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Role of lipid peroxidation and the glutathione-dependent antioxidant system in the impairment of endothelium-dependent relaxations with age

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1 Age-related changes in the blood prooxidant-antioxidant state, as well as its influence on the relaxant responses to acetylcholine (ACh) were studied in the tail artery from 6-, 24- and 30-month-old Sprague-Dawley (SD) rats.

2 Malondialdehyde (MDA) plasma levels increased 2 and 3 times in 24- and 30-month-old rats, respectively, when compared with 6-month-old rats (0.43 ± 0.09 μM). This increase was accompanied by an induction of 6-phosphogluconate dehydrogenase (6PG-DH) and glutathione reductase (GR) activities in red blood cells from 24-month-old rats. In 30-month-old rats, a further induction of these enzymatic activities, as well as glucose-6-phosphate dehydrogenase (G6P-DH) and glutathione peroxidase (GPx) activities was observed.

3 No differences with age were found in the concentration-response curves to ACh in isolated tail artery segments from 6- and 24-month-old rats precontracted with 0.3 μM noradrenaline (NA). However, a decrease in sensitivity to ACh-induced relaxation was observed in 30-month-old rats; EC_{30} values were 3.5 $(1.3-8.0) \times 10^{-7}$ M and 18.1 $(8.9-30.1) \times 10^{-7}$ M for 6- and 30-month-old rats, respectively. Moreover, a decrease in maximum ACh relaxation (10 μM) was found in 30-month-old rats in comparison with that obtained in 6-month-old rats ($58.5 \pm 3.9\%$ and $42.5 \pm 3.4\%$ of previous NA contraction, respectively).

4 Incubation of tail artery segments with MDA (0.5, 1 or 10 μM) caused a reduction of ACh-induced relaxations that was different in the three ages. Thus, the reduction of ACh-induced relaxations became significant with 0.5 μM MDA in 6-, with 1 μM MDA in 24-, and with 10 μM MDA in 30-month-old rats. In addition, MDA did not cause a shift in the concentration-response curve to ACh, but a decrease in the maximum response.

5 Superoxide dismutase (SOD; 150 u ml^{-1}, a superoxide anion scavenger) reversed the inhibitory effect of MDA on ACh-induced relaxations at all ages studied.

6 We conclude that: (1) ageing produces an increase in lipid peroxidation process, as indicated by the increase in MDA plasma levels, that is accompanied by an induction of lipid peroxide detoxification enzymes; (2) the changes in prooxidant-antioxidant equilibrium with age contribute, at least partially, to the impairment of the relaxant responses evoked by ACh; and (3) the effect of MDA appears to be mediated by superoxide anion at all ages studied.

Keywords: Malondialdehyde; lipid peroxidation; acetylcholine; endothelium-dependent relaxation; glutathione-dependent enzymes; glutathione reductase; glutathione peroxidase; superoxide dismutase; ageing; rat tail artery

Introduction

Oxidation of polyunsaturated fatty acids of membranes is a common process in living organisms, since they are the target of oxygen-derived free radicals produced during mitochondrial electron transport (Porter *et al*., 1995). Lipid peroxidation has been involved in the pathogenesis of clinically significant diseases such as atherosclerosis and diabetes, as well as in the vascular damage associated with oxidative stress (Griesmacher *et al*., 1995; Holvoet *et al*., 1995; Díaz-Vélez *et al*., 1996). Moreover, lipid peroxidation has been extensively involved in aging and age pigment formation (Kikugawa *et al*., 1981; Emerit & Chaudiere, 1989). To protect against the deleterious effects of oxyradicals and to prevent lipid peroxidation process, both non-enzymatic and enzymatic antioxidant defense systems exist. The first one includes compounds such as α-tocopherol, ascorbate, carotenoids and uric acid (Cutler, 1991; Sohal & Orr, 1992; Sies, 1993). The second includes enzymes such as catalase, superoxide dismutase (SOD) and the glutathione-dependent enzymatic system, where the key protecting enzyme is glutathione peroxidase (GPx) (Little & O'Brien, 1968; Chance *et al*., 1979). Several other enzymes, such as glutathione reductase (GR) (Fridovich, 1975; Marklund, 1984), glucose-6-phosphate dehydrogenase (G6P-DH) and 6-phosphogluconate dehydrogenase (6PG-DH) support the primary antioxidant enzymes by supplying them with either substrates or reducing equivalents. Nevertheless, protection against free radical reactions is not complete, so that changes, defining a normal ageing pattern, are continuously produced (Harman, 1984; 1992).

Malondialdehyde (MDA) is a lipid peroxidation derivative resulting from oxidation of fatty acids with three or more double bounds such as arachidonic acid. It has been extensively used as a marker of lipid peroxidation, mainly in processes associated with oxidative stress and vascular injury. Thus, an increase of either MDA vascular tissue content (Mooradian & Uko-enin, 1995) or MDA plasma level

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(Rodríguez-Martínez & Ruíz-Torres, 1992) has been found with age. In the latter case, the increase in human MDA plasma levels with age was accompanied by an induction of the erythrocyte antioxidant enzymes (Rodríguez-Martínez & Ruíz-Torres, 1992). In addition, an age-related reduction of the relaxation to acetylcholine (ACh) has been found in different vascular beds of different species, such as human brachial (Taddei *et al*., 1995) and coronary (Egashira *et al*., 1993) arteries; rat aorta (Shirasaki *et al*., 1986; Küng & Lüscher, 1995), carotid (Hongo *et al*., 1988; Paternò *et al*., 1994) and mesenteric (Shirasaki *et al*., 1986; Atkinson *et al*., 1994; Amerini *et al*., 1994) arteries; and beagles mesenteric (Shimizu & Toda, 1986) and femoral (Haidet *et al*., 1995) arteries. Recently, we have shown, for the first time, that MDA is, in addition to a marker of lipid peroxidation, an agent that causes inhibition of the endothelium-dependent relaxations to ACh in the tail artery from adult rats (Rodríguez-Martínez *et al*., 1996). Moreover, rats deprived of dietary vitamin E show increased MDA levels in plasma and aorta, this has been correlated with a decrease in the relaxation response to ACh (Hubel *et al*., 1989).

We present here a study on the changes that age produces in the blood prooxidant-antioxidant state and its influence on the impairment of the relaxation caused by ACh. For this purpose, we analysed in Sprague-Dawley (SD) rats from 6-, 24- and 30-month-old the influence of age on: (1) MDA plasma levels; (2) erythrocyte enzymatic activities of GPx, GR, G6P-DH and 6PG-DH; (3) relaxations induced by ACh; and (4) the effect of MDA in the ACh-evoked relaxations and the possible mechanisms involved.

Methods

Animals

The study was performed in 45 SD male rats (Iffa-Creddo SA, Domaine des Oncins, L'Abresle, France) of 6, 24 and 30 months, which were fed with regular chow at the facilities of the Facultad de Medicina of the Universidad Autónoma of Madrid. Animals were anaesthetized with diethyl ether (Panreac, Barcelona, Spain). A randomly selected group of 7 animals of each age was weighed, and mean blood pressure and heart rate measured in right carotid artery, and recorded by means of a pressure transducer (Letica, Barcelona, Spain) connected to a polygraph (Letica Polygraph 2006).

In the same animals, plasma MDA concentrations and erythrocyte enzymatic activities were determined in a spectrophotometer (Shimadzu, UV-160 model, Kyoto, Japan) connected to a temperature controller (Shimadzu-CPS-240A model), as described below.

MDA assay

To measure MDA plasma levels, blood obtained by cardiac puncture was collected in vacuum tubes with 3.8% sodium citrate (Venoject, Terumo Europe, Leuven, Belgium). MDA plasma concentrations were determined by the thiobarbituric acid (TBA) method modified as previously described (Rodríguez-Martínez & Ruiz-Torres, 1992), to prevent the formation of either lipid hydroperoxide or MDA by oxygenation of polyunsaturated fatty acids present in the blood samples. All assays were carried out within 2–4 h of blood extraction and the samples of reactive substances were bubbled with nitrogen before use. After blood centrifugation at $1500 \times g$ for 15 min, the plasma obtained was mixed with 20% trichloracetic acid in 0.6 M HCl (1 : 1, v/v) and the tubes were kept on ice for 20 min, so that plasma components were precipitated to avoid any possible interference. Samples were centrifuged at $1500 \times g$ for 15 min, TBA (120 mM in Tris 260 mM, pH 7) was added to the supernatant in the proportion of 1 : 5, v/v, and the mixture boiled at $97 \pm 1^{\circ}C$ for 30 min. The reaction between MDA and TBA produces a stable red pigment which is a 1 : 2-MDA:TBA adduct having a molar absorption coefficient of 1.56×10^5 M^{-1} cm^{-1} in the visible spectrum (535 nm). Spectrophotometric measurements were made at 20°C. This method is highly reproducible, as shown by the intraassay variation coefficient (4.4%, $n = 6$, 0.64 μM MDA).

Enzymatic activities

To determine erythrocyte enzymatic activities, blood samples were collected in vacuum tubes containing 1.7 mg ml^{-1} K_3 EDTA as anticoagulant. After centrifugation at $1500 \times g$ for 15 min plasma and buffy coat were removed. The pellet containing red blood cells was washed twice with an ice-cold isotonic sodium chloride solution (1 : 10, v/v), and the packed cells obtained were resuspended in the washing solution to give a 50% suspension. Haemolysis of the washed cell suspension was achieved by mixing 1 volume with 9 volumes of distilled water. The haemolysate obtained was used for the spectrophotometric determination of either haemoglobin concentration or the enzymatic activities. Haemoglobin concentration was determined according to the cyanomethaemoglobin method of Tentori & Salvati (1981). The reaction mixture contained 120 mM KH_2PO_4 (pH 7.4), 0.60 mM potassium ferrycianide, 0.77 mM potassium cyanide and the haemolysate.

G6P-DH and 6PG-DH activities were determined according to the method of Beutler (1984). The reaction mixture contained 100 mM Tris (pH 7.5), 0.5 mM K_3EDTA, 6.3 mM $Cl_2Mg.6H_2O$, 0.60 mM β-nicotinamide adenine dinucleotide phosphate (NADP) and haemolysate. To determine G6P-DH activity, 5 mM maleimide and 0.80 mM D-glucose 6-phosphate were also added to the reaction mixture, and to determine 6PG-DH activity, 0.80 mM 6-phosphogluconic acid was added to the reaction mixture. GR activity was measured according to the procedure described by Goldberg & Spooner (1983), containing the reaction mixture 100 mM Tris (pH 7.5), 0.5 mM K_3EDTA, 2.2 mM oxidized glutathione, 0.2 mM reduced NADP (NADPH) and haemolysate. GPx activity was determined according to the method of Nakamura *et al*. (1974) by using coupling of hydrogen peroxide reduction to oxidation of NADPH. Both, the blank and the sample cuvette contained 100 mM Tris (pH 7.5), 0.5 mM K_3EDTA, 2 mM reduced glutathione, 1 u ml^{-1} GR, 1 mM sodium azide and 0.2 mM NADPH. The sample cuvette contained, in addition, the haemolysate. After 5 min preincubation, the reaction was started, in both cases, by the addition of 0.2 mM hydrogen peroxide. The blank cuvette, which indicates the spontaneous oxidation of GSH in the absence of the enzyme, was subtracted from the sample cuvette containing the haemolysate to obtain the real GPx activity. One unit of enzymatic activity was defined as the amount of enzyme capable of reducing or oxidizing 1 μmol NADP or NADPH min^{-1} g^{-1} haemoglobin at 30°C and pH 7.5.

Reactivity experiments

A length of about 10 cm of the proximal end of the tail artery was carefully dissected out, cleaned of connective tissue and

kept in Krebs-Henseleit solution (KHS) at 4°C for a period less than 30 min; time necessary to divide the artery into segments of 3 mm in length and to place them in the organ bath. For isometric tension recording, each segment was set up in an organ bath that contained 5 ml of KHS at 37°C continuously bubbled with a 95% O_2-5% CO_2 mixture, which gave a pH of 7.4. Two horizontally arranged stainless steel pins, 75 μm in diameter, were passed through the lumen of the vascular cylinder. One pin was fixed to the organ bath wall, while the other one was vertically connected to a strain gauge for isometric tension recording. The isometric contraction was recorded through a force-displacement transducer (Grass FTO3C; Quincy, Mass., U.S.A.) connected to a polygraph (Grass, model 7D). Segments were subjected to a tension of 0.5 g (optimal resting tension), which was readjusted every 15 min during a 60 min equilibration period before drug administration. Vessels were previously exposed to 75 mM K^+ to check their functional integrity. After a washout period, to confirm the presence and functionality of the vascular endothelium, each segment was contracted with 0.3 μM noradrenaline (NA) and once a stable plateau had been reached, 10 μM ACh was added to analyse its ability to induce relaxation.

Experimental protocol for reactivity

To study the effect of age on ACh-induced responses, once the presence of endothelium had been demonstrated as previously described, segments from 6-, 24- and 30-month-old rats were contracted with 0.3 μM NA and when a stable plateau had been reached, a concentration-response curve to ACh (10 nM – 10 μM) was carried out. Concentration-response curves to ACh from 24- and 30-month-old rats were compared with those obtained from 6-month-old rats. In another set of experiments, the effect of MDA on the relaxations to ACh was studied in the different age groups. Thus, after a control concentration-response curve to ACh had been carried out, different concentrations of MDA (0.5, 1 and 10 μM) were added to the bath, and 60 min later a second curve to ACh was obtained.

To analyse whether the relaxant machinery is altered with age, the relaxations elicited by the nitric oxide donor sodium nitroprusside (SNP, 1, 10 and 100 μM) were studied in precontracted (0.3 μM NA) endothelium-denuded segments from 6- and 30-month-old rats. Moreover, the effect of MDA (1 and 10 μM in 6- and 30-month-old rats, respectively) on the relaxations induced by SNP was also studied.

To assess whether SOD could prevent the effect of MDA on the ACh-induced relaxation, after a control curve to ACh had been obtained, segments from 6-, 24- and 30-month-old rats were incubated with either 150 u ml^{-1} SOD for 75 min, or 150 u ml^{-1} SOD for 15 min, followed by addition of MDA (1 μM in 6- and 24-month-old rats, or 10 μM in 30-month-old rats) for 60 min. Then, a second curve to ACh was carried out in both cases.

Solutions and drugs

The composition of KHS (mM) was: NaCl 115, $CaCl_2$ 2.5, KCl 4.6, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.2, $NaHCO_3$ 25, glucose 11.1 and Na_2EDTA 0.03.

(−)-NA hydrochloride, ACh chloride, SNP, 1,1,3,3-tetramethoxypropane, TBA, Tris, potassium ferrycianide, potassium cyanide, maleimide anhydrous, sodium azide, trichloracetic acid, D-glucose 6-phosphate, 6-phosphogluconic acid, glutathione oxidized, glutathione reduced, hydrogen peroxide, NADP, NADPH, bovine erythrocyte SOD (EC 1.15.1.1.), bakers yeast GR (EC 1.6.4.2) were purchased from Sigma Chemical Co (St. Louis, MO, U.S.A.).

For reactivity experiments, stock solutions (10 mM) of drugs were made in distilled water, except NA, which was prepared in saline (0.9% NaCl)-ascorbic acid (0.01% w/v) solution. These solutions were kept at −20°C and appropriate dilutions were made in distilled water on the day of the experiment. When MDA was used, synthetic MDA was obtained by hydrolysing 82 μl of 1,1,3,3-tetramethoxypropane with 20 μl of concentrated hydrochloric acid, and then diluting to a final volume of 5 ml with KHS. The stock solution of MDA (100 mM) was diluted with KHS and the concentrations of MDA were checked spectrophotometrically as previously described.

Statistical analysis

Results are expressed as mean ± s.e.mean. Student's t test, and one-tail analysis of variance (ANOVA) were used to determine significant differences between means. In the reactivity experiments, a vertical pairwise contrast was also used to determine the ACh concentrations at which a difference between treatment groups or ages was first apparent; P values were adjusted by the Dunn-Sidàk procedure (Ludbrook, 1994). A P value less than 0.05 was considered significant. More than 3 rats were used in each set of vascular reactivity experiments.

The sensitivity to ACh was measured in the different age groups by the ACh concentration that produces a 30% inhibition of 0.3 μM NA contraction (EC_{30}). These values were determined according to the method of Fleming *et al.* (1972), and expressed as mean and 95% confidence interval.

In the reactivity experiments, the relaxant responses are expressed as a percentage of the previous contraction induced by 0.3 μM NA.

Results

MDA plasma levels and biological variables

A randomly-selected group of 7 SD rats of each age group was weighed, and mean blood pressure and heart rate measured in right carotid artery. There was no significant differences in either the mean blood pressure or the heart rate with age. Nevertheless, the weight of animals increased with age, this increase being greater in 24- than 30-month-old rats (Table 1).

MDA plasma levels were 0.43 ± 0.09 μM in 6-month-old rats. These levels increased 2 and 3 times in 24- and 30-month-old rats, respectively (Figure 1).

Table 1 Variations with age of weight, mean arterial pressure (MAP) and heart rate (HR) of the SD rats

	6 Months	*24 Months*	*30 Months*
Weight (g)	442 ± 24	664 ± 30 *	556 ± 45 *†
HR (beats min^{-1})	352 ± 11	365 ± 22	348 ± 14
MAP (mmHg)	117 ± 3	131 ± 18	113 ± 5

Results from 6–7 rats are expressed as mean ± s.e.mean. *$P<0.05$ vs 6-month-old rats. †$P<0.05$ vs 24-month-old rats.

Variations in the erythrocyte activities of the glutathione-dependent oxidative detoxification enzymes with age

The enzymatic activities in red blood cells, used as a measure of the detoxification capacity of lipid peroxidation derivatives, showed an age-dependent variation (Figure 2). In 24-month-old rats, 6PG-DH and GR, that regulate the velocity of function of this detoxification enzymatic system, showed an induction in their activities. At 30 months, the enzymatic activities of G6P-DH, 6PG-DH, GR and GPx were induced in red blood cell. Likewise, in 30-month-old rats, 6PG-DH, GR and GPx showed an increase in their activities in comparison with those found in 24-month-old rats (Figure 2).

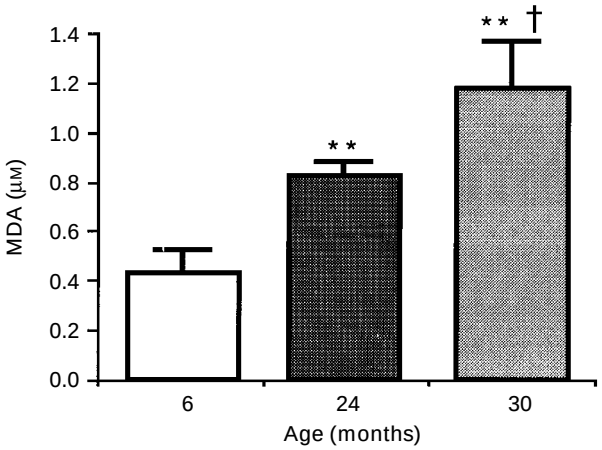

Figure 1 Changes in malondialdehyde (MDA) plasma levels with age. Results from 6–7 rats of each age group are expressed as mean ± s.e.mean. $^{**}P<0.001$ vs 6-month-old rats. †$P<0.05$ vs 24-month-old rats.

Role of MDA in the changes with age in the ACh-induced relaxation

ACh (10 nM – 10 μM) caused endothelium-dependent relaxation in tail artery segments precontracted with NA (0.3 μM). The contractions elicited by 0.3 μM NA (525 ± 91, 435 ± 60 and 486 ± 63 mg in 6-, 24- and 30-month-old rats, respectively) as well as the contractions induced by 75 mM KCl (1016 ± 77, 990 ± 90 and 1095 ± 69 mg in 6-, 24-, and 30-month-old rats, respectively) were similar at all ages studied. To analyse the changes in the relaxations to ACh with age, concentration-response curves to ACh were performed in tail artery segments from 6-, 24- and 30-month-old rats. There were not significant differences in the concentration-response curves to ACh in 6- and 24-month-old rats (Figure 3). However, a decrease in sensitivity to ACh-induced relaxation was observed in 30-month-old rats. The EC_{30} values were: 3.5 $(1.3-8.0)\times10^{-7}$ M, 4.1 $(1.6-8.1)\times10^{-7}$ M, and 18.1 $(8.9-30.1)\times10^{-7}$ M for 6-, 24- and 30-month-old rats, respectively. Moreover, a decrease in the maximum response to ACh (10 μM) was found in 30-month-old rats in comparison with that obtained in 6-month-old rats (58.5 ± 3.9% and 42.5 ± 3.4%, respectively) (Figure 3). In addition, there were not significant differences between three consecutive control curves to ACh in each age group (data not shown).

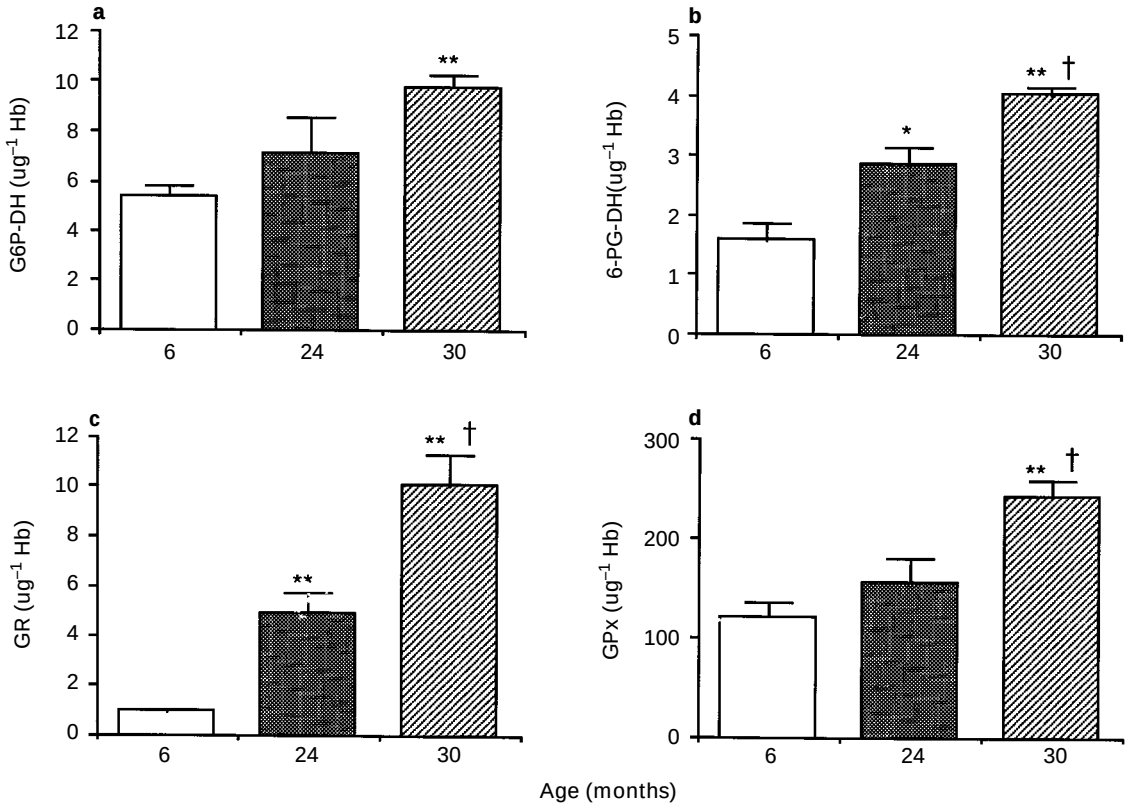

Figure 2 Influence of age on the erythrocyte enzymatic activities of (a) glucose-6-phosphate dehydrogenase (G6P-DH), (b) 6-phosphogluconate dehydrogenase (6PG-DH), (c) glutathione reductase (GR) and (d) glutathione peroxidase (GPx). Results from 6–7 rats of each age group are expressed as mean ± s.e.mean. $^{*}P<0.05$ and $^{**}P<0.001$ vs 6-month-old rats. †$P<0.05$ vs 24-month-old rats.

MDA (0.5, 1 and 10 μM) did not alter either the basal tone of segments or the tone elicited by 0.3 μM NA (data not shown). Preincubation of segments from 6-, 24- and 30-month-old rats for 60 min with MDA produced a reduction of the relaxation induced by ACh; and the MDA concentration necessary to produce this reduction was different depending on age. In 6-month-old rats, a significant reduction of ACh-induced relaxation started at 10 μM ACh with 0.5 μM MDA, at 1 μM ACh with 1 μM MDA, and at 0.1 μM ACh with 10 μM MDA (Figure 4). In 24-month-old rats, this reduction started at 1 μM ACh with 1 μM MDA, and at 0.1 μM ACh with 10 μM MDA; no effect was observed with the lowest concentration of MDA (Figure 5). In 30-month-old rats, the reduction started at 1 μM ACh with 10 μM MDA, whereas lower MDA concentrations did not produce an effect (Figure 6). In addition, the inhibitory effect of MDA on the relaxations evoked by ACh did not disappear after repeated washing of segments with KHS (data not shown).

The relaxant responses to SNP (1, 10 and 100 μM) were studied in endothelium-denuded segments from 6- and 30-month-old rats. SNP produced similar concentration-dependent relaxations in segments of both age groups. Thus, the relaxations caused by these SNP concentrations were: 1 μM, $21\pm5\%$; 10 μM, $10\pm4\%$; and 100 μM, $2\pm4\%$ in 6-month-old rats, and 1 μM, $25\pm4\%$; 10 μM, $13\pm3\%$; and 100 μM, $0.1\pm3\%$ in 30-month-old rats. In addition, neither 1 μM MDA in 6- nor 10 μM MDA in 30-month-old rats modified the relaxant responses caused by SNP (data not shown).

To assess whether free radical production could be implicated in the mechanism of action of MDA, tail artery segments from 6-, 24- and 30-month-old rats were incubated with either 150 u ml^{-1} SOD (a superoxide anion scavenger) for 75 min or with 150 u ml^{-1} SOD for 15 min followed by the addition of MDA for 60 min. In these experiments, while segments from 6- and 24-month-old rats were incubated with 1 μM MDA, segments from 30-month-old rats were incubated with 10 μM MDA due to their reduced sensitivity to the inhibitory effect of MDA on ACh-induced relaxations, as previously shown. The impairment of endothelium-dependent relaxations to ACh by MDA was completely reversed by SOD in all age groups (Figure 7). The incubation of segments with only 150 u ml^{-1} SOD did not modify the relaxant responses to ACh at any age studied (data not shown).

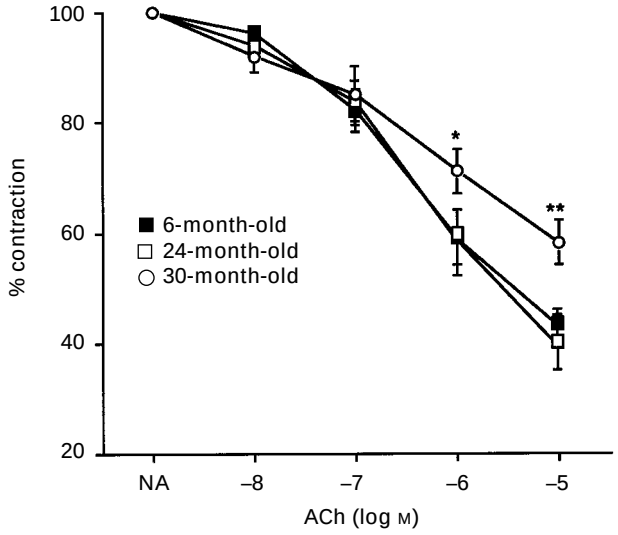

Figure 3 Effect of age on relaxant responses to acetylcholine (ACh) in the rat tail artery. Results from 10–14 arterial segments studied in each age group are expressed as a percentage of the previous contraction to 0.3 μM noradrenaline (NA). The contractions induced by NA in 6-, 24- and 30-month-old rats were: 525 ± 91 mg, 435 ± 60 mg and 486 ± 63 mg, respectively. ANOVA, $P<0.05$, 30- vs 6- and 24-month-old rats. Vertical pairwise contrast: $^{*}P<0.05$ and $^{**}P<0.001$. Vertical lines show s.e.mean.

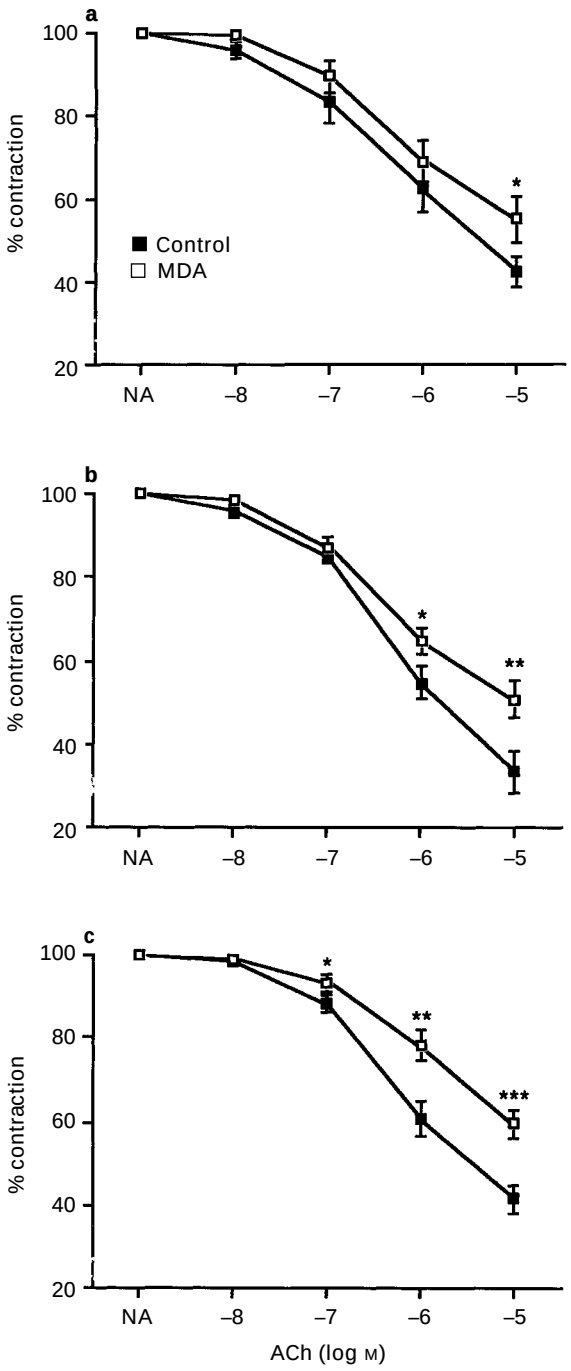

Figure 4 Effect of 60 min preincubation with malondialdehyde (MDA) on the relaxations induced by acetylcholine (ACh) in tail artery segments from 6-month-old rats. (a) MDA 0.5 μM vs control, ANOVA, $P=0.03$. (b) MDA 1 μM vs control, ANOVA, $P<0.001$. (c) MDA 10 μM vs control, ANOVA, $P<0.001$. Results from 10–14 arterial segments studied in each set of experiments are expressed as a percentage of the previous contraction to 0.3 μM noradrenaline (NA). Vertical pairwise contrast: $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. Vertical lines show s.e.mean.

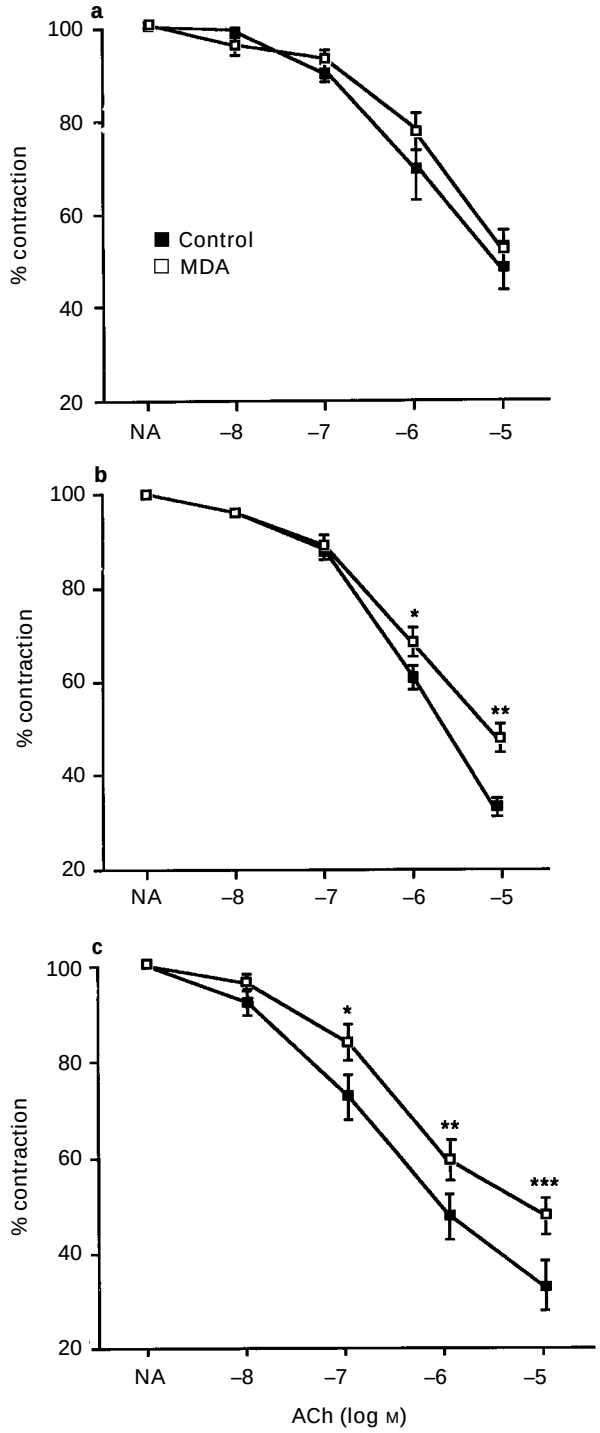

Figure 5 Effect of 60 min preincubation with malondialdehyde (MDA) on the relaxations elicited by acetylcholine (ACh) in tail artery segments from 24-month-old rats. (a) MDA 0.5 μM vs control, ANOVA, $P=0.86$. (b) MDA 1 μM vs control, ANOVA, $P<0.05$. (c) MDA 10 μM vs control, ANOVA, $P<0.001$. Results from 10–14 arterial segments studied in each set of experiments are expressed as a percentage of the previous contraction to 0.3 μM noradrenaline (NA). Vertical pairwise contrast: $*P<0.05$, $**P<0.01$ and $***P<0.001$. Vertical lines show s.e.mean.

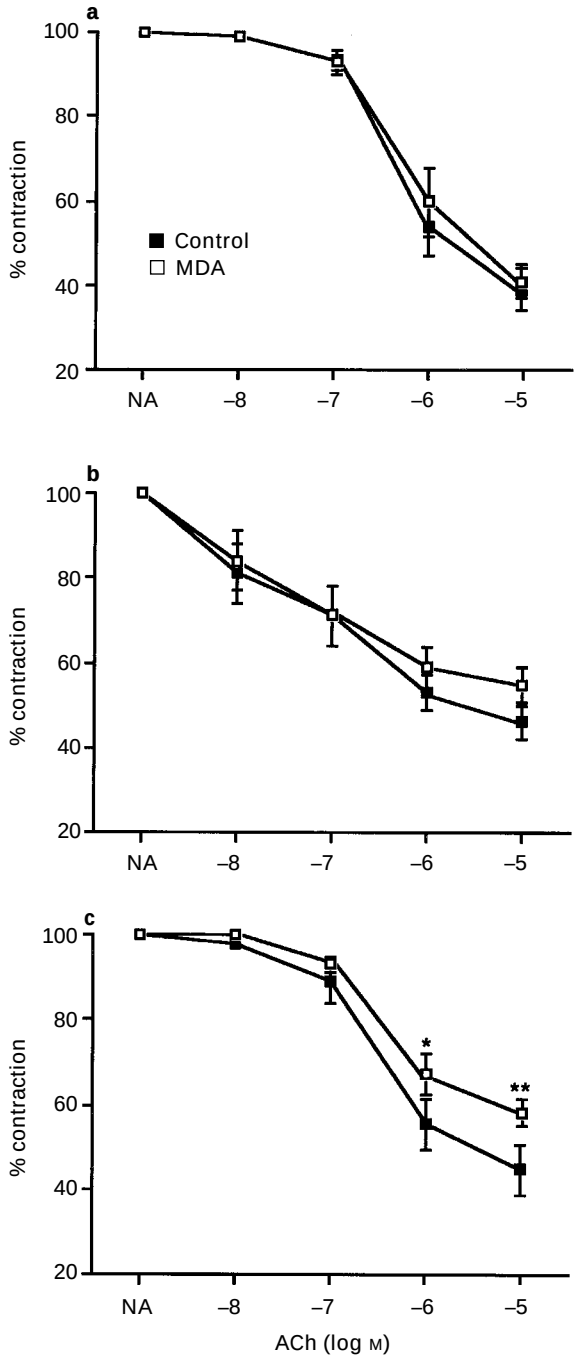

Figure 6 Effect of 60 min preincubation with malondialdehyde (MDA) on the relaxations induced by acetylcholine (ACh) in tail artery segments from 30-month-old rats. (a) MDA 0.5 μM vs control, ANOVA, $P=0.86$. (b) MDA 1 μM vs control, ANOVA, $P=0.30$. (c) MDA 10 μM vs control, ANOVA, $P<0.05$. Results from 8–10 arterial segments studied in each set of experiments are expressed as a percentage of the previous contraction to 0.3 μM noradrenaline (NA). Vertical pairwise contrast: $*P<0.05$ and $**P<0.01$. Vertical lines show s.e.mean.

Discussion

The present study shows that MDA plasma levels increased 2 and 3 times in 24- and 30-month-old rats, respectively, in comparison with 6-month-old rats. Since MDA is an end product of the metabolically uncoupled peroxidation of polyunsaturated fatty acids, stimulated by free radicals such as hydroxyl radical, the increase in MDA plasma levels with age could be interpreted as a greater production of free radicals during the aging process. Increased MDA plasma levels with age in man have been demonstrated previously (Rodríguez-Martínez & Ruíz-Torres, 1992; Congy *et al.*, 1995; Sanderson *et al.*, 1995). In addition, an increase with age in either MDA production or MDA-modified protein content in vascular and other tissues from rats has also been

found (Mooradian *et al*., 1994; Mooradian & Uko-eninn, 1995).

Enhancement of the lipid peroxidation process appears to be a common finding during the aging process. Nevertheless, the rate of occurrence of lipid peroxidation process with age seems to be dependent on the antioxidant capacity of either the organ or the species studied (Harman, 1984; López-Torres *et al*., 1993). We have analysed the effect of age on the activities of the glutathione-dependent enzymes in erythrocytes, since red blood cells are especially well protected against oxidative events, and could be like a 'sink' of circulating free radicals (Sweder Van Asbeck *et al*., 1985). Furthermore, we had found in man a good correlation with age between changes in MDA plasma levels and changes in glutathione-dependent enzymatic activities in red blood cells (Rodríguez-Martínez & Ruíz-Torres, 1992). In 24-month-old rats we have observed an induction of the erythrocyte activities of 6PG-DH and GR. It is an interesting finding since 6PG-DH is the enzyme of the penthose phosphate shunt, that limits the velocity of production of reducing equivalents such as NADPH, and GR is the enzyme that provides the GSH for the glutathione-dependent system. The induction of both enzymatic activities could be interpreted as a greater demand of reductant power with age, probably to compensate for the increased free radical production, as reflected by increased MDA plasma levels in these animals. In 30-month-old rats, all the erythrocyte enzymatic activities studied were induced, in parallel with the additional increase in MDA plasma levels. This induction of the lipid peroxide detoxification system with age could indicate the existence of a greater antioxidant capacity in response to an increased lipid peroxidation with age. These results agree with the hypothesis that in a normal ageing pattern a relatively stable equilibrium between free radical production and detoxification by the antioxidant systems should be expected to maintain homeostasis (Harman, 1984). Nevertheless, whereas the increase in lipid peroxidation process with age is extensively accepted, the increase in the antioxidant potential with age is a controversial point. Thus some studies, conducted on the effect of age on antioxidant defences, have demonstrated a mixed age-related pattern depending on both tissues and species (Sohal *et al*., 1990; Pérez *et al*., 1991; Rodríguez-Martínez & Ruiz-Torres 1992; López-Torres *et al*., 1993; Mo *et al*., 1995; Christon *et al*., 1995; Azhar *et al*., 1995). Recently, some authors have postulated that alterations in the balance between the first and second step of the antioxidant pathway correlate with cellular damage during ageing (de-Haan *et al*., 1995; Cristiano *et al*., 1995). Hence, isolated changes in some enzymatic activities with age probably do not reflect the real antioxidant potential with age. Our results show that the induction of the glutathione-dependent enzymes is followed by induction of dehydrogenase activities of the penthose phosphate shunt with age, which indicates that both enzymatic pathways act in a coordinated manner during the ageing process.

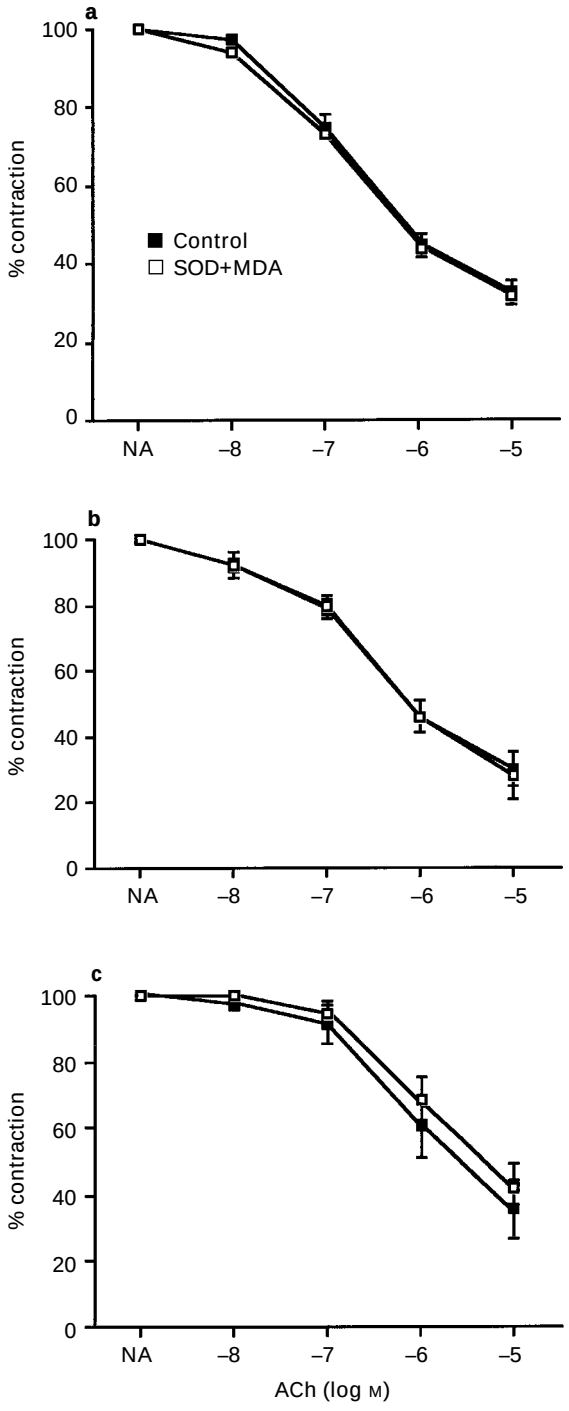

Figure 7 Effect of superoxide dismutase (SOD) on the inhibition of ACh-evoked relaxations caused by malondialdehyde (MDA). ACh-induced relaxations in the absence (control) or presence of: (a) 150 u ml^{-1} SOD + 1 μM MDA in tail artery segments from 6-month-old rats, ANOVA, $P = 0.50$. (b) 150 u ml^{-1} SOD + 1 μM MDA in tail artery segments from 24-month-old rats, ANOVA, $P = 0.98$. (c) 150 u ml^{-1} SOD + 10 μM MDA in tail artery segments from 30-month-old rats, ANOVA, $P = 0.30$. Results from 8–10 arterial segments studied in each set of experiments are expressed as a percentage of the previous contraction to 0.3 μM noradrenaline (NA). Vertical lines show s.e.mean.

Impairment of vascular responses to different vasoactive agents with age is a well-known phenomenon, especially with respect to endothelium-dependent relaxations, such as those evoked by ACh (Marín, 1995). We found no variations in the relaxations caused by ACh in tail arteries from 24-month-old SD rats in comparison with those from young animals (6 months). Nevertheless, in arteries from 30-month-old rats a reduction in ACh-evoked relaxant responses was observed. This reduction was not due to an enhancement in the ability of aged arterial segments to develop a contractile response, since the responses to both 75 mM KCl and 0.3 μM NA were not modified with age. A decrease in the relaxations induced by ACh with age has been found in different vascular beds of different species (Shimizu & Toda, 1986; Paternò *et al*., 1994; Atkinson *et al*., 1994; Taddei *et al*., 1995; Küng & Lüscher, 1995; Haidet *et al*., 1995). It is interesting to note, that the majority of studies analysing the effect of age on the relaxant

responses have been done in Wistar or Wistar-Kyoto rats, in which the impairment of these responses appears between 18 and 22 months of age. In our case, the impairment of ACh relaxations appeared in very old (30 months) SD rats, in which high MDA plasma levels were observed despite the induction of the detoxification enzymes. A possible explanation could be the existence of differences in the blood prooxidant-antioxidant state among different rat strains, although this point remains to be clarified.

Recently, we demonstrated, for the first time, that MDA produces a reduction of endothelium-dependent relaxations to ACh in the tail artery from adult SD rats (6 months) (Rodríguez-Martínez *et al*., 1996). In the present study, we have assessed the effect of age on the vascular actions of MDA, associated with age-related changes in the blood prooxidant-antioxidant state of the animals. In 6-month-old rats, MDA produced an inhibition of ACh-induced relaxations to concentrations either similar (0.5 μM) or higher (1 and 10 μM) to those found in plasma (0.43 ± 0.09 μM) of the animals. In addition, as the MDA concentration increased, its ability to inhibit the relaxations to lower ACh concentrations also increased. In 24-month-old rats, the inhibitory effect of MDA on ACh-induced relaxations was not apparent with a MDA concentration lower (0.5 μM MDA) than that found in the plasma (0.82 ± 0.06 μM MDA) of rats, whereas similar or higher MDA concentrations (1 and 10 μM MDA, respectively) to those found in plasma produced inhibition of the ACh-induced relaxations. In 30-month-old rats, the reduction in ACh relaxation was observed only at 10 μM MDA. These results indicate that the exposure of the tail artery to MDA produces an inhibition of endothelium-dependent relaxations caused by ACh in young (6 months), old (24 months) and very old (30 months) SD rats. Therefore, MDA is, in addition to a marker of changes in lipid peroxidation process with age, an agent that contributes to the impairment of relaxations induced by ACh at any age, although the MDA sensitivity of the tail artery is reduced with age. The fact that the *in vitro* sensitivity to the vascular effect of MDA decreases with age could reflect an adaptation of the vascular wall *in vivo* to the continuous exposure to MDA plasma levels, which increase with age. The induction of lipid peroxide detoxification enzymes in red blood cells, as a compensatory mechanism, supports this conclusion.

In addition, the fact that the relaxations caused by the nitric oxide donor SNP were not modified by either age or MDA suggests that: (1) the relaxant machinery is not altered with age; (2) the effect of MDA on the relaxations elicited by ACh is not due to an alteration of such a machinery, and (3) MDA probably acts at endothelial level.

As MDA is involved in chains of membrane lipid peroxidation in which free radicals play an important role, and as free radical production is also involved in the ageing process, we studied the effect of SOD (an enzymatic antioxidant that scavenges superoxide anion) on the reduction of ACh-evoked relaxations caused by MDA. Tail artery segments from all ages were incubated with MDA, at concentrations greater than those found in the plasma at each age to assure that the inhibitory effect of MDA on ACh relaxations was produced. We observed that 150 u ml^{-1} SOD completely reversed the inhibitory effect of MDA at all ages studied. The fact that SOD prevents the inhibitory effect of MDA on ACh-induced relaxations in young (6 months), old (24 months) and very old (30 months) rats indicates that the effect of MDA is mediated by superoxide anion generation in all ages. We hypothesize that MDA could contribute to the maintenance of the lipid peroxidation process at the endothelial membrane level, by favouring an imbalance between intrinsic SOD and superoxide anion generation. Since SOD activity in endothelial cells is necessary for the release of biologically active nitric oxide (Mügge *et al*., 1991), the imbalance between SOD and superoxide anion generation produced by MDA could justify the impairment of ACh-induced relaxations.

In summary, the results from the present studies suggest that: (1) ageing produces an increase in the lipid peroxidation process, as indicated by the increase in MDA plasma levels, that is accompanied by an induction of lipid peroxide detoxification enzymes; (2) the changes in the blood prooxidant-antioxidant equilibrium with age contribute, at least partially, to the impairment of the relaxant responses evoked by ACh; and (3) the effect of MDA is mediated by superoxide anion at all ages studied. These results open new persepectives to the understanding of the role that lipid peroxidation derivatives, such as MDA, play in the regulation of vascular responses in either ageing or diseases associated with oxidative stress.

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