USE OF HYDROGEN PEROXIDE AND PEROXYL RADICALS TO INDUCE OXIDATIVE STRESS IN NEURONAL CELLS

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ABSTRACT

Reactive oxygen species accumulation has an established role in aging-related diseases, particularly in neurodegenerative diseases; however, its role remains incompletely elucidated. Considering the increasing elderly population, especially in developed countries, proper management of aging-related diseases has become essential and, research on antioxidant therapies is flourishing. Neuronal cells are at the center of oxidative stress research, where studies are being conducted to develop preventive or curative treatments against neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. A panel of pro-oxidants can be used to induce oxidative damage in neuronal cells *in vitro*. In this mini-review, the use of hydrogen peroxide and 2,2'-azobis (2-amidinopropane) dihydrochloride -generated peroxy radicals to induce oxidative stress in neuronal cells is discussed.

Keywords: Aging, Hydrogen peroxide, Neuronal cells, Oxidative stress, Peroxyl radical.

Introduction

Free radicals were first reported in 1900 by Moses Gomberg, and linked to aging 56 years later when Denham Harman (1956) proposed that free radicals were a vital factor in the aging process (Friedman, 2011a; Lushchak, 2014). Today, the role of free radicals and oxidative stress in aging-related disease is widely recognized and largely studied. Oxidative stress-induced damage to biomolecules is a central feature of the pathology of a broad spectrum of human diseases including neurodegenerative diseases, cardiovascular diseases, diabetes, and cancer (Halliwell, 2005; Cheli and Baldi, 2011).

Oxidative stress in living cells is defined as the imbalance between the oxidants generated by cellular respiration and metabolism, and the cell's antioxidant defense system in favor of the oxidants. Reactive oxygen species (ROS) are continuously generated by the mitochondrial electron-transport chain. In healthy cells, ROS levels are under homeostatic control by the endogenous antioxidant defense system that includes biological antioxidants such as glutathione (GSH), vitamin E, and vitamin C and antioxidant enzymes including catalase, superoxide dismutase (SOD), glutathione peroxidase (GPx), and heme-oxygenase I (HO-1). Thus, oxidative stress occurs when levels of ROS generated exceed the cellular antioxidant capacity (Finkel and Holbrook,

2000; Klein and Ackerman, 2003; Cheli and Baldi, 2011).

Neuronal cells are more sensitive to oxidative stress than other cells for a number of reasons, including their high rate of oxygen consumption, which generates a significant amount of ROS, and a modest antioxidant defense. Neuronal cell membranes are also rich in polyunsaturated fatty acids (PUFAs), which makes them highly sensitive to oxidation by extracellular ROS (Friedman, 2011b).

Considering the sensitivity of neuronal cells to oxidative stress and its implication in neurodegenerative disease, many have attempted to determine the mechanism of ROS-induced oxidative damage and the protective effect of antioxidants. Such studies often use primary neuronal cells cultures or neuronal cell lines and oxidative stress is induced *in vitro* using oxidants such as H₂O₂ and free radical initiators as a source of peroxy radicals. Neurotoxins such as 6-hydroxydopamine (6-OHDA) (Lopes *et al.*, 2010) or secondary lipid peroxidation products such as aldehyde-4-hydroxy-2-nonenal (HNE) (Schneider *et al.*, 2011) have also been used to mimic the oxidative stress observed in neurodegenerative disease.

This review will focus on the use of H₂O₂ and the free radical-generating azo compound 2,2' - azobis (2-amidinopropane) dihydrochloride (AAPH) to induce oxidative stress in neuronal cells. The mechanisms of action and uses of both oxidants will be discussed.

H₂O₂-Induced Oxidative Stress in Neuronal Cells

In eukaryotic organisms, ROS are continuously produced by the mitochondrial electron transport chain where molecular oxygen is reduced to O₂^{•−} due to the escape of an active electron. As illustrated in Figure 1, the generated O₂^{•−} is spontaneously or enzymatically converted to H₂O₂. Thereafter, H₂O₂ can be converted to OH[•] and OH[−] by accepting an electron in a reaction catalyzed by transition metal ions (Fe²⁺ or Cu⁺). The generated hydroxyl radicals are highly reactive and are believed to cause significant oxidative damage. In order to prevent the formation of these harmful hydroxyl radicals, H₂O₂ is converted to water via reactions catalyzed by enzymes such as catalase and GPx (Lushchak, 2014). The intracellular concentration of H₂O₂ is therefore tightly controlled by the cellular antioxidant defense system. The homeostatic concentration of H₂O₂ ranges between 1 and 700 nM, and an intracellular concentration above 1 μM is considered to induce oxidative stress (Gülden *et al.*, 2010).

H₂O₂ is therefore used as a model to investigate the oxidative stress mechanisms within neuronal cells. Whittemore *et al.* (1995) showed that exposure to low concentrations of H₂O₂ (10-100 μM) induced apoptosis of neuronal cells. Specifically, they reported that exposure to 30 μM H₂O₂ for only 3 h induced nuclear changes characteristic of apoptosis, including chromatin condensation,

nuclear pyknosis, and fragmentation.

There is wide variation in the H₂O₂ concentration thought to be cytotoxic, with estimates ranging from 10 to 1000 μM. We collected estimates from recent studies that investigated H₂O₂-induced oxidative stress in PC-12 and SH-SY5Y cells; some examples are given in Table 1. PC-12 is a rat adrenal pheochromocytoma cell line, and the human neuroblastoma SH-SY5Y cell line is a cloned subline of SK-N-SH cells originally derived from a bone marrow neuroblastoma. Both cell lines are often used in studies of neurodegenerative disease, owing to their neuronal cell-like properties (Xie *et al.*, 2010; Cheng *et al.*, 2013). According to Gülden and co-workers (2010), differences in cell plating density contribute to the variation in H₂O₂ cytotoxic concentration reported in different studies. They showed that the short-term exposure of high-density C6 glioma cells in culture to high concentrations of H₂O₂ is equivalent to the long-term exposure of a low-density cell culture to a low H₂O₂ concentration. Thus, they recommended that the H₂O₂ concentration used for cytotoxicity studies be reported as μmol/10⁷ cells or μmol/mg cell protein rather than μmol/L. However, the cell density used for experiments is often omitted in papers, which makes it difficult to compare data obtained from different studies.

It is important to consider the role of metabolic H₂O₂ in redox

Fig.1. ROS generation and neutralization in neuronal cells and their role in neurodegenerative diseases. ROS, reactive oxygen species; mito-ETC, mitochondrial electron transport chain; SOD, superoxide dismutase; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione.

Table 1 Variation in H₂O₂-induced cytotoxicity in rat adrenal pheochromocytoma (PC-12) and human neuroblastoma (SH-SY5Y) cell lines.

Cell line	H ₂ O ₂ Concentration	Treatment duration	Cell viability	Reference
PC-12 cells	100 μ M	24 h	~50%	Jin <i>et al.</i> (2013)
	200 μ M	24 h	~40%	Lou <i>et al.</i> (2012)
	300 μ M	24 h	~50%	Cheng <i>et al.</i> (2013)
	300 μ M	24 h	~60%	Pavlica and Gebhardt (2010)
	400 μ M	24 h	~50%	Crispo <i>et al.</i> (2010)
	400 μ M	1 h	~70%	Liu <i>et al.</i> (2011)
SH-SY5Y cells	1 mM	16 h	~15%	Ju <i>et al.</i> (2012)
	1 mM	16 h	~25%	Ruffels <i>et al.</i> (2004)
	750 μ M	16 h	~80%	Ruffels <i>et al.</i> (2004)
	200 μ M	24 h	~70%	Nakano <i>et al.</i> (2011)
	200 μ M	24 h	~60%	Xiao <i>et al.</i> (2013)
	200 μ M	24 h	~50%	Zhang <i>et al.</i> (2007)
	100 μ M	24 h	~65%	Zhang <i>et al.</i> (2007)
	100 μ M	24 h	~40%	Suematsu <i>et al.</i> (2011)

signaling. H₂O₂ is being increasingly recognized as a messenger molecule. It is involved in signaling pathways involved in changes to cell shape, the formation of actomyosin, and the recruitment of immune cells (Sies, 2014). Oxidative stress induced by H₂O₂ was first thought to cause only lipid peroxidation, DNA and protein damage, but it is now known that H₂O₂ activates various intracellular signaling pathways closely associated with neuronal cell death and survival (Ruffels *et al.*, 2004). The cellular response to H₂O₂ depends on its intracellular concentration: low levels (3-15 μ M) stimulate growth, whereas increasing concentrations induce cell growth arrest, apoptosis, and finally necrosis (at levels above 1 mM) (Gülden *et al.*, 2010). The turning point between signaling and toxicity is still to be determined and may vary depending on the cell type and metabolic conditions (Saito *et al.*, 2006; Sies, 2014). Therefore, choosing the appropriate experimental conditions when using H₂O₂ to study oxidative damage (and potential protective treatments) is paramount.

AAPH-Induced Oxidative Stress in Neuronal Cells

Azo compounds are commonly used as free radical initiators. AAPH is a hydrophilic radical initiator. As shown in Figure 2, AAPH decomposes spontaneously at 37°C to yield molecular nitrogen and two carbon radicals, R[•]. The generated radicals react rapidly with oxygen to form peroxy radical ROO[•] (Niki, 1990; Werber *et al.*, 2011). Peroxy radicals were initially considered the predominant radical generated by AAPH, but Werber and co-workers (2011) recently showed by using LC-MS/MS that the predominant radical species generated by AAPH is in fact alkoxyl

radical RO[•]. Although ROO[•] must be produced to generate RO[•], the researchers postulate that it is short-lived in solution.

While AAPH has been used to induce oxidative stress in neuronal cell cultures, it is less commonly used than H₂O₂. AAPH was reported to induce the oxidation of membrane lipids and proteins (Niki, 1990). PUFAs are the most oxygen-sensitive constituents of cells, and the lipid peroxy radicals (LOO[•]) they produce are very reactive and have been associated with the development of neurodegenerative diseases (Spiteller, 2006; Friedman, 2011a). Lipid peroxidation is involved in AAPH-induced oxidative damage in PC-12 cells, resulting in both apoptosis and necrosis, as reported by Piga *et al.* (2007). This study also indicated that AAPH-induced apoptosis occurs via a caspase-independent pathway, such as the mitochondrial apoptosis-inducing factor (AIF) pathway. AAPH was also reported to induce DNA damage (Kim *et al.*, 2012) but it is unclear if such damage is directly induced by peroxy radicals or if intermediate products are involved.

Ca²⁺ homeostasis is an important factor in protecting neuronal cells and preserving their functionality (Sharma *et al.*, 2014). The treatment of synaptic plasma membranes from rat brains with AAPH *in vitro* was shown to inactivate the plasma membrane Ca²⁺ ATPase (PMCA) by PMCA protein oxidation and aggregation (Zaidi and Michaelis, 1999). Thus, loss of Ca²⁺ homeostasis may contribute to AAPH-induced neuronal cell death.

Unlike H₂O₂, which can be transported across the cell membrane via simple diffusion or aquaporins (Sies, 2014) and induce different signaling pathways, AAPH generates peroxy and alkoxyl radicals that react with cell membrane components (primarily PUFAs),

Fig.2. AAPH thermal degradation scheme. This figure is a simplified version of the reaction scheme published by Werber *et al.* (2011). XH and X'H refer to hydrogen donors in solution.

initiating an oxidative chain reaction resulting in cell death.

Conclusion

Since being associated with aging-related diseases, oxidative stress has been extensively studied with a view to developing preventive and curative treatments for such diseases. However, the exact role of ROS in inducing oxidative damage is still not completely elucidated which makes it more difficult to interpret the precise mechanism of action of antioxidants in protecting cells against oxidative stress.

As reviewed here, oxidative stress depends on the nature of the oxidant and its concentration in the cell. Lushchak (2014) classified oxidative stress based on its intensity, ranging from basal oxidative stress to high-intensity oxidative stress. He proposed that this classification be used to describe experimental data in oxidative stress studies. Moreover, in a more recent commentary, Sies (2015) emphasized the importance of specifying the exact molecular condition used to induce oxidative stress, as the cell response depends on the nature of the oxidant, its

concentration, and the cell culture conditions.

Further studies regarding the interactions between ROS and cell components, and the mechanisms of the resulting cellular signaling and damage, are crucial for the development of novel antioxidant therapies for aging-related diseases.

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