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Original Contribution

Modification of endoplasmic reticulum Ca^{2+} stores by select oxidants produces changes reminiscent of those in cells from patients with Alzheimer disease

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Abstract

Abnormalities in calcium homeostasis and oxidative processes occur in fibroblasts from patients with Alzheimer disease (AD) and in fibroblasts and neurons from transgenic mice bearing a presenilin-1 (PS-1) mutation. Bombesin-releasable endoplasmic reticulum Ca^{2+} stores (BRCS) are exaggerated in all of these cells. Our previous studies show that H_2O_2 exaggerates BRCS. The goal of the present study was to determine whether select reactive species exaggerate BRCS in cultured human fibroblasts and to determine if the ability of fibroblasts to handle these specific oxidant species is altered in cells from AD patients. Two fluorescent indicators were used to distinguish different reactive oxygen species (ROS): 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester) (c-DCF) and 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM). ROS were produced by a variety of oxidants, including *tert*-butyl-hydroxyperoxide (*t*-BHP), hypoxanthine/xanthine oxidase, *S*-nitroso-*N*-acetylpenicillamine, 3-morpholinosydnonimine, and sodium nitroprusside. Different oxidants selectively induced various ROS in distinct patterns. These oxidants also induced selective modification in $[Ca^{2+}]_i$ and/or BRCS. Of the several oxidants tested, *t*-BHP was most specific for exaggerating BRCS without affecting basal $[Ca^{2+}]_i$ and inducing only c-DCF-detectable ROS. On the other hand, the results show that NO that reacted with DAF-FM was not responsible for alterations in BRCS. Furthermore, the c-DCF-detectable ROS production induced by *t*-BHP was higher in fibroblasts from AD patients bearing a PS-1 mutation ($n = 7$) than in those from aged controls ($n = 8$). The higher production of c-DCF-detectable ROS may underlie the exaggeration of BRCS in fibroblasts from AD patients. Thus, these results are consistent with the hypothesis that abnormalities in selective cellular ROS cause AD-related changes in intracellular calcium regulation.

Keywords: Alzheimer's disease; Endoplasmic reticulum Ca^{2+} stores; Fibroblasts; Free radicals; Presenilin-1; Oxidants; Oxidative stress; Reactive oxygen species

Mitochondrial dysfunction and oxidative stress occur in neurodegenerative diseases. Mitochondria are the primary sites of oxygen utilization and the major source of free radicals. During respiration, electrons transferred from oxidation of NADH to molecular O_2 are coupled to a proton gradient to produce ATP in mitochondria. Reactive oxygen species (ROS) including superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) are products of respiration [1,2]. Oxidation of nitric oxide ($NO^{\bullet}$) produces the very toxic peroxynitrite ($ONOO^-$) [3]. ROS are physiological modulators of multiple cellular functions, but excess ROS damage cells and induce cell toxicity.

Abbreviations: BSS, balanced salt solution; BRCS, bombesin-releasable calcium store; *t*-BHP, *tert*-butyl-hydroxyperoxide; BK, bradykinin; c-DCF, 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (acetoxymethyl ester); $[Ca^{2+}]_i$, cytosolic free calcium concentration; DAF-FM, 4-amino-5-methylamino- 2′,7′-difluorofluorescein diacetate; ER, endoplasmic reticulum; Fura-2AM, Fura-2-acetoxymethyl ester; H_2O_2, hydrogen peroxide; $^{\bullet}OH$, hydroxyl radical; HX/XO, hypoxanthine/xanthine oxidase; $InsP_3R$, inositol trisphosphate receptor; KMV, α-keto-β-methyl valeric acid; NO nitric oxide; NO^-, nitroxyl ion; NO^+, nitrosyl ion; ROS, reactive oxygen species; RyR, ryanodine receptor; SIN-1, 3-morpholinosydnonimine; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside; $O_2^{\bullet -}$, superoxide radical.

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Abnormalities in calcium homeostasis occur in fibroblasts from patients with Alzheimer disease (AD) and these changes may be due to modification by select oxidants. Fibroblast calcium uptake [4] and bradykinin (BK)-mediated inositol trisphosphate ($InsP_3$) production are altered with AD [5,6]. In addition, the numbers of BK receptors are increased in fibroblasts from AD patients compared to controls [7]. Ca^{2+} release from bombesin-releasable Ca^{2+} stores (BRCS) in the endoplasmic reticulum (ER) is mediated by $InsP_3$ and is greatly enhanced in fibroblasts from AD patients [8] and in fibroblasts [9] and neurons [10] isolated from presenilin-1 (PS-1)-transgenic mice. Transfection of mutant PS-1 into PC12 cells exaggerates the Ca^{2+} release from the ER in response to carbachol or BK [10,11], and this elevation may be due to the increased expression of ryanodine receptors (RyR) [10] or to changes in the capacitative calcium entry refilling mechanism [9,12]. Treatment of cells with H_2O_2 exaggerates ER calcium stores and mimics the changes that occur in cells from AD patients [13]. Other data suggest that hydroxyl radicals generated by H_2O_2 react with SH groups of the RyR to increase Ca^{2+} release from the ER [14]. Thus, one goal of the current study was to define the oxidant species that can exaggerate ER calcium stores.

Diverse oxidants induce different ROS although considerable overlap occurs in the precise species that is produced (Table 1). *tert*-Butyl-hydroperoxide (*t*-BHP) produces the radicals *tert*-butyloxyl (*t*-bu-O$^\bullet$) and *t*-butylperoxyl (*t*-bu-OO$^\bullet$) [15] (Table 1, Reactions 1.1-1.3) and activates glutathione peroxidase (Gpx) to oxidize glutathione (GSH) to form GSSG while converting H_2O_2 to H_2O (Table 1, Reactions 1.4 and 1.5) [16,17]. The radicals subsequently react with other biological compounds to produce alkyl (R$^\bullet$) and thiol (RS$^\bullet$) radicals (Table 1, Reactions 1.6 and 1.7). During the oxidation, the hypoxanthine/xanthine oxidase (HX/XO) system produces H_2O_2 (Table 1, Reactions 2.1 and 2.2), $^\bullet$OH (Reaction 2.3), and $O_2^{\bullet-}$ (Reaction 2.4) [18]. NO$^\bullet$ and several other reactive nitrogen species, including nitrosonium ion (NO^+), nitroxyl anion (NO^-), $ONOO^-$, and *S*-nitrosothiols (RSNO) [19–21], have potent biological effects. *S*-Nitroso-*N*-acetylpenicillamine (SNAP), 3-morpholinosydnonimine (SIN-1), and sodium nitroprusside (SNP) are commonly used to produce various forms of nitrogen monoxides [19]. SNAP is a RSNO that can release NO$^\bullet$ (Table 1, Reaction 3.1). The resulting NO$^\bullet$ can react with $O_2^{\bullet-}$ to form $ONOO^-$ (Reaction 3.2). Decomposition of RSNOs can also release nitroxy anion (NO^-), which reacts with thiol-containing proteins (R′SH) to form a disulfide link (RSSR′) (Reaction 3.3). RSNOs can participate in transnitrosation reactions, whereby the NO^+ group is transferred to another R′SH to form R′SNO (Reaction 3.4) [20,22]. NO^-, which combines with O_2, forms $ONOO^-$ (Reaction 3.5). In the presence of O_2, SIN-1 releases both NO$^\bullet$ and $O_2^{\bullet-}$, which generate $OONO^-$ rapidly (Reactions 4.1 and 4.2) [22-24], and may be regarded as a $OONO^-$ donor [25]. SNP, a cyanoferrate, generates NO$^\bullet$ and releases cyanide anion (CN^-), Fe^{2+}, and $O_2^{\bullet-}$ (Reactions 5.1–5.4) [26]. These reactive oxygen species are difficult to distinguish experimentally in cells by current techniques. The current studies have begun to do this by testing the effects of multiple oxidants on the temporal profiles of reactive oxygen species as detected by two intracellular indicators and on a select target (i.e., BRCS).

Two indicators of different oxidant species were used to distinguish different ROS: 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester) (c-DCF) and 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM). The goal of the current studies was to determine whether the combination of the use of these probes and the temporal profile of the ROS induction in response to oxidants could be used to distinguish select ROS within the cells after treatment with diverse oxidants. The second goal was to determine which of these oxidants altered BRCS in an AD-like fashion. The third goal was to determine whether the production of c-DCF-detectable ROS and BRCS in response to oxidants was different in fibroblasts from AD patients bearing the PS-1 mutation compared to appropriate controls.

Table 1
Different oxidants induce different patterns of ROS as revealed by different fluorescent probes

(1)	(1.1)	*t*-bu-OOH + Fe^{2+} → *t*-buO$^\bullet$ + Fe^{3+} + H_2O
	(1.2)	*t*-buO$^\bullet$ + *t*-buOOH → *t*-buOO$^\bullet$ + *t*-buOH
	(1.3)	*t*-bu-OOH + Fe^{3+} → *t*-buOO$^\bullet$ + Fe^{2+}
	(1.4)	
	(1.5)	2GSH + H_2O_2 → GSSG + 2H_2O
	(1.6)	*t*-buOO$^\bullet$ + RH → *t*-bu-OOH + R$^\bullet$
	(1.7)	*t*-buOO$^\bullet$ + RSH → *t*-bu-OOH + RS$^\bullet$
(2)	(2.1)	Hypoxanthine + H_2O + O_2 → xanthine + H_2O_2
	(2.2)	Xanthine + H_2O + O_2 → uric acid + H_2O_2
	(2.3)	H_2O_2 + Fe^{2+} → $^\bullet$OH + OH^- + Fe^{3+}
	(2.4)	O_2 + Fe^{2+} → Fe^{3+} + $O_2^{\bullet-}$
(3)	(3.1)	2 RSNO → RSSR + 2 NO$^\bullet$
	(3.2)	NO$^\bullet$ + $O_2^{\bullet-}$ → $ONOO^-$
	(3.3)	RSNO + R′SH → RSSR′ + H^+ + NO^-
	(3.4)	RSNO + R′SH → RSH + R′SNO
	(3.5)	NO^- + O_2 → $ONOO^-$
(4)	(4.1)	SIN-1 + O_2 → NO$^\bullet$ + $O_2^{\bullet-}$
	(4.2)	NO$^\bullet$ + $O_2^{\bullet-}$ → $ONOO^-$
(5)	(5.1)	$[Fe(CN)_5NO]^{2-}$ + H_2O → $[Fe(CN)_5H_2O]^{2-}$ + NO$^\bullet$
	(5.2)	$[Fe(CN)_5NO]^{2-}$ + reductants → $[Fe(CN)_5NO]^{3-}$
	(5.3)	3 $[Fe(CN)_5NO]^{3-}$ → 2 $[Fe(CN)_6]^{4-}$ + $3CN^-$ + Fe^{2+} + 3NO$^\bullet$
	(5.4)	$[Fe(CN)_5NO]^{3-}$ + O_2 → $[Fe(CN)_5NO]^{2-}$ + $O_2^{\bullet-}$

Materials and methods

The supplies were from the indicated companies: cell culture reagents, GIBCO (Grand Island, NY, USA); c-DCF,