Neurochemistry International 92 (2016) 1–12

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/nci

Real-time quantification of oxidative stress and the protective effect of nitroxide antioxidants

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ARTICLE INFO

Article history:
Received 28 August 2015
Received in revised form
28 October 2015
Accepted 10 November 2015
Available online 22 November 2015

Keywords:
Antioxidant
Ischemia
Retina
Nitroxide
Neuroprotection
Radical

ABSTRACT

Nitroxides have been exploited as profluorescent probes for the detection of oxidative stress. In addition, they deliver potent antioxidant action and attenuate reactive oxygen species (ROS) in various models of oxidative stress, with these results ascribed to superoxide dismutase or redox and radical-scavenging actions. Our laboratory has developed a range of novel, biostable, isoindoline nitroxide-based antioxidants, DCTEIO and CTMIO. In this study we compared the efficiency of these novel compounds as antioxidant therapies in reducing ROS both *in vivo* (rat model) and *in vitro* (661W photoreceptor cells), with the established antioxidant resveratrol. By assessing changes in fluorescence intensity of a unique redox-responsive probe in the rat retina *in vivo*, we evaluated the ability of antioxidant therapy to (1) *ameliorate* ROS production and (2) *reverse* the accumulation of ROS after complete, acute ischemia followed by reperfusion (I/R). I/R injury induced a marked decrease in fluorescence intensity over 60 min of reperfusion, which was successfully ameliorated with each of the antioxidants. DCTEIO and CTMIO reversed the accumulation of ROS when administered intraocularly *post* ischemic insult, whereas, the effect of resveratrol was not significant. We also investigated our novel agents' capacity to prevent ROS-mediated metabolic dysfunction in the 661W photoreceptor cell line. Cellular stress induced by the oxidant, *tert*-butyl hydroperoxide, resulted in a loss of spare mitochondrial respiratory capacity (SMRC) and in the extracellular acidification rate in 661W cells. DCTEIO antioxidant administration successfully reduced the loss of SMRC. Together, these findings show we can quantify dynamic changes in cellular oxidative status *in vivo* and suggest that nitroxide-based antioxidants may provide greater protection against oxidative stress than the current state-of-the-art antioxidant treatments for ROS-mediated diseases.

1. Introduction

Changes to the redox status of biological systems have been implicated in the pathogenesis of numerous neurodegenerative diseases (Aliev et al., 2013; Ray et al., 2014; Schwarz et al., 2014), including the major visual degenerative disease, glaucoma (Almasieh et al., 2012; Chrysostomou et al., 2013; Yuki et al., 2011). The mechanisms underlying retinal degeneration in glaucoma are complex, however, vascular insufficiency, hypoxic-ischemic injury, mitochondrial dysfunction, reactive oxygen species (ROS) and oxidative stress are considered primary factors (Almasieh et al., 2012; Beal, 1995; Yuki et al., 2011). ROS are natural by-products of aerobic respiration by the mitochondria and at low levels under normal conditions are essential signalling molecules in many

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http://dx.doi.org/10.1016/j.neuint.2015.11.003
0197-0186/

Abbreviations

ROS	reactive oxygen species
I/R	ischemia-reperfusion
PFN	profluorescent nitroxide
ME-TRN	methyl ester tetraethylrhodamine nitroxide
SOD	superoxide dismutase
DCTEIO	5,6-dicarboxy-1,1,3,3-tetraethylisoindolin-2-yloxyl
CTMIO	5-carboxy-1,1,3,3-tetramethylisoindolin-2-yloxyl
OCR	oxygen consumption rate
SMRC	spare mitochondrial respiratory capacity
ECAR	extracellular acidification rate
DMSO	dimethyl sulphoxide
i.p.	intraperitoneal
i.o.	intraocular
IOP	intraocular pressure
*t*BuOOH	*tert*-butyl hydroperoxide
FCCP	carbonyl cyanide 4-trifluoromethoxy-phenylhydrazone

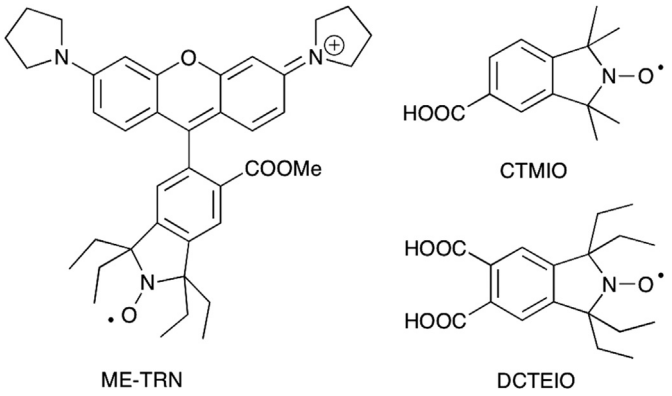

Fig. 1. Structures of the nitroxide compounds used in this study. Redox-responsive fluorescent probe, methyl ester tetraethylrhodamine nitroxide (ME-TRN); antioxidants 5-carboxy-1,1,3,3-tetramethylisoindolin-2-yloxyl (CTMIO) and 5,6-dicarboxy-1,1,3,3-tetraethylisoindolin-2-yloxyl (DCTEIO).

physiological processes, including redox homeostasis and cellular signal transduction (Cui et al., 2012; Dröge, 2002; Halliwell, 1991; Tezel, 2006; Wang et al., 2013). However, in times of environmental stress, such as ischemic-reperfusion injury (I/R), ROS production overwhelms the cell's intrinsic antioxidant capacity i.e. oxidative stress, which may induce irreversible damage to vital cellular components including mitochondria, resulting in cell death (Cutler, 1984; Jarrett et al., 2008; Tezel, 2006). Experimental neuronal cell death can be prevented, at least in part, by reducing the toxicity of the environment with exogenous substances, possibly by altering the apoptotic cell death cascade (Chidlow et al., 2002; Dilsiz et al., 2006; Li et al., 2009; Maher and Hanneken, 2005; Vidal-Sanz et al., 2000).

Mitochondrial dysfunction is considered an essential component in ROS-mediated neurodegeneration (Chrysostomou et al., 2013; Osborne and del Olmo-Aguado, 2013), with recent studies suggesting this may be due to a loss in spare mitochondrial respiratory capacity (SMRC) (Perron et al., 2013). Mitochondrial dysfunction can result from oxidative modification of the respiratory chain complexes, reducing ATP and increasing ROS-production, ultimately amplifying and promoting further oxidative damage (Reily et al., 2013). The mitochondria are particularly vulnerable to reactive lipid species (formed by lipid peroxidation) (Higdon et al., 2012a), which induce the formation of superoxide and hydrogen peroxide, resulting in bioenergetic dysfunction, cellular stress and mitophagy/autophagy (Higdon et al., 2012b; Landar et al., 2006). To understand the overall contribution of ROS and mitochondrial dysfunction to cellular damage and to produce targeted compounds that provide the most effective therapeutic strategies, techniques capable of detecting and quantifying ROS *in vivo* and in real-time, are essential.

Fluorescent redox-responsive probes, particularly fluorophores covalently linked to nitroxide radicals (i.e. profluorescent nitroxide probes, PFN), have emerged as important, versatile analytical tools for the detection of oxidative stress (Blinco et al., 2011; Blough and Simpson, 1988; Hirosawa et al., 2012; Morrow et al., 2010; Yapici et al., 2011). We have developed and applied a novel, reversible, PFN probe molecule based on the rhodamine class of fluorescent dyes (methyl ester tetraethylrhodamine nitroxide, ME-TRN, Fig. 1) (Rayner et al., 2014), to an established model of complete, acute I/R injury: an *in vivo*, pro-oxidant condition known to generate ROS (Tong et al., 2012). This unique, reversible property of the nitroxide moiety allows the probe to shuttle between the reduced form (called a hydroxylamine, generated by the normal metabolism of healthy cells) and the more oxidized nitroxide free radical form (driven by ROS accumulation), turning the probe 'on' and 'off' respectively (Blough and Simpson, 1988; Green et al., 1990). Being rhodamine based and hence positively charged, this probe is selectively accumulated by the mitochondria (Johnson et al., 1980; Morrow et al., 2010). The fluorescence signal is therefore a direct representation of the retinal cells responding to changes in their cellular environment, providing a real-time insight into mechanisms underlying diseases of oxidative stress in the retina.

Nitroxide chemistry can also be exploited to delivery potent antioxidant action. Nitroxides are cell permeable, stable, free radical scavengers that have multiple biological effects (Blinco et al., 2011; Hahn et al., 1994; Kajer et al., 2014). They have shown great potential as protective agents against oxidative damage *in vivo* (Gelvan et al., 1991; McDonald et al., 1999; Rak et al., 2000; Wilcox and Pearlman, 2008) and *in vitro* (Hosokawa et al., 2004; Liang et al., 2005; Lipman et al., 2006; Mohsen et al., 1995; Samuni et al., 2002) as they are able to react with and detoxify harmful radical species. Nitroxides have been purported to act either as superoxide dismutase (SOD) mimetics, catalyzing the dismutation of superoxide anion (Krishna and Samuni, 1994; Samuni et al., 1988) or reversible or irreversible radical scavengers (e.g. carbon, oxygen, nitrogen, sulphur, and protein radicals) (Krishna and Samuni, 1994; Lam et al., 2008; Linares et al., 2008; Pattison et al., 2012), removing free radicals by reacting directly with ROS or by oxidizing the reduced metals thus inhibiting the Fenton and metal-catalyzed Haber–Weiss reactions (reactions key to the oxidation of membrane lipids and amino acids) (Mohsen et al., 1995). The terminal products produced from the reaction of nitroxides with free radicals (hydroxylamine derivatives, aldehydes and amines) are also less damaging to cells than the free radicals themselves (Chamulitrat et al., 1993; Kotake and Janzen, 1991), potentially making them ideal therapeutic antioxidants.

As nitroxides can modulate a wide range of metabolic processes and deliver therapeutic potential, a number of studies have examined possible structural modifications that afford greater biological stability (Kajer et al., 2014; Mitchell et al., 2003; Soule et al., 2007; Wilcox and Pearlman, 2008). Our study is novel in that we have recently developed a range of chemically modified, isoindoline nitroxide-based, antioxidant compounds: 5,6-dicarboxy-1,1,3,3-tetraethylisoindolin-2-yloxyl (DCTEIO) and 5-