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Effect on rat arterial blood pressure of chemically generated peroxyl radicals and protection by antioxidants

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Abstract

Convincing evidence suggests that blood redox changes play a role in the development of various cardiovascular disorders including hypertension. Nutritional antioxidants have been suggested to play a role in cardiovascular disease prevention. In this study, we investigated *in vivo* changes in rat arterial blood pressure induced by acute exposition to an increased load of peroxyl radicals and by the administration of selected antioxidants after chemically induced oxidative stress. Hydrosoluble and liposoluble peroxyl radicals, generated by 2,2'-azobis-(2-amidinopropane) dihydrochloride and 2,2'-azobis 2,4-di-methylvaleronitrile, induced a dose-dependent decrease in rat blood pressure. All antioxidants tested (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, vitamin C, glutathione and dithiothreitol) returned peroxyl radical–induced hypotension to normal. Of the various antioxidants tested, glutathione was the most effective in restoring blood pressure after peroxyl radical generation. Treatment of rats with a thiol-chelating agent (*N*-ethylmaleimide) and an oxidizing agent (5,5'-dithiobis-2-nitrobenzoic) inhibited peroxyl radical–mediated hypotension. Our results suggest that acute exposition to peroxyl radicals have a hypotensive effect on blood pressure and that thiols play an active role in the redox regulation of blood pressure. Other experiments are needed to clarify the role played by oxidative potentials on blood pressure and the mechanism of action of nutritional antioxidants.

Keywords: Blood pressure; Peroxyl radicals; Antioxidants; Rats; Thiols; Hypotension

1. Introduction

Among the various factors responsible for the development of disease related to hypertension and atherosclerosis are redox changes originating from oxidative stress, a change in the dynamic balance between production of free radicals and antioxidant defenses [1,2]. In hypertensive patients and rats, antioxidant supplementation with vitamin C or E decreases lipid peroxidation and restores plasma and tissue antioxidant levels to normal [3–7]. Whether antioxidants prevent the early effects of oxidative stress associated with endothelial dysfunction remains unclear [8,9].

During the atherosclerotic process, acetylcholine-induced endothelium-dependent relaxation is impaired [10]. Some evidence suggests that superoxide anion ($O_2^{-\bullet}$), a by-product of oxygen metabolism, plays a role in the pathophysiology of arterial wall by inactivating nitric oxide and decreasing endothelium-dependent vascular relaxation [11–13]. Conversely, other studies have shown that the peroxynitrite ($ONOO^-$), the product of the reaction between anion superoxide and nitric oxide, exerts a vasorelaxant action *in vitro* [14,15] as well as in anesthetized rats [16]. Despite mounting evidence that free radicals substantially alter blood pressure, the mechanism underlying these phenomena is unclear.

In this study *in vivo*, we investigated early changes in rat arterial blood pressure in response to increased peroxyl radical loading and to the administration in bolus to rats of selected antioxidants before chemically induced oxidative stress. We used two generators because previous evidence suggests that the toxicity of the azocompounds may also arise through non-radical mechanisms owing to the different stable products generated by 2,2'-azobis-(2-amidinopropane) dihydrochloride (ABAP) and 2,2'-azobis 2,4 di-metil-valeronitrile (AMVN) [17].

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2. Methods and materials

2.1. Chemicals

Sodium pentobarbitone (Sigma), norepinephrine hydrochloride (NA; Sigma), 2,2'-azobis-(2-amidinopropane) dihydrochloride (ABAP; Wako), L-ascorbic acid (Sigma), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX; Sigma), glutathione (GSH; Sigma), dithiothreitol (DTT; Sigma), N-ethylmaleimide (NEM; Sigma), and 5,5'-dithiobis-(2-nitrobenzoic) (DTNB; Sigma) were dissolved in a fresh saline solution (0.9% sodium chloride). 2,2'-Azobis 2,4-di-methylvaleronitrile (AMVN, Wako) and α-tocopherol (Sigma) were dissolved in ethylene glycol and diluted with fresh saline (0.9% sodium chloride) at a final concentration (of 0.3%) that left blood pressure unchanged.

2.2. Experiments in anesthetized rats

Adult male Wistar rats, weighing 0.3-0.35 kg and acclimatized for 1 week to an illumination cycle of 12 hours light and 12 hours dark at 23°C, with food and water provided *ad libitum*, were anesthetized with an intraperitoneal injection of sodium pentobarbitone (60 mg/kg). The trachea was exposed and cannulated to facilitate spontaneous respiration. Arterial blood pressure was measured from the carotid artery through a heparinized polyethylene cannula PE-50 connected to a pressure transducer calibrator (Ugo Basile 2900) coupled with a pen-recorder (Ugo Basile Unirecord 7050). Drugs were given as a bolus injection (0.1 mL) through a cannula inserted into the external jugular vein and the vein was flushed with saline (0.2 mL). Animals were allowed to equilibrate for at least 30 minutes before administration of two identical control doses of norepinephrine (1 μg/kg). ABAP, AMVN, or DTT was then administered in increasing doses. The antioxidants and DTT were infused intravenously 5 minutes before ABAP. Changes in blood pressure were calculated and expressed as percentages of control values obtained immediately before the administration of the test substances (baseline).

2.3. Statistical analysis

All data are presented as mean ± SEM. Statistical evaluations were performed using Sigmastat software (Jandel Scientific Inc., San Rafael, CA). One-way analysis of variance (ANOVA) with the Student-Newman-Keuls post-test and linear regression analysis were used to compare concentration–effect curves. The Student *t* test was used to compare one control with several experimental groups. Results presented below are given with 95% confidence intervals.

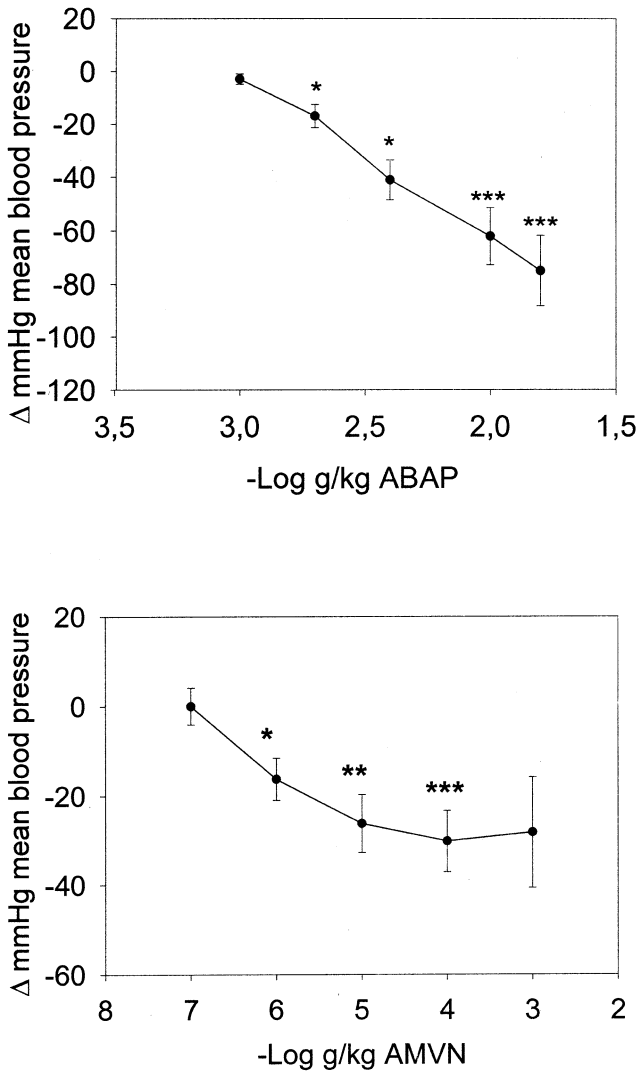


Fig. 1. 2,2'-Azobis-(2-amidinopropane) dihydrochloride (ABAP) and 2,2'-azobis 2,4 di-metilvaleronitrile (AMVN) were administered by intravenous bolus infusion in single doses. Results are expressed as ΔmmHg of mean arterial blood pressure; each point represents the mean ± SEM of four different animals. ABAP-induced (A) and AMVN-induced (B) dose-dependent hypotension. Analysis of variance by Student-Newman-Keuls test *vs* controls: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ ($n = 4$ rats).

3. Results

Although the peroxyl radical generator ABAP at low doses (from 1 ng/kg to 100 μg/kg), left rat arterial blood pressure unchanged (data not shown), higher doses significantly reduced mean blood pressure. Cumulative doses from 1 mg/kg to 32 mg/kg induced dose-dependent hypotension (Fig. 1A) with animal death occurring at 32 mg/kg. AMVN induced dose-dependent hypotension even at low doses (1 ng/kg to 1 mg/kg) (Fig. 1B).

In preliminary experiments investigating the effect of antioxidants and DDT bolus infusion on rat arterial blood pressure, α-tocopherol (1 mg/kg) and TROLOX (1 mg/kg) induced slight although not significant hypotension ($-25 \pm$

20.4 mm Hg and −28.75 ± 17.84 mm Hg), whereas ascorbic acid (1 mg/kg) and GSH (10 mg/kg) slightly increased blood pressure (13 ± 6.25 mm Hg and 8.75 ± 11.25 mm Hg; not significant). After all antioxidant treatments blood pressure fully recovered within 2 minutes (data not shown).

At concentrations up to 1 mg/kg, DDT left rat arterial blood pressure significantly unchanged (1 μg/kg: 13.52 ± 8.90 mm Hg; 10 μg/kg: 3.12 ± 3.13 mm Hg; 100 μg/kg: 3.12 ± 3.13 mm Hg; and 1 mg/kg: 9 ± 7.13 mm Hg). After DDT injections blood pressure fully recovered within 2 minutes (data not shown). At higher doses, DTT produced irreversible hypotension (10 mg/kg: −23.5 ± 9.87 mm Hg), probably due to its toxic denaturizing activity.

All three antioxidants and DTT significantly protected against hypotension induced by peroxyl radicals (ABAP and AMVN) (Fig. 2). GSH and DTT induced similar effects, and both were more effective than TROLOX and ascorbic acid in protecting against ABAP-induced hypotension (Fig. 2A). On AMVN-induced hypotension the order of protection was GSH > α-tocopherol > ascorbic acid = DTT (Fig. 2B). DTT and GSH showed a similar effect also on ΔmmHg systolic/diastolic blood pressure (Fig. 3), inhibiting the reduction of the difference in maximal and minimal blood pressures better than TROLOX or ascorbic acid. After ABAP infusion, mean blood pressure returned to baseline within about 10 minutes (10 ± 0.82 min). When antioxidants (TROLOX 1 mg/kg, GSH 10 mg/kg, ascorbic acid 1 mg/kg) or DTT (1 mg/kg) were administered, mean blood pressure returned to baseline earlier (mean blood pressure recovery: TROLOX 2.62 ± 0.75 minutes, GSH 2.25 ± 0.48 minutes, ascorbic acid 2.25 ± 0.75 minutes, and DTT 3.0 ± 0.4 minutes).

Both the thiol-oxidizing (5,5'-dithiobis-2-nitrobenzoic, DTNB) and thiol-chelating (N-ethylmaleimide, NEM) agents tested clearly induced a marked dose-dependent decrease in rat blood pressure (Figs. 4A and 4B) mirroring ABAP-induced and AMVN-induced hypotension. When AMVN (Fig. 5) was injected after NEM, its hypotensive effect drastically diminished (before NEM: ΔmmHg = −39 ± 10; after NEM: ΔmmHg = −18 ± 5). Similarly NEM diminished ABAP hypotensive effect (before NEM: ABAP ΔmmHg = −66 ± 12, after NEM: ABAP ΔmmHg = −30 ± 10) (not shown).

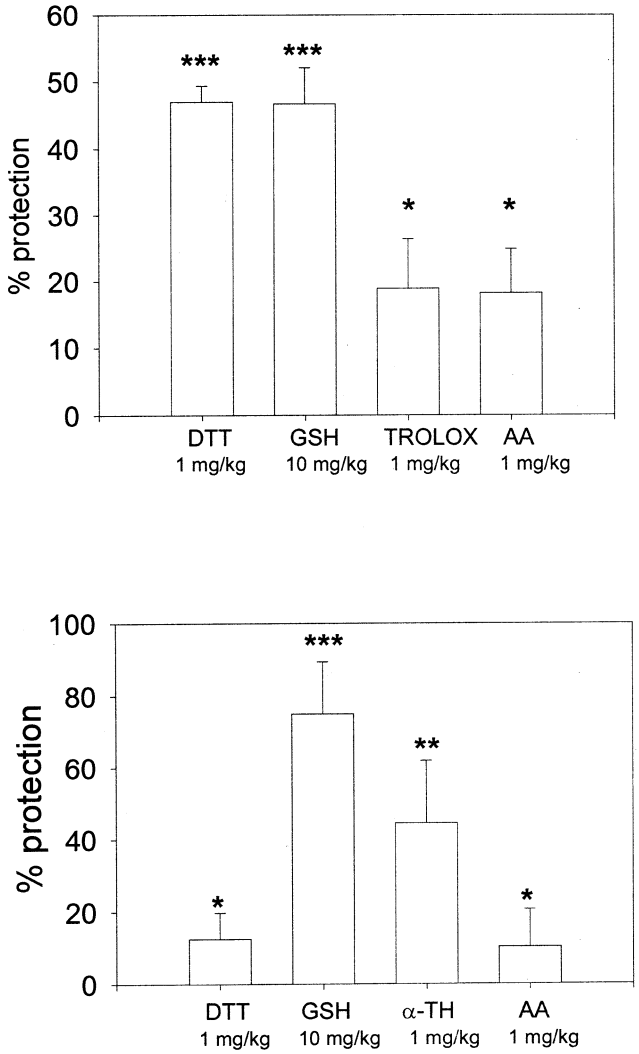


Fig. 2. Effect of antioxidants (glutathione [GSH], 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid [TROLOX], ascorbic acid [AA], α-tocopherol [α-TH]), and dithiothreitol [DTT] on hypotension induced by (A) 2,2'-azobis-(2-amidinopropane) dihydrochloride (ABAP) and (B) 2,2'-azobis 2,4 di-metilvaleronitrile (AMVN). Antioxidants and DTT were administered intravenously 5 minutes before ABAP. Values are expressed as percentage protection compared with ABAP-and AMVN-induced hypotension. Each bar represents the mean ± SEM of four different experiments. Results of *t* test *vs* ABAP 10 mg/kg: $^{*}P < 0.05$; $^{**}P < 0.001$. *t* test *vs* AMVN 100 μg/kg: $^{*}P < 0.05$ $^{**}P < 0,01$ $^{***}P < 0.001$.

4. Discussion

In the present study, the effect of free radicals on blood pressure was investigated by treatment of anesthetized rats with a hydrosoluble azocompound (ABAP) and a liposoluble azocompound (AMVN), both extensively used as peroxyl radical generators *in vivo* [17-19]. In our experiments, high intravenous doses of ABAP were fatal, in accordance with previous findings after intraperitoneal injection in rats [17]. Problems related to solubility prevented us from determining the lethal dose of AMVN.

In our *in vivo* study, ABAP and AMVN bolus infusions induced no consistent changes in pulse pressure or heart rate and left NA control doses unchanged, thus excluding an oxidation of endogenous catecholamine [20]. Hence, hypotension induced by azocompounds is presumably an early cardiovascular effect rather than an indirect effect on circulating catecholamines.

In this study, when the blood redox status shifted through oxidizing potentials, after peroxyl radical production, blood pressure clearly decreased. This hypotensive effect is in agreement with findings obtained by Jacinto et al. in a study using anion superoxide as the oxidizing agent [21].

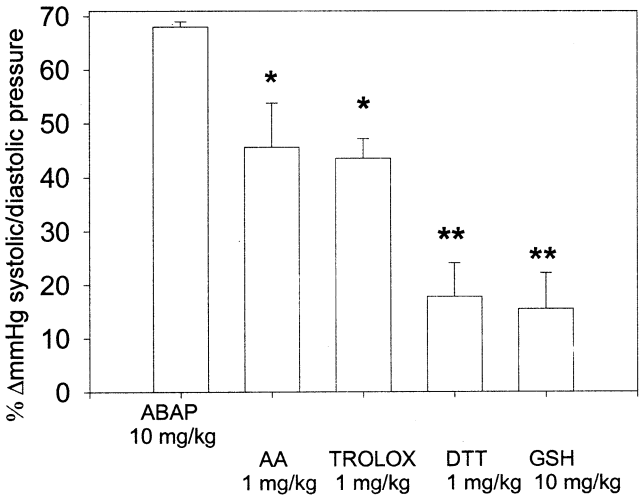
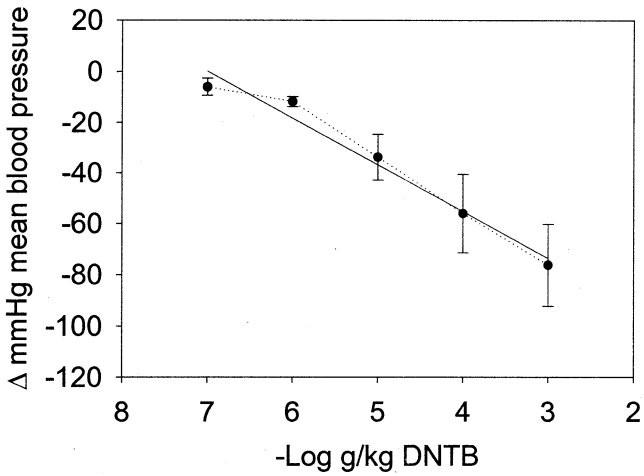


Fig. 3. Effect of antioxidants (ascorbic acid [AA], 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid [TROLOX], and glutathione [GSH]) and dithiothreitol (DTT) bolus infusion on 2,2'-azobis-(2-amidinopropane) dihydrochloride (ABAP)–induced reduction of ΔmmHg systolic/diastolic pressure (ΔmmHg systolic/diastolic blood pressure represents the differences between systolic and diastolic arterial pressure and maximal and minimal blood pressures). Antioxidants and DTT were administered intravenously 5 minutes before ABAP 10 mg/kg. Values are expressed as percentage reduction in ΔmmHg systolic/diastolic pressure before treatment. Results of *t* test *vs* ABAP 10 mg/kg: $*P < 0.05$; $**P < 0.001$. Each bar represents the mean ± SEM of four different experiments.



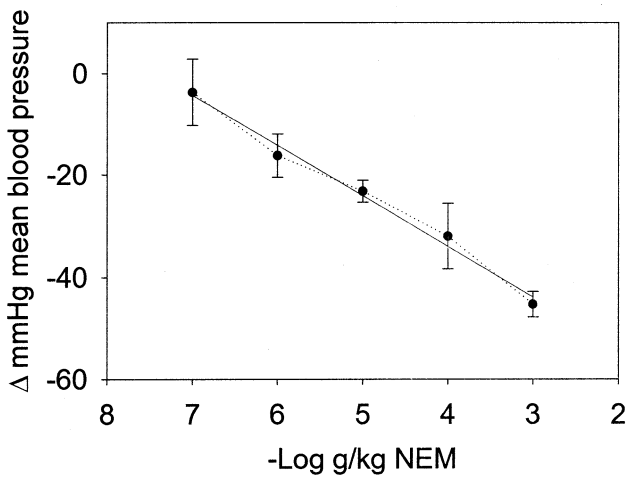


Fig. 4. 5,5'-Dithiobis-(2-nitrobenzoic) (DTNB) and N-ethylmaleimide (NEM) were administered by intravenous bolus infusion in single doses. (A) DTNB-induced and (B) NEM-induced dose-dependent hypotension. Linear regression: DTNB (A) $y = -129.125X + 18.475$, $r = 0.66$ (95% confidence intervals); NEM (B) $y = -73.45X + 9.875$, $r = 0.736$ (95% confidence intervals). Each point represents the mean ± SEM of four experiments.

To investigate whether the hypotensive effect of peroxyl radicals could be prevented by antioxidant bolus infusion, we selected three different types of antioxidants: α-tocopherol, ascorbic acid, and glutathione (GSH) at concentrations similar to physiological values. We chose nutritional antioxidants, α-tocopherol and ascorbic acid, because of their radical scavenging properties and GSH for its reduction properties. We also used a synthetic reduction agent (DTT) to reduce protein disulfur (S-S) to thiol groups (S-H). In the experiment with ABAP, we replaced α-tocopherol with its hydrosoluble analogue, TROLOX.

Ascorbic acid and TROLOX are well known antioxidants; both are extremely efficient in scavenging radicals in general and in neutralizing peroxyl radicals in particular. Despite this well recognized chain-breaking property, they nonetheless displayed a weak effect in restoring blood pressure after ABAP infusion. On the other hand, GSH, a weaker peroxyl radical scavenger than TROLOX, displayed the strongest effect. The involvement of thiols in peroxyl radicals induced hypotension is further supported by the comparable effect in restoring blood pressure shown by DTT, a molecule able to reduce protein thiols from oxidized disulfides.

To test whether changes in thiols could underlie peroxyl radical–induced hypotension, we investigated the effect of DTNB, a thiol oxidizing agent, and NEM, a chelating agent. Both induced a dose-dependent decrease in blood pressure. These results agree well with data obtained on isolated arteries [22], showing that thiol oxidation elicited by diamide activates a novel redox-regulated vasodilatory mechanism that inhibits extracellular Ca^{+2} influx.

When we infused AMVN or ABAP after NEM, the hypotensive effects of these agents diminished. This finding suggests that the fall in blood pressure induced by peroxyl radicals depends on thiol oxidation and that thiol chelation induced by NEM prevents ABAP and AMVN effects. Our findings therefore imply that for compensating the hypotensive effect of ABAP, the redox properties of thiols are more important than the specific radical scavenging properties of ascorbic acid and TROLOX. We suggest that the early cardiovascular effects of free radicals may involve the thiol-regulated vasodilatory mechanism proposed by others [22] and that thiols are more prone to a redox shift than other plasma antioxidants.

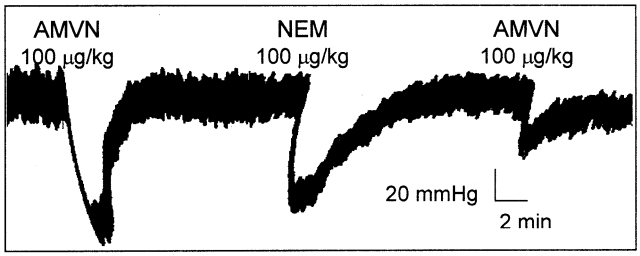


Fig. 5. Typical blood pressure tracing recorded during bolus infusion of 2,2'-azobis 2,4 di-metilvaleronitrile (AMVN; 100 μg/kg), before and after infusion of N-ethylmaleimide (NEM; 100 μg/kg).

Various hypotheses have been postulated to explain arterialendothelial dysfunction; among them, decreased vasorelaxation due to nitric oxide and, in some arteries, endothelium-derived hyperpolarizing factors as well as increased vasoconstriction mediated by cyclooxygenase products are likely to occur in impairment of endothelial vasodilation. Their relative contribution to endothelial dysfunction depends strictly on the species and type of vascular bed [23].

In addition, vascular smooth muscle cell hyperproliferation [24] induced by chronic oxidative stress may alter arterial functionality thus leading to hypertension. Our findings suggest that the early cardiovascular effects of free radicals may involve not a quenching activity on NO but the thiol-regulated vasodilator mechanism recently proposed by Iesaki and Wolin [22]. Free radicals could well exert their cardiovascular effects before hyperproliferation [24] or apoptosis of vascular smooth muscle cells [24,25] related to cardiovascular disease and associated with the systemic redox status.

In conclusion, our findings provide evidence that acute peroxyl loading induce marked hypotensive effects, and that the addition of selected antioxidants after chemically induced oxidative stress on rat prevents early free radical mediated arterial blood pressure alteration *in vivo*. Other experiments are needed to clarify the role played by oxidative potentials on blood pressure and the mechanism of action of nutritional antioxidants.

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