Free Rad. Res., Vol. 23, No. 3, pp. 245–254
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INCREASE IN ANTILIPOPEROXIDANT ACTIVITY OF PLASMA AS A CONSEQUENCE OF AN INFLAMMATORY REACTION INDUCED BY SUBCUTANEOUS TURPENTINE IN THE RABBIT

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(Received March 30th, 1994; in revised form, November 7th, 1994)

In the rabbit, an acute inflammatory reaction triggered by the subcutaneous administration of turpentine induces in hepatic tissues an oxidative stress, as well as a decrease in activity of enzymatic scavengers of reactive oxygen species (ROS). The objective of this study was to investigate, the repercussions of a local inflammatory reaction on the antioxidant capacity and markers of systemic oxidative stress in plasma. To this purpose, rabbits received a s.c. injection of turpentine (5 mL/kg) or NaCl 0.9% (w/v). Blood samples were collected at different times during the 48 hours of the experiment to evaluate: firstly, the antilipoperoxidant activity of plasma by measuring the inhibition of autoxidation of brain homogenate, and the concentrations of tocopherol and ascorbic acid; secondly, the severity of oxidative stress in plasma by assaying the concentration of thiobarbituric acid reactive substances (TBARS), and the concentration of ascorbyl radical. The results show that the antilipoperoxidant capacity of plasma gradually increased to be 167% higher than baseline values ($p < 0.05$) after 48 hours of experiment. α-Tocopherol and ascorbic acid levels increased by 49% and 80%, respectively ($p < 0.05$) during the first 24 hours. Lipid peroxidation continuously increased to be 98% higher than baseline values ($p < 0.05$) at 48 hours, while ascorbyl radical levels were not modified ($p < 0.05$). In summary, an acute local inflammatory reaction causes a steady progression of oxidative stress, while it stimulates the antilipoperoxidant activity of plasma, to which α-tocopherol and ascorbic acid appear to contribute, essentially early in the inflammation.

KEY WORDS: Inflammation, reactive oxygen species, antioxidants, plasma, lipid peroxidation

[a]Abbreviations: ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances; MDA-TBA, malondialdehyde-thiobarbituric acid; Khz, Kilohertz; G, Gauss; mW, milliwatt; s, second.

INTRODUCTION

For several years, reactive oxygen species (ROS) have been known to be implicated in the evolution of an inflammatory reaction.[1–3] The beneficial role of ROS as oxidant in the host's defence against bacterial infection and the associated inflammatory disorders is well established.[3,4] On the other hand, since this activity of the ROS[a] is not selective,

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their presence can produce deleterious effects on surrounding tissues.[5,6] The deleterious effects of ROS on tissues are usually prevented by a rise in the activity of antioxidants.[7,8] However, when the presence of ROS is excessive, the activity of the antioxidant systems is decreased.[9,10] For instance, an inflammatory reaction induced by the s.c. administration of turpentine increases hepatic lipid peroxidation and reduces the antioxidant activity of scavenger enzymes.[11]

Based on the observation that plasma concentrations of tocopherol and ascorbic acid are decreased in patients with a chronic inflammatory process,[12,13] it has been proposed that the total antioxidant activity of the plasma is reduced during an oxidative stress induced by a chronic inflammation. In contrast, there are some evidences suggesting that an acute inflammatory reaction may be able to promote the antioxidant activity of plasma, as demonstrated by the increase in plasma ceruloplasmin concentrations induced by the subcutaneous administration of turpentine.[14]

The main objective of the present study were to document, in rabbits, the effect of an acute inflammatory reaction induced by the subcutaneous administration of turpentine on a) plasma markers of systemic oxidative stress, such as lipid peroxidation and ascorbyl radical,[15,17] and on b) the total antilipoperoxidant activity of the plasma. Secondary goals were to assess the role of α-tocopherol and ascorbic acid in the plasma antilipoperoxidant activity during an acute inflammatory reaction.

MATERIAL AND METHODS

Animals

Male New Zealand rabbits (IFFA CREDO, L'Abresle, France) weighing 2.0–2.2 Kg were maintained on Purina Laboratory Chow pellets and water *ad libitum* for one week, as an acclimatization period, and for the 48 hours the experiment lasted.

The inflammatory reaction was induced locally by injecting turpentine (5 mL/Kg) s.c., at two distinct sites on the back of ten rabbits, as described elsewhere.[18,19] A control group of eleven rabbits received s.c. the same volume of sterile NaCl 0.9% (w/v) solution. Arterial blood samples (8 mL) were withdrawn at 0, 6, 24, 48 hours in heparinized syringes. Blood samples, were centrifuged at 2,700 × g for 5 minutes at 4°C, and aliquots of plasma were kept frozen at –80°C, until assayed. In order to evaluate the effect of the inflammatory reaction on the number of circulating monocytes, blood was collected at 0, 6, 24, and 48 hours from two rabbits with a turpentine-induced inflammatory reaction, and monocytes were counted automatically with a Technicon apparatus.

Assays

To evaluate the severity of the oxidative stress, lipid peroxidation and concentration of ascorbyl radical were assessed in plasma. Lipid peroxidation was assessed in plasma by determining spectrofluorometrically the concentration of thiobarbituric acid reactive substances (TBARS),[20] according to the method modified by Buege and Aust.[21] Since the main component of TBARS is malondialdehyde-thiobarbituric acid (MDA-TBA), the concentration of TBARS reflects lipid peroxidation.[22] The concentration of ascorbyl radical, the oxidized form of ascorbic acid, was assayed by electron paramagnetic resonance (EPR) spectroscopy.[16] EPR spectra were recorded with a Bruker ESP 300 E spectrometer using a TM_{110} cavity and a flat-type quartz cell with the following

instrument settings: modulation frequency = 100 Khz, modulation amplitude = 0.5 G, microwave power 4 mW, time constant 0.16 s, scan rate 10 G/84 s. The results for this assay are given with arbitrary units.

To document the total antilipoperoxidant activity of plasma, the ability of plasma to inhibit spontaneous autoxidation of rat brain homogenates was measured. Briefly, rat brain (10% w/v) were homogenized in 0.05 M phosphate buffer pH 7, containing 0.05 M KCl and 0.015 M NaCl. An aliquot of the plasma (10μL) obtained from the blood samples withdrawn at 0, 6, 24 and 48 hours was incubated for 30 minutes at 37°C with 1 mL of 10% (w/v) rat brain homogenate. Following the incubation period, 25 μL of the homogenate were immediately removed and added to a thiobarbituric acid reactive solution, to measure the concentration of TBARS.[20,21,23] Brain homogenates incubated without plasma allowed to estimate the degree of inhibition of spontaneous autoxidation.

The concentrations of specific antioxidants, such as tocopherols and ascorbic acid, were also assessed. The assay of α, β, τ, and δ isomers of tocopherol in plasma were assayed by high-performance liquid chromatography.[24] The method of Roe and Kuether was used to measure spectrophotometrically the concentration of ascorbic acid.[25]

Statistical analysis

The results obtained in animals with an inflammatory reaction were compared to those from control animals using a one-way analysis of variance, and the difference was determined using Dunnett's distribution table. To determine the association between the changes in plasma antilipoperoxidant activity and plasma markers of systemic oxidative stress, regression analysis was performed.[26] The significance criteria was established at $p < 0.05$.

RESULTS

The turpentine-induced inflammatory reaction induced a progressive increase in the concentration of plasma TBARS, to reach values 98% higher than baseline ($p < 0.05$), 48 hours after the initiation of the experiment (Figure 1). In the group of control rabbits receiving a s.c. injection of NaCl 0.9%, the concentration of TBARS remained stable all along the protocol.

Under the above mentioned conditions, EPR spectrum of ascorbyl radical depicted a characteristic doublet signal with a g factor of 2.005 and a splitting of 1.8 G as observed in the study of Roginsky and Stegmann.[17] An example of this signal appears in Figure 2. The concentration of ascorbyl radical, another potential marker of oxidative stress, was however not significantly modified during the 48 hours of the experiment in either group (Figure 1).

In control rabbits, plasma total antilipoperoxidant activity was stable for the 48 hours during the experiment. However, in animals with an inflammatory reaction, the antilipoperoxidant activity increased as time elapsed, as reflected by an increment in the inhibition of spontaneous autoxidation of rat brain homogenate, i.e. from 24 ± 6% to 64 ± 5% ($p < 0.05$) (Figure 1). By comparison to control rabbits, the antilipoperoxidant activity in rabbits with an inflammatory reaction was 167% higher 48 hours after the beginning of the experiment.

In the animals with an inflammatory reaction, the increases in antilipoperoxidant

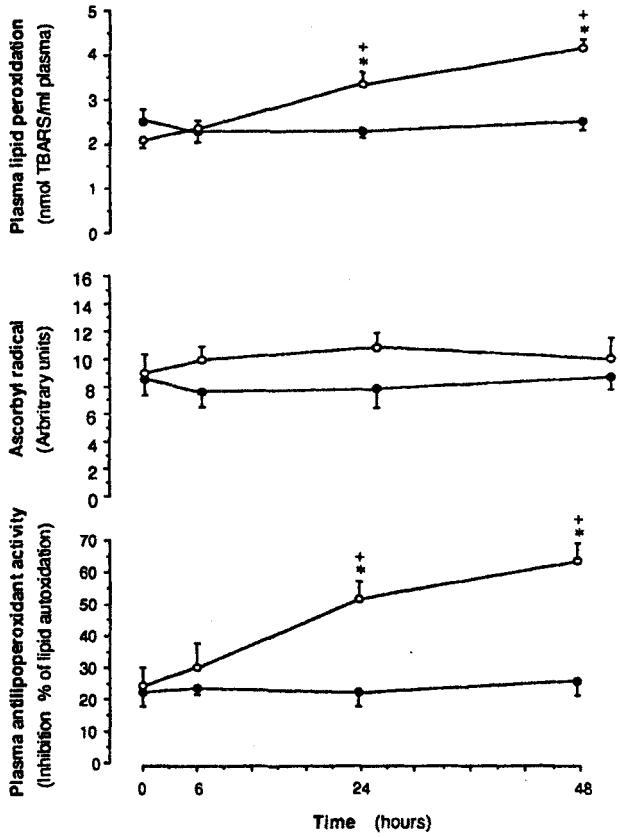

FIGURE 1 Lipid peroxidation (thiobarbituric acid reactive substances-TBARS), ascorbyl radical concentration and antilipoperoxidant activity (inhibition % of lipid autoxidation in rat brain homogenate) in plasma, as a function of time, in rabbits (n = 10) with an inflammatory reaction induced by s.c. turpentine (○) and control rabbits (n = 11) receiving s.c. NaCl 0.9% (●). Where $^{+}$ indicates $p < 0.05$ compared to baseline values, and * $p < 0.05$ compared to values of control animals. Each point represents the mean ± S.E.

activity of plasma appeared associated with the changes in plasma lipid peroxidation ($r = 0.772$, $p < 0.05$) (Figure 3).

The effect of the inflammatory reaction on circulating monocytes was assessed in two rabbits 48 hours after the injection of turpentine. Inflammation increased baseline values (before the injection of turpentine) of 1 and 2×10^8 monocytes/L of blood to 5 and 9×10^8 monocytes/L, respectively.

Only α-tocopherol was detected in plasma. The concentrations of this lipid-soluble antioxidant showed a significant increase by 49% in the rabbits with an inflammatory reaction, 24 hours after the onset of the experiment ($p < 0.05$) (Figure 4). Control values of α-tocopherol remained relatively stable during the experiment. Ascorbic acid in plasma also showed a significant increment, i.e. by 80% ($p < 0.05$), at the twenty-fourth hour (Figure 4) and then, tended to decrease.

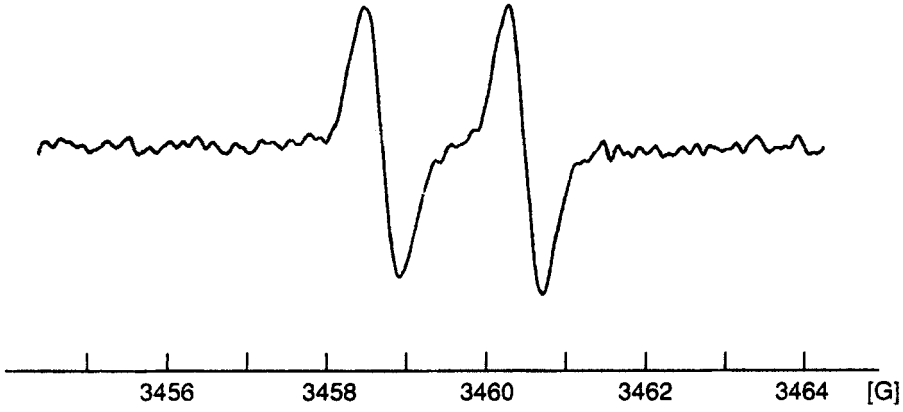

FIGURE 2 Example of the characteristic ascorbyl radical doublet EPR signal.

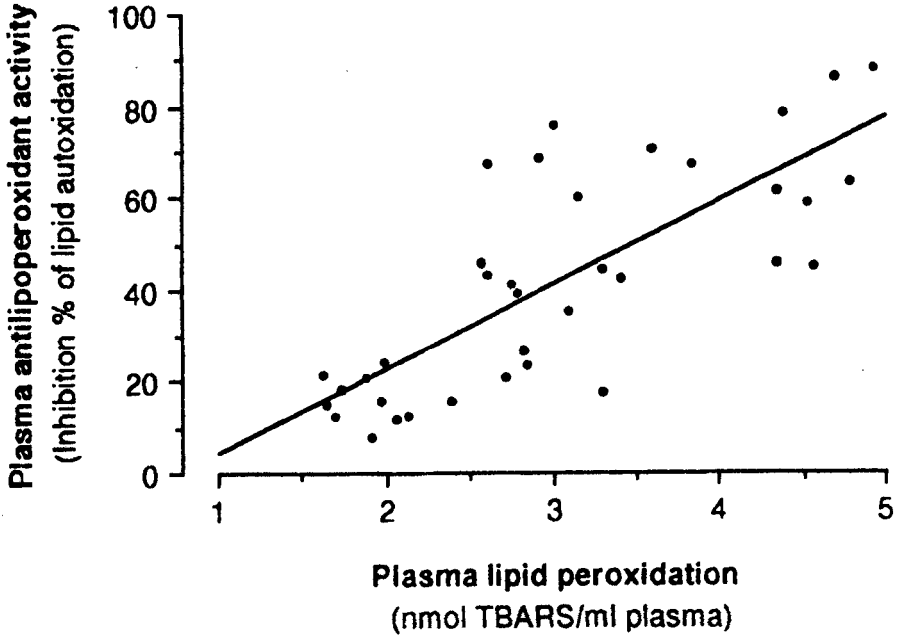

FIGURE 3 Changes in plasma antilipoperoxidant activity as a function of plasma thiobarbituric acid reactive substances (TBARS).

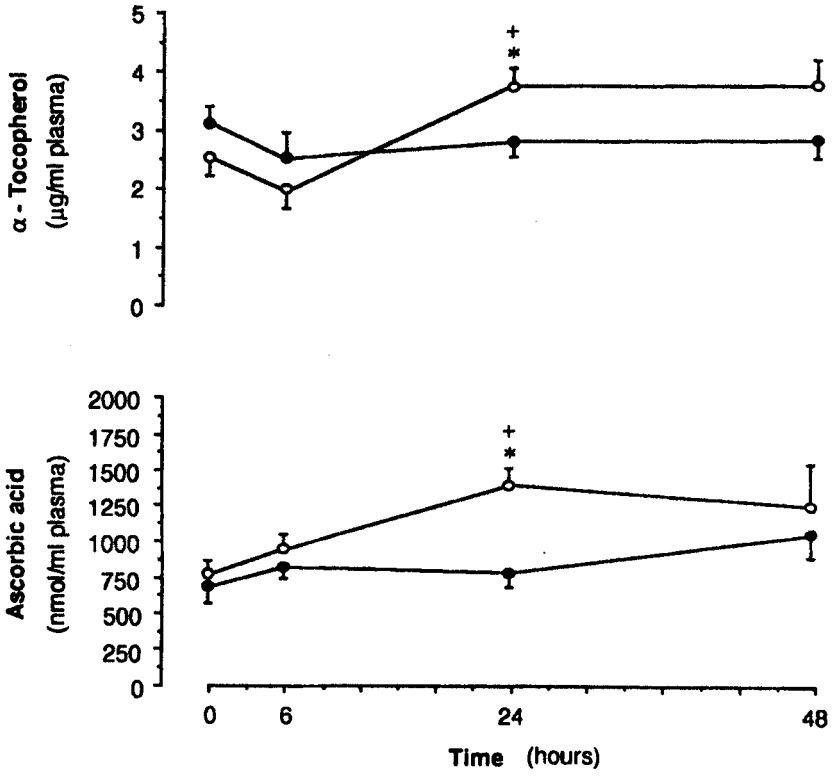

FIGURE 4 Concentration of α-tocopherol and ascorbic acid in plasma, as a function of time, in rabbits (n = 10) with an inflammatory reaction induced by s.c. turpentine (○) and control rabbits (n = 11) receiving NaCl s.c. 0.9% (●). Where $^{+}$ indicates $p < 0.05$ compared to baseline values, and * $p < 0.05$ compared to values in control animals. Each point represents the mean ± S.E.

DISCUSSION

The present study demonstrates that an acute inflammatory reaction, provoked by the s.c. injection of turpentine, induces a progressive increase in plasma TBARS concentrations, suggesting that the severity of the oxidative stress, i.e. lipid peroxidation, increases as a function of time. In parallel to the enhancement of lipid peroxidation, the total antilipoperoxidant activity of the plasma also increases, suggesting that plasma has a protective role.

The progressive increase in TBARS concentrations in plasma with time could be the result of tissue damage induced by ROS generated during the acute inflammatory reaction. This hypothesis is supported by the fact that in the rat, hepatic lesions induced during a granulomatous inflammation promotes an increase in plasma concentrations of TBARS.[15] The progressive increase in TBARS concentrations in plasma suggest that this parameter may be used as marker of the evolution of an oxidative stress caused by an acute localized inflammatory process. The origin of ROS remains uncertain, but tissues like liver and blood cells may contribute to their production.[27-30] Hepatocytes

can release nitric oxide in response to endotoxins or factors secreted by Kupffer cells.[27,28] On the other hand, in response to an acute or chronic inflammation, monocytes, polymorphonuclear leukocytes and macrophages represent a potential source of ROS in blood.[3,13,31] Monocytes can produce hydrogen peroxide, which cross cell membranes readily[3,29] and react with transition metals to generate highly reactive oxygen species able to boost an oxidative stress.[30] In the present study, in two rabbits, the number of circulating monocytes in blood increased almost five times, 48 hours after the induction of the inflammatory reaction.

Turpentine-induced inflammatory reaction elicited a progressive increase in the total antilipoperoxidant capacity of plasma. Our results are in accordance with the ones of Gitlin[14] showing an increment in plasma ceruloplasmin concentration in hamsters, during an acute inflammatory reaction induced by turpentine. In the present study, total antilipoperoxidant activity of plasma was directly correlated with TBARS concentrations, suggesting that the increase in antilipoperoxidant activity in plasma is a response to the oxidative stress.

While plasma levels of β-carotene are too low to make a relevant antioxidant contribution to antilipoperoxidant activity of plasma,[32,33] other antioxidants such as α-tocopherol, which is recognized to possess the highest lipid antioxidant efficacy,[34] or ascorbic acid, are part of the antilipoperoxidant activity of plasma. Our results show a significant increment in α-tocopherol and ascorbic acid plasma concentrations between the sixth and the twenty-fourth hour of the experiment, to remain rather stable thereafter. The mechanism underlying the increase in tocopherol concentrations remains uncertain, because tocopherols are essential nutrients, i.e. they are not synthesized by the organism, which possesses limited labile reserves of this antioxidant.[35] We may speculate that the accumulation of lipids in plasma during an acute inflammatory reaction[36] is accompanied by a transfer of tocopherol from the membrane stocks. Since tocopherols bound to lipoproteins or phospholipids can trap radical oxygen species and as a consequence, reduce lipid peroxidation,[34,37,38] we hypothesize that the increase in plasma tocopherol, and perhaps also in plasma lipids, is part of the defence mechanism of the organism against an acute inflammatory injury.

The increase in plasma levels of ascorbic acid observed during the inflammatory reaction may be secondary for its transfer from tissue reserves to plasma.[39] Ascorbic acid, a water-soluble substance, has the ability to trap directly peroxyl radicals, and can also reduce α-tocopheroxyl radical to regenerate α-tocopherol; the result of these reactions is the formation of ascorbyl radical.[37,40] The fact that in the present study, ascorbyl radical plasma concentrations were not modified, do not invalidate the contribution of ascorbic acid to antioxidant capacity of plasma, since ascorbyl radical could be reduced at the expense of glutathione or other reducers.[41] The complex regulatory mechanisms of ascorbyl radicals make the relationship between this radical and the evolution of oxidative stress[42] obscure.

Regardless of the contribution of α-tocopherol and ascorbic acid to the increase in the antilipoperoxidant activity of plasma, they do not account for the entire capacity of plasma, since their concentrations were stabilized or tended to diminish at 48 hours, while the total antilipoperoxidant activity continued to increase in plasma. Contrasting with the intracellular compartment, where high levels of scavenger enzymes exist, the plasma compartment contains little or no catalase activity, and only low activities of superoxide dismutase and selenium-dependent glutathione peroxidase.[43,44] Metal-binding proteins, particularly ceruloplasmin or transferrin could contribute to the increase in antilipoperoxidant activity of the plasma. Indeed, transferrin and ceruloplasmin can inhibit hydroperoxide decomposition to radicals, by binding iron or

copper ion. In addition, ceruloplasmin can oxidize Fe^{2+} to Fe^{3+}, a less powerful transition metal implicated in the oxidative stress.[14,45,46] However, using another experimental model, i.e. rats with acute inflammation induced by carrageenan, serum transferrin and ceruloplasmin decreased.[47] Further studies are required to better characterize the systems contributing to the total antilipoperoxidant capacity of plasma.

In conclusion, an acute inflammatory reaction induced by s.c. turpentine generates an oxidative stress with systemic consequences, as suggested by the progressive increase in TBARS plasma concentrations and a gradual increment in total antilipoperoxidant capacity of the plasma. α-Tocopherol and ascorbic acid probably contribute, as well as other antioxidants, to the antilipoperoxidant capacity of plasma, at least early in the evolution of the inflammatory process.

Acknowledgements

This work was supported by the Medical Research Council of Canada (Grant # MA-7834), by Conseil Régional de Bourgogne and INSERM (Grant # CRE-900911) M. Proulx was recipient of a fellowship of the Faculté des Etudes Supérieures of the Université de Montréal (Grant # P-339).

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Accepted by Professor Ed. Janzen