirror installed in the light source box. An XF04 dichroic cube (Omega Optical, Brattleboro, VT, USA) with no excitation filter, dichroic 400 nm, and emission filter 510 nm, band pass 40 nm, was installed in an Olympus IMT-2 microscope with a 10×DAPO UV340 objective lens. Pairs of fluorescent images at excitation 350/378 nm were taken with a Hamamatu C2400 SIT camera at 5 s intervals. Each image was the average of 32 images taken within 2 s. Standard images of fura-2 solutions with minimum (0) and maximum (>0.1 mM) [Ca^{2+}] were taken during each day’s experiment for calculation of intracellular free calcium within the PTI program according to the method of Grynkiewicz et al. [35].

2.4. Bombesin-releasable [Ca^{2+}]_i pool

Fibroblasts in calcium free BSS buffer have basal [Ca^{2+}]_i of about 66 nM. This value did not change significantly at the 4 min time point of the measurement (i.e., after 7 min of incubation in calcium free buffer). Bombesin (1 μM) increased [Ca^{2+}]_i in about half of the cells by over 100% of the value before bombesin addition. The bombesin-induced change of [Ca^{2+}]_i was expressed as the integration of [Ca^{2+}]_i over the 3 min interval of 240 to 420 s (Fig. 3A).

2.5. Measurement of ROS in fibroblasts by fluorescence imaging

Fibroblasts were loaded with 10 μM 6-c-H2DCF-AM (Molecular Probes, Eugene, OR, USA) in BSS at room temperature for 60 min. During this time the AM group is cleaved to leave the c-H2DCF trapped in the cells. Cells were then rinsed in BSS and transferred onto the microscope stage for measurements. The fluorescence of the oxidized form of c-H2DCF (c-DCF) was measured with an excitation wavelength of 488 nm (band pass 3 nm), emission at 535 nm (band pass 45 nm) with the PTI system using a 20× UV Fluor objective. Composite images (i.e., the average of 64 images taken within 2 s) were taken every 10 s. An image of BSS buffer in a Petri dish with same quality as those used for the experiments was taken as background for each day’s experiment. Another image of 2 ml of 100 nM c-DCF in BSS in the same dish was also taken. The concentration of c-DCF provided optimal images to correct for background, uniformity and to calibrate intensity for all the images taken on that day. All images were divided by this 100 nm c-DCF image after background subtraction. The ratioed images were then multiplied by 100. The derived values therefore represent the fluorescence of equal concentration of c-DCF in nM.
2.6. Measurement of ROS in fibroblasts with a fluorescent plate reader

Fibroblasts were grown on 96 well cell culture plates to confluence in 7 days. The cells were rinsed once with BSS buffer and incubated with 10 μM c-H$_2$DCF AM in BSS buffer for 60 min at room temperature to load the dye. The cells were then rinsed and incubated with 1 mM H$_2$O$_2$ in BSS with various treatments at 37°C in a 5% CO$_2$ incubator. Cells were rinsed once at the end of treatments and 100 μl of BSS was added into each well. c-DCF fluorescence of 485/538 nm (Ex/Em) was measured in a Molecular Devices (Sunnyvale, CA, USA) GEMINI fluorescence plate reader. 100 μl of 500 nM c-DCF was measured along with each time’s measurement as a control. The day-to-day variation of the fluorescence of the c-DCF solution was less than 10%. The c-DCF signal can be converted to concentration by the relation that one relative fluorescence unit (RFU) equals 0.07 pmol.

2.7. Measurements of KGDHC activity

Fibroblasts were sub-cultured on 6 well culture plates to reach confluence in 7 days. After the indicated treatments, cells were collected by scraping in a lysis buffer [26] and sheared by passing through a 27 G needle five times. KGDHC activities of the cell lysate were measured by the conversion rate of NAD to NADH with a 96 well fluorescence plate reader (SpectraMax GEMINI, from Molecular Devices, Sunnyvale, CA, USA). Briefly, the emission fluorescence at 460 nM was measured after excitation at 340 nm at 30°C in a reaction mix (pH 7.8) containing in mM: MgCl$_2$ 1, CaCl$_2$ 1, EDTA 0.5, TPP 0.3, DTT 1, β-NAD 1, Coenzyme A 0.163, α-ketoglutarate 1, Tris–HCl 50, and 0.1% Triton X-100. The fluorescence was converted into NADH with an NADH standard on the day of experiment. An aliquot of the cell lysate was saved for measurement of protein concentration with a Bio-Rad 500-006 kit based on the Bradford dye-binding procedure that utilizes the color change of Coomassie brilliant blue G-250 [36].

2.8. Dilution of drugs

H$_2$O$_2$ was diluted with H$_2$O. Thus, equally small amounts of water were added to the controls. The antioxidants and bombesin were all dissolved in BSS.

2.9. Data analysis and statistics

All imaging experiments were repeated in three dishes each day on at least 2 separate days. Typically, about 30–50 cells per image field were measured for calcium studies and 10–20 cells per image field for ROS measurements. Cell values ([Ca$^{2+}$], in nM, or fluorescence equivalent to nM c-DCF, respectively) were averaged on per cell basis to derive mean and standard error of mean. ANOVA and Student’s t-tests were performed with Excel. Other measurements were run in triplicates and repeated at least one more time on a separate day.

3. Results

3.1. ROS generation in fibroblasts and the effects of antioxidants

The metabolism of ROS was measured by monitoring the oxidation of c-H$_2$DCF. Cells were loaded with c-H$_2$DCF AM. Once inside the cell the AM group is hydrolyzed to give c-H$_2$DCF. The c-H$_2$DCF serves as an oxidizable substrate for cellular peroxidases and the oxidized product, c-DCF, is highly fluorescent. H$_2$O$_2$ produced a time- and concentration-dependent increase in c-DCF-detectable ROS formation in fibroblasts, whether the response was monitored with a microscope (Fig. 1) or with 96 well plates. On the microscope stage, concentrations of H$_2$O$_2$ ranging from 88 μM to 88 mM produced a robust increase in ROS over the whole 3 min interval. When the analysis was done on the plate reader, 1 mM H$_2$O$_2$ for 2 h increased the c-DCF signal from 20.7 ± 0.4 to 68.1 ± 6.3 RFU/well (P < 0.001). Subsequent studies were monitored with the plate reader.

3.2. Acute and chronic effects of antioxidants on H$_2$O$_2$-induced ROS generation

To assess acute effects of antioxidants, several concentrations of Tx, NAC and DMSO were incubated with the fibroblasts for 1 h before measurements, and...
the effects on H$_2$O$_2$-induced fluorescence were monitored in the presence of the antioxidants (Top panel of Fig. 2). Tx reduced the H$_2$O$_2$-induced ROS in a concentration-dependent fashion. The inhibition at the maximal Tx concentration (2 mM) was 84%. NAC at the highest concentration (10 mM) diminished ROS by about 90%. DMSO (0.5%) reduced the H$_2$O$_2$-induced response by about 60%, and higher DMSO concentrations produced only slightly greater effects. The above values were obtained using the plate reader, and similar values were obtained with the microscope.

To assess chronic effects of antioxidants, cells were pretreated with antioxidants for 5 days and the effects on H$_2$O$_2$-induced ROS formation was determined in the absence of antioxidants (Fig. 2, bottom panel). Tx (200 µM) increased the H$_2$O$_2$-
induced elevation in ROS, while 2 mM Tx reduced the H$_2$O$_2$ effect on ROS by more than 50%. Pre-treatment with 1 mM NAC did not affect H$_2$O$_2$-induced ROS. Many cells treated with 10 mM NAC did not adhere to the dish so those data are not included. Chronic treatment with DMSO was more effective than the acute treatment and reduced the H$_2$O$_2$-induced ROS more than 80%. Thus, Tx and NAC are more effective at diminishing H$_2$O$_2$-induced ROS with acute, than chronic treatment.

The reverse is true for DMSO. Thus, chronic treatment with antioxidants provided a unique pattern of protection against H$_2$O$_2$-induced ROS as determined with c-DCF.

3.3. H$_2$O$_2$ and cellular calcium dynamics

H$_2$O$_2$-induced changes in basal [Ca$^{2+}$], and in BRCS. Bombesin releases calcium from internal calcium stores to increase [Ca$^{2+}$]. The concentrations of H$_2$O$_2$ that increased c-DCF-sensitive ROS (see Fig. 1) proportionally elevated basal cytosolic free calcium ([Ca$^{2+}$]) and BRCS (Fig. 3). Basal [Ca$^{2+}$], was minimally, but significantly, affected by 88 and 880 μM. Higher concentrations of H$_2$O$_2$ (8.8 mM) produced larger changes in [Ca$^{2+}$]. H$_2$O$_2$ at very high concentration (88 mM) increased the basal calcium nearly 5-fold. H$_2$O$_2$ concentrations as low as 88 W formed nearly tripled BRCS. At the highest H$_2$O$_2$ concentration the bombesin-induced change was similar to that in controls. Thus, except at the highest concentrations, H$_2$O$_2$ proportional increased c-DCF-detectable ROS and BRCS.

3.4. Effect of antioxidants on fibroblasts calcium dynamics (Fig. 4)

Before assessing the interaction of antioxidants and H$_2$O$_2$ with calcium dynamics, the effects of the antioxidants on cellular calcium pools were determined. The effects of the antioxidants were studied in the presence of the antioxidant after a 1 h pre-treatment (acute), or in the absence of the antioxidant after a 5-day pretreatment (chronic). Basal calcium was measured for 1 min and then H$_2$O was added (as a control for the experiments in the following paragraph) and bombesin was added 3 min later. Acute treatment of fibroblasts with antioxidants had minimal effects on BRCS (Fig. 4). However, chronic treatment of fibroblasts had a striking effect on BRCS, and the highest concentration (2 mM) more than doubled the BRCS. On the other hand, NAC and DMSO both diminished the BRCS (Fig. 4).
3. Antioxidants and the \( H_2O_2 \)-induced changes in bombesin-sensitive calcium stores (Fig. 5)

To test the acute effects of antioxidants on \( H_2O_2 \)-induced changes in BSCS, the drugs were added 1 h before measuring calcium and were present during the measurements. One h pretreatment with 20 \( \mu M \) or 200 \( \mu M \) Tx exaggerated the increase in BRCS due to 88 \( \mu M \) \( H_2O_2 \). Indeed, the higher concentration (200 \( \mu M \)) nearly quadrupled the BRCs over the control values. Neither the highest Tx concentration (2 mM), nor any concentration of NAC, altered the \( H_2O_2 \)-induced change. However, DMSO exaggerated the \( H_2O_2 \)-induced change in BSCS by approximately 4-fold. Thus, antioxidant treatments that diminish c-DCF-sensitive ROS exaggerate the effects of \( H_2O_2 \) on BRCS.

Fig. 4. Antioxidants and BRCS. Calcium measurements followed the paradigm in Fig. 3. For acute antioxidant treatments, drugs were present during the 1 h fura-2AM loading period in BSS and during the subsequent \([Ca^{2+}]_i\) measurements in calcium free BSS. \( H_2O \) (10 \( \mu l \)) was added 1 min after the initiation of the calcium measures (as a control for studies in Fig. 5). For chronic studies, antioxidants were incubated with the cells in culture media for 5 days. Drugs were absent during the fura-2AM loading in BSS and subsequent \([Ca^{2+}]_i\) measurements were in calcium free BSS. \( H_2O \) (as a control for Fig. 5) was added after 1 min measurement of basal calcium. The bars show the integrated \([Ca^{2+}]_i\) peak (nM min) of the bombesin response as shown in the bottom right of Fig. 3. A control group was included with each drug because different drugs were tested on separate days. Note that NAC at 10 mM for 5 days or 100 mM for 1 h were not included in the graph, because they slowed the cell growth or killed the cells when cells were seeded at a light density for imaging. Data are means \( \pm \) S.E.M. Each column represents the measurement of 250–350 cells in at least six dishes measured on two separate days. Asterisks indicate difference from the control group: \( *P < 0.05, **P < 0.01, ***P < 0.001 \).

Fig. 5. Antioxidants selectively alter the \( H_2O_2 \)-induced increase in BSCS. The experimental paradigm for both the acute and chronic treatment was identical to that in Fig. 4, except that 10 \( \mu l \) of \( H_2O_2 \) (88 \( \mu M \) final) was added in place of \( H_2O \). However, the presentation is different. Each bar indicates the percent increase in BSCS induced by \( H_2O_2 \) over each group’s \( H_2O \) control (in Fig. 4). Data were calculated on per cell basis and are means \( \pm \) S.E.M. Each column represents the measurement of 250–350 cells in at least six dishes measured on 2 separate days. Asterisks indicate difference from the control group: \( *P < 0.05, **P < 0.01, ***P < 0.001 \).
To test the chronic effects of antioxidants, they were added 5 days before testing, and the drugs were removed before the calcium measurements. Only the highest concentration of Tx (2 mM) diminished the H$_2$O$_2$ exaggeration in the BRCS under these conditions (Fig. 5, bottom panel). Although Tx diminished the H$_2$O$_2$-induced change in BRCS, the level of BRCS was still higher than control because of the very large induction of BRCS by 2 mM TX alone (see Fig. 4). On the other hand, long-term treatment with 1 mM NAC exaggerated the H$_2$O$_2$ response. Five-day treatments with NAC at higher concentrations (10 or 100 mM) caused cells to detach so those results are not presented. DMSO had no effect on the H$_2$O$_2$-induced changes in calcium (Fig. 5, bottom panel). Thus, although chronic treatment with Tx (2 mM) and DMSO (2%) diminished H$_2$O$_2$-induced, DCF-sensitive ROS (see Fig. 2), Tx reversed the resulting calcium deficit, but DMSO did not.

3.6. H$_2$O$_2$ inactivates KGDHC activities in fibroblasts (Fig. 6)

Fibroblasts were incubated with 1 or 10 mM H$_2$O$_2$ for 3 min or 2 h. The cells were then rinsed, lysed and their KGDHC activity was determined immediately. H$_2$O$_2$ reduced KGDHC activity in fibroblasts in a concentration-dependent fashion at both 3 min and 2 h.

3.7. Effect of antioxidants on KGDHC and H$_2$O$_2$-induced reductions in KGDHC (Fig. 7)

Short-term treatment (1 h) with the antioxidants that diminish H$_2$O$_2$-induced ROS selectively affected KGDHC activities. In the absence of H$_2$O$_2$, the antioxidants had almost no effect on KGDHC. Surpris-
ingly, the H$_2$O$_2$-induced reductions of KGDHC activities were only minimally altered by concentrations of Tx that significantly diminished H$_2$O$_2$-induced ROS. Furthermore, concentrations of NAC that reduced ROS exaggerated the H$_2$O$_2$-induced reduction in KGDHC. DMSO did not alter KGDHC in the presence or absence of H$_2$O$_2$.

Chronic treatment with the antioxidants produced a very striking response on KGDHC. All concentrations of Tx increased KGDHC activities in the absence of H$_2$O$_2$. The elevation was maximal at 2 mM and the effect was nearly 3-fold. Following H$_2$O$_2$ treatment, all Tx-treated cells had higher KGDHC activities than the H$_2$O$_2$ alone. The intermediate concentration of Tx diminished the H$_2$O$_2$-induced reduction in KGDHC activities by 50%, but the activity was still higher than the non-H$_2$O$_2$-treated control, because of the very large induction of KGDHC by 2 mM Tx. On the other hand, NAC exaggerated H$_2$O$_2$-induced deficits on KGDHC activities and DMSO was ineffective.

3.8. H$_2$O$_2$ and cell death

The effects of H$_2$O$_2$ on KGDHC and BRCS were not related to cell death. Cells were treated with either 1 or 10 mM H$_2$O$_2$ for 2 h. The ability of the cells to exclude ethidium bromide was used to determine cell death. No cell death was observed at 1 mM H$_2$O$_2$, which approximates the concentration used in most of our experiments. Even 10 mM H$_2$O$_2$ only produced minimal cell death (Fig. 8).

4. Discussion

The experiments demonstrate that BRCS and KGDHC are cellular targets of H$_2$O$_2$-induced ROS, but the mechanism(s) for the H$_2$O$_2$-induced change is unknown. The current studies used antioxidants in the presence or absence of H$_2$O$_2$ to demonstrate that different H$_2$O$_2$-derived oxidative species act on the two cellular targets, BRCS and KGDHC. As expected, antioxidants diminished the H$_2$O$_2$-induced formation of c-DCF-sensitive ROS. Unexpectedly, the reduction in c-DCF-sensitive ROS did not predict the beneficial effects on the cellular targets following either chronic or acute treatments. The results are consistent with the following three part hypothesis: (i) The diminution of the c-DCF signal by the antioxidants suggests that they are better substrates for cellular peroxidases than c-DCF. (ii) The reaction of these antioxidants with H$_2$O$_2$-derived species converted them to specific oxidants. (iii) The resulting oxidants select between the two cell targets KGDHC and BRCS. These suggestions are developed in the following paragraphs.

The interaction of cellular peroxidases with NAC, Tx, DMSO and c-DCF provides a plausible explanation for the ability of the antioxidants to diminish the c-DCF signal and to subsequently alter cell processes. In cells, H$_2$O$_2$ is catabolized by either catalase or peroxidases that reduce H$_2$O$_2$ at the expense of an oxidizable substrate (RH; c-H$_2$CDF, Tx, NAC, DMSO), as shown below:

$$2RH \rightarrow 2R^+ + 2H^+ + 2e^-$$

$$H_2O_2 + 2e^- + 2H^+ \rightarrow H_2O$$

Since the identity of the peroxidase is unknown, subsequent discussion will refer to enzyme as c-H$_2$DCF-oxidizing peroxidase. NAC, Tx [37-44], DMSO [45,46] and c-H$_2$DCF [47-49] are oxidizable substrates for the cellular c-H$_2$DCF-oxidizing peroxidase. Thus, the diminution of the c-DCF signal by acute exposure to NAC, Tx and DMSO suggests that these antioxidants are better substrates for the cellular c-H$_2$DCF-oxidizing peroxidase than c-H$_2$DCF. As indicated by the equation, the diminished c-
DCF signal by each antioxidant indicates the production of an oxidant. The oxidation of Tx and NAC by peroxidases generates phenoxy and thyl radicals, respectively [38-44,50,51]. The thyl and phenoxy radicals generated from NAC and Tx can then react with oxygen to produce a spectrum of radical species [48]. DMSO can also generate a variety of radical species [52,53] and von Sonntag [54] has described several prooxidant effects of DMSO in irradiation studies. The utilization of NAC, Tx and DMSO by a c-H$_2$DCF-oxidizing peroxidase could also account for the ability of these compounds, which markedly differ on the basis of structure and reactivity with H$_2$O$_2$, to produce similar effects on the oxidation of c-H$_2$DCF.

The response of BRCS to H$_2$O$_2$ with and without the antioxidants demonstrates that these calcium stores are sensitive to oxidative stress. The response of the BRCS to H$_2$O$_2$ and the antioxidants suggests some regulator of BRCS is an oxidizable substrate for the peroxidase that oxidizes c-H$_2$DCF. Moreover, the data suggest that the oxidation of this regulator stimulates BRCS. Thus, the addition of H$_2$O$_2$ oxidizes c-H$_2$DCF and the regulator of BRCS. The inability of Tx, DMSO or NAC to protect this process from H$_2$O$_2$ suggests that the BRCS regulator is a better substrate for the c-H$_2$DCF-oxidizing peroxidase than any of these antioxidants. Thus, the substrate preference for this peroxidase would be BRCS regulator $>$ Tx, NAC, DMSO $>$ c-H$_2$DCF. The exaggeration of the H$_2$O$_2$-induced response by Tx and DMSO suggests that the reaction of Tx or DMSO with peroxidase produces products that further oxidize the regulator of BRCS.

The relative abilities of these antioxidants and c-H$_2$DCF to act as substrates for the cellular c-H$_2$DCF-oxidizing peroxidase could explain the effects of H$_2$O$_2$ and the antioxidants on KGDHC. Although the antioxidants diminished the H$_2$O$_2$-induced changes in c-H$_2$DCF, as described above, they did not diminish the H$_2$O$_2$-induced deficit in KGDHC activities. This suggests that the oxidant-sensitive component of KGDHC is a better substrate for the c-H$_2$DCF-oxidizing peroxidase than any of the antioxidants. A likely candidate is the lipoamide component of the KGDHC reaction. High concentrations of NAC exaggerated the H$_2$O$_2$-induced deficits in KGDHC, whereas DMSO and Tx were ineffective. This suggests that other actions of NAC contribute to its effects on KGDHC. One possibility is that the stimulation of cellular GSH production by NAC. Svensson has shown that GSH stimulates the oxidation of the thiol substrates [37]. Thus, the effects of NAC on KGDHC may be due the combined actions of NAC and NAC-derived GSH. The effect of NAC, DMSO and Tx is a different than the effect that these antioxidants have on BRCS, which were altered by DMSO and Tx.

The chronic pretreatment with these drugs and the subsequent interaction with H$_2$O$_2$ suggests that the oxidant species produced from these antioxidants selectively affect the cells’ processes. Chronic Tx treatment increased BRCS whereas the NAC and DMSO reduced the BRCS. The results suggest that the regulator of BRCS that was sensitive to the Tx-derived oxidant species was already maximally oxidized following chronic Tx, so that the BRCS was no longer increased by H$_2$O$_2$ in combination with Tx. Indeed, it was diminished. This suggests that the same oxidant species was involved in the chronic treatment and the acute treatment in the presence of H$_2$O$_2$. On the other hand, chronic incubation with NAC produced a species that reacted with H$_2$O$_2$ to produce a species that oxidized (i.e., enhanced) the process leading to BSCR process. DMSO was ineffective. These responses are just reverse to those occurring with acute antioxidant treatment in the presence of H$_2$O$_2$, which suggests that different oxidants are involved. Thus, each of the antioxidants produced selective changes in calcium depending upon the length of the incubation and the presence or absence of H$_2$O$_2$. Just as in the acute experiments, the effects of antioxidant pretreatment on c-DCF fluorescence did not predict the response of the cellular markers.

Chronic treatment with the antioxidants had a selective effect on KGDHC that differed from the effect on BRCS. Chronic treatment with Tx generated a species that increased KGDHC activities 3-fold, but neither NAC nor DMSO affected KGDHC. KGDHC activities respond like an antioxidant enzyme. In the short term, it is oxidized, but low-grade oxidative stress over time leads to its induction. Once induced it is still sensitive to H$_2$O$_2$, but a large reserve is now present so that in spite of the reduction, the remaining activity is still higher than the non-stressed control. The KGDHC activities are mea-
sured in the presence of the reducing agent dithiothreitol, so the Tx-induced increase is not just an effect on the oxidation state of KGDHC.

Interactions of oxidative stress with internal calcium stores, especially in the endoplasmic reticulum, may be a common link in a variety of neurodegenerative processes, but the mechanism may vary between disorders (see review in [55,56]). For example, BRCS are altered in fibroblasts from AD patients, as well as in fibroblasts and neurons from transgenic mice bearing gene mutations that lead to AD [5]. In these cells the alteration is due to an increase in the size of the endoplasmic reticulum calcium store [5]. In some tissues, oxidative stress including H$_2$O$_2$ inactivates the endoplasmic reticulum Ca-ATPase leading to impaired calcium re-uptake into the endoplasmic reticulum [57,58]. H$_2$O$_2$ had only small effects on resting [Ca$^{2+}$]i, whereas the increase in the calcium response following bombesin was large, which suggests that release from the internal stores were selectively increased.

Reductions in KGDHC have been implicated in AD and other neurodegenerative disorders [59], and KGDHC is particularly sensitive to oxidative stress [32]. The current studies show these effects can be specific for various ROS. Our previous studies show that peroxynitrite and NO both inactivate the isolated enzyme and the intracellular enzyme [60]. Others have shown that hydroxynonenal inactivates KGDHC in mitochondria [61], and that H$_2$O$_2$ inactivates KGDHC in isolated nerve endings [62]. The current data suggest that low-grade oxidative stress induces KGDHC activity, while higher levels of oxidative stress inactivate it. The current studies expand those studies by showing that it occurs in yet another cell type, and by demonstrating the relation of the changes in KGDHC to those in alterations of calcium pools. In addition, there studies reveal a selective action with specific antioxidants.

H$_2$O$_2$-induced oxidative stress produces changes in cellular signaling and metabolism that resemble the abnormalities that occur in fibroblasts from patients with AD. However, beneficial effects cannot be predicted from changes in c-DCF, since antioxidant treatments are complex and antioxidants can exaggerate rather than diminish the H$_2$O$_2$-induced abnormalities.

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