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Novel mechanism of increased Ca^{2+} release following oxidative stress in neuronal cells involves type 2 inositol-1,4,5-trisphosphate receptors

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Abstract

Dysregulation of Ca^{2+} signaling following oxidative stress is an important pathophysiological mechanism of many chronic neurodegenerative disorders, including Alzheimer's Disease, age-related macular degeneration, glaucomatous and diabetic retinopathies. However, the underlying mechanisms of disturbed intracellular Ca^{2+} signaling remain largely unknown. We here describe a novel mechanism for increased intracellular Ca^{2+} release following oxidative stress in a neuronal cell line. Using an experimental approach that included quantitative polymerase chain reaction, quantitative immunoblotting, microfluorimetry and the optical imaging of intracellular Ca^{2+} release, we show that sub-lethal *tert*-butyl hydroperoxide-mediated oxidative stress result in a selective up-regulation of type-2 inositol-1,4,5-trisphosphate receptors. This oxidative stress mediated change was detected both at the transcriptional and translational level and functionally resulted in increased Ca^{2+} release into the nucleoplasm from the membranes of the nuclear envelope at a given receptor-specific stimulus. Our data describe a novel source of Ca^{2+} dysregulation induced by oxidative stress with potential relevance for differential subcellular Ca^{2+} signaling specifically within the nucleus and the development of novel neuroprotective strategies in neurodegenerative disorders.

1. Introduction

Intracellular free calcium (Ca^{2+}) is a second messenger of pivotal importance and involved in a variety of cellular processes, including neurotransmitter release, gene expression and apoptosis (Berridge et al., 2003; Koulen and Thrower, 2001). As such, the maintenance of the intracellular Ca^{2+} homeostasis is critical for cellular function and survival; therefore, the concentration of Ca^{2+} is tightly controlled and regulated (Berridge et al., 2003; Koulen and Thrower, 2001). Ca^{2+} can enter the intracellular milieu either from the extracellular space through voltage, ligand, store or second messenger-activated Ca^{2+} channels (Berridge et al., 2003; Koulen and Thrower, 2001), or from intracellular stores, such as the endoplasmic

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reticulum. The release of Ca^{2+} from intracellular stores is mediated by three types of intracellular Ca^{2+} release channels: inositol-1,4,5-trisphosphate receptors (IP_3Rs), ryanodine receptors (RyRs) or polycystin-2 (pc-2; Berridge et al., 2003; Koulen et al., 2002; Koulen and Thrower, 2001). In addition to Ca^{2+} entering the cell, mechanisms controlling Ca^{2+} uptake into intracellular stores or extrusion to the extracellular space critically shape cellular Ca^{2+} transients and Ca^{2+} homeostasis (Berridge et al., 2003; Koulen and Thrower, 2001).

Dysregulation of Ca^{2+} is an important pathophysiological mechanism in aging and many neurodegenerative diseases, including Alzheimer's Disease (AD; (Berridge, 2010; Khachaturian, 1994), many ocular diseases such as age-related macular degeneration, glaucomatous and diabetic retinopathies (Rhodes and Sanderson, 2009; Schmidt et al., 2008) as well as the ensuing oxidative stress (Ohia et al., 2005). Cellular oxidative stress as the result of the formation of Reactive Oxygen Species (ROS) can alter Ca^{2+} homeostasis by a variety of mechanisms that include plasma membrane proteins, mitochondria as well as intracellular Ca^{2+} channels (Duchen et al., 2008; Foster, 2007; Ohia et al., 2005; Wojda et al., 2008). Mechanisms by which oxidative stress affects Ca^{2+} signaling in neurons are largely unknown (Sayre et al., 2008). Furthermore, chronically elevated levels of oxidative stress may result in subtle yet significant changes in Ca^{2+} homeostasis that ultimately cause or exacerbate disease pathophysiology.

We here investigated the hypothesis that oxidative stress alters intracellular Ca^{2+} signaling through an IP_3R -controlled pathway.

The group of IP_3Rs consists of three members ($\text{IP}_3\text{R1}$, $\text{IP}_3\text{R2}$ and $\text{IP}_3\text{R3}$), which each have distinct physiological, pharmacological and biophysical properties (Koulen and Thrower, 2001). Expressed predominantly on the membranes of the endoplasmic reticulum (ER) and contingent membranes, such as the nuclear envelope, IP_3Rs are differentially localized in different tissues and different compartments of cells in general (Leite et al., 2003; Rodrigues et al., 2009) and neurons in particular (Berridge, 1998; Berridge, 2006; Berridge, 2009; Koulen and Thrower, 2001).

HT-22 cells are a cell line derived from mouse hippocampus that is commonly used as a model for studying neuroprotection against oxidative stress (Berry and Toms, 2006; Duncan et al., 2007; Fu and Koo, 2006; Rybalchenko et al., 2009; Sagara et al., 1998; Sagara and Schubert, 1998). We have previously shown that all three subtypes of IP_3Rs are expressed in HT-22 cells (Duncan et al., 2007). Specifically, we showed a preferential distribution of $\text{IP}_3\text{R1}$ s and $\text{IP}_3\text{R3}$ s on ER membranes within the cytosol, whereas $\text{IP}_3\text{R2}$ s are localized to the membranes of the nuclear envelope (Duncan et al., 2007) and mediate Ca^{2+} release into the nucleoplasm (Leite et al., 2003).

In order to induce cellular oxidative stress without initiating cell death pathways we determined sub-lethal concentrations of *tert*-butyl hydroperoxide (tBHP). tBHP is a substrate for glutathione peroxidase that induces oxidative stress by increasing the levels of oxidized glutathione at the expense of reduced glutathione (Kurz et al., 2004). Furthermore, tBHP is more stable in aqueous solution than hydrogen peroxide (H_2O_2) providing a longer and more sustained effect on the cells, and reducing the variability in the levels of oxidative stress induced (Alia et al., 2005). Use of these conditions of sub-lethal oxidative stress in a neuronal cell line allowed us to biochemically and functionally analyze subsequent cellular responses that do not involve the initiation of apoptotic and/or necrotic pathways.

We show that sub-lethal concentrations of tBHP cause a distinct and significant increase in intracellular Ca^{2+} release into the nucleoplasm mediated selectively by $\text{IP}_3\text{R2}$ s, which are located in the membranes of the nuclear envelope. Sub-lethal tBHP exposure did not alter Ca^{2+} release from ER stores into the cytosol. Our data describe a novel mechanism for

oxidative stress-induced disturbances of intracellular Ca^{2+} signaling that is of high relevance for the development of novel neuroprotective strategies for AD and pathophysiologically related and similar disorders.

2. Experimental Methods

2.1. Cell culture and preparation

HT-22 cells (passages 7–25) were maintained in a humidified 37 °C/5% CO_2 incubator in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin (all from Lonza, Walkersville, MD). For immunocytochemistry and Ca^{2+} imaging experiments, cells were seeded on 12 mm glass cover slips coated with 0.01% poly-L-lysine (Sigma-Aldrich, St. Louis, MO) two days before the experiment and grown to about 40% confluency. For immunoblotting and quantitative polymerase chain reaction experiments, cells were grown in 150 cm^2 flasks (TPP, MidSci, St. Louis, MO) to about 60% confluency. Media was then changed to either contain 2 μM or 10 μM tBHP or vehicle, diluted from a 10 mM aqueous stock solution, or vehicle control (dH_2O). Cells were washed with and scraped in 15 ml ice-cold phosphate-buffered saline (PBS). Following centrifugation (2 min at $200 \times g$ at 4 °C), protein was isolated using the ProteoJET Mammalian Cell Lysis reagent for total protein and ProteoJET Cytoplasmic and Nuclear Protein Extraction kit (both from Fermentas, Glen Burnie, MD), according to the manufacturer's recommendations. Protein concentration was determined using the method of Lowry in microtiter plate format (BioRad Laboratories, Hercules, CA), using bovine serum albumin as protein standard, and a FlexStation3 plate reader (Molecular Devices, Sunnyvale, CA).

2.2. Cell viability assays and measurements of oxidative stress

Fluorimetric calcein-AM and colorimetric MTT viability assays were conducted in 96 well plates. Cells were seeded at a density of 2,000 cells/well. 24 hrs after seeding, cells were incubated with tBHP (1–100 μM) or vehicle (dH_2O) and maintained overnight (16–20 hrs.). For the calcein-AM assay, media was removed from cells and replaced with pre-warmed Hank's balanced salt solution (HBSS, Mediatech, Manassas, VA) supplemented with 2 mM CaCl_2 and calcein-AM dye (Axxora, San Diego, CA) at a final concentration of 4 μM for 20 minutes. Calcein fluorescence was measured using a fluorimetric plate reader (FlexStation3, Molecular Devices, Sunnyvale, CA).

The MTT assay was conducted according to manufacturer's instructions (Invitrogen/Molecular Probes, Eugene, OR) and was measured at an absorbance wavelength of 560 nm using a FlexStation3 plate reader (Molecular Devices, Sunnyvale, CA).

In order to estimate the level of oxidative stress we performed the DCFDA assay. The DCFDA assay was conducted by incubating HT-22 cells (2,000 cells/well) in complete DMEM containing 10 μM carboxy-2', 7' dichlorodihydrofluorescein diacetate (carboxy- H_2 DCFDA) for 30 min. Media was removed and cells were then incubated for 6 hrs. in complete DMEM containing various concentrations of tBHP (1–100 μM) or vehicle at 37°C. Cells were washed twice with Hank's Balanced Salt Solution (HBSS) containing 2 mM CaCl_2 and read in a fluorimetric plate reader at 485/530nm (excitation/emission).

2.3. Total RNA isolation, cDNA synthesis and quantitative polymerase chain reaction

Total RNA was isolated from HT-22 cell pellets after 16 hour incubation with tBHP or vehicle using the Ambion RNaqueous4PCR kit (Applied Biosciences, Foster City, CA) according to the manufacturer's recommendations. Quantity and quality of total RNA was assessed using a NanoVue spectrophotometer (GE Healthcare, Piscataway, NJ). cDNA was

synthesized from 2 µg total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations. The quantitative polymerase chain reaction was performed using a StepOne Plus PCR System and gene-specific FAM-labeled Taqman[®] gene expression assays (both from Applied Biosystems, Foster City, CA) using a total reaction volume of 20 µl and the equivalent of 100 ng total RNA transcribed to cDNA per well. Mouse β-actin (mACTB) was used as endogenous control and a VIC-labeled mACTB assay was used in parallel in every well. All assays were validated using standard curve analyses. We performed relative expression analysis according to the $2^{-\Delta\Delta CT}$ method by Livak (Livak and Schmittgen, 2001). Data was exported and plotted in Origin 8 (OriginLab Corporation, Northampton, MA). Statistical significance was performed using Analysis of Variance (ANOVA).

2.4. Immunocytochemistry

Effects of oxidative stress on IP₃ receptor immunoreactivity were assessed after overnight incubation with tBHP or vehicle. Immunocytochemistry was performed as described previously (Duncan et al., 2007). Briefly, cells were fixed for 15 min with 4% paraformaldehyde in PBS and then washed three times for 5 min with PBS. Cells were then blocked with pre-incubation solution (10% normal goat serum, 1% BSA, 0.05% tween-20 in PBS) for 1 h. Primary antibodies against IP₃R receptor subtypes were obtained from Calbiochem (IP₃R1 and IP₃R2) and BD Transduction Laboratories (IP₃R3) and diluted in incubation buffer (3% normal goat serum, 1% BSA, 0.05% tween-20 in PBS) and added to cells and incubated at 4 °C overnight and then washed three times for 5 min with PBS. Fluorescent Alexa488- or Alexa594-labeled goat anti-rabbit or goat anti-mouse IgG secondary antibody (Invitrogen, Carlsbad, CA) was diluted in incubation solution (1:1000) and applied to cells for 1 h at room temperature in the dark. Coverslips were then washed in PBS three times for 5 min with PBS and then mounted onto glass slides using ProLong anti-fade reagent with DAPI (Invitrogen) and left to cure overnight at 4 °C. Slides were viewed within 5 days after mounting. Samples were visualized at ambient temperature (21–23 °C) under a fluorescence microscope (Olympus IX70, Tokyo, Japan) using a 20× interference contrast or fluorescence microscopy. A constant exposure time was maintained among images to facilitate the comparison among images.

2.5. Immunoblotting

For semi-quantitative immunoblotting, HT-22 cells were grown in 75 cm² flasks (TPP, MidSci, St. Louis, MO). Cells were seeded at a density of 10⁵ cells. 24 hrs. after seeding cells were treated with tBHP or vehicle and maintained in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin (all from Lonza, Walkersville, MD) for 16 hrs, reaching about 80% confluency. 20 µg protein were denatured in 2×SDS loading buffer (500 mM Tris-HCl pH 6.8 containing 5% SDS, 20% glycerol, 2% β-mercaptoethanol and 0.004% bromophenol blue) and loaded on a 4–12% Tris-HCl SDS Criterion Midi Gel (Biorad Laboratories, Hercules, CA) using Spectra Multicolor High Range Protein Ladder (Fermentas, Glen Burnie, MD) as size standard. Proteins were transferred to nitrocellulose membranes (0.22 µm; BioRad Laboratories, Hercules, CA), blocked in PBS with 5% milk (BioRad Laboratories, Hercules, CA) and 0.2% Tween-20 (EMD Chemicals, Gibbstown, NJ) for 1 hr at ambient temperature. Membranes were then incubated in primary antibody diluted in PBS with 2.5% milk, overnight, gently shaking at 4° C. Primary antibodies specific for IP₃R1 (diluted 1:20,000), IP₃R2 (diluted 1:50,000) and IP₃R3 (diluted 1:50,000) were from Chemicon (AB9072, AB9074 and AB9076, respectively; Millipore, Billerica, MA). The membrane was then washed three times with PBS containing 2.5% milk and 0.2% Tween-20, before addition of horseradish peroxidase-labeled goat anti-rabbit secondary antibody (diluted at 1:10,000; GE Biosciences, Piscataway, NJ), for 1 hr at ambient

temperature. Membranes were washed three times in PBS with 2.5% milk, 0.2% Tween-20 and two times in PBS alone. Membrane were subsequently developed with ImmunStar Western ECL kit (BioRad Laboratories) and imaged on CL-Xposure light-sensitive film (Pierce/ThermoScientific, Rockford, IL).

2.6. Optical imaging of intracellular Ca^{2+} concentrations

Optical imaging was performed as described previously (Duncan et al., 2007). Briefly, HT-22 cells were plated on poly-L-lysine-coated 15 mm glass coverslips at a density of 5,000 cells per coverslip, and grown for 24 h as described above in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin (all from Lonza, Walkersville, MD). Cells were then incubated with either tBHP or vehicle overnight (16–20 hrs.). Subsequently, HT-22 cells were incubated with 4 μM of the fluorescent cell-permeant Ca^{2+} indicator dye Fluo-3 acetoxymethylester (AM) in Leibovitz L-15 Medium (both Invitrogen, Grand Island, NY) for 30 min at 37 °C and subsequently washed with L-15 medium. Coverslips were transferred to a perfusion chamber containing extracellular solution (in mM: NaCl 137, KCl 5, Na_2HPO_4 1, HEPES 10, glucose 22, EGTA 5, pH 7.4) and placed on an inverted microscope (Olympus IX70, Tokyo, Japan) at ambient temperature. Fluorescence intensity was recorded using time-lapse video microscopy using Simple PCI software (Hamamatsu, Bridgewater, NJ). For every cell, mean fluorescence intensity was determined by averaging the responses of five randomly selected regions of interest (ROIs) of equal size in the nucleus and cytosol. Fluorescence was quantified by dividing the measured fluorescence intensity during drug application (F) by the baseline fluorescence intensity (F_0). Cells were treated with pipette-applied 1 μM cell-permeant IP_3 -AM (A.G. Scientific, Inc., San Diego, CA). Pharmacological agents xestospongine D and dantrolene were obtained from EMD Chemicals (Gibbstown, NJ). The slope of Ca^{2+} transients was calculated by plotting the mean fluorescence intensity within the 20–80% range of the maximum fluorescence intensity over time followed by addition of a linear trend line.

3. Results

3.1. Identification of sub-lethal tBHP concentrations

Oxidative stress under pathophysiological conditions does not typically result in acute cell death. Therefore, determination of sub-lethal concentration of the oxidant in a cell-based experimental system is critical. We chose the neuronal HT-22 cell line for our studies due to their suitability for oxidative stress studies (Berry and Toms, 2006; Duncan et al., 2007; Fu and Koo, 2006; Rybalchenko et al., 2009; Sagara et al., 1998; Sagara and Schubert, 1998). Furthermore, IP_3 Rs are highly expressed in hippocampus-derived HT-22 cells and show a subtype-specific expression pattern, as described by us previously (Duncan et al., 2007). In order to assess sub-lethal concentrations of tBHP on HT-22 cells, we performed both calcein-AM and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays as correlates for cell viability. Exposure of HT-22 cells to tBHP concentrations of up to 10 μM had no significant effect on calcein fluorescence (Fig. 1A). The fact that fluorescence intensity of calcein was unaffected under oxidative conditions indicates that the endogenous cellular esterases critical for conversion of cell membrane permeable chemicals are not affected by sublethal oxidative stress. Similarly, absorbance as determined by the MTT assay was similar to control up to concentrations of approx. 10 μM tBHP (Fig. 1B). As determined by both assays, the approximate LC_{50} of tBHP in HT-22 cells is around 25–50 μM (Fig. 1), which is in accordance with previous reports for neuron-derived cells (Zitzler et al., 2004). Based on these findings we chose 2 μM and 10 μM as representative conditions for mild and elevated sub-lethal oxidative stress. Morphology of HT-22 cells is unaffected by sub-lethal concentrations of tBHP-induced oxidative stress (Fig. 1C). In contrast, cells

experiencing higher tBHP concentrations exhibit a more spherical morphology characteristic of cells undergoing apoptosis or necrosis, rendering them unsuitable for subcellular analysis of intracellular Ca^{2+} .

An intact complement of intracellular esterases is critical for the subsequent functional assays as non-invasive stimulation of IP_3Rs with the membrane-permeable IP_3 -acetoxymethylester ($\text{IP}_3\text{-AM}$) and delivery of the Ca^{2+} indicator dye Fluo-3 acetoxymethylester (Fluo3-AM) rely on the same principle. As a measure of tBHP-induced oxidative stress in HT-22 cells we used carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy- H_2 DCFDA) conversion. 10 μM tBHP caused a significant 4.2% increase in DCFDA fluorescence, compared with control ($n=3$, $P<0.05$; Fig. 1D), confirming that 10 μM tBHP indeed subjects HT-22 cells to conditions of oxidative stress in our experimental system. Increasing tBHP concentrations correlated with increasing DCFDA fluorescence in our assay system (Fig. 1D).

3.2 Increased IP_3R -mediated Ca^{2+} release from intracellular stores following oxidative stress

Given the pivotal role of IP_3Rs in controlling intracellular Ca^{2+} homeostasis (Berridge, 1998; Koulen and Thrower, 2001) and the well-established expression pattern and functional characteristics of IP_3Rs in HT-22 cells (Duncan et al., 2007), we hypothesized that Ca^{2+} dysregulation in response to tBHP-mediated oxidative stress may occur through an IP_3R -dependent mechanism by altering the response of IP_3Rs to physiological concentrations of their ligand.

In order to measure IP_3R mediated intracellular Ca^{2+} release, we loaded cells with Fluo3-AM and stimulated Ca^{2+} release with the IP_3R agonist IP_3 -acetoxymethylester ($\text{IP}_3\text{-AM}$; 1 μM). This concentration is within the physiological range of concentrations to stimulate IP_3R Ca^{2+} channel activity (Berridge et al., 2003; Leite et al., 2003). As tBHP treatment may affect the endogenous esterase activity and thereby influence cleavage of the AM-ester precursors, we compared esterase activity by comparing calcein-AM fluorescence in a plate-reader based assay in the presence and absence of tBHP. No differences in fluorescence were found (data not shown), suggesting that tBHP treatment resulting in sub-lethal oxidative stress does not compromise endogenous esterase activity.

Using this approach of optical imaging of intracellular Ca^{2+} , we found a small non-significant increase in overall Ca^{2+} release from stores in the presence of 10 μM tBHP compared with control (Fig. 2A, B). We performed control experiments in the presence of xestospongine D (10 μM) and dantrolene (20 μM) to verify the specificity of the agonist, $\text{IP}_3\text{-AM}$. Cellular responses to $\text{IP}_3\text{-AM}$ in the presence of the IP_3R blocker xestospongine D were completely abolished (Fig. 2C). The RyR blocker dantrolene had no effect on tBHP-mediated increases of intracellular Ca^{2+} release in HT-22 cells (Fig. 2C), excluding RyR-mediated contributions to the observed effect.

There is currently no selective pc-2 inhibitor available, limiting our ability to test any nonspecific contribution of pc-2 to $\text{IP}_3\text{-AM}$ induced Ca^{2+} responses using pharmacological means. However, pc-2 requires significant, prolonged depolarization of the membranes of the endoplasmic reticulum in addition to Ca^{2+} induced activation to generate Ca^{2+} release (Koulen et al., 2002), making their involvement unlikely. In order to exclude effects on the size of the total ER releasable Ca^{2+} pool, we compared the effect of 2 μM thapsigargin in the presence and absence of oxidative stress. In Ca^{2+} -free extracellular medium, the area under the curve of the normalized Ca^{2+} response to thapsigargin was 176.2 ± 20.6 after incubation with vehicle, compared with 161.6 ± 9.6 following overnight incubation with 8 μM tBHP ($n=8$; $P=0.53$). Our further subcellular analysis revealed a specific 32% increase

in nuclear Ca^{2+} release (Fig. 2D, E) in the presence of 10 μM tBHP compared with control or the 2 μM tBHP conditions (peak F/F_0 were 5.8 ± 0.4 , 4.5 ± 0.5 and 4.4 ± 0.8 , respectively; $n=6$ experiments, ANOVA $P<0.05$). Release from cytosolic stores was similar between control and tBHP conditions (3.5 ± 0.5 , 3.7 ± 0.8 and 3.9 ± 0.3 , respectively; $n=6$ experiments, ANOVA $P=0.84$; Fig. 2D, E) although a statistically not significant trend towards higher Ca^{2+} release in the 10 μM tBHP condition was observed. Increased intracellular Ca^{2+} release through IP_3Rs may be the result of either some or all of an increase in the number of receptors, a change in the localization of IP_3R subtypes and a change in biophysical and physiological properties such as ligand binding. Given the preferential localization of $\text{IP}_3\text{R2s}$ on membranes of the nuclear envelope, versus $\text{IP}_3\text{R1s}$ and $\text{IP}_3\text{R3s}$ on ER membranes within the cytosol and the difference in IP_3Rs ' affinities for their ligand IP_3 with $\text{IP}_3\text{R2}$ having the highest and $\text{IP}_3\text{R3}$ the lowest, a change in subtypes would likely encompass a change in the kinetics of the Ca^{2+} responses. Analysis of the response kinetics yielded no differences between control and tBHP treated conditions (Fig. 2C). The slope of the rising phase of the Ca^{2+} release (calculated for 20–80% of the peak response) was similar between all conditions (control, 2 μM tBHP and 10 μM tBHP), in the nucleus (0.44 ± 0.11 , 0.39 ± 0.10 and 0.47 ± 0.09 , respectively; $n=6$, ANOVA $P=0.84$; Fig. 2B) and the cytosol (0.29 ± 0.10 , 0.29 ± 0.06 and 0.35 ± 0.09 , respectively; $n=6$, ANOVA $P=0.68$; Fig. 2B). Based on the localization of the increased Ca^{2+} signal, and the unaltered kinetics of the response, we concluded that $\text{IP}_3\text{R2s}$ are the likely targets of tBHP-mediated Ca^{2+} dysregulation in neuronal cells and hypothesized that this response likely is due to a change in receptor number.

3.3 $\text{IP}_3\text{R2}$ is up-regulated in response to oxidative stress

We used immunocytochemistry, microfluorimetric analysis, qPCR and immunoblotting to test our hypothesis that an up-regulation of $\text{IP}_3\text{R2s}$ is responsible for increased nuclear Ca^{2+} release in the presence of sub-lethal oxidative stress. In the nucleus, relative fluorescence as a correlate of $\text{IP}_3\text{R2}$ protein concentration was similar between control and 2 μM tBHP conditions, however, increased by 62% in 10 μM ($100 \pm 6\%$, $99 \pm 6\%$ and $162 \pm 11\%$, respectively; $n=3$ experiments; ANOVA $P<0.05$; Fig. 3A, B). In the cytosol, a trend towards increased $\text{IP}_3\text{R2}$ immunoreactivity was seen (approximately 30%), however, this increase did not reach statistical significance. In order to verify our observations from microfluorimetric analysis, we performed qPCR using $\text{IP}_3\text{R2}$ specific Taqman[®] probes and mouse β -actin (mACTB) as endogenous control (Fig. 3C). Relative $\text{IP}_3\text{R2}$ gene expression was increased significantly 3-fold in the presence 10 μM tBHP, compared with control and 2 μM tBHP ($n=3$ experiments, ANOVA $P<0.05$, Bonferroni post-hoc test $P<0.01$ for 10 μM vs. control; Fig. 3C). Oxidative stress did not affect the expression levels of the endogenous control (mACTB; Fig. 3C, right panel). Furthermore, we also used ribosomal protein 18S and glyceraldehyde-3-phosphate dehydrogenase as endogenous controls, yielding the same result (data not shown). We also performed immunoblotting, measuring IP_3R protein isolated from total HT-22 cell lysate. Using 25 μg of total protein as assessed by Lowry assay with BSA as a reference (Lowry et al., 1951), we found a qualitative, dose-dependent increase in $\text{IP}_3\text{R2}$ -specific signal. A representative experiment is shown in Fig. 3D.

3.4 $\text{IP}_3\text{R1}$ and $\text{IP}_3\text{R3}$ levels are unaffected by sub-lethal oxidative stress

Intracellular Ca^{2+} signaling is often mediated by a combination of types of IP_3R receptors that show a distinct subcellular localization and, therefore, have different downstream effects (Duncan et al., 2007; Koulen and Thrower, 2001; Leite et al., 2003; Rodrigues et al., 2009). In order to assess the possible involvement of increased levels of the other two types of IP_3R , $\text{IP}_3\text{R1}$ and $\text{IP}_3\text{R3}$, to increased Ca^{2+} release following tBHP-mediated oxidative stress, we performed the same analyses as for $\text{IP}_3\text{R2}$. Overall immunoreactivity for $\text{IP}_3\text{R1s}$ was similar following oxidative stress compared with control; microfluorimetry revealed no

statistically significant differences between the different conditions ($n=3$, $P=0.81$; Fig. 4A). Similar results were obtained for IP₃R3s ($n=3$, $P=0.92$; Fig. 4B). The absence of up-regulation of IP₃R1 (Fig. 4C) and IP₃R3 (Fig. 4D) was corroborated by immunoblotting experiments: expression was similar between the control, 2 μ M and 10 μ M tBHP treatment groups ($n=3$, $P=0.92$ for IP₃R1, and $n=3$, $P=0.55$ for IP₃R3, respectively; Fig. 4C, D). Similarly, qPCR analysis did not reveal a statistically significant increase of the mRNA for *IPTR1* and *IPTR3* genes, encoding IP₃R1s and IP₃R3s, respectively (Table 1) under conditions of chemically-induced oxidative stress of 10 μ M tBHP. In order to investigate the possible transcriptional response of other intracellular Ca²⁺ channels to tBHP-induced oxidative stress in HT-22 cells, we performed a qPCR study. mRNA expression levels for all genes encoding intracellular Ca²⁺ channels – with the exception of *ITPR2* (Fig. 3C) – were similar between vehicle and oxidative stress conditions (Table 1).

4. Discussion

4.1. Relevance for neurodegenerative disease

We here present a novel mechanism of how oxidative stress leads to Ca²⁺ dysregulation in a neuronal cell line, by causing an up-regulation of IP₃R2 expression on the nuclear envelope and an increase in Ca²⁺ release into the nucleus, as demonstrated by Ca²⁺ imaging, immunocytochemistry, qPCR and immunoblotting.

Ca²⁺ is tightly controlled in neuronal cells (Berridge, 1998) and a variety of mechanisms may compensate for dysregulated Ca²⁺ homeostasis in response to oxidative stress, including other types of intracellular Ca²⁺ channels as well as store-filling mechanisms (Bezprozvanny and Mattson, 2008; Foster, 2007; Ohia et al., 2005; Sagara et al., 1998; Sayre et al., 2008; Wojda et al., 2008). Numerous studies have indicated the critical role for regulation of the intracellular Ca²⁺ concentration in the pathogenesis of age-related neuronal dysfunction with a variety of techniques and human disease model systems (Landfield, 1987; Mattson and Chan, 2001; Mattson et al., 1989; Missiaen et al., 2000; Squier and Bigelow, 2000; Thibault et al., 1998). Perturbations in the intracellular Ca²⁺ homeostasis are often a result of oxidative stress and are typically correlated with deficits in nerve cell function, increased cell damage and cell death of neurons and require control of the intracellular Ca²⁺ concentration to achieve improvement of the conditions (Baskys and Adamchik, 2001; Butterfield et al., 2001; Chen, 1998; Chen and Fernandez, 1999; Hall et al., 2001; Kontush, 2001; Leissring et al., 2000; Massheimer et al., 2000; Mattson and Chan, 2001; Mattson et al., 1989; Mattson et al., 2000; Missiaen et al., 2000; O'Neill et al., 2001; Squier and Bigelow, 2000; Thibault et al., 2001; Thibault et al., 1998; Tuppo and Forman, 2001; Veinbergs et al., 2002). IP₃Rs are pivotal to the regulation of intracellular Ca²⁺ and neuronal function (Berridge, 2009) and have been found involved in a variety of pathophysiological mechanisms, including those leading to AD (for review see, Stutzmann, 2005), by either direct or indirect modulation (Cheung et al., 2010; Crews et al., 1994; Ferrari-DiLeo and Flynn, 1993; Ferreira et al., 2006; Garlind et al., 1995; Kasri et al., 2006; Kurumatani et al., 1998; Stokes and Hawthorne, 1987; Stutzmann et al., 2004; Young et al., 1988).

Our novel finding that the IP₃R2 subtype mediates increases in intracellular Ca²⁺ release in response to oxidative stress is of high (patho-) physiological relevance: IP₃Rs are expressed in the central nervous system (Berridge, 1998); IP₃R2 has the highest affinity for its ligand among all IP₃Rs rendering it most sensitive to activation by low ligand concentrations when IP₃R1 and IP₃R3 remain inactive (Koulen and Thrower, 2001), and unlike IP₃R1, which is inhibited by high cytosolic Ca²⁺ concentrations, IP₃R2 and IP₃R3 do not inactivate in the continuing presence of high intracellular Ca²⁺ concentrations (Koulen and Thrower, 2001). Therefore, an induction of elevated IP₃R2 expression has the potential to directly result in

abnormally high intracellular Ca^{2+} concentrations that can affect a variety of down-stream pathways, including gene expression, and ultimately lead to calcium toxicity and cell death. The significant effect observed at low, sublethal levels of oxidative stress suggests that this mechanism may not only be relevant during disease states associated with increased oxidative stress, such as AD (Berridge, 2010; Khachaturian, 1994), but also occur during normal aging (Kregel and Zhang, 2007).

The significance of our finding of the involvement of IP_3Rs in the cellular response to oxidative stress and its implications for AD and other neurodegenerative disorders is further highlighted by a recently published study demonstrating gain-of-function enhancement of IP_3Rs in familial AD (Cheung et al., 2010).

It remains to be verified whether this mechanism of $\text{IP}_3\text{R2}$ up-regulation in response to oxidative stress is also present in more physiologically relevant systems, such as primary cultured neuronal cells and *in vivo*. If so, the novel mechanism described here represents a potential target for the development of urgently-needed neuroprotective strategies for CNS aging, cognitive decline, AD and related age-related neurodegenerative diseases.

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Abbreviations List

AD	Alzheimer's Disease
Carboxy-H_2 DCFDA	carboxy-2', 7'-dichlorodihydrofluorescein diacetate
ER	endoplasmic reticulum
IP_3	inositol-1,4,5-trisphosphate
IP_3R	inositol-1,4,5-trisphosphate receptor
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
PBS	phosphate buffered saline
ROS	Reactive Oxygen Species
tBHP	<i>tert</i> -butyl hydroperoxide

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Figure 1. Identification of sub-lethal tBHP concentrations

(A) Exposure of HT-22 cells to tBHP concentrations of up to 10 μM for 16–20 hrs. had no significant effect on normalized calcein-AM fluorescence. (B) Absorbance of the MTT assay was similar to control up to concentrations of 10 μM tBHP. As determined by both assays, the approximate LC_{50} of tBHP in HT-22 cells is around 25 μM . (C) Representative images of HT-22 cell culture (passage 14), taken with an inverted tissue culture microscope with phase contrast, shows that morphology of HT-22 cells *in vitro* is unaffected by sublethal oxidative stress with 10 μM tBHP. There is no evidence of rounded cell morphology, typically observed at higher tBHP concentrations. (D) Level of oxidative stress was measured after 6 hrs. exposure to tBHP or vehicle using carboxy-2', 7'-dichlorodihydrofluorescein diacetate (carboxy- H_2 DCFDA). 10 μM tBHP resulted in a significant 4.2% increase in DCFDA fluorescence compared with control ($n=3$; $P<0.05$). Higher concentrations of tBHP correlated with higher DCFDA fluorescence, indicating increased levels of oxidative stress. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Figure 2. tBHP-mediated oxidative stress causes increased nuclear Ca²⁺ release

(A) Representative example of IP₃-AM stimulated Ca²⁺ release in HT-22 cells, following 16–20 hrs. exposure to either vehicle or 10 μM tBHP. Fluorescence is higher in the nucleus in the tBHP condition compared with control. (B) Overall magnitude of the response, expressed as increases in the amplitude of Ca²⁺ transients and presented as peak normalized change in fluorescence (F/F₀) is statistically not significantly different following exposure to oxidative stress (n=6, ANOVA P=0.096). Data is shown as mean ± s.e.m. (C) The specificity of the agonist (IP₃-AM) for IP₃Rs is confirmed by using Ca²⁺ channel blockers. The RyR blocker dantrolene (20 μM) did not affect overall Ca²⁺ release following IP₃-AM stimulation, whereas the IP₃R antagonist Xestospongin D (10 μM) abolished the response (n=2). Data is shown as mean ± s.d. (D) Subcellular analysis and quantification revealed a significant 32% increase in Ca²⁺ release into the nucleoplasm from membranes of the nuclear envelope (n=6, P<0.0%). No difference in Ca²⁺ release was found in the cytosol (n=6, P=0.84). Data is presented as peak normalized change in fluorescence (F/F₀). (E) The slope of the rising phase of the Ca²⁺ transient (20–80% of the peak) did not change in the presence of tBHP, suggesting the normal complement of IP₃Rs. * P<0.05. Scale bar: 10 μM.

Figure 3. Selective up-regulation of IP₃R2s following oxidative stress

(A) Specific immunoreactivity for IP₃R2s is increased following overnight (16–20 hrs.) treatment with 10 μM tBHP. Scale bar 25 μm. (B) Microfluorimetric analysis reveals a significant 62% increase of specific IP₃R2 immunoreactivity following 10 μM tBHP treatment, but not 2 μM or control. We observed a small, non-significant increase of immunoreactivity in the cytosol (n=3, P<0.05). (C) Using Taqman chemistry, we observed a three-fold increase in IP₃R2 mRNA levels following 10 μM tBHP oxidative stress for overnight (n=3, P<0.01). A modest increase following 2 μM treatment did not reach statistical significance. The endogenous control, mouse β-actin, did not change in response to oxidative stress. A representative experiment is shown. (D) Representative example of immunoblotting of total protein lysate against IP₃R2s shows an increase of specific IP₃R2 immunoreactivity at the expected size of around 270 kDa. Mouse β-actin was used as endogenous control. Quantification of three separate experiments revealed a statistically significant doubling of IP₃R2-specific signal. * P<0.05; ** P<0.01.

Figure 4. Absence of IP₃R1 and IP₃R3 involvement

(A/B) Immunocytochemistry (left) and microfluorimetric analysis (right) did not reveal any changes in specific immunoreactivity for IP₃R1s ($n=3$, $P=0.81$) and IP₃R3s ($n=3$, $P=0.92$). Scale bar: 25 μ M. (C/D) Similarly, immunoblotting against IP₃R1 and IP₃R3 was similar following oxidative stress, compared with control. Representative blots are shown. Mouse β -actin was used as endogenous control. Quantification of three separate experiments revealed no statistically significant difference between sham and tBHP treatments.

Table 1

Effect of tBHP-induced oxidative stress on mRNA levels of intracellular Ca^{2+} channels.

Target name	Gene name	Mean C_T	Normalized expression level following 10 μM tBHP	Statistics
type 1 IP3R	ITPR1	27.22 ± 0.30	1.11 ± 0.18	n=3, P=0.31
** type 2 IP3R	ITPR2	29.92 ± 0.11	3.12 ± 0.26	n=3, P<0.01
type 3 IP3R	ITPR3	26.98 ± 0.12	1.07 ± 0.14	n=3, P=0.73
type 1 RyR	RyR1	25.92 ± 0.06	0.99 ± 0.10	n=3, P=0.68
type 2 RyR	RyR2	not detectable	not detectable	
type 3 RyR	RyR3	34.87 ± 0.24	1.12 ± 0.19	n=3, P=0.86
polycystin-2	PKD2	24.92 ± 0.10	0.94 ± 0.11	n=3, P=0.51

IP3Rs and pc-2 are abundantly expressed in HT-22 cells. Of the RyRs, only type 1 RyRs are expressed at significant levels, whereas mRNA levels for type 3 RyRs are very low. Type 2 RyR expression was beyond the detection threshold of our assay. Overnight exposure (16–20 hrs.) to 10 μM tBHP selectively increased the mRNA levels of IP3R2s (see also Fig. 3C). Expression levels for all other intracellular Ca^{2+} channels were similar between vehicle and oxidative stress conditions.