Tempol protects blood proteins and lipids against peroxynitrite-mediated oxidative damage

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Abstract

Oxidative stress is characterized by excessive production of various free radicals and reactive species among which, peroxynitrite is most frequently produced in several pathological conditions. Peroxynitrite is the product of the superoxide anion reaction with nitric oxide, which is reported to take place in the intravascular compartment. Several studies have reported that peroxynitrite targets red blood cells, platelets and plasma proteins, and induces various forms of oxidative damage. This *in vitro* study was designed to further characterize the types of oxidative damage induced in platelets and plasma proteins by peroxynitrite. This study also determined the ability of tempol to protect blood plasma and platelets against peroxynitrite-induced oxidative damage. The ability of various concentrations of tempol (25, 50, 75, and 100 μ M) to antagonize peroxynitrite-induced oxidation was evaluated by measuring the levels of protein carbonyl groups and thiobarbituric-acid-reactive substances in experimental groups. Exposure of platelets and plasma to 100 μ M peroxynitrite resulted in an increased levels of carbonyl groups and lipid peroxidation ($P < 0.05$). Tempol significantly inhibited carbonyl group formation in plasma and platelet proteins ($P < 0.05$). In addition, tempol significantly reduced the levels of lipid peroxidation in both plasma and platelet samples ($P < 0.05$). Thus, tempol has antioxidative properties against peroxynitrite-induced oxidative damage in blood plasma and platelets.

Keywords: Carbonyl groups, peroxynitrite, plasma, platelets, tempol, thiobarbituric acid

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Introduction

Oxidative stress is associated with many diseases such as diabetes, hypertension, heart failure, dyslipidemia, and atherosclerosis.^{1–4} It is defined as a state in which reactive species and free radicals are in excess of the endogenous antioxidant defenses in the body to the point that cellular homeostasis is disrupted.⁵ Although most of the reactive oxygen species (ROS) and free radicals are produced as by-products of important physiological pathways, they are still involved in several pathological conditions.^{6–8} Furthermore, free radicals and ROS can be generated in living cells by exogenous factors such as ultraviolet light, ionizing radiation, inflammatory cytokines, and environmental toxins.⁹

Peroxynitrite is the most regularly produced oxidant in several pathological conditions⁶ and is the product of the superoxide anion reaction with nitric oxide^{10,11} that occurs in the intravascular compartment.^{6,7} Peroxynitrite decomposes to yield three highly reactive free radicals: the hydroxyl radical, carbonyl radical, and nitrogen dioxide radical.⁶

These potent oxidizing species are capable of inducing cytotoxicity in body cells, tissues, and fluids via protein nitration and carbonylation, lipid peroxidation, and DNA nitration and deamination.^{12,13}

Tempol is a nitroxide antioxidant with properties similar to those of a potent metal-independent superoxide dismutase.¹⁴ However, the membrane permeability and potency of tempol are superior to those of superoxide dismutase.¹⁴ It has been reported that tempol catalytically decomposes the peroxynitrite-derived free radicals, namely, nitrogen dioxide ($\bullet\text{NO}_2$), carbonate ($\bullet\text{CO}_3$), and superoxide (O_2^-) with a rapid rate constant.¹⁵ These properties increase the therapeutic value of tempol in protection against peroxynitrite-mediated oxidative damage. Indeed, tempol has been shown to mitigate peroxynitrite-mediated damage in neural tissues by several studies.^{16–18} In this study, we aimed to explore the ability of tempol to attenuate oxidative damage in the form of protein carbonylation and lipid peroxidation in blood platelets and plasma proteins and lipids exposed to peroxynitrite.

Materials and methods

Samples

The study was approved by the Institutional Research Board at Jordan University of Science and Technology (JUST), and the University Review Committee for Research on Humans at JUST and in accordance with the National Institutes of Health guidelines. Three units of blood were obtained from young non-smoking voluntary donors (age 22–25 years) with no personal history of diabetes, hypertension, atherosclerosis, or dyslipidemia. None of the donors received any pharmacological therapy. The blood samples were collected individually into tubes containing CPDA-1 anticoagulant.

Sample treatment

Blood units were centrifuged at 450g for 6.20 min to obtain platelet-rich plasma. Platelets were then sedimented by centrifugation at 2500g for 5.40 min at room temperature, and the platelet pellet was washed twice with Tyrode's buffer. Finally, the platelets were suspended in Tyrode's buffer, and the platelet count was approximately 10^9 per milliliter of platelet suspension.¹⁹

Aliquots of platelets and plasma were randomly distributed into each of the following experimental groups:

1. Control group: The samples did not receive any further treatment.
2. Peroxynitrite-treated group: Samples were incubated for 20 min at room temperature with 100 μ M peroxynitrite.
3. Tempol+peroxynitrite-treated group: Samples were pre-incubated with 25, 50, 75, or 100 μ M tempol for 2 min, and these aliquots were incubated with peroxynitrite for 20 min at 37°C.
4. Tempol-treated group: Samples were incubated with 25, 50, 75, and 100 μ M tempol at 37°C for 2 min.

Estimation of plasma protein concentration by bicinchoninic acid protein assay and platelet counting

The samples and standards (50 μ L each) were pipetted into duplicate wells in a clear-bottom 96-well plate, and then 100 μ L of working solution was added to each well containing the samples and standards. This method utilizes a copper salt that can be reduced to cuprous ion by the action of proteins. The generated Cu^{2+} ion forms an intensely colored complex with bicinchoninic acid reagent that can be detected on the basis of the absorbance at 562 nm.

Detection of carbonyl group by colorimetry

Each sample (100 μ L) was mixed with dinitrophenylhydrazine at a volume ratio of 1:1 and then vortexed and incubated for 10 min at room temperature. The protein was then precipitated by adding 30 μ L of trichloroacetic acid to each sample. The precipitate was washed three times with 500 μ L of cold acetone and redissolved in guanidine hydrochloride. The supernatant was loaded in a 96-well plate and the

absorbance at 375 nm was measured to estimate the content of carbonyl groups in each sample.

Detection of thiobarbituric acid reactive substances as an index of lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) were detected by colorimetry. Samples and standards (100 μ L each) were added to appropriately labeled 5-mL vials, and 100 μ L of sodium dodecyl sulfate was then added to each vial and swirled to mix, after which 4 mL of the color reagent was added to each vial. The color reagent was a solution containing 530 mg of thiobarbituric acid added to 50 mL of diluted acetic acid (consisted of 40 mL acetic acid and 160 mL HPLC-grade water) and 50 mL of diluted sodium hydroxide (consisted of 20 mL sodium hydroxide and 180 mL HPLC-grade water). This color reagent is stable for 24 h. After addition of the color reagent, the vials were incubated in boiling water for 1 h. After boiling, the vials were immediately removed and placed in an ice bath for 10 min to stop the reaction. The vials were then centrifuged for 10 min at 1600g at 4°C. Finally, 150 μ L of each sample and standard was loaded in duplicate wells and the absorbance was read at 530 nm.

Statistical analysis

Experimental groups were compared using an analysis of variance (ANOVA), followed by Fisher's *post hoc* test. $P < 0.05$ was used to define significant differences between the groups.

Results

Carbonyl group formation

Peroxynitrite treatment (100 μ M) induced a significant increase ($P < 0.05$) in carbonyl group formation in both plasma and platelet proteins (Figures 1 and 2). However, pre-incubating the samples with tempol reduced protein oxidative damage in the form of carbonyl group formation. Tempol, at concentrations of 50, 75, and 100 μ M, significantly reduced ($P < 0.05$) peroxynitrite-mediated carbonyl group formation in plasma proteins (Figure 1). Moreover, 75 and 100 μ M tempol significantly reduced ($P < 0.05$) peroxynitrite-mediated carbonyl group formation in platelet proteins (Figure 2).

TBARS formation

Peroxynitrite treatment (100 μ M) significantly increased ($P < 0.05$) TBARS formation in both plasma and platelet proteins (Figures 3 and 4). However, pre-incubating the samples with tempol reduced lipid peroxidative damage in the form of TBARS formation. Tempol, at all concentrations, significantly reduced ($P < 0.05$) peroxynitrite-mediated TBARS formation in plasma proteins (Figure 3). Moreover, all concentrations of tempol significantly reduced ($P < 0.05$) peroxynitrite-mediated TBARS formation in platelet proteins (Figure 4).

Figure 1 The ability of tempol to reduce peroxynitrite-induced carbonyl group formation in plasma proteins. The results are expressed as nano-moles of protein carbonyl group formation per milligram of protein. The results are representative of three independent experiments done in triplicates and are expressed as means $\pm$ SD. Pn: 100 μ M peroxynitrite, T1: 25 μ M tempol, T2: 50 μ M tempol, T3: 75 μ M tempol, T4: 100 μ M tempol. All tempol-treated groups received 100 μ M peroxynitrite after treatment with tempol. * $P < 0.05$: significant difference compared to control group; ** $P < 0.05$: significant difference compared to peroxynitrite treated group

Figure 2 The ability of tempol to reduce peroxynitrite-induced carbonyl group formation in platelets. The results are expressed as nano-moles of protein carbonyl group formation per milligram of protein. The results are representative of three independent experiments done in triplicates and are expressed as means $\pm$ SD. Pn: 100 μ M peroxynitrite, T1: 25 μ M tempol, T2: 50 μ M tempol, T3: 75 μ M tempol, T4: 100 μ M tempol. All tempol-treated groups received 100 μ M peroxynitrite after treatment with tempol. * $P < 0.05$: significant difference compared to control group; ** $P < 0.05$: significant difference compared to peroxynitrite treated group

Discussion

In this study, we have shown that exposing proteins and lipids in plasma and platelets to exogenous peroxynitrite significantly increases protein oxidation in the form of protein carbonyl formation and significantly increases lipid peroxidative damage in the form of TBARS formation. In addition, our study is the first to show that pre-incubating platelets and plasma with tempol significantly reduces peroxynitrite-mediated oxidative damage to platelets and plasma proteins and lipids. Not only equimolar concentrations but also lower concentrations (25, 50, and 75 μ M) of tempol reduced peroxynitrite-mediated oxidative damage.

Figure 3 The ability of tempol to reduce peroxynitrite-induced thiobarbituric acid reactive substances (TBARS) formation in plasma samples. The results are expressed as micro-molar concentration of TBARS. The results are representative of three independent experiments done in triplicates and are expressed as means $\pm$ SD. Pn: 100 μ M peroxynitrite, T1: 25 μ M tempol, T2: 50 μ M tempol, T3: 75 μ M tempol, T4: 100 μ M tempol. All tempol treated groups received 100 μ M peroxynitrite after treatment with tempol. * $P < 0.05$: significant difference compared to control group; ** $P < 0.05$: significant difference compared to peroxynitrite treated group

Figure 4 The ability of tempol to reduce peroxynitrite-induced thiobarbituric acid reactive substances (TBARS) formation in platelets samples. The results are expressed as micro-molar concentration of TBARS. The results are representative of three independent experiments done in triplicates and are expressed as means $\pm$ SD. Pn: 100 μ M peroxynitrite, T1: 25 μ M tempol, T2: 50 μ M tempol, T3: 75 μ M tempol, T4: 100 μ M tempol. All tempol-treated groups received 100 μ M peroxynitrite after treatment with tempol. * $P < 0.05$: significant difference compared to control group; ** $P < 0.05$: significant difference compared to peroxynitrite treated group

This finding was expected because the antioxidant action of tempol is catalytic. Indeed, previous work on isolated brain mitochondria showed a similar profile where lower concentrations of tempol were effective in reducing the oxidative effects of peroxynitrite.¹⁷ Catalytic scavenging of peroxynitrite-derived radicals by tempol has been suggested.¹⁵ It has also been suggested that tempol reacts with nitrogen dioxide radicals to form a nitrosylated tempol that reacts with water to release nitrite anions. By undergoing this reaction, tempol is converted to tempol peroxide, which reacts with a carbonate radical to induce the release of bicarbonate (HCO_3^-) and a peroxytempol. Two molecules of peroxytempol combine

and then break apart to generate one molecule of oxygen and one molecule of regenerated tempol.¹⁵

Some previous reports indicated that nitroxide compounds such as tempol can act as oxidants at certain concentrations.¹⁶ However, this study provides solid evidence of the antioxidant properties of tempol. Tempol antagonized the oxidative damage inflicted by peroxynitrite at concentration ranges from 25 to 100 μ M. We also compared the level of oxidative markers in control samples with that in samples incubated with tempol alone (data not shown), and found that tempol, at the aforementioned concentrations, has no inherent oxidative properties.

It is worth mentioning that the concentration of peroxynitrite used in our study (100 μ M) falls in a range likely to occur *in vivo*.^{19,20} Our findings were in agreement with previous reports documenting that such concentrations of peroxynitrite are sufficient to induce oxidative damage in blood proteins and lipids.¹⁸ Collectively, our study showed that tempol, especially at the two highest concentrations (75 and 100 μ M) significantly and consistently reduced peroxynitrite-mediated carbonylation and TBARS production in blood platelets and plasma.

This promising outcome for using tempol to antagonize oxidative damage in blood components could be further tested in an *in vivo* experimental design. In fact, it has been documented that intraperitoneal injections of tempol reduced traumatic brain-injury-associated oxidative damage in the neural tissues.¹⁶ Likewise, we could test the ability of tempol injections to reduce oxidative damage in blood components which result from systemic and metabolic disorders such as dyslipidemia, hypertension and diabetes.⁶

Conclusion

Our results demonstrate the antioxidative action of tempol against peroxynitrite-induced oxidative stress in plasma proteins and platelets. We have shown that tempol reduces oxidative damage to blood platelets and plasma components such as proteins and lipids.

Author Contributions: All authors participated in the design, interpretation of the studies, and analysis of the data and review of the manuscript. AGM, MAB, and AQJ conducted the experiment; AGM and MZA supplied critical input to experimental design and data interpretation; AGM and MAB provided statistical analysis and interpretation; AGM, AQJ and MZA were responsible for writing the manuscript, and MZA provided editorial comments.

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