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# Consequences of Treatment with Dexamethasone in Rats on the Susceptibility of Total Plasma and Isolated Lipoprotein Fractions to Copper Oxidation

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**According to the oxidative hypothesis of atherosclerosis, a hyperoxidizability of lipoproteins could favor the development of the atherosclerotic process. Besides, it has been recently reported that models of elevated very-low-density-lipoprotein (VLDL) levels in rats resulted in an increased susceptibility of these VLDL to oxidation. Treatment with dexamethasone classically induces an increase in plasma VLDL concentration. The aim of our study was thus to assess the effects of a treatment with dexamethasone in rats on the susceptibility to copper oxidation, both on total plasma and on isolated lipoproteins.**

**Male Sprague-Dawley rats aged three months were treated with a daily intraperitoneal injection of dexamethasone (1.5 mg per kg) for five days (DEX group), whereas control rats were fed *ad libitum* (AL group). In order to take into account the decrease of food intake induced by dexamethasone treatment, a group of *pair-fed* rats was constituted (PF group). These rats had the same food intake as rats of the DEX group and were treated with a daily isovolumic intraperitoneal injection of NaCl for 5 d. After 5 d treatment, rats were fasted overnight, then killed, and blood was collected on EDTA. Low-density lipoproteins (VLDL + LDL) and high-density lipoproteins (HDL) were isolated by ultracentrifugation. A copper oxidation was conducted both on total plasma and on isolated lipoproteins.**

**As expected, after treatment with dexamethasone, plasma exhibited increased triglyceride and glucose levels. Similarly, VLDL + LDL of rats from the DEX group were enriched with triglycerides, when compared with VLDL + LDL of the other two groups of rats. Our major finding was a marked increase in the susceptibility of total plasma of the DEX group to copper oxidation, in comparison with the other two groups of rats. This oxidizability was assessed by the maximal level of oxidation products absorbing at 234 nm and classically considered to be conjugated dienes ($7.46 \pm 0.70$ μmol $L^{-1}$ in the DEX group vs $3.36 \pm 0.40$ and $2.05 \pm 0.60$ μmol $L^{-1}$ in the AL and PF groups, respectively). Nevertheless, this higher oxidizability was not observed in the isolated lipoprotein fractions, as shown by the formation of lipid peroxidation products such as conjugated dienes, thiobarbituric-acid reactive substances, hydroperoxides, 7-ketocholesterol, and dienals. This is not in agreement with other models of hypertriglyceridemia that have been reported to induce a hyperoxidizability of lipoproteins in rats. Our results led us to hypothesize that other plasma components such as proteins could be involved in this susceptibility to oxidation. Indeed, the severe protein catabolism induced by dexamethasone treatment could support this hypothesis, by forming protein components that are more susceptible to oxidation, as shown by an increased carbonyl formation upon plasma copper oxidation.**

**Key Words:** Carbonyl formation; copper oxidizability; dexamethasone; lipoproteins; plasma.

**Abbreviations:** d = density; HDL = high density lipoprotein; LDL = low density lipoprotein; mRNA = messenger ribonucleic acid; $Na_2EDTA$ = disodium ethylene diamine tetraacetate; TBARS = thiobarbituric acid-reactive substances; VLDL = very low density lipoprotein.

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## Introduction

Dexamethasone (9α-fluoro 16α-methylprednisolone) is a commonly used antiinflammatory and immunosuppressive corticoid. Treatment with dexamethasone in humans results in metabolic disorders close to those observed in Cushing's syndrome *(1)*. It induces hyperglycemia *(2)* and an enhancement of plasma protein concentration partly related to increased liver biosynthesis due to muscle catabolism *(3)*. Moreover, treatment with dexamethasone has been shown to lead to hypertriglyceridemia *(2,4–6)* associated with a rise in the concentration of very-low-density lipoproteins (VLDL), which are the main triglyceride carriers in plasma *(2,4,5,7)*. This rise could result from an increased VLDL production by the liver *(4,6,8)* and/or from a decreased VLDL catabolism *(3,4)* due to lipoprotein lipase inactivation *(9)*. According to the oxidative hypothesis of atherosclerosis *(10)*, which involves oxidized lipoproteins in the development of atherosclerotic plaques, such a hypertriglyceridemia could favor the atherosclerotic process. Indeed, it has recently been reported that other models of hypertriglyceridemia in rats led to an increased susceptibility of VLDL to copper oxidation when compared with control rats without hypertriglyceridemia *(7,11,12)*.

In contrast, other studies reported a protective effect of dexamethasone. In addition, dexamethasone has been shown to increase the activity of some antioxidant enzymes such as catalase, glutathione peroxidase, and superoxide dismutase in the adult rat lung, via a mechanism of enzyme induction *(13)*. Similarly, dexamethasone could induce suppression of aortic atherosclerosis in cholesterol-fed rabbits by decreasing macrophage recruitment in the vascular wall, despite inducing a hypertriglyceridemia *(3)*. Indeed, it is possible that dexamethasone inhibits the chemotaxis of oxidized β-VLDL toward monocytes *(14)* and decreases the internalization of β-VLDL, which could thus inhibit formation of foam cells by decreasing β-VLDL uptake by macrophages *(3)*.

Thus, despite its hyperlipidemic effect and a possible enhancement of coronary risk factors in long-term treatment when used in diseases such as systemic lupus erythematosus or rheumatoid arthritis *(15–17)*, dexamethasone could exhibit some antioxidant and antiatherogenic properties. However, no data are available about the consequences of dexamethasone-induced hypertriglyceridemia on the susceptibility of lipoproteins to oxidation, whereas, as reported above, other models of hypertriglyceridemia induced an increased oxidizability of lipoproteins *(7,11,12)*. Therefore, the aim of our study was to assess the consequences of a treatment with dexamethasone in rats on the susceptibility to copper oxidation, both on total plasma and on isolated plasma lipoproteins.

## Results

### *Plasma Analyses and Oxidation*

Table 1 shows the plasma glucose, protein, triglyceride, and phospholipid concentrations of the three groups of rats (AL, PF, and DEX). As expected, the glycemia of the DEX rats (11.2 mmol $L^{-1}$) was enhanced by 64% when compared to that of the AL rats (6.8 mmol $L^{-1}$) and by 20% in comparison with the PF rats (9.3 mmol $L^{-1}$). Dexamethasone-treated rats also exhibited a 10% higher protein concentration than the AL or PF animals. With regard to the lipid status, the main effect of dexamethasone treatment appeared on triglycerides, whose concentration was 5.2-times and 2.5-times higher in the DEX group than in the PF and AL groups, respectively. In contrast, plasma phospholipid levels were significantly lower in the DEX group than in the others. Cholesterol concentration was not significantly different among the three groups of rats. Plasma agarose gel electrophoresis provided data in agreement with this plasma lipid status. Indeed, a similar proportion of β-lipoproteins was observed in the three groups (15.4 ± 2.3%, 18.5 ± 3.4%, and 18.3 ± 2.6% in the AL, PF, and DEX groups, respectively). In contrast, pre-β-lipoprotein proportion was markedly increased in the DEX group (36.7 ± 3.2%) in comparison with the AL group (25.2 ± 3.8%, $p < 0.05$) and the PF group (18.4 ± 3.3%, $p < 0.05$). Consequently, less α-lipoproteins were found in DEX rats than in AL and PF rats (44.8 ± 5.5% vs 59.4 ± 4.0%, and 63.3 ± 2.8% respectively). Plasma thiobarbituric acid-reactive substances (TBARS), α-tocopherol, and β-carotene levels of the three groups of rats are given in Table 2. Plasma TBARS concentrations were similar in all groups, whereas the DEX and PF groups exhibited higher levels of α-toco-

Table 1
Plasma Concentrations of Glucose, Total Proteins, Total Cholesterol, Triglycerides, and Phospholipids in the Three Groups of Rats[d]

| | Glucose (mmol $L^{-1}$) | Total proteins (g $L^{-1}$) | Total cholesterol (mmol $L^{-1}$) | Triglycerides (mmol $L^{-1}$) | Phospholipids (mmol $L^{-1}$) |
|---|---|---|---|---|---|
| AL ($n = 3$) | $6.8 \pm 0.4^{a,b}$ | $60 \pm 2^{b}$ | 1.90 ± 0.20 | $0.72 \pm 0.20^{a,b}$ | $1.51 \pm 0.06^{a,b}$ |
| PF ($n = 6$) | $9.3 \pm 0.5^{a,c}$ | $62 \pm 1^{c}$ | 2.09 ± 0.20 | $0.35 \pm 0.05^{a,c}$ | $1.29 \pm 0.09^{a,c}$ |
| DEX ($n = 3$) | $11.2 \pm 1.0^{b,c}$ | $68 \pm 1^{b,c}$ | 2.00 ± 0.80 | $1.82 \pm 0.10^{b,c}$ | $1.03 \pm 0.14^{b,c}$ |

[a] $p < 0.05$ (AL vs PF)
[b] $p < 0.05$ (AL vs DEX)
[c] $p < 0.05$ (DEX vs PF)
[d] AL: fed *ad libitum*; PF: *pair-fed*; and DEX: dexamethasone-treated. Results are means ± SD of *n* pools of plasmas.

Table 2
Plasma Concentrations of Thiobarbituric Acid-Reactive Substances (TBARS), α-Tocopherol and β-Carotene, and α-Tocopherol/Total Lipid Ratio in the Three Groups of Rats[e]

| | TBARS (μmol $L^{-1}$) | α-tocopherol (μmol $L^{-1}$) | α-tocopherol/total lipids (μmol $g^{-1}$) | β-carotene (μmol $L^{-1}$) |
|---|---|---|---|---|
| AL ($n$ = 3) | 2.13 ± 0.10 | 9.9 ± 0.7[a,b] | 3.38 ± 0.26[a] | n.d.[d] |
| PF ($n$ = 6) | 2.13 ± 0.28 | 17.7 ± 1.8[a] | 5.41 ± 0.42[a,c] | n.d. |
| DEX ($n$ = 3) | 2.13 ± 0.20 | 14.5 ± 3.4[b] | 4.04 ± 0.56[c] | n.d. |

[a] $p < 0.05$ (AL vs PF)
[b] $p < 0.05$ (AL vs DEX)
[c] $p < 0.05$ (DEX vs PF)
[d] n.d.: not detectable
[e] AL: fed *ad libitum*; PF: *pair-fed*; and DEX: dexamethasone-treated. Results are means ± SD of $n$ pools of plasmas.

pherol than that of the AL group. This latter feature was also observed with regard to the α-tocopherol/total lipid ratio, especially for the PF group. It is noteworthy that no β-carotene concentration was detectable in the plasma.

Figure 1 shows differential absorbance at 234 nm in plasma as a function of the oxidation time (150-fold diluted plasma in $10^{-2}$ mol $L^{-1}$ PBS, 50 μmol $L^{-1}$ $CuSO_4$, 37°C), for the three groups of rats. No lag time could be determined, given the very quick oxidation of plasma under these experimental conditions. The main feature was related to the plasma of rats treated with dexamethasone, which, after about 150 min oxidation, exhibited a markedly higher oxidation plateau-level than those of the two other groups (Δ absorbance = 0.22 ± 0.02 vs 0.10 ± 0.01 and 0.09 ± 0.02 for AL and PF groups, respectively). Given a molar extinction coefficient $\varepsilon_{234}$ = 29,500 $mol^{-1}$ L $cm^{-1}$ *(18)*, these oxidation plateaus corresponded to maximum theoretical conjugated diene concentrations of 3.36 ± 0.46, 2.05 ± 0.60, and 7.46 ± 0.70 μmol $L^{-1}$ in the AL, PF, and DEX groups, respectively. Treatment with dexamethasone thus clearly induced a much higher oxidizability of the plasma, in comparison with the plasma of the AL and PF groups whose behavior was similar.

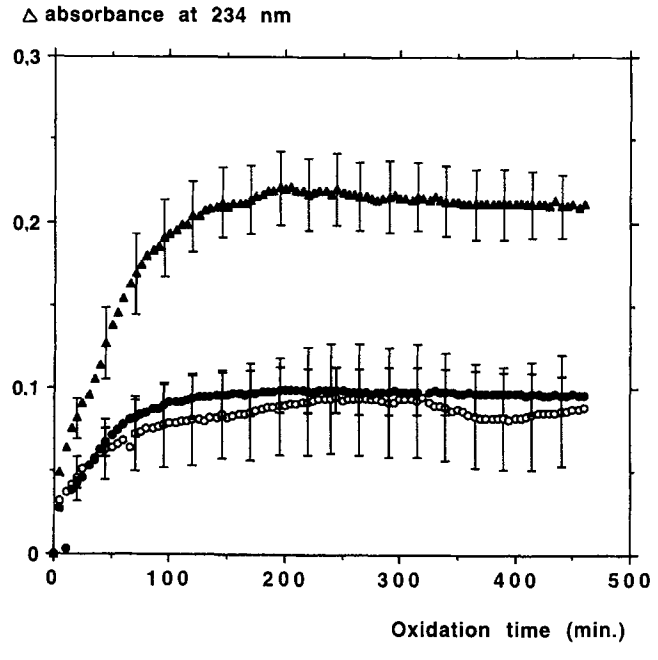


**Fig. 1.** Continuous monitoring of the differential absorbance at 234 nm of plasma as a function of the oxidation time (150-fold diluted plasma in $10^{-2}$ mol $L^{-1}$ PBS, 50 μmol $L^{-1}$ $CuSO_4$, 37°C), for the three groups of rats [i.e., AL (●): fed *ad libitum*, PF (○): *pair-fed*, and DEX (▲): dexamethasone-treated]. Results are means of three pools (AL and DEX groups) or of six pools (PF group) of plasmas. To improve the clarity of the figure, only some SD are shown.

Plasma carbonyl content determined on the same plasma samples before and after 150 min. of copper oxidation showed a significantly higher carbonyl formation in the DEX group than in the AL and PF groups (3.50 ± 1.48 nmol $mg^{-1}$ protein vs 1.18 ± 0.50 and 1.23 ± 0.48 nmol $mg^{-1}$ protein, respectively, given a molar extinction coefficient of 22,000 $mol^{-1}$ L $cm^{-1}$) *(19)*.

### *Lipoprotein Analyses and Oxidation*

The chemical composition of VLDL + LDL and of HDL is shown in Tables 3 and 4, respectively. With regard to VLDL + LDL, lipoproteins from the DEX group exhibited a marked increase in triglyceride content (+ 26% and +98% when compared to those of the AL and PF groups, respectively) and a loss in cholesteryl esters (–39% and –68%, when compared to those of the AL and PF groups, respectively). In contrast, the protein content of VLDL + LDL was similar in the three groups. VLDL + LDL from the DEX plasma also showed a 34% decrease of their unesterified cholesterol percentage in comparison with those from the PF plasmas, and they exhibited a significantly lower phospholipid content than that of the PF VLDL + LDL. As can be observed in Table 4, the HDL from the DEX group showed a lower unesterified cholesterol content than the HDL from the two other groups (1.8 ± 0.0% vs 3.0 ± 0.6 and 2.3 ± 0.3% for PF and AL groups, respectively). This observation was also true for phospholipids (16.4 ± 2.0% for DEX, vs 20.3 ± 0.8% and 19.6 ± 0.7% for PF and AL, respectively). In contrast, HDL from the DEX

Table 3
Chemical Composition (Expressed as a Percentage of Total Lipoprotein Mass) of the VLDL + LDL Fraction Isolated From the Plasmas of the Three Groups of Rats[c]

| | Total protein (%) | Unesterified cholesterol (%) | Cholesteryl esters (%) | Triglycerides (%) | Phospholipids (%) |
|---|---|---|---|---|---|
| AL ($n = 3$) | 13.7 ± 0.1 | 4.4 ± 0.1[a] | 17.3 ± 0.6[a] | 49.4 ± 1.2[a] | 15.3 ± 0.3 |
| PF ($n = 6$) | 12.8 ± 3.0 | 6.2 ± 0.7[a,b] | 33.2 ± 2.9[a,b] | 31.3 ± 5.8[a,b] | 16.5 ± 1.3[b] |
| DEX ($n = 3$) | 11.0 ± 0.4 | 4.1 ± 0.3[b] | 10.5 ± 3.2[b] | 62.0 ± 1.4[b] | 12.4 ± 2.1[b] |

[a] $p < 0.05$ (AL vs PF)
[b] $p < 0.05$ (DEX vs PF)
[c] AL: fed *ad libitum*; PF: *pair-fed*; and DEX: dexamethasone-treated. Results are means ± SD of $n$ pools of plasmas.

Table 4
Chemical Composition (Expressed as a Percentage of Total Lipoprotein Mass) of the HDL Fraction Isolated from the Plasmas of the Three Groups of Rats

| | Total protein (%) | Unesterified cholesterol (%) | Cholesteryl esters (%) | Triglycerides (%) | Phospholipids (%) |
|---|---|---|---|---|---|
| AL ($n = 3$) | 50.5 ± 6.8[a] | 2.3 ± 0.3 | 27.1 ± 6.8[a] | 0.50 ± 0.20 | 19.6 ± 0.7 |
| PF ($n = 6$) | 42.4 ± 1.0[a,b] | 3.0 ± 0.6[b] | 33.5 ± 0.7[a,b] | 0.50 ± 0.20 | 20.3 ± 0.8[b] |
| DEX ($n = 3$) | 54.3 ± 3.1[b] | 1.8 ± 0.0[b] | 27.1 ± 1.3[b] | 0.45 ± 0.05 | 16.4 ± 2.0[b] |

[a] $p < 0.05$ (AL vs PF)
[b] $p < 0.05$ (DEX vs PF)
[c] AL: fed *ad libitum*; PF: *pair-fed*; and DEX: dexamethasone-treated). Results are means ± SD of $n$ pools of plasmas.

Table 5
Oxidation Features of the VLDL + LDL Fraction Isolated From the Plasma of the Three Groups of Rats[b]

| | Lag phase (min) | Propagation rate (μmol conjugated dienes $L^{-1}$ $min^{-1}$) | TBARS formation after 5 h oxidation (μmol $L^{-1}$) |
|---|---|---|---|
| AL ($n = 3$) | 17.2 ± 0.0 | 1.14 ± 0.15 | 13.2 ± 1.4 |
| PF ($n = 6$) | 48.1 ± 10.8 | 0.83 ± 0.09 | N.D.[a] |
| DEX ($n = 3$) | 20.3 ± 2.7 | 1.12 ± 0.13 | 14.5 ± 0.0 |

[a] N.D.: not determined, due to the low volume of lipoproteins available
[b] AL: fed *ad libitum*; PF: *pair-fed*; and DEX: dexamethasone-treated. 0.5 g $L^{-1}$ total lipoprotein, $10^{-2}$ mol $L^{-1}$ PBS, 5 μmol $L^{-1}$ $CuSO_4$, 37°C. Results are means ± SD of $n$ pools of plasmas.

group had a higher protein content (54.3 ± 3.1%, vs 42.4 ± 1.0% and 50.5 ± 6.8% for PF and AL groups, respectively).

Oxidation experiments (0.5 g $L^{-1}$ total lipoprotein, $10^{-2}$ mol $L^{-1}$ PBS, 37°C, 5 μmol $L^{-1}$ $CuSO_4$) were performed on both VLDL + LDL and HDL. Results related to VLDL + LDL oxidation are reported in Table 5. As described in the "Materials and Methods" section, continuous monitoring of VLDL + LDL oxidation by the measurement of the variation of the differential absorbance at 234 nm allowed us to determine two parameters, namely the lag phase (minutes) and the propagation rate (μmol conjugated dienes $L^{-1}$ $min^{-1}$). VLDL + LDL from the AL and DEX groups exhibited very similar lag phases (17.2 ± 0.0 and 20.3 ± 2.7 min, respectively), whereas the lag phase of VLDL + LDL from the PF group was markedly longer (48.1 ± 10.8 min.). With regard to the propagation rate, VLDL + LDL from the PF group were also less oxidizable than lipoproteins from the other two groups, as sensed by their lower propagation rate (0.83 ± 0.09 μmol conjugated dienes $L^{-1}$ $min^{-1}$ for the PF group vs 1.14 ± 0.15 and 1.12 ± 0.13 μmol conjugated dienes $L^{-1}$ $min^{-1}$ for the AL and DEX groups, respectively). Thus, VLDL + LDL from the AL and DEX groups exhibited a very similar behavior toward copper oxidation. This was also supported by the level of TBARS obtained after 5 h oxidation, which was about 14 μmol $L^{-1}$ in both the AL and DEX groups.

With regard to HDL oxidation, data reported in Table 6 show that, as previously observed for VLDL + LDL, the lag phase preceding oxidation was approximately twice as long in the PF group as in the other two groups (18.2 ± 2.1 min in the PF group vs 10.5 ± 1.4 and 9.9 ± 1.9 min in the AL and DEX groups, respectively). The propagation rate was also lower in the PF group than in the others whose propagation rates were similar. With regard to both TBARS, and more specific markers such as 7-ketocholesterol, hydroperoxides, and dienals formed after 5 h oxidation, no signifi-

Table 6
Oxidation Features of the HDL Fraction Isolated from the Plasma of the Three Groups of Rats[a]

| | Lag phase (min) | Propagation rate (μmol conjugated dienes $L^{-1}$ $min^{-1}$) | TBARS formation after 5 h oxidation (μmol $L^{-1}$) | 7-keto-cholesterol formation after 5 h oxidation (μmol $L^{-1}$) | Hydro-peroxide formation after 5 h oxidation (μmol $L^{-1}$) | Dienal formation after 5 h oxidation (μmol $L^{-1}$) |
|---|---|---|---|---|---|---|
| AL ($n$ = 3) | 10.5 ± 1.4 | 1.09 ± 0.10 | 14.1 ± 0.2 | 100.5 ± 1.4 | 10.3 ± 0.9 | 30.5 ± 0.7 |
| PF ($n$ = 6) | 18.2 ± 2.1 | 0.79 ± 0.08 | 12.9 ± 0.5 | 88.6 ± 9.1 | 9.2 ± 0.2 | 26.0 ± 1.1 |
| DEX ($n$ = 3) | 9.9 ± 1.9 | 1.09 ± 0.10 | 11.5 ± 0.9 | 92.8 ± 4.2 | 4.3 ± 1.9 | 26.0 ± 3.1 |

[a]AL: fed *ad libitum*; PF: *pair-fed*; and DEX: dexamethasone-treated. 0.5 g $L^{-1}$ total lipoprotein, $10^{-2}$ mol $L^{-1}$ PBS, 5 μmol $L^{-1}$ $CuSO_4$, 37°C. Results are means ± SD of $n$ pools of plasmas.

cant difference could be observed in the oxidation behavior of HDL from the DEX group as compared with the other two groups. Only the level of hydroperoxides attained after 5 h oxidation was lower in the DEX group (4.3 ± 1.9 μmol $L^{-1}$) than in the AL and PF groups (10.3 ± 0.9 and 9.2 ± 0.2 μmol $L^{-1}$, respectively).

### In vitro *Effect of Dexamethasone on Copper Oxidation of Rat Plasma and Lipoproteins*

Dexamethasone did not exhibit any prooxidant or protective effect when the plasma was oxidized (150-fold diluted plasma in $10^{-2}$ mol $L^{-1}$ PBS, 37°C, 50 μmol $L^{-1}$ $CuSO_4$) with or without ethanol + dexamethasone at the final concentrations tested ($10^{-7}$, $10^{-6}$, $10^{-5}$, $10^{-4}$ mol $L^{-1}$), and continuously monitored by measuring differential absorbance at 234 nm (data not shown).

This absence of effect of dexamethasone was also noted on lipoprotein oxidation. VLDL + LDL and HDL were isolated from rats fed *ad libitum* and were then oxidized by copper for 4 h (0.5 g $L^{-1}$ total lipoprotein, $10^{-2}$ mol $L^{-1}$ PBS, 37°C, 5 μmol $L^{-1}$ $CuSO_4$), in the absence or presence of ethanolic solutions with or without dexamethasone. TBARS formation in these lipoproteins is shown on Fig. 2, a,b. After 4 h oxidation (plateau of TBARS concentration), no significant difference in the TBARS concentration was observed in the VLDL + LDL oxidized with or without ethanol + dexamethasone at the same final concentrations ($10^{-7}$, $10^{-6}$, $10^{-5}$, $10^{-4}$ mol $L^{-1}$). The same conclusion could be drawn from the data related to the HDL. Thus, under our experimental conditions, dexamethasone did not exhibit any effect on the oxidation of rat lipoprotein fractions in vitro. It is of note that ethanol had a slight protective effect toward HDL oxidation. Moreover, after 1 h oxidation of VLDL + LDL, or after 30 min oxidation of HDL (propagation phase), no significant difference was observed between lipoproteins oxidized in the absence or presence of dexamethasone (data not shown).

## Discussion

Under our experimental conditions (Sprague-Dawley rats treated for 5 d with a daily intraperitoneal injection of 1.5 mg per kg dexamethasone), treatment with dexamethasone resulted in classically described modifications in plasma parameters (especially hypertriglyceridemia and hyperglycemia) and in lipoprotein composition (enrichment of VLDL + LDL in triglycerides). However, an unusual observation appeared with regard to the oxidizability of total plasma. Indeed, the plasma of the rats treated

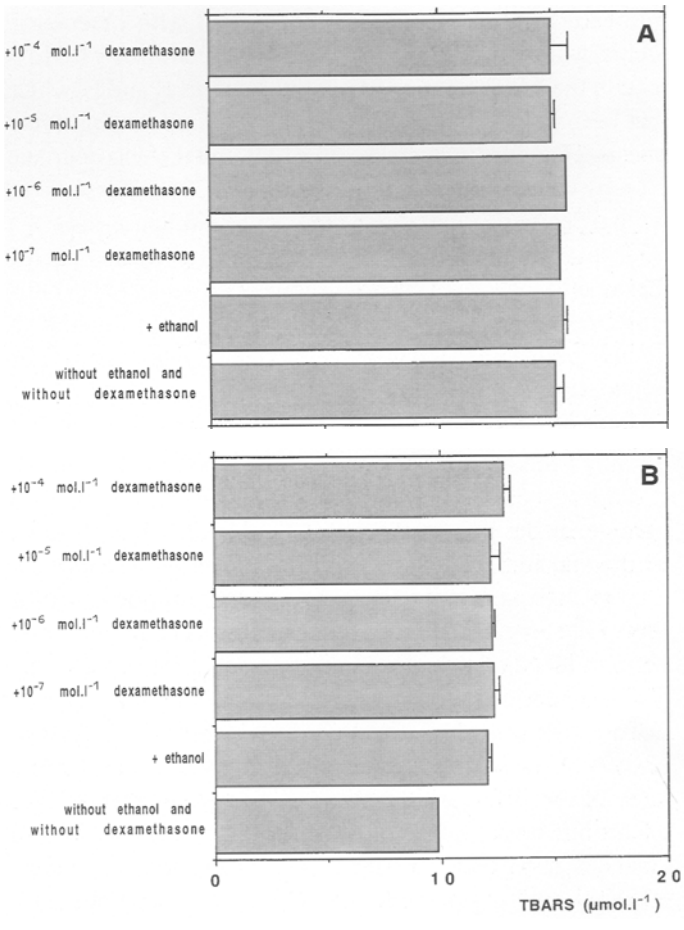


**Fig. 2.** TBARS formation in VLDL + LDL (a) and in HDL (b) isolated from rats fed *ad libitum* and oxidized by copper for 4 h (0.5 g $L^{-1}$ total lipoprotein, $10^{-2}$ mol $L^{-1}$ PBS, 5 μmol $L^{-1}$ $CuSO_4$, 37°C), in the absence or presence of ethanolic solutions (1/100, v/v) with or without dexamethasone. Results are means of two pools of plasmas.

with dexamethasone exhibited a much higher oxidizability by $CuSO_4$ than the plasma of the control rats (fed *ad libitum* or *pair-fed*), as shown by the differential absorbance at 234 nm. Nevertheless, this increased susceptibility to oxidation was not observed in the isolated lipoprotein fractions, as shown by the formation of lipid peroxidation products such as conjugated dienes, TBARS, hydroperoxides, 7-ketocholesterol, and dienals. It could thus be hypothesized that this hyperoxidizability could result from the oxidation of nonlipid plasma components, such as protein components, since protein catabolism was enhanced by treatment with dexamethasone.

With regard to the main effects of the dexamethasone treatment on plasma and lipoprotein composition, our study showed a marked increase of plasma triglyceride concentration and of the VLDL proportion in plasma (VLDL are the major triglyceride carriers in plasma). These effects have been widely described after treatment with glucocorticoids in humans (*20–22*) as well as in animals (*2–4,9*). According to published data, the rise in the proportion of VLDL could result from an enhancement of VLDL production (*3–5*). Indeed, Reaven et al. (*6*) and Garfield et al. (*8*) reported liver ultrastructural alterations after treatment with glucocorticoids, which supported an increased production of VLDL. A rise in the liver secretion of apolipoproteins B and E, which has been reported in rats (*5,23*), is also in line with this hypothesis. However, a decrease of VLDL uptake and degradation by cells would also be involved (*3–5,9*) and associated with a decreased activity of the lipoprotein lipase (*24*).

The second major modification induced by treatment with dexamethasone was a hyperglycemia (11.2 mmol $L^{-1}$ in DEX group vs 9.3 mmol $L^{-1}$ and 6.8 mmol $L^{-1}$ in the PF and AL groups, respectively). This effect is well known and has been reported to be related to an insulin resistance syndrome (*2,23*).

The hyperproteinemia found in the DEX group has already been described in another study (*3*). In our model, it did not seem to be due to hemoconcentration, since no change in the hematocrite was noted (data not shown). Rather, it seemed due to an increased liver protein biosynthesis subsequent to the efflux of muscle amino acids, since under our experimental conditions dexamethasone treatment induced an enhancement of muscle proteolysis (*25*).

The chemical composition of VLDL + LDL of the AL and PF rats was similar to that described in the literature (*2,4*). With regard to the chemical composition of VLDL + LDL of the DEX rats, the rise in the proportion of triglycerides has been widely reported in the literature (*2,4–6*). Cole et al. (*4*) isolated VLDL from rats treated with dexamethasone (0.5 mg per kg). These VLDL exhibited a 30% loss in cholesteryl esters and an enrichment in triglycerides when compared with those of *pair-fed* rats, which is in total accordance with the chemical composition of VLDL + LDL isolated from the DEX rats in our study. The HDL chemical composition of rats from the AL group was in agreement with the data of Rayssiguier et al. (*7*). The dexamethasone-induced modifications in the chemical composition of HDL from DEX group were also in line with those of Cole et al. (*4*), with a 10% increase in the proportion of proteins and a similar decrease in the proportion of total lipids, in comparison with *pair-fed* rats.

The "prooxidant/antioxidant" balance of the rats included in our study was assessed by determining plasma TBARS and α-tocopherol concentrations. Plasma TBARS are nonspecific lipid peroxidation endproducts. Their concentration is enhanced in several pathologies involving free radicals, especially in diabetes whose biological features (hyperglycemia, hypertriglyceridemia) could be considered close to those induced by dexamethasone treatment (*26*). The three groups of rats exhibited a similar plasma TBARS concentration which was close to that reported in rats by others (*27*). Thus, dexamethasone treatment did not induce an increased plasma peroxidation level. Moreover, the plasma of the DEX and PF groups contained much more α-tocopherol than that of the AL group. It is to be noted that these α-tocopherol values were close to those reported by others (*27,28*). Since rats of the DEX and PF groups had food intakes that were twice as low as rats of the AL group, the higher α-tocopherol level in plasmas of the DEX and PF groups could not be due to a higher food intake. A mobilization of α-tocopherol from adipose tissue and liver could therefore explain the difference in plasma α-tocopherol concentration between the DEX and PF groups, on one hand, and the AL group, on the other hand. It is noteworthy that the undetectable level of β-carotene in the plasmas of the three experimental groups of rats has already been reported by others (*29*) and would be due to the presence of a very efficient enzyme which cleaves the carotenes in the rat intestine, thus resulting in very low plasma carotenoid levels (*29*).

With regard to the oxidation experiments, our results clearly showed that plasma exhibited an increased oxidizability after treatment with dexamethasone. This phenomenon could not be due to the effect of dexamethasone by itself. Indeed, in our study, the plasma of rats treated with dexamethasone was collected 24 h after the last injection and no longer contained any detectable concentration of this drug (half-life of dexamethasone = 4–5 h). Moreover, dexamethasone by itself did not exhibit in vitro any prooxidant effect on the copper oxidation of plasma of rats fed *ad libitum*. Only two studies conducted in vitro (*30,31*) showed a poor protective effect of dexamethasone toward oxidative processes. Our results showed that under our experimental conditions, dexamethasone by itself was not responsible for the increased susceptibility of the plasma to oxidation in rats treated with dexamethasone.

In order to explain this specific oxidizability of the plasma, we studied the susceptibility of isolated lipoprotein fractions (VLDL + LDL and HDL) to copper oxidation. Surprisingly, continuous monitoring at 234 nm did not reveal any enhanced oxidizability of VLDL + LDL or HDL

from the DEX group, when compared to those of the AL group, despite the changes in the composition of these lipoproteins, especially the enrichment of VLDL + LDL in triglycerides. Formation of TBARS, dienals, and 7-ketocholesterol was also similar in the three groups of rats. It is noteworthy that, when compared to the other two groups, the apparent lower oxidizability of VLDL + LDL and of HDL from the PF group could be related to the higher α-tocopherol/lipid ratio in PF rats. Thus, the hypertriglyceridemia induced by dexamethasone treatment did not result in an increased susceptibility of lipoprotein fractions to copper oxidation, as has been previously reported in other models of hypertriglyceridemia *(7,11,12)* or even in experimental diabetes induced by streptozotocin in rats *(32)*.

Plasma hyperoxidizability after treatment with dexamethasone was thus not related to the lipoprotein fractions. It is well known that this treatment induces metabolic disturbances, especially a high level of protein catabolism *(3)*. Thus it is hypothesized that treatment with dexamethasone at a dosage inducing a severe protein catabolism in our model (*25*) could have resulted in a modified protein composition that might have led to an increased susceptibility of plasma proteins and/or their metabolites to copper-induced oxidation and that could account for the overestimated absorbance at 234 nm. This hypothesis was supported by the fact that the carbonyl content detected in the oxidized plasma was markedly higher in the DEX group than in the others. Indeed, metal ion-catalyzed oxidation of proteins is mainly a site-specific process in which one or a few amino acids at metal-binding sites on proteins are preferentially oxidized and converted to carbonyl derivatives (*33*). Thus, assay for the carbonyl content is a convenient method for assessing metal-catalyzed oxidation of proteins (*19*).

In conclusion, this study allowed us to observe an original consequence of the dexamethasone treatment in rats, as we noted a marked increase in the susceptibility of their plasma to copper oxidation, in comparison to that of nontreated rats. Nevertheless, this higher oxidizability was not observed in the isolated lipoprotein fractions, despite the hypertriglyceridemia that was induced by dexamethasone treatment and was responsible in other models for an increased susceptibility to oxidation. Our observation would thus imply that other plasma components, such as proteins or amino acids, could be involved. This is strongly supported by the fact that treatment with dexamethasone induced metabolic disturbances, especially a high level of protein catabolism, which could favor oxidizability of nonlipid plasma components, as shown by an increased carbonyl formation upon plasma copper oxidation.

## Materials and Methods

### *Animals*

Male Sprague-Dawley rats (IFFA-CREDO, L'Arbresle, France) aged 3 mo (300 g mean weight) were acclimatized for 7 d with a 12:12 light:dark cycle. During this period, rats were maintained on a standard diet (AO4, Villemoisson-sur-Orge, France) and water *ad libitum*. Animal care and experimentation complied with the rules of our institution and one of us (MPV) was authorized by the French Ministry of Agriculture and Forestry.

The rats were then divided at random into three groups:

1. The AL group, consisting of six rats that did not receive any treatment and were fed *ad libitum*.
2. The DEX group, consisting of six rats fed *ad libitum* that were treated with a daily intraperitoneal injection of dexamethasone (1.5 mg per kg) for 5 d. It is noteworthy that the dexamethasone dosage (i.e., 1.5 mg per kg and per day) was close to that used in acute therapy in humans (1–2 mg per kg and per d), whereas the dosage used for a long-term treatment is 0.1–0.5 mg per kg and per d. The treatment we chose was thus an acute treatment known to induce a severe catabolic state in adult rats *(25,34)*.
3. The PF group, consisting of 12 rats which were *pair-fed* to rats from the DEX group and were treated with a daily isovolumic intraperitoneal injection of NaCl solution for five days. The rationale for the study of a PF group was the fact that dexamethasone treatment induced anorexia. Therefore, studying a PF group allowed us to discriminate between the effect of dexamethasone itself and the effects induced by anorexia *(35,36)*.

On d 5, 24 h after the last intraperitoneal injection and after 12 h fasting, the rats were anesthetized with diethyl ether and beheaded. Blood was collected on EDTA (1g $L^{-1}$ final concentration) to prevent lipid peroxidation. All further experiments were carried out on fresh plasma. Plasmas from two rats in each group were pooled in order to obtain a sufficient volume of plasma. This procedure has been commonly reported in studies conducted on rats *(7,11,37)*. Pools of plasma from each rat group were then divided into aliquots used for plasma assays, plasma copper oxidation, and isolation of lipoprotein fractions for their analysis and copper oxidation.

### *Methods*

#### *Plasma Analyses*

Plasma triglyceride, phospholipid, and cholesterol concentrations were determined by enzymatic methods *(38–40)*. Total lipid concentration in plasma was calculated as the sum (expressed in g $L^{-1}$) of unesterified cholesterol, cholesteryl esters, phospholipids, and triglycerides. The amount of cholesteryl esters was estimated as 1.67 x esterified cholesterol, this factor representing the ratio of the average molecular weight of cholesteryl ester to unesterified cholesterol. The proportion of each lipoprotein fraction in plasma was assessed by plasma lipid electrophoresis. Migration was conducted on 1 μL plasma for 30 min at 90 V in a Tris-veronal buffer (1.84 g $L^{-1}$ veronal, 10.3 g $L^{-1}$ sodium veronal, 20 g $L^{-1}$ trishydroxymethylaminometh-

ane) pH 9.2 (Sebia). After drying, the gels were stained with Fat Red B and integrated (Fat Red B and precast agarose gels were from the Ciba Corning Diagnostics Corp.). Total plasma protein and glucose concentrations were also measured, in order to get an evaluation of the effect of dexamethasone treatment on the two other major metabolisms.

In order to globally assess the plasma lipid peroxidation status of the rats studied, a spectrofluorimetric determination of the TBARS was carried out according to the spectrofluorometric Yagi's method *(41)*. After extraction with hexane, two lipophilic antioxidant systems, namely, α-tocopherol (the main lipophilic antioxidant carried by lipoproteins) and β-carotene, were assayed by high-performance liquid chromatography and detected at 292 and 450 nm, respectively. Tocopherol acetate was used as an internal standard *(42)*. These results provided an assessment of the prooxidant/antioxidant balance in plasma.

*Continuous Monitoring of Plasma Copper Oxidation*

One hundred fifty microliters of plasma was dialyzed in $10^{-2}$ mol $L^{-1}$ phosphate buffer saline (PBS) pH 7.4 for 18 h in the dark at 4°C. Oxidation was carried out on 20 µl dialyzed plasma, which was 150-fold diluted with $10^{-2}$ mol $L^{-1}$ PBS, according to the procedure of Spranger et al. *(43)*. Oxidation was conducted at 37°C, with 50 µmol $L^{-1}$ $CuSO_4$ (final concentration). Plasma oxidation was continuously monitored by measuring absorbance at 234 nm on a Beckman (Fullerton, CA) DU 640 spectrophotometer (one measurement every 300 s for 460 min). Results were expressed as differential absorbance (reference = absorbance at 234 nm before oxidation).

*Determination of Carbonyl Content in Plasma upon Copper Oxidation*

Carbonyl content was determined in 150-fold diluted plasma before oxidation and after 150 min of copper oxidation conducted as described above *(43)*. Carbonyls were measured spectrophotometrically with the use of the carbonyl-specific reagent 2,4-dinitrophenylhydrazine (D-2630, Sigma, Saint-Quentin Fallavier, France) *(19)*. For each sample, a blank was prepared by treatment with 2 mol $L^{-1}$ HCl instead of 2,4-dinitrophenylhydrazine in 2 mol $L^{-1}$ HCl. Concentration of the carbonyl content was calculated using a molar absorption coefficient of 22,000 $mol^{-1}$ L $cm^{-1}$ at 370 nm *(19)*. Carbonyl formation upon the 150 min of copper oxidation was calculated as the difference between carbonyl concentration after and before oxidation, and was expressed as nmol carbonyls $mg^{-1}$ protein.

*Isolation of Lipoproteins*

It is noteworthy that rats possess a poor proportion of LDL, which does not allow an easy, reproducible isolation of these LDL by ultracentrifugation *(44)*. Therefore, most authors isolated both LDL and VLDL *(11,12,26,31,45)*. In the present study, VLDL + LDL were classically isolated between d = 1.006 and d = 1.050 *(7)*, and high-density lipoproteins (HDL) between d = 1.050 and d = 1.210, with a Beckman XL-80 ultracentrifuge and a 6513 Kontron rotor. Chylomicrons (d < 1.006) were discarded (upper phase) in a first step after two 30-min ultracentrifugations at 15°C and at 13,200 rpm (12,000*g*). The lower phase was then adjusted with solid KBr to a density of 1.050 and centrifuged for 20 h at 15°C and at 42,000 rpm (100,000*g*). The upper phase contained VLDL and LDL. Isolation of HDL was obtained by ultracentrifugation of the latter lower phase at d = 1.21 after density adjustment with KBr, for 20 h at 15°C and at 42,000 rpm (100,000*g*). HDL were collected as the upper phase.

*Chemical Composition and Electrophoresis of Lipoprotein Fractions*

Lipids (i.e., total and unesterified cholesterol, phospholipids, and triglycerides) were assayed in each lipoprotein fraction (VLDL + LDL and HDL) by enzymatic methods as previously described for plasma. Proteins were determined by using a pyrogallol red technique (Elitech Diagnostics, Sees, France) *(46)*. The concentration of total lipoprotein (expressed in g $L^{-1}$) was calculated as the sum of the lipid and protein concentrations (expressed in g $L^{-1}$) in each fraction. Percentages of each lipid and protein component in the lipoprotein fractions allowed us to obtain their chemical composition. The purity of each lipoprotein fraction was checked by carrying out an agarose gel electrophoresis as previously described for plasma.

*Continuous Monitoring of Lipoprotein Copper Oxidation*

As described for plasma, lipoprotein fractions were dialyzed in $10^{-2}$ mol $L^{-1}$ PBS pH 7.4 for 18 h at 4°C in the dark; 0.5 g $L^{-1}$ (final concentration expressed as total lipoprotein) of each lipoprotein fraction was oxidized at 37°C with 5 µmol $L^{-1}$ $CuSO_4$ (final concentration). This oxidation was continuously monitored by measuring the differential absorbance at 234 nm every 300 s for 460 min, according to the procedure of Esterbauer et al. *(18)*. The rise of this differential absorbance as a function of the oxidation time corresponds classically to the formation of conjugated dienes which are early products of lipid peroxidation. During the lag phase, it is commonly admitted that the lipophilic antioxidants (such as α-tocopherol) protect the polyunsaturated fatty acids of lipoproteins against oxidation. After consumption of these antioxidants, the lipid peroxidation process can begin the propagating chain reaction phase. A tangent to the curve was drawn during the propagation phase and extrapolated to the time axis. The time interval between the addition of copper ions (time 0) and the intersection point of the tangent on the time axis was defined as the lag phase (expressed in minutes). The propagation rate was calculated from the slope of the tangent, using a molar extinction coefficient for conjugated dienes at 234 nm equal to 29,500 $mol^{-1}$ L $cm^{-1}$ *(18)*, and was expressed as µmoles of conjugated dienes formed per liter and per minute.

*Determination of TBARS, 7-Ketocholesterol, Hydroperoxides and Dienals in Lipoproteins after 5 h Copper Oxidation*

Experimental conditions for oxidation were the same as those described for the continuous monitoring of lipoprotein oxidation (0.5 g $L^{-1}$ total lipoprotein, 5 μmol $L^{-1}$ $CuSO_4$, $10^{-2}$ mol $L^{-1}$ PBS, 37°C). Oxidation was stopped after 5 h by adding $Na_2EDTA$ (final concentration = 200 μmol $L^{-1}$) and cooling (4°C). TBARS concentration was determined to assess a global lipid peroxidation level in lipoproteins. TBARS formed in each sample were then calculated by substracting the initial TBARS value before oxidation. Moreover, the volume of the HDL sample was sufficient to allow us to assess other more specific peroxidation products formed during copper oxidation, namely hydroperoxides, 7-ketocholesterol, and dienals. For these determinations, we used a simultaneous measurement of the differential absorbance of the samples at 220, 234, 250, and 268 nm and calculations recently proposed by Pinchuk et al. *(47)*.

*In Vitro Effect of Dexamethasone on Copper Oxidation of Rat Lipoproteins*

The VLDL + LDL and HDL isolated from the two pools of plasma of rats fed *ad libitum* were oxidized under the same experimental conditions as those described above (0.5 g $L^{-1}$ total lipoprotein, 5 μmol $L^{-1}$ $CuSO_4$, $10^{-2}$ mol $L^{-1}$ PBS, 37°C), in the absence or presence of dexamethasone. The final dexamethasone concentrations tested were $10^{-7}$, $10^{-6}$, $10^{-5}$, and $10^{-4}$ mol $L^{-1}$. For this purpose, dexamethasone (D-1756, Sigma, Saint-Quentin Fallavier, France) was solubilized in ethanol and added to the lipoproteins (1 μL ethanolic solution for 1 mL lipoprotein). A control sample with ethanol without dexamethasone was simultaneously tested. Given the low volume of lipoproteins available, only two oxidation times were studied: one corresponding to the half-propagation phase (1 h for VLDL/LDL and 30 min for HDL), and one corresponding to the maximum oxidation level (4 h for both VLDL + LDL and HDL). TBARS were assayed in each lipoprotein sample in order to determine the lipid peroxidation level.

*Statistical Analysis*

Statistical comparison between the means obtained from the three groups of rats was first carried out by Kruskal-Wallis' test (Apple Macintosh computer, Statview software). Significantly different values ($p < 0.05$) were then studied by Conover's procedure *(48)*.

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