Redox Biology 4 (2015) 355–362

Contents lists available at ScienceDirect

Redox Biology

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

Research Paper

Antihypertensive effect of mitochondria-targeted proxyl nitroxides

Anna E. Dikalova [a], Igor A. Kirilyuk [b,c], Sergey I. Dikalov [a,*]

[a] Vanderbilt University Medical Center, 2200 Pierce Avenue, Nashville, TN 37232, USA
[b] Novosibirsk Institute of Organic Chemistry, Lavrentieva 9, Novosibirsk 630090, Russia
[c] Novosibirsk State University, Pirogova 2, Novosibirsk 630090, Russia

ARTICLE INFO

Article history:
Received 19 December 2014
Accepted 22 December 2014
Available online 24 December 2014

Keywords:
Mitochondria
Antioxidant
Superoxide
Endothelial cells
Nitroxide
Hypertension

ABSTRACT

Superoxide ($O_2^{-\bullet}$) has been implicated in the pathogenesis of many human diseases including hypertension. Mitochondria-targeted superoxide scavenger mitoTEMPO reduces blood pressure; however, the structure–functional relationships in antihypertensive effect of mitochondria-targeted nitroxides remain unclear. The nitroxides are known to undergo bioreduction into hydroxylamine derivatives which reacts with $O_2^{-\bullet}$ with much lower rate. The nitroxides of pyrrolidine series (proxyls) are much more resistant to bioreduction compared to TEMPOL derivatives suggesting that mitochondria-targeted proxyls can be effective antioxidants with antihypertensive activity. In this work we have designed and studied two new pyrrolidine mitochondria targeted nitroxides: 3-[2-(triphenyphosphonio)acetamido]- and 3-[2-(triphenyphosphonio) acetamidomethyl]-2,2,5,5-tetramethylpyrrolidine-1-oxyl (mCP2) and (mCP1). These new mitochondria targeted nitroxides have 3- to 7-fold lower rate constants of the reaction with $O_2^{-\bullet}$ compared with mitoTEMPO; however, the cellular bioreduction of mCP1 and mCP2 was 3- and 2-fold slower. As a consequence incubation with cells afforded much higher intracellular concentration of mCP1 and mCP2 nitroxides compared to mitoTEMPO nitroxide. This has compensated for the difference in the rate of $O_2^{-\bullet}$ scavenging and all nitroxides similarly protected mitochondrial respiration in H_2O_2 treated endothelial cells. Treatment of hypertensive mice with mCP1 and mCP2 (1.4 mg/kg/day) after onset of angiotensin II-induced hypertension significantly reduced blood pressure to 133 ± 5 mmHg and 129 ± 6 mmHg compared to 163 ± 5 mmHg in mice infused with angiotensin II alone. mCP1 and mCP2 reduced vascular $O_2^{-\bullet}$ and prevented decrease of endothelial nitric oxide production. These data indicate that resistance to bioreduction play significant role in antioxidant activity of nitroxides. Studies of nitroxide analogs such as mCP1 and mCP2 may help in optimization of chemical structure of mitochondria-targeted nitroxides for improved efficacy and pharmacokinetics of these drugs in treatment of hypertension and many other conditions including atherosclerosis, diabetes and degenerative neurological disorders in which mitochondrial oxidative stress seems to play a role.

Introduction

Clinical data show that 26% of adult population has hypertension [1]. This disease represents a major risk factor for stroke, myocardial infarction, and heart failure [2]. Hypertension is a multifactorial disorder involving perturbations of the vasculature, the kidney and the central nervous system [3]. Despite treatment with multiple drugs, 37% of hypertensive patients remain hypertensive [4], likely due to the mechanisms contributing to blood pressure elevation that are not affected by current treatments. New classes of antihypertensive agents could therefore add to the currently available therapeutic armamentarium to improve treatment of hypertension.

In the past decade it has become clear that vascular superoxide ($O_2^{-\bullet}$) production contributes to hypertension [5]. In almost all experimental models of hypertension $O_2^{-\bullet}$ production is increased in multiple organs, including vasculature where $O_2^{-\bullet}$ promote vasoconstriction and remodeling, increasing systemic vascular resistance. In the past several years, we have shown that the mitochondria become dysfunctional in hypertension and have defined novel role of mitochondrial $O_2^{-\bullet}$ in this disease [6,7]. The mitochondria are an important source of $O_2^{-\bullet}$ and we have shown that scavenging mitochondrial $O_2^{-\bullet}$ improves endothelial function and attenuates hypertension [6].

Common antioxidants like ascorbate and vitamin E have proven ineffective in preventing cardiovascular diseases and hypertension [8]. These agents unlikely reach important sites of ROS production such as the mitochondria. Experimental studies have shown an important role of mitochondrial reactive oxygen species in the development of endothelial dysfunction, hypertension and

* Correspondence to: Division of Clinical Pharmacology, VUMC, 2200 Pierce Avenue, PRB 554, Nashville, TN 37232-6600, USA.
E-mail address: sergey.dikalov@Vanderbilt.edu (S.I. Dikalov).

http://dx.doi.org/10.1016/j.redox.2014.12.012

atherosclerosis [9,10]. Indeed, we have shown that SOD2 overexpression attenuates hypertension and treatment of hypertensive mice with mitochondria-targeted antioxidants reduces blood pressure [6,7].

The membrane potential of mitochondria within living cells is negative inside (−140 mV). As this membrane potential is far larger than in other organelles within cells, lipophilic cations such as triphenylphosphonium (TPP) selectively accumulate within mitochondria [11]. Antioxidants conjugated to TPP, therefore can be targeted to mitochondria and may be concentrated in the mitochondrial matrix by 1000-fold [12]. The pharmacology of mitochondria-targeted antioxidants is not well understood. For example, previously described mitoquinone ($MitoQ_{10}$) [12] may have prooxidant and proapoptotic properties due to redox cycling and generation of $O_2^{-\bullet}$ by quinone [13,14]. Nitroxides are well-known SOD mimetics [15,16]. They are not consumed in the reaction of superoxide dismutation, and this makes their use advantageous over many other antioxidants. Nitroxides have very low toxicity and this makes them perfect candidates for conjugation with lipophilic cations (e.g., triphenylmethylphosphonium) for *in vivo* use [17]. Furthermore, some phosphonium ions may inhibit oxidation of pyruvate, malate, 2-oxoglutarate and glutamate in heart mitochondria at micromolar concentrations [18] suggesting that triphenylmethylphosphonium conjugates should be used at submicromolar concentrations and tested for side effect on respiration. Indeed, low doses of mitoTEMPO did not affect mitochondrial respiration in control cells but improved mitochondrial function in the presence of angiotensin II [6].

Recently, it has been shown that pretreatment of endothelial cells with the mitochondria-targeted SOD mimetic Mito-CP significantly reduces H_2O_2- and lipid peroxide-induced cellular oxidative stress [19]. Mito-CP inhibits peroxide-induced inactivation of complex I and aconitase, while restoring the mitochondrial membrane potential. In contrast, the "untargeted" carboxy proxyl (CP) did not protect the cells from peroxide-induced oxidative stress and apoptosis. However, in Mito-CP nitroxide is connected to triphenylmethylphosphonium cation via ester spacer and ester group can be potentially hydrolyzed to give inactive 3-carboxyproxyl (CP).

The main disadvantage of nitroxides is their rapid reduction with cellular antioxidants and enzymatic systems. For example, mitoTEMPO is readily reduced in mitochondria to corresponding hydroxylamine [20]. Hydroxylamine derivatives react with $O_2^{-\bullet}$ much slower compared with nitroxides [6] therefore bioreduction of nitroxides can significantly reduce antioxidant potentials of nitroxides.

We have hypothesized that increased resistance to bioreduction will be beneficial to mitochondria-targeted nitroxides. The structure-functional relationships in antioxidant properties of mitochondria-targeted nitroxides however remain unclear. In this work we have designed and studied two new pyrrolidine mitochondria targeted nitroxides. The nitroxides of pyrrolidine series (proxyls) are weaker oxidants as compared to TEMPOL derivatives, and they demonstrate higher stability in biological samples [21]. We hypothesized that mitochondria targeted proxyl derivatives are much more resistant to bioreduction compared to TEMPOL and this may improve antioxidant and antihypertensive properties of new nitroxide derivatives. In this work we have designed two new pyrrolidine mitochondria targeted nitroxides mCP1 and mCP1 (Fig. 1), and studied their antioxidant properties, cellular accumulation, their bioreduction and antihypertensive properties.

Materials and methods

Reagents

Xanthine oxidase was purchased from Roche Molecular Biochemicals (Indianapolis, IN). All other reagents were obtained from Sigma (St Louis, MO).

Synthesis of mCP1 and mCP2

3-Amino-PROXYL (**1a**) and 3-aminomethyl-PROXYL (**1b**) were prepared according to the literature methods described by Rozantsev and Hankowsky [22,23]. The nitroxides mCP1 and mCP2 were synthesized according to Fig. 2 similarly to the procedure previously described for mitoTEMPO (mT) [24].

3-((2-Chloroacetamido)methyl)-2,2,5,5-tetramethylpyrrolidin-1-oxyl (**2b**). A solution of 3-aminomethyl-PROXYL (0.9 g or 0.005 mol) and dry triethylamine (1.7 ml or 0.0117 mol) in dry chloroform (20 ml) was placed into 50 ml flat-bottom flask and cooled to − 5 °C. The cold solution was placed into ice bath and chloroacetyl chloride (0.42 ml or 0.005 mol) was added dropwise upon stirring. The resulting dark solution was washed with water (3 × 5 ml), and dried with $MgSO_4$. The chloroform was removed under reduced pressure and the residue was separated by column chromatography (Kieselgel 60, Merck, eluent chloroform) to give **2b**. Yellow oil, Found: C, 53.06; H, 8.08; N, 11.56; Cl 14.10. Calculated for $C_{11}H_2ON_2O_2Cl$: C, 53.33; H, 8.14; N, 11.31; Cl 14.31 %; ν_{max} (KBr)/см$^{-1}$ 3306 br, 3084 br, 2972, 2934, 2872, 1668, 1543, 1462, 1364, 1313, 1252, 1178, 1159, 1107, 1057, 789, 764, 691 br, 600, 571, 525, 476.

Similarly, 3-(2-chloroacetamido)-2,2,5,5-tetramethylpyrrolidin-1-oxyl (**2a**) have been prepared from 3-amino-PROXYL (**1a**), (this compound was first described in [Mao-Man-Jun; Tian, Xuan; Chen, Yao-Zu; Gaodeng Xuexiao Huaxue Xuebao (1998), 19(3), 395–398.], but this publication is not available to authors): yellow crystals, m.p. 130–131°C (hexane-ethyl acetate). Found: C, 51.31; H, 7.66; N, 11.87; Cl 15.10. Calculated for $C_{10}H_{18}N_2O_2Cl$: C, 51.30; H, 7.76; N, 11.99; Cl 15.17 %; ν_{max} (KBr)/см$^{-1}$ 3319, 3076 br, 2982, 2938, 2876, 1676, 1553, 1485, 1408, 1366, 1331, 1299, 1259, 1229, 1196, 1165, 1111, 1045, 806, 772, 687 br, 572, 555, 547, 530, 500.

3-[(2-(Triphenyphosphonio)acetamido)methyl]-2,2,5,5-tetramethylpyrrolidin-1-oxyl (mCP1). The **2b** (1 g, 0.004 mol) was placed into 50 ml flask containing toluene (15 ml) and triphenylphosphine (2.7 g, 0.01 mol). The mixture was heated to reflux under nitrogen for 15 hours and then cooled in ice bath the

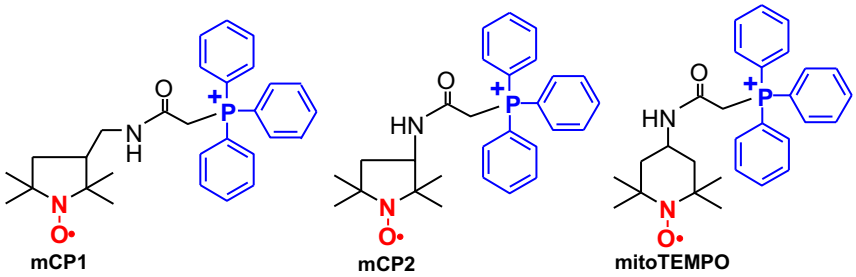

Fig. 1. Structures of mitochondria-targeted nitroxides.