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Protective role of melatonin and retinol palmitate in oxidative stress and hyperlipidemic nephropathy induced by adriamycin in rats

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Abstract: We have studied the effects of melatonin and retinol palmitate (RP) on the nephropathy and oxidative stress induced by a single and high dose of adriamycin (AD) in Wistar male rats. A dose of melatonin (75 μg/kg/day) and a dose of RP (0.25 g oily solution/kg/day, sc) were injected 3 and 9 days before and after the administration of AD (25 mg/kg, i.p.), respectively. After the decapitation, samples were taken from the neck vascular trunk in order to determine the triglycerides, total cholesterol, phospholipids, HDL-cholesterol, total proteins, urea, lipoperoxides, and reduced glutathione (GSH). We estimated the lipoperoxide and glutathione (GSH) contents in renal homogenates, and the excretion of proteins in urine over a 24 hr period. The administration of AD caused significant increases in proteinuria and in the other parameters studied [lipids (triglycerides, total cholesterol, phospholipids, and HDL-cholesterol), non-protein nitrogen compounds, and lipoperoxides]. AD increased the lipoperoxide content, but it decreased the GSH content in the kidney. Both melatonin and RP, although melatonin more significantly, decreased the intensity of the changes produced by the administration of AD alone. In fact, melatonin was quite efficient in reducing the formation of lipoperoxides, restoring renal GSH content and decreasing remarkably the severity of proteinuria. These results support the powerful antioxidant action of melatonin at renal level and a lower antioxidant action of retinol. Likewise, these data reinforce the hypothesis which supports the pathogenetic role and the close relation between the oxidative stress and the expression of the nephropathy induced by AD. However, in spite of this obvious antioxidant effect of melatonin in the kidney, additional studies are required to establish accurately the role of this pineal indole in the regulation and dynamics of the antioxidative defense enzyme system, which neutralizes the damaging effect of free radicals, both endogenous and exogenous, in this organ.

Pedro Lopez Montilla, Isaac Fiñana Túnez, Carmen Muñoz de Agueda, Felix Luna Gascón, and Juan Vicente López Soria

Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad de Córdoba, 14004 Córdoba, Spain

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Address reprint requests to Pedro Lopez Montilla, Departamento de Bioquímica y Biología Molecular, Facultad de Medicina—Universidad de Córdoba, Avada. Menéndez Pidal s/n, 14004, Córdoba, Spain.

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Introduction

Adriamycin (AD), commonly used as an antitumoral antibiotic in humans, given by different routes of administration, provides an ideal model in rats and other rodents, of oxidative stress accompanied by a type of hyperlipidemic nephropathy quite similar to human focal glomerulesclerosis from a histologic point of view [Beretazolli et al., 1972; Suzuki et al., 1978; Fajardo et al., 1980; Bertani et al., 1982; Okuda et al., 1986; Canepa et al., 1992]. In the pathology of this nephropathy, there are increasing experimental and clinical data, suggesting a prominent role at free radicals of oxygen in the disease [Weening et al., 1983; Bertani et al., 1996a,b; Zima et al., 1997]. This hypothesis is indirectly supported by the discovery of a specific and active defense

enzymatic system, which protects against these radicals in renal tissue [Ichikawa et al., 1994]. This idea suggests, both from the standpoint of a therapeutic approach and an explanation of pathogenetic mechanisms, the use of natural or synthesis antioxidants in models of nephropathy induced both by AD and by other nephrotoxic agents that produce oxidative stress, such as puromycin, ciclosporine A, cephaloridine, and heavy metals [Bristow et al., 1981; Bertani et al., 1986a; Diamond et al., 1986; Thakur et al., 1988].

Among the biological antioxidants, N-acetyl-5-methoxytryptamine or melatonin, the main product of the pineal gland, has acquired a notable role. Melatonin is a molecule present naturally, from dinoflagellata (*Gonyallaux polyedra*) and bacteria to higher vertebrates [Poeggeler et al., 1991; Manchester et al., 1995; Tilden et al., 1997]. This fact suggests that this substance may have an extensive and varied biological function. The relationship between the pineal gland and melatonin reproduction, photoperiod, circadian rhythms, animal behavior, sleep, and immune system are well established and some are in the final stage of an investigation, which began 30 years ago [Reiter, 1973, 1991; Arendt, 1989; Maestroni, 1993; Dawson et al., 1993]. As of 1991, melatonin was identified as an antioxidant, as shown by Ianas et al. [1991]. This action of melatonin has been intensively investigated [Reiter, 1995, 1996; Reiter et al., 1997]. Studies to date indicate that melatonin's powerful biological antioxidant in the neutralization of the hydroxyl radical (•OH), the most toxic and reactive free radical of oxygen in cells [Tan et al., 1993; Poeggeler et al., 1993, 1994]. This molecule has at least two properties that make it suitable as an antioxidant: 1) its easy diffusion through cellular membranes, due to its lipophylic nature; and 2) the molecule does not require cells with specific receptors since it is a direct free radical scavenger [Reiter et al., 1993].

The protective effect of melatonin on the hyperlipidemic nephropathy and against renal and cerebral oxidative stress induced by AD in rats was previously reported [Montilla et al., 1997a,b]. The main purpose of the present study was to compare the effects of melatonin and retinol, another natural antioxidant, on adriamycin nephropathy [Ciaccio et al., 1993; Livrea et al., 1995]. Although the scavenging action of retinol's predecessor, β-carotene, has been well documented, there are still doubts concerning the antioxidant function of this vitamin [Sies et al., 1995; Nakagawa et al., 1996]. It is important to note that together with studies that demonstrate the antioxidant effect of retinol [Ciaccio et al., 1993; Livrea, 1995], recent data also show that this vitamin and several compounds of its family (all-trans-retinoic and 13-cis-retinoic acid) act as powerful inhibitors of lipoxygenases 1 and 2, two enzymes that catalyse the dioxygenation of polyenoic acids such as linoleic acid and the arachidonic acid [Goldreich et al., 1997]. These reactions can lead to a prooxidant stage, and they are involved in the synthesis of prostaglandins, a process that generates free radicals, and in the formation of lipoperoxides in cellular membranes of different tissues.

Materials and methods

Animals

Male Wistar rats weighting 200 g were purchased from Charles Rivers, Barcelona, Spain. They were housed at 21–23°C under a light regime of 14:10 (14 hr light/12 hr darkness) with food and water ad libitum.

Experimental procedures

The following groups of rats were arranged: 1) control, 2) vehicle for melatonin, 3) oil vehicle, 4) melatonin injected, 5) retinol palmitate (RP) injected, 6) adriamycin (AD), 7) AD + melatonin, and 8) AD + RP.

AD was administered ip at a single dose (25 mg/kg), whereas melatonin was injected ip at 75 μg/kg, and RP (oily solution of retinol palmitate) was administered at a dose of 0.25 g/kg sc. Melatonin and RP, both alone and when combined with AD, were injected daily for a period of 12 days, beginning before 3 and for 9 days after the administration of AD. Each day, a fresh solution of melatonin was prepared by dissolving the indole in 30% of ethanol and normal saline. The final concentration of melatonin was 75 μg/0.1 ml.

Nine days after the injection of AD, all animals under light anesthesia by ether were decapitated, being recollected the samples of blood from trunk vascular of the neck in order to determine the lipoperoxide and GSH contents, lipids, urea, creatinine, and total protein in plasma. Immediately, both kidneys were removed and homogenized as previously described [Ciaccio et al., 1993]. Crude extracts were used to determine the total protein, lipoperoxide, and GSH contents. One day before the sacrifice, urine was collected from rats, which had been housed in cages for this purpose. Proteinuria was determined in the urine samples. During the urine collection, the animals had water ad libitum, whereas food was withdrawn 12 hr before they were killed.

Table 1. Effect of melatonin and RP alone on the levels of lipoperoxides (MDA) and GSH content in plasma and renal tissue[a]

	MDA			GSH	
	Plasma (μmol/L)	*P*	Renal tissue (μmol/mg protein)	Plasma (μmol/L)	Renal tissue (μmol/mg protein)
Control	8.43 ± 0.90		1.61 ± 0.05	8.10 ± 0.75	20.14 ± 1.95
Melatonin vehicle	7.75 ± 0.85		1.42 ± 0.07	9.03 ± 0.90	21.19 ± 2.24
Oil vehicle	8.20 ± 0.80		1.75 ± 0.10	8.60 ± 0.79	19.80 ± 1.75
Melatonin	6.44 ± 0.75	<0.05[b]	1.32 ± 0.08	9.75 ± 0.85	21.85 ± 2.00
RP	7.06 ± 0.69		1.53 ± 0.15	9.15 ± 0.85	21.90 ± 2.50

[a]Data are mean values ± S.E. n = 6 animals per group.
[b]Versus control group; all other values do not differed significantly from any other value.

Biochemical determinations

The lipoperoxides, relative to malonaldehyde (MDA), were measured in the samples, and GSH contents, relative to mg protein, were, respectively, determined by colorimetric assay (LPO-586 and GSH-400), with kits purchased from Bioxytech S.A. Urea, creatinine, total protein, and lipid profile (triglyceride, TG; total cholesterol, TC; phospholipids, PL; and HDL-cholesterol, HDL-c) were determined enzymatically by kits and procedures supplied by Boehringer Mannheim, Barcelona. Proteins in urine were measured by the biuret reaction (Bio-Mérieux, España, S.A.).

Administered products

Melatonin and retinol palmitate were supplied by Sigma Chemical Co., St. Louis, USA, while AD in its chlorohydrate form (doxorubicin) was supplied by Lab Farmitalia-Carlo Erba S.A. Madrid.

Statistics

The results are expressed as mean ± SE. Significance has been calculated using the Student's t test.

Results

Effects of melatonin and RP in rats not treated with AD

The data related MDA and GSH values in plasma and renal tissue are shown in Table 1, lipidic profile in Table 2, and urea, creatinine, total protein levels, and proteinuria are shown in Table 3. There were no significant changes, except for the small MDA decrease in the plasma of the melatonin treated ($P < 0.05$).

Effect of melatonin and RP in rats treated with AD

The changes induced by melatonin and RP in the animals injected with AD are shown in the corresponding figures: MDA and GSH changes in plasma and tissue (Fig. 1); lipids (Fig. 2); and urea, creatinine, total proteins, and proteinuria (Fig. 3). AD alone caused significant increases in MDA in plasma and kidney. Also, the drug caused a weak GSH increase in plasma ($P < 0.05$), whereas it induced a significant decrease in renal tissue ($P < 0.01$). It is worth noting the significance that AD caused increases in TG, TC, and PL ($P < 0.001$), while HDL-c significantly decreased. Additionally, the administration of AD did not significantly change the plasmatic total proteins values, but BUN, creatinine in plasma, and the excretion of urinary proteins underwent highly significant increases. Finally, the simultaneous administration of melatonin or RP reversed the changes produced by AD in terms MDA and GSH in plasma and renal tissue, lipidic profile, BUN, creatinine, and excretion of urinary proteins. Melatonin was more effective in reversing these changes than was RP.

Table 2. Effect of melatonin and RP alone on the changes lipid profile (TG, TC, HDL-c, and PL)[a]

	TG (mg/dl)	TC (mg/dl)	HDL-c (mg/dl)	PL (mg/dl)
Control	74.13 ± 8.50	85.00 ± 7.93	35.00 ± 2.97	120 ± 13.45
Melatonin vehicle	75.13 ± 7.52	83.18 ± 8.65	33.18 ± 2.55	122 ± 12.54
Oil vehicle	76.80 ± 6.95	84.81 ± 9.32	34.80 ± 3.67	118 ± 14.25
Melatonin	75.55 ± 7.82	87.55 ± 8.45	37.55 ± 4.53	129 ± 13.32
RP	74.95 ± 7.49	86.95 ± 8.10	36.95 ± 3.35	127 ± 12.50

[a]Data are mean values ± SE. n=6 animals per group. No value differed significantly from any other value.

Table 3. Effect of melatonin and RP alone on BUN, cretatinine, total protein and urinary protein excretion[a]

	BUN (mg/dl)	Creatinine (mg/dl)	Total protein (g/dl)	Proteinuria (mg/24 hr)
Control	28.00 ± 3.00	0.66 ± 0.05	6.10 ± 0.65	7.00 ± 0.8
Melatonin vehicle	27.00 ± 2.50	0.70 ± 0.07	5.90 ± 0.91	7.50 ± 0.6
Oil vehicle	27.50 ± 2.90	0.69 ± 0.05	6.05 ± 0.75	6.95 ± 0.8
Melatonin	26.90 ± 3.01	0.72 ± 0.06	6.25 ± 0.62	8.25 ± 0.5
RP	26.85 ± 2.60	0.71 ± 0.08	6.18 ± 0.69	7.18 ± 0.7

[a]Data are mean values ± SE. n=6 animals per group. No value differed significantly from any other value.

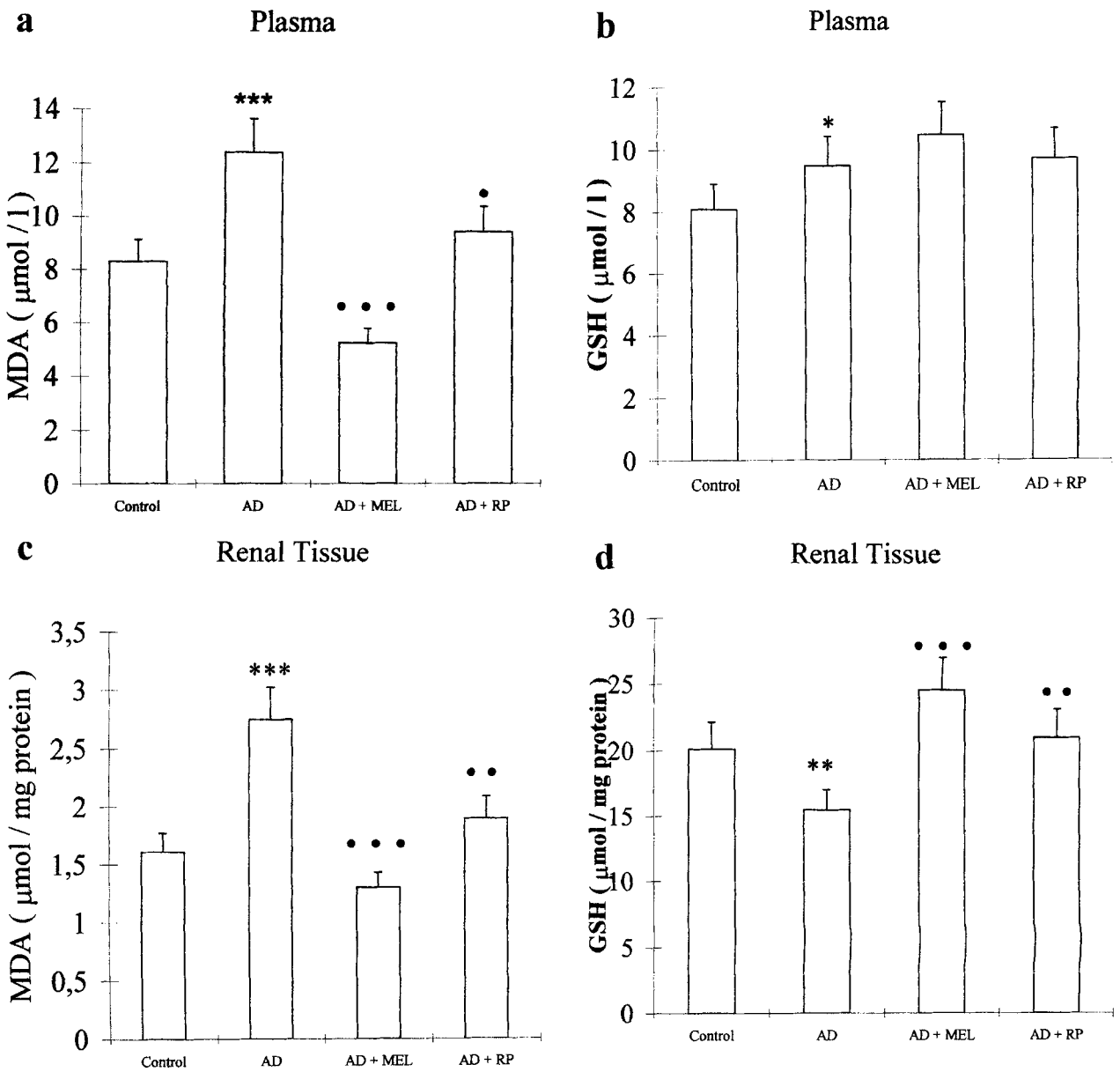

Fig. 1. Effect of melatonin (MEL) or retinol palmitate (RP) on changes in lipoperoxide (MDA) and glutathione (GSH) in plasma and renal tissue in rats treated with adriamycin (AD). Bars represent mean values ± SE. n = 6 animals per group. * $P < 0.05$ vs. control, ** $P < 0.01$ vs. control, *** $P < 0.001$ vs. control, • $P < 0.05$ vs. AD, •• $P < 0.01$ vs. AD, ••• $P < 0.001$ vs. AD.

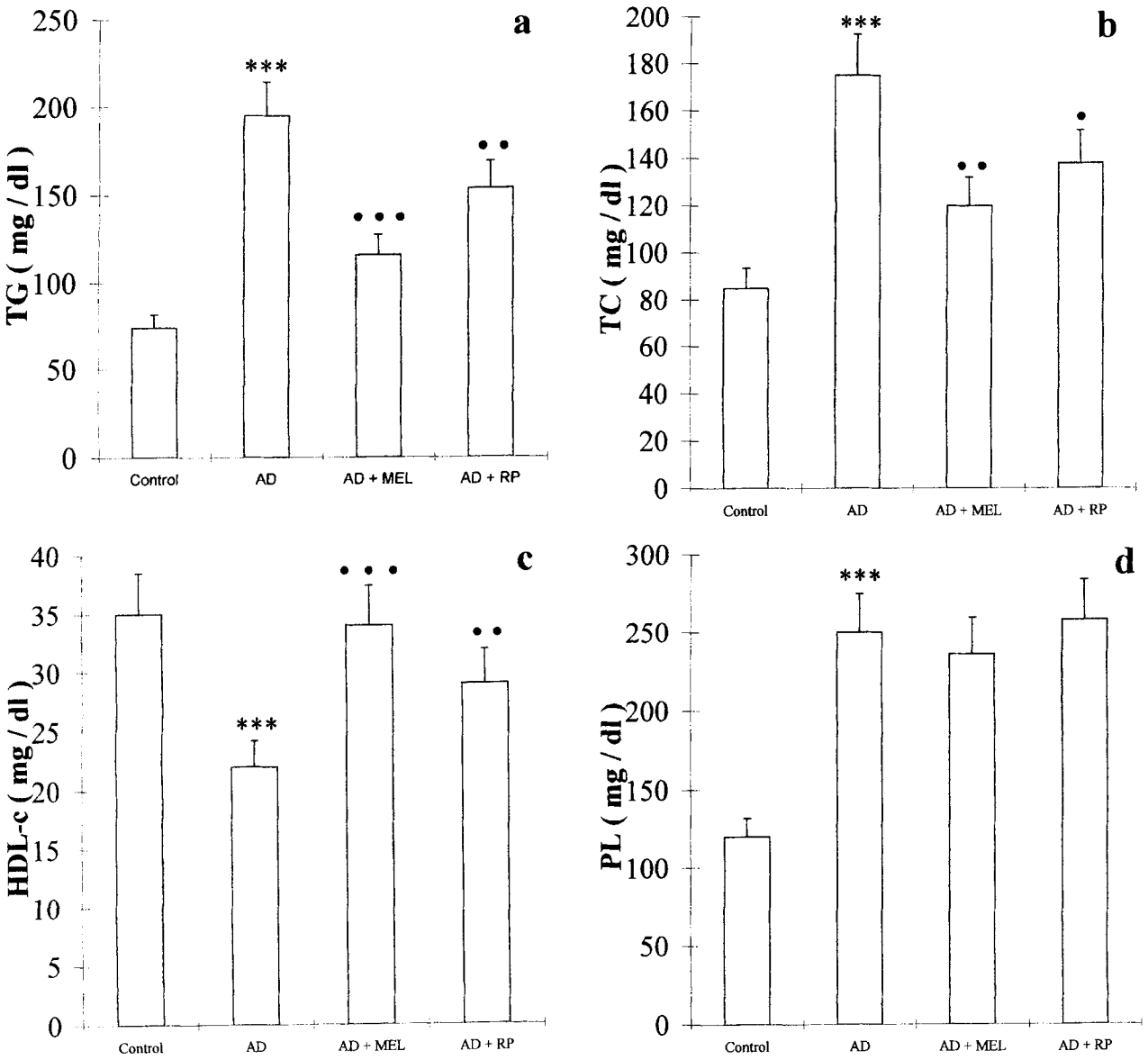

Fig. 2. Effect of melatonin (MEL) or retinol palmitate (RP) on changes in lipid profiles (TG, TC, HDL-c and PL) in rats treated with adriamycin (AD). Bars represent mean values ± SE. n = 6 animals per group. *** $P < 0.001$ vs. control, • $P < 0.05$ vs. AD, •• $P < 0.01$ vs. AD, ••• $P < 0.001$ vs. AD.

Discussion

Once again, these results confirm the high efficiency of AD to produce a state of oxidative stress with biochemical, biological, and clinical changes characteristic of a hyperlipidemic nephropathy in rodents [Beretazolli et al., 1972; Suzuki et al., 1978; Fajardo et al., 1980; Bertani et al., 1982; Okuda et al., 1986; Canepa et al., 1992]. The high degree of lipoperoxidation in plasma and renal tissues, together with GSH content in the latter organ, show clearly the existence of oxidative stress in this model. This oxidative stress is also shown by the renal dysfunction: alterations in lipid profiles (CT, TG, and PL increases), intense proteinuria, and high concentrations of urea and creatinine in the plasma.

These data also support the hypothesis that suggests a close relation between the degree of oxidative stress and the severity of the nephropathy produced both by AD and by other agents. The specific toxicities of these agents are basically correlated with the degree of oxidative stress that they produce, since it has been confirmed that the toxicity of these substances is not sufficient by itself to explain satisfactorily many of the pathogenetic aspects of these nephropathies [Okuda et al., 1986; Canepa et al., 1992]. In support of such a hypothesis, the present study shows that the administration

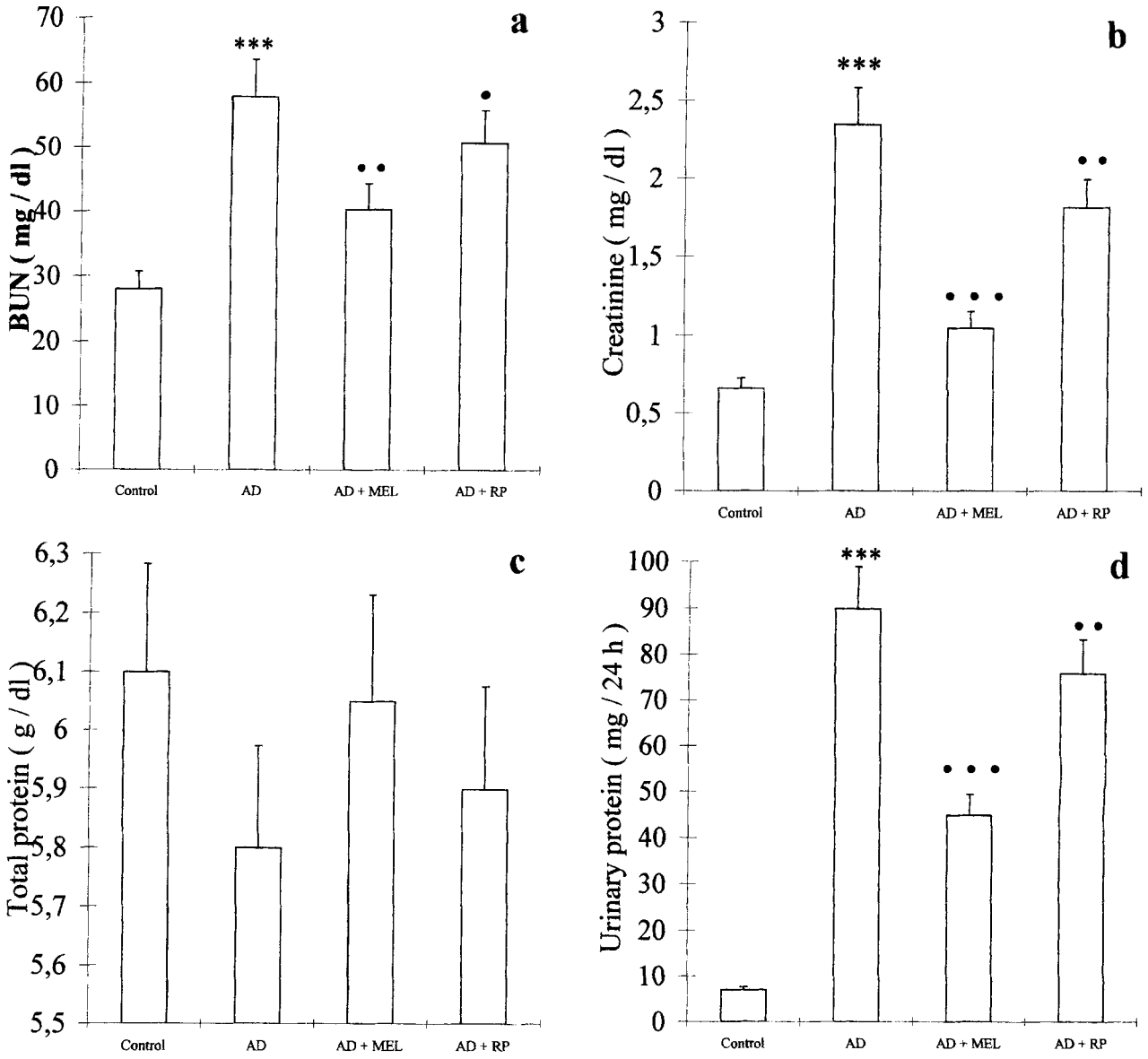

Fig. 3. Effect of melatonin (MEL) or retinol palmitate (RP) on changes in BUN, creatinine, total protein and urinary protein excretion in rats treated with adriamycin (AD). Bars represent mean values ± SE. n = 6 animals per group. *** $P < 0.001$ vs. control, • $P < 0.05$ vs. AD, •• $P < 0.01$ vs. AD, ••• $P < 0.001$ vs. AD.

of antioxidants (superoxide dismutase, alpha-tocopherol, and allopurinol) reduces significantly the nephrotoxicity, hyperlipidemia, and hyperproteinuric signs [Beretazolli et al., 1972; Suzuki et al., 1978; Fajardo et al., 1980; Bertani et al., 1982; Okuda et al., 1986; Canepa et al., 1992; Ghiggeri et al., 1990]. In the particular case of allopurinol, an antioxidant that inhibits the activity of the renal xanthine oxidase, the proteinuria induced by AD decreases by more than 70% [Ghiggeri et al., 1990], a decrease quantitatively similar to that observed after the administration of melatonin in the present adriamycin model.

Both melatonin and RP show their efficiency to counteract many of the signs of the nephropathy induced by AD. We observed that melatonin is more effective as inferred from the biochemical changes and other clinical aspects discussed below. These results support a previous study carried out by the present group of researchers [Montilla et al., 1997a], which differed in that the quantity of melatonin administered was lower and period of administration was shorter. Nevertheless, despite these methodological differences, the results obtained in the studies are equivalent with regard to the restoration to normality of the markers of oxidative stress (MDA and GSH), plasma lipids, BUN, creatinine, and proteinuria. It is also important to note that the clinical aspects previously studied, such as degree ascites,

accumulation of peritoneal fat, lethality index, and hepatonephrotoxicity (data not presented in this study), were reduced, by virtually 100%, to following the injection of higher doses of melatonin. According to these data, the retinol palmitate could be considered an acceptable antioxidant, since it reduces significantly the lipoperoxidation in both tissues, restores the GSH content, reduces the TG, TC, BUN, and creatinine levels, and produces a moderate reduction in proteinuria.

With regard to melatonin, it would seem to have powerful properties: melatonin reversed the high MDA values caused by AD in plasma and renal tissue below those values detected in the untreated and vehicle injected controls. The same melatonin prevented GSH renal depletion caused by this antibiotic. Finally, with respect to the biochemical signs indicative of hyperlipidemic nephropathy, significant decreases of TG, TC, BUN, creatinine, and proteinuria are obvious. Particularly important is the nearly 50% decrease in proteinuria, a primary biochemical sign in the evaluation of the intensity and course of this type of nephrosis.

In the light of these results, the role of melatonin as an antioxidant and inhibitor of lipoperoxidation becomes obvious at the renal level. These effects have been extensively confirmed in other organs by using varied experimental models in vivo and in vitro by other researchers [Pierrifiche et al., 1995; Melchiorri et al., 1995, 1996a,b]. This pronounced effect of melatonin on the markers of oxidative stress and on the biochemical parameters indicative of renal dysfunction suggests that this pineal indole does not simply neutralize free radicals. There are two facts that lead us to believe that melatonin performs a modulating action on the antioxidative defense enzymes in the kidney, as well as in other tissues: 1) melatonin is present and seems to function as an antioxidant in primitive unicellular organisms to vertebrata and 2) the synergism in exhibits with other antioxidants (GSH, alpha-tocopherol, etc.) in the neutralization of the •OH radical [Poeggeler et al., 1994]. In fact, in other organs, the activities of glutathione peroxidase (GPX), glutathione reductase (GPR), nitric oxide synthase, and 5-lipoxygenase are modified by melatonin in a positive or a negative sense, depending on the pro-oxidant or antioxidant action that these enzymes carry out [Samuelsson et al., 1987; Pozo et al., 1994; Barlow-Walden et al., 1995]. This last aspect is in turn intimately related with indeed the activities of GPX and GPR correlates with the high and low levels of melatonin in its circadian rhythm [Pablos et al., 1995a].

In brief, these results support once again the protector action of melatonin in this nephropathy induced by AD. Likewise, although less efficient retinol palmitate deserves to be included as a renal antioxidant as suggested by others [Ciaccio et al., 1993; Livrea et al., 1995], who postulate that the accumulation of vitamin A in the membranes protects them from lipoperoxidation, since it highly resists the oxidative agents. This assumption has been confirmed by the verification that the retinol is more effective than β-carotene in preventing the oxidation of the human LDL exposed to metals which stimulate oxidation [Livrea et al., 1995]. With regard to the antioxidant effect of retinol, the inhibition that this vitamin exerts on two enzymes, lipoxygenases 1 and 2, both of which possess a marked prooxidant character [Goldreich et al., 1997], should not be ruled out. Finally, it should be mentioned that the potent protective effect of melatonin, due to its possible clinical utility, may provide a basis for new developments in those renal pathological processes in which the free radicals of oxygen are involved. For this reason it would seem important to investigate the effects of melatonin in antioxidative enzymes in the kidney; these results will come from future studies.

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