

CONCENTRATION-EFFECT RELATIONSHIP FOR THE RADICAL SCAVENGING ACTIVITY OF TELMISARTAN IN NITRITE-INDUCED HEMOGLOBIN OXIDATION: *In-vitro* STUDY

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ABSTRACT

Several free radical species are generated during the course of nitrite-induced oxidation of hemoglobin. Many chemical substances, including drugs with pleotropic activity demonstrate inherited radical scavenging activity. The present study was designed to evaluate the dose-effect relationship for the radical scavenging activity of the angiotensin receptor blocker telmisartan in models of nitrite-induced oxidation of hemoglobin (Hb) and formation of methemoglobin (MetHb) in hemolysate and intact erythrocytes. Blood samples were obtained from a healthy volunteer for preparation of hemolysate and erythrocytes suspension, and the effect of different concentrations of telmisartan on nitrite-induced oxidation of Hb and MetHb formation was monitored spectrophotometrically at 631 nm. Incubation of both intact erythrocytes and the lysate with different concentrations of telmisartan resulted in a concentration-dependent slowing in the rate of Hb oxidation, as predicted by the increment in the time required to form 50% MetHb. In conclusion, telmisartan protects against nitrite-induced methemoglobin formation in hemolysate and intact erythrocytes in a concentration-dependent manner.

Keywords: Telmisartan, radical scavenger, hemoglobin oxidation.

INTRODUCTION

Free radicals are responsible for cellular damage and are involved in many pathological conditions.^{1,2} The occurrence of oxidative stress may be a consequence of a primary decrease in the antioxidant defense system activity or an elevation of reactive oxygen species (ROS) concentration.^{3,4} When erythrocytes are exposed to oxidative stress, an alteration of the cellular proteins and plasma membranes were observed; the free radicals are responsible for lipid peroxidation and protein oxidation that leads to the formation of hemolytic holes.⁵ Hemoglobin (Hb) is the primary oxygen-transport protein in vertebrate organisms; it can be converted into methemoglobin by multiple pharmacological and chemical insults, including the nitrites⁶, with consequent loss of its oxygen-carrying capacity which may predispose to hypoxia.⁷ Moreover, several free radical species are generated during the course of nitrite-induced oxidation of hemoglobin^{8,9}; the elevated ROS formation in the vascular wall is a key feature of cardiovascular diseases and contributes to endothelial dysfunction and vascular inflammation.^{10,11} Many antihypertensive drugs are proven to have antioxidant properties and the ability to lower oxidative stress^{12,13}, offering good chances for additional cytoprotective effects against free radical-mediated vascular injury.¹⁴ Telmisartan is a non-peptide angiotensin II (ATII) receptor antagonist that selectively

and insurmountably inhibits the angiotensin II AT1 receptor subtype without affecting other receptor systems involved in cardiovascular regulation.¹⁵ Although considerable evidence exists that angiotensin II receptor blockers (ARBs) protect against the oxidative stress of angiotensin II^{16,17} the mechanism of this effect remains unclear. However, telmisartan was reported to inhibit intracellular oxidative stress, at least in part, in a receptor-independent manner, possibly owing to its lipophilic and antioxidant structure.¹⁸ The present study was designed to investigate the free radical scavenging effects of different concentrations of telmisartan in the *in vitro* model of free radical-induced erythrocyte damage.

MATERIALS AND METHODS

Blood sample collection and preparation of lysate

Blood was obtained by vein puncture from healthy volunteers, who are not taking any kinds of drugs and/or supplements that may affect the physiological condition of RBCs, in ethylene diamine tetra-acetic acid (EDTA) tubes. Blood samples were centrifuged at 2500 rpm and 4°C for 10 min to remove the plasma and buffy coat of white cells. The erythrocytes were washed thrice with Phosphate Buffer Saline (PBS; pH 7.4) and lysed by suspending in 20 volumes of 20mM phosphate buffer (PB; pH 7.4) to yield the required hemolysate concentration of 1:20, then hemolysate was centrifuged at 10000 rpm for 10 min and supernatant has been utilized for the study.¹⁹

Effect of different concentrations of telmisartan on nitrite-induced Hb in hemolysate

In-vitro model for oxidation of Hb with sodium nitrite was

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utilized for production of MetHb²⁰ and the antioxidant effect of different concentrations of telmisartan (Boehringer Ingelheim, Germany) was evaluated. To 1.0 ml of freshly prepared hemolysate, 1.0 ml of each concentration of telmisartan (0.0004, 0.004, 0.04, 0.4 mg/ml) was added concomitantly with 1.0 ml sodium nitrite (E. Merck Ag, Darmstadt, Germany) (final concentration 1.0 mM) and the formation of MetHb was monitored spectrophotometrically at 631 nm for 30 minutes using Specord 40 spectrophotometer. Additionally, the effect of telmisartan on MetHb formation at various time intervals from nitrite addition was evaluated. To 1.0 ml of freshly prepared hemolysate, 1.0ml of the highly effective concentration of telmisartan (0.4 mg/ml, as predicted from the previous experiment) was added either 10 min before, or at 5 and 10 min after the addition of sodium nitrite to the hemolysate solution, and the formation of MetHb was monitored spectrophotometrically as mentioned before.

Effect of different concentration of telmisartan on nitrite-induced Hb in intact erythrocytes

Erythrocytes suspension was prepared by mixing a volume of fresh blood with 20 volumes of PBS (pH 7.4). The erythrocyte suspension was incubated with different concentrations of telmisartan (0.004, 0.04, 0.4 mg/ml) for 30 min, followed by addition of sodium nitrite (final concentration 1.8 mM) for further 120 min. The suspension was centrifuged at 2500 rpm for 20 min to remove excess telmisartan and nitrite. The cells were

Table 1. Effect of different concentrations of telmisartan (0.0004, 0.004, 0.04, 0.4 mg/ml) on the time-course of nitrite-induced Hb oxidation and MetHb formation in erythrocytes lysate.

Telmisartan (mg/ml)	% MetHb formation	% MetHb inhibition	Time to form 50% MetHb (t _{1/2}) (min)
Control	100	0	15
0.0004	87	13	19
0.004	45	55	26
0.04	29.5	70.5	42.4
0.4	10.089	89.9	123.89

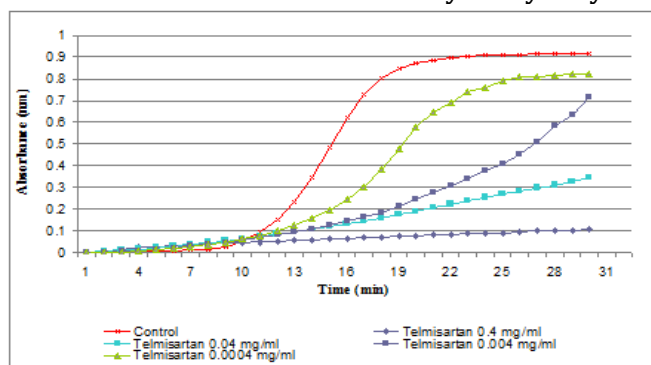
Values represent the mean of three experiments

Table 2. Effect of incubation with telmisartan (0.4 mg/ml) at different time intervals on the time course of Hb oxidation and formation of MetHb in erythrocyte lysate.

Time-course for addition of 0.4 mg/ml telmisartan	% MetHb formation	% MetHb inhibition	Time to form 50% MetHb (t _{1/2}) (min)
Control	100	0	15
Incubation before 10 min	19.72	80.27	65.9
Addition after 5 min	92.59	7.4	19
Addition after 10 min	93.89	6.11	20

Values represent the mean of three experiments

Figure 1. Effect of different concentrations of telmisartan on the time-course of nitrite-induced Hb oxidation and MetHb formation in erythrocytes lysate.



Effect of telmisartan on nitrite-induced Hb oxidation and MetHb formation in erythrocytes lysate at different time intervals

Addition of the highly effective concentration of telmisartan (0.4 mg/ml), identified from the previous experiment, to the hemolysate either 10 min before

washed thrice with PBS (pH 7.4) and lysed by suspending in 20 volumes of 20mM PB (pH 7.4). The hemolysate was then centrifuge at 12500 rpm for 60 min to remove the membrane, and the clear supernatant was removed and light absorbance at 631 nm was measured.²⁰

Statistical Analysis

The results were expressed as mean±S.D. The statistical analysis was performed using one tailed unpaired Student's *t*-test. Differences are considered significant when *P*<0.05.

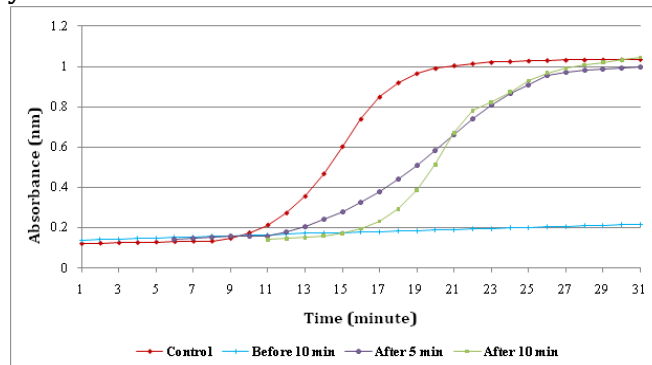
RESULTS AND DISCUSSION

Effect of different concentrations of telmisartan on the time-course of nitrite-induced Hb oxidation in erythrocytes lysate

In the present study, incubation with different concentrations of telmisartan (0.0004, 0.004, 0.04, 0.4 mg/ml) resulted in a concentration-dependent slowing in the rate of Hb oxidation and MetHb formation (13%, 55%, 70.5%, and 89.9%; respectively), as shown in table 1 and figure 1.

In the absence of telmisartan (control), the time required to form 50% MetHb was 15 min; in presence of telmisartan, the time required to form 50% MetHb was increased to 19, 26, 42.4, and 123.89 min respectively, and the concentration-effect curve shows some sort of linearity when log concentration was plotted against the time required to induce 50% Hb oxidation (Table 2, Figure 2).

Figure 2. Effect of telmisartan (0.4 mg/ml) on nitrite-induced Hb and MetHb formation in erythrocytes lysate at different time intervals.



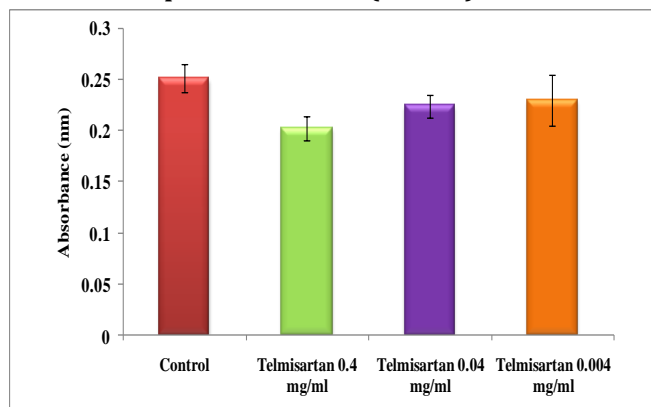
addition of sodium nitrite, and 5 min and 10 min after nitrite addition (i.e. during the autocatalytic phase) resulted in reduction of MetHb formation (19.72%, 92.59%, and 93.89%, respectively) (Figure 2). The addition of telmisartan (0.4 mg/ml) before 10 min produced remarkable decrease in MetHb formation. In the

absence of telmisartan (control), the time required to convert 50% of the Hb to MetHb was 15 min; this time was increased to 65.9, 19, and 20 min respectively (Table 2).

Effect of different concentrations of telmisartan on nitrite-induced Hb oxidation and MetHb formation in intact erythrocytes

Addition of telmisartan (0.4 and 0.04 mg/ml) to the medium that contain intact erythrocytes resulted in a significant ($P<0.05$) reduction of MetHb formation in presence of sodium nitrite (18 mM) compared with control. Meanwhile, telmisartan in a concentration of 0,004 mg/ml reduced MetHb formation but non-significantly different ($P>0.05$) compared with control (Figure 3).

Figure 3. Effect of different concentrations of telmisartan on nitrite-induced Hb oxidation and MetHb formation in intact erythrocytes, * Significantly different compared to control ($P<0.05$).



Erythrocytes are highly prone to oxidative damage, probably due to exposure to high oxygen tension, high content of peroxidizable polyunsaturated fatty acids (PUFAs) in their membranes, and their high level of hemoglobin-bound iron.²¹ In experiments where oxidative stress is evaluated, erythrocytes appear to be excellent model because of their simple structure and the relatively large amounts of polyunsaturated fatty acids in their membranes.²² Due to their very high ferrous iron concentration, human erythrocytes might be exposed to risks of increased oxidative stress, mainly through the formation of ferryl-hemoglobin,²³ and, in part, through the Fenton reaction of hydrogen peroxide with Fe^{2+} of hemoglobin, which generates the powerful oxidant hydroxyl radical.²⁴ Despite its therapeutic value at given doses, supernormal concentrations of sodium nitrite in living organisms may cause life-threatening states due to excessive MetHb formation and consequent hypoxia.²⁵ Increased formation of methemoglobin inhibits oxygen transport in blood, and its saturation leads to ischemia, cyanosis, and irreversible damage in the tissues and ultimately to mortality.²⁶ In addition to strong methemoglobinemia, nitrite induced a net depletion of reduced glutathione (GSH) in the intracellular medium associated with membrane lipid peroxidation.²⁷ In the present study, addition of sodium nitrite to the hemolysate caused Hb oxidation and MetHb formation; this process was characterized by a lag phase followed by an autocatalytic phase and appeared consistent with previously reported data.^{28,20} Nitrite-induced Hb oxidation is a potent process involving free radical generation²⁵; sodium nitrite, as a pro-oxidant, induces a primary extensive MetHb formation as a result of generating several types of free radical species like superoxide anion, hydroxyl, peroxy nitrite and nitrogen oxide radicals which

are implicated in promoting the autocatalytic stage of Hb oxidation.⁹ Telmisartan, a lipophilic and highly selective ARB²⁹, has received attention because of an array of pharmacological properties unrelated to AT1R antagonism, including partial agonism for nuclear PPAR- γ system and other pleiotropic actions like modulation of oxidative stress, inhibition of pro-inflammatory stimuli³⁰ and anti-proliferative effects on the vascular wall cells.³¹ The present study has shown that telmisartan, when early added to the incubation mixture, can protect against Hb oxidation by sodium nitrite in a concentration-dependent manner in the hemolysate. However, it did not reverse the effect of sodium nitrite when added at the later stage (after 5 and 10 min). Furthermore, it was found that telmisartan reduce nitrite-induced oxidation of Hb and MetHb formation in intact erythrocytes also in a concentration-dependent manner, thus protecting against oxidative damage. It is well established that oxidation of Hb by nitrite takes place in two stages. It has been reported previously that nitrite treatments in intact RBCs causes a noticeable oxidation of oxy-hemoglobin to MetHb by radical generation along with a decrease in glutathione level in the intracellular medium and associated with membrane lipid peroxidation; this oxidative reactivity induced by nitrite alters cellular ionic flux.²⁷ Moreover, uptake of nitrite by the RBCs involves both HNO_2 diffusion and facilitated diffusion of NO_2^- via the anion-exchange (AE-1) protein. Once inside erythrocytes, nitrite reacts with oxy-hemoglobin, which leads to the formation of nitrate and MetHb.³² The extent to which nitrite is reduced by the major low-molecular-weight antioxidants within erythrocytes is uncertain; nitrite has been reported to oxidize GSH in rat erythrocytes.²⁷ However, concern had been previously raised that GSH loss in such type of studies was due to its destruction after cell lysis.³³ According to our result, telmisartan had the ability to prevent the onset of the autocatalytic stage. Since superoxide anion is implicated in the autocatalytic stage¹⁹ and telmisartan markedly reduced superoxide production³⁴, it can be suggested that this effect may be attributed to the scavenging superoxide anions generated during Hb oxidation. Additionally, superoxide anion, the most potent member of ROS, was inactivated by superoxide dismutase, and both ACE inhibitors and ARBs have been shown to stimulate superoxide dismutase³⁵, which may play a role in the reported attenuation of nitrite-induced Hb oxidation. Also, telmisartan has been proven to inhibit intracellular oxidative stress, at least in part, in a receptor-independent manner, possibly owing to its lipophilic and inherited anti-oxidant structure³⁶; it inhibits tumor necrosis factor (TNF)- α induced vascular cell adhesion molecules (VCAM-1) expression and modulates hydrogen peroxide-induced cell damage possibly by acting as a hydroxyl radical scavenger in human umbilical vein endothelial cell (HUVEC).³⁷ Several *in-vitro* and preclinical data³⁸ suggest that ACE inhibitors and ARBs protect vasculature from inflammation and oxidative stress by inhibiting the ATII-mediated stimulation of inflammatory cell functions, such as the promotion of oxidative stress by the membrane-bound NADPH-dependent oxidase and the over expression of inflammatory adhesion molecules, chemokines, and cytokines.³⁹

CONCLUSION

Dose-effect relationship was studied for the radical scavenging activity of the angiotensin receptor blocker

telmisartan in models of nitrite-induced oxidation of hemoglobin (Hb) and formation of methemoglobin (MetHb) in hemolysate and intact erythrocytes. In conclusion, telmisartan, in concentration dependent pattern, can protect erythrocytes against nitrite-induced Hb oxidation and MetHb formation in hemolysate and intact erythrocytes.

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