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Changes in the Ascorbic Acid Levels of Peritoneal Lymphocytes and Macrophages of Mice with Endotoxin-induced Oxidative Stress

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Ascorbic acid (AA) is an important cytoplasmic antioxidant that mice synthesize in the liver, the intracellular levels of which decrease in an oxidative stress situation such as endotoxic shock. The present work deals with the changes in AA levels, that modulate the immune function, in the two main immune cells, namely macrophages and lymphocytes, from female BALB/c mice suffering endotoxic shock caused by intraperitoneal injection of *Escherichia coli* lipopolysaccharide (LPS) (100 mg/kg). The intake by cells of this antioxidant present *in vitro* at different concentrations was also studied. The animals show an oxidative stress, standardized in previous studies, that causes mortality at 30 h after LPS injection. The cells were obtained from the peritoneum at 2, 4, 12 and 24 h after LPS or PBS (control) injections and were incubated without or with AA at 0.01, 0.1 and 1 mM for 10, 30, 60, 120 or 180 min. The hepatic AA levels were also studied at 0, 2, 4, 12 and 24 h after LPS injection. The peritoneal cells obtained from animals injected with LPS showed increased AA levels in relation to the control cells at all times after LPS injection, with maximal effect at 12 h. The AA levels decreased after this time, in agreement with changes in the AA hepatic levels. The increase was due to the AA of lymphocytes since macrophages showed a decrease in AA at different times after LPS injection. Both cells showed an increase in the intracellular levels of AA when this antioxidant was added *in vitro*. This takes place mainly at 30–60 min of incubation in cells from controls and at 10 min in cells from treated mice 12–24 h after LPS injection. The incorporation decreased at these times of endotoxic shock, a few hours before death. In all cases AA levels were higher in lymphocytes than in macrophages, and 1 mM was the most effective concentration. These results suggest that the immune cells need appropriate levels of antioxidants, such as AA, under oxidative stress conditions, and that while lymphocytes take and accumulate AA, macrophages use it.

Keywords: Ascorbic acid; Macrophages; Lymphocytes; Oxidative stress

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INTRODUCTION

Several studies have shown that oxidative stress occurs during endotoxic shock,[1–3] as the result of an imbalance between the generation of reactive oxygen species (ROS) and the antioxidant levels in favor of the former. The activation of immune cells in response to endotoxin causes an increase in the ROS involved in host defense mechanisms, and thereafter these ROS contribute to the development of the pathological state of endotoxic shock.[1,2,4–8] Since immune cell functions are specially linked to ROS generation[9] and are strongly influenced by the redox potential,[10] the antioxidant/oxidant balance is an important determinant of immune cell activity. Thus, the antioxidant levels in these cells play a pivotal role protecting them from oxidative stress and therefore preserving their adequate function.[11] Antioxidants maintain the integrity and function of membrane lipids, cellular proteins, and nucleic acids and the control of signal transduction of gene expression in immune cells.[12] For this reason the immune cells are particularly sensitive to changes in their antioxidant status.[12,13] Moreover, the cells of the immune system have a high percentage of polyunsaturated fatty acids in their plasma membrane, and therefore it is not surprising that these cells usually contain higher concentrations of antioxidants than do other cells.[14,15]

Ascorbic acid (AA) plays an important role in cellular defense against oxidative damage[16] specially in macrophages and lymphocytes. The main role of AA in the organism is linked to its function as a reductor[17] but it also participates in the modulation of complex biochemical pathways which are an essential part of the normal metabolism of immune cells.[18] AA is transported across cellular membranes[19] and it protects the phagocytes from oxygen radicals that enter the cytoplasm from the phagosome. Further, secreted AA can protect against extracellular free radicals at inflammation sites.[16] Thus, AA is an antioxidant with a protector effect on the phagocytic process of peritoneal immune cells during endotoxic shock.[8] Moreover, this antioxidant inhibits the activation of the nuclear transcription factor NF-κB produced by endotoxin,[20] which could result in a decrease of the tumor necrosis factor alpha (TNFα) synthesis, a key cytokine involved in the endotoxic shock,[21] with effects on the different steps of the phagocytic process[22] and implication in ROS production.[23]

In view of the above, the aim of the present study was to investigate the changes in the AA intracellular levels of peritoneal macrophages and lymphocytes from BALB/c mice with endotoxin-induced oxidative stress, as well as the changes in the presence of several *in vitro* concentrations of AA. These studies could provide a basis for future attempts to *in vivo* improvement of immune competence by administration of antioxidants in oxidative stress situations such as endotoxic shock, since adequate levels are essential for phagocyte and lymphocyte functions and counteract excessive ROS production.

MATERIALS AND METHODS

Animals

Adult female BALB/c mice (*Mus musculus*) (Harlan Interfauna Ibérica, Barcelona, Spain) 24 ± 2 weeks old, were maintained at a constant temperature (22 ± 2°C) in sterile conditions inside an aseptic air negative-pressure environmental cabinet (Flufrance, Cachan, France) on a 12 h light/dark cycle and fed Sander Mus pellets (Panlab L.S. Barcelona, Spain) and water *ad libitum*. The animals used did not show any sign of malignancy or other pathological processes. Mice were treated according to the guidelines of the European Community Council Directives 86/6091 EEC. Although we have previously observed that the oestrous cycle phase of the mice has no effect on this experimental assay, all

females used in the present study were at the beginning of dioestrous.

Oxidative stress was induced by intraperitoneal injection of *Escherichia coli* lipopolysaccharide (LPS) (055:B5, Sigma, St Louis, MO, USA) at a concentration of 100 mg/kg following an irreversible endotoxic shock model induced in this and other murine strain.[5,24] Each animal was injected with LPS between 9:00 and 10:00 a.m.

Mortality Experiment and Preparation of Liver Samples

In order to confirm again that this injection of LPS produces an irreversible endotoxic shock,[5] a group of 10 mice was used to observe mortality after endotoxin administration.

Another group of animals was sacrificed by cervical dislocation at 0, 2, 4, 12 or 24 h after LPS injection and their livers were quickly extracted, rinsed and homogenized in 20 vol of cold 50 mM-percloric acid, using a Wheaton homogenizer wth a Teflon pestle. The homogenates were centrifuged at 5°C and 3000*g* for 30 min, and the supernatants were stored at −20°C until their use.

Collection of Cells

At 0, 2, 4, 12 and 24 h after LPS injection, peritoneal suspensions were obtained by a procedure previously described.[25] Briefly, 3 ml of Hank's solution, adjusted to pH 7.4, were injected intraperitoneally, then the abdomen was massaged and the peritoneal exudate cells were collected allowing recovery of 90–95% of the injected volume containing lymphocytes and macrophages, which were identified by morphologic and cytometric assay. Each peritoneal suspension was divided in 2 aliquots. In one of them the cells were adjusted to 1×10^6 cells/ml of Hank's solution. In the other aliquot the cells were incubated at 37°C for 45 min in eppendorf tubes in the presence of 0.5% of trypsin (Sigma, St Louis, MO, USA) to allow adherence to the plastic of macrophages, which were separated from the lymphocytes.

Incubation of Cells in Presence of AA

In order to follow the AA accumulation in the total peritoneal cells, lymphocyte and macrophage samples, these cells, adjusted at 1×10^6 cells/ml of Hank's solution, were incubated in eppendorf tubes (0.5 ml) at 37°C in a humidified atmosphere of 5% CO_2 for 10, 30, 60 or 180 min in the absence or presence of 0.01, 0.1 or 1 mM of AA (Sigma, St Louis, MO, USA).

Ascorbic Acid Determination

To each leukocyte sample 0.5 ml 1 N trifluoroacetic acid (Merck, Darmstadt, FRG) and 50 µl 1 mM 1,4-dithioeryhritol as antioxidant (Boehringer, Mannheim, FRG) were added. When the extracts were not going to be immediately analyzed the supernatants were stored at −20°C.

The AA determination in cells was carried out following the method of Hernanz.[26] Briefly, AA was measured by high-performance liquid chromatography (HPLC) and UV spectrophotometric detection at 265 nm using a Waters liquid chromatograph and a reverse-phase Novapak C18 column (Waters). As mobile phase, 1 mmol/l ammonium formate (Carlo Erba, Milano, Italy) (pH 6.0), containing 3 mmol/l tetrahexylamonium chloride (Fluka, Steinheim, Switzerland) as a paired-ion reagent in water/methanol (55/45 by vol) was used. The addition of tetrahexylammonium chloride to the mobile phase allows ionic compounds, such as AA, to be separated in C18 reverse-phase columns, eliminating the problems of precise pH and temperature control, reproducibility and short column life associated with ion exchange. Further filtration was performed with a 0.5 µm FHUP filter (Millipore, Bedford, MA, USA). The flow rate of the mobile phase was adjusted to 0.8 ml/min. AA working standards were prepared fresh daily in the mobile phase.

For the determination of hepatic AA, we used disodic phosphate 4.3 mM (Sigma, St Louis, MO, USA), containing tetradecyltrimethyl-amonium-bromide 99% Myristrimethylamonium bromide 1.07 mM (Sigma, St Louis, MO, USA) in acetonitrile/water (12.5/87.5 by vol) as the mobile phase.

Statistical Analysis

The data are expressed as the mean ± standard deviation (S.D.) of the values from the number of experiments shown in the tables and figures. The data were analyzed by two-way repeated measures analysis of variance (ANOVA) in the different groups of mice at 0, 2, 4, 12 and 24 h after LPS injection, and one-way ANOVA for hepatic AA content. The Student–Newman–Keuls test with a minimum level of significance set at $P < 0.05$ was used for posthoc comparisons.

RESULTS

Figure 1 shows the intracellular AA levels in total peritoneal cells, in lymphocytes and in macrophages from animals at 0, 2, 4, 12 and 24 h after LPS injection. In total cells and lymphocytes the AA levels were increased significantly, but at 24 h there was a decrease with respect to 12 h. In peritoneal macrophages the intracellular AA levels decreased at 4, 12 and 24 h after LPS injection. In all cases the AA levels were higher in total cells and peritoneal lymphocytes than in peritoneal macrophages.

Figure 2 shows the hepatic AA levels at different times after LPS injection. The statistical analysis shows an increase at 4 and 12 h, while at 24 h there is a decrease, with values near zero.

The results obtained on the AA intracellular levels in the total peritoneal cells, in lymphocytes and in macrophages from mice at 0, 2, 4, 12 and 24 h after LPS injection and incubated for 10, 30,

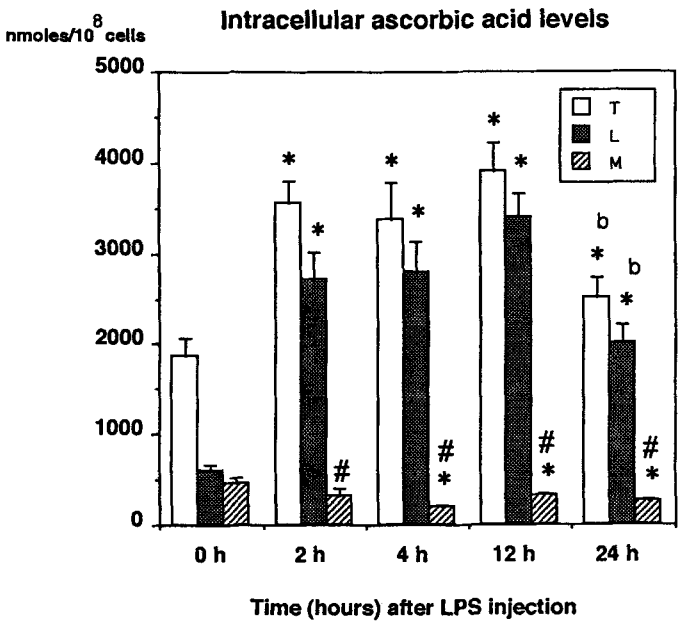

FIGURE 1 Intracellular AA levels of T (total peritoneal cells), L (lymphocytes) and M (macrophages). In all cases, the cells were obtained at 0, 2, 4, 12 and 24 h after LPS injection. Each column represents the mean ± S.D. of eight values corresponding to eight animals, with each value being the mean of duplicate assays. *$P < 0.05$ with respect to the corresponding values in the control group (0 h). a $P < 0.05$ comparing the results of lymphocytes with those of macrophages. b $P < 0.05$ comparing the results at 12 and 24 h.

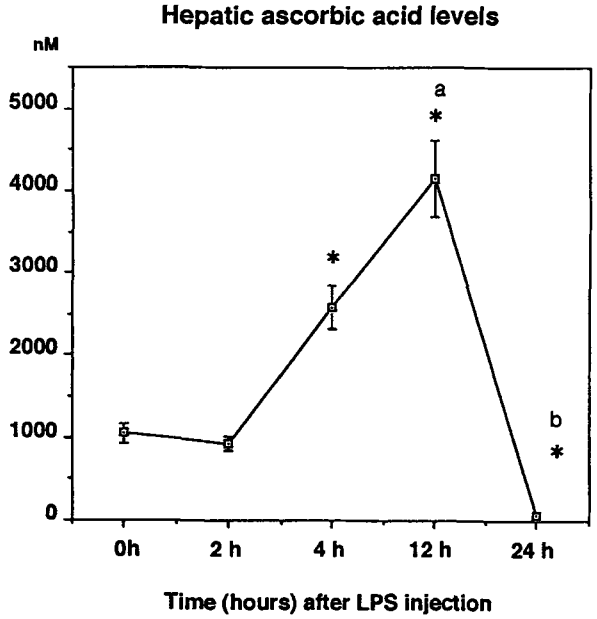

FIGURE 2 Hepatic AA levels at 2, 4, 12 and 24 h after injection. Each column represents the mean ± S.D. of eight values corresponding to eight animals, with each value being the mean of duplicate assays. *$P < 0.05$ with respect to the corresponding values in the control group (0 h). a $P < 0.05$ comparing the results at 4 with those at 12 h. b $P < 0.05$ comparing the results at 12 and 24 h.

60 or 180 min in presence of 0.01, 0.1 and 1 mM or in absence of AA are shown in Tables I–III, respectively. In the absence of AA the intracellular levels of this compound did not change with time of incubation, and the highest concentration of AA used (1 mM) was the most effective for increasing intracellular AA levels. In the total peritoneal cells (Table I), the presence of the lowest concentration of AA 0.01 mM in control cells (0 h) increased the intracellular levels of AA only at 60 min of incubation, whereas with 0.1 mM the increase occurred at 30 and 60 min, with 30 min being the most effective time. With the highest concentration of AA (1 mM) at 30, 60 and 180 min of incubation an increase of AA levels was also observed, with 30 min being the most effective time. In cells from mice at 2 and 4 h after LPS injection the presence of AA at 0.01 mM did not change their levels, while at 12 h after LPS the cells increased their AA levels after 30 and 60 min of incubation, and at 24 h the levels increased at 10 and 30 min. With the highest doses of AA (0.1 and 1 mM), the increase of AA levels occurred at almost all times of incubation, and after LPS injection. In general a 30 min incubation time was the most suitable for increasing the AA levels.

Considering the results obtained on the AA intracellular levels in lymphocytes (Table II), the presence of AA at all concentrations (0.01, 0.1 and 1 mM) increased the intracellular levels of AA at all times of incubation in cells from control mice (0 h) and from animals injected with LPS. The concentration of 1 mM increased the levels of AA, with 30 min being the most effective time at 2 h, 60 min at 4 h, and 10 and 30 min at 12 and 24 h after LPS injection. With the lowest concentrations (0.01 and 0.01 mM) the increase of AA took place mainly at 30 min of incubation in cells from mice after 2 and 4 h of LPS injection, and at 10 and 30 min after 12 and 24 h of injection.

The results obtained on the AA intracellular levels in macrophages are shown in Table III. The presence of AA at all concentrations (0.01, 0.1 and 1 mM) increased the intracellular levels of AA. In macrophages from control mice the increase of AA occurred mainly at 60 and 180 min of incubation, and in cells from animals after LPS injection this increase was mainly at 60 min at 2 h and at 10–30 min at 4 and 12 h after LPS injection.

DISCUSSION

Previous studies have shown the central role of ROS and proinflammatory cytokines, such as TNFα, generated by peritoneal macrophages and lymphocytes in the pathogenesis of irreversible endotoxic shock.[2,6,8] Thus, antioxidants, which act by trapping or metabolizing free radicals, inhibiting the enzymes involved in free radical production, as well as preventing the activation of nuclear transcription factors such as NF-κB involved in TNFα synthesis, could improve

TABLE I Intracellular levels of AA in the total peritoneal cells from mice at different times after LPS injection and in the presence of several AA concentrations (the results are the mean ± S.D. of eight values corresponding to eight animals, with each value being the mean of duplicate assays)

Time after LPS injection (h)	AA (mM)	Time of incubation (min)			
		10	30	60	180
0	None	2203 ± 371	1877 ± 195	1884 ± 277	1961 ± 215
	0.01	1907 ± 178	2331 ± 267*	2641 ± 289*,†,‡	2087 ± 267§
	0.1	2214 ± 234	3401 ± 304*,†,‡	2800 ± 325*,†,‡	2133 ± 400¶
	1	2350 ± 336	11724 ± 1593†,‡	839 ± 1007†,‡,¶	2507 ± 215†,¶,§
2	None	3757 ± 224	3563 ± 177	3706 ± 251	3394 ± 347
	0.01	3483 ± 354*	4046 ± 367*	4046 ± 339*	3433 ± 357*
	0.1	4346 ± 445*,†	5894 ± 601*,†,‡	4340 ± 410*,†,¶	3843 ± 421*,†,¶
	1	8300 ± 831†	16489 ± 1286†,‡	9279 ± 891†,¶	5760 ± 894†,‡,¶,§
4	None	3243 ± 268	3365 ± 168	3418 ± 278	2924 ± 216
	0.01	3308 ± 315	3423 ± 371*	3806 ± 391*	3494 ± 313*
	0.1	4510 ± 332†	4483 ± 298†	4332 ± 419†	3911 ± 342*,†
	1	4177 ± 455†	4365 ± 428†	5676 ± 403†,‡	5171 ± 399†,‡
12	None	4132 ± 295	3900 ± 176	4252 ± 367	3867 ± 431
	0.01	4439 ± 412*	5218 ± 464*,†,‡	4876 ± 427*,†	4488 ± 401*
	0.1	8152 ± 632*,†	6764 ± 400*,†,‡	5832 ± 412*,†,‡,¶	5218 ± 442*,†,‡,¶
	1	19687 ± 2134†	10829 ± 540†,‡	7077 ± 887†,‡,¶	6889 ± 365†,‡,¶
24	None	2727 ± 322	2503 ± 221	2551 ± 234	2329 ± 171
	0.01	377 ± 311*,†	3528 ± 312*,†	2545 ± 265*,‡,¶	2613 ± 254*,‡,¶
	0.1	5322 ± 510*,†	3843 ± 331*,†,‡	2938 ± 301*,‡,¶	2858 ± 303*,‡,¶
	1	19956 ± 711†	4160 ± 159†,‡	3854 ± 205†,‡	3595 ± 600†,‡

* $P < 0.05$ with respect to the corresponding value with 1 mM.
† $P < 0.05$ with respect to the corresponding control group (none).
‡ $P < 0.05$ with respect to 10 min of incubation.
¶ $P < 0.05$ with respect to 30 min of incubation.
§ $P < 0.05$ with respect to 60 min of incubation.

survival in animal models of septic shock and chronic bacteremia.[3,6,27–29] In fact, the presence of antioxidants such as *N*-acetylcysteine *in vivo*[6,29] or AA *in vitro*[8] improve the immune cell functions in animals with endotoxic shock, bringing them to values more similar to controls and increasing survival.

AA, that is a vitamin for man but not for other mammals such as mouse, is an important cellular antioxidant and it seems to play a crucial role in the function of immune cells.[12,13,30–33] The measure of AA has been used to assess the effects of oxidative stress in other pathological conditions such as parasitic infections[34] and ischaemia reperfusion.[35] In the present work we have observed that peritoneal cells, and specifically the lymphocytes, increase the levels of AA at all times after LPS injection, with 12 h being the most effective time. These data are in agreement with the hepatic AA levels found in our experiments. Thus, lymphocytes show a marked ability to concentrate AA, as suggested by other reports and its concentration is higher in those cells than in granulocytes or platelets.[36] This high concentration of AA in lymphocytes suggests its important role in the physiological function of these cells. In fact lymphocytes are a marker of general tissue reserves of this antioxidant.[37] On the contrary, we have observed that macrophages have decreased levels of AA at all times after LPS injection, as it has also been shown to occur during phagocytosis.[38] We have described previously an activation of the different functions of macrophages during endotoxic shock,[5,6,8] while we have observed in lymphocytes a decrease in the lymphoproliferative response to the mitogen concanavalin A (unpublished data). These data reveal the different behavior of these cell types during oxidative stress induced by

TABLE II Intracellular levels of AA in the peritoneal lymphocytes from mice at different times after LPS injection and in the presence of several AA concentrations (the results are the mean ± S.D. of eight values corresponding to eight animals, with each value being the mean of duplicate assays)

Time after LPS injection (h)	AA (mM)	Time of incubation (min)			
		10	30	60	180
0	None	732 ± 69	599 ± 82	597 ± 98	573 ± 570
	0.01	1171 ± 161*,†	1777 ± 78*,†,‡	1726 ± 183*,†,‡	1367 ± 149*,†
	0.1	1565 ± 270*,†	1962 ± 96*,†	1793 ± 130*,†	1452 ± 95*,†
	1	2112 ± 238†	4135 ± 309†,‡	3391 ± 454†,‡	2102 ± 173†,¶,§
2	None	2566 ± 316	2708 ± 272	2525 ± 278	2878 ± 154
	0.01	2586 ± 300*	4034 ± 518*,†,‡	3540 ± 293*,†,‡	2977 ± 291*,¶
	0.1	2639 ± 278*	5415 ± 432*,‡	3282 ± 301*,†,‡,¶	3380 ± 292*,†,¶
	1	5597 ± 877†	12221 ± 2008†,‡	6488 ± 162†,¶	4074 ± 241†,‡,¶,§
4	None	2847 ± 207	2796 ± 310	3061 ± 155	2781 ± 120
	0.01	2983 ± 321*	3674 ± 314†	3251 ± 361*	3077 ± 299*
	0.1	3139 ± 265*	3782 ± 301†,‡	3502 ± 325*,†	3365 ± 249*,†
	1	3752 ± 216†	3892 ± 486†	4629 ± 324†,‡,¶	4005 ± 205†,§
12	None	3092 ± 251	3387 ± 321	2983 ± 106	3033 ± 247
	0.01	4083 ± 421*,†	4274 ± 431*,†	3939 ± 345*,†	3477 ± 314¶
	0.1	4539 ± 455*,†	4612 ± 503*,†	3902 ± 387†,‡	3485 ± 400‡,¶
	1	14422 ± 395†	12161 ± 780†,‡	4486 ± 377†,‡,¶	3474 ± 400‡,¶,§
24	None	2094 ± 143	2017 ± 217	2066 ± 173	1948 ± 102
	0.01	3083 ± 345*,†	2774 ± 321†	2456 ± 324‡	2477 ± 214‡
	0.1	4938 ± 419*,†	812 ± 459†,‡	2578 ± 367†,‡	2285 ± 254‡
	1	16495 ± 661†	2837 ± 330†,‡	2610 ± 187†,‡	2752 ± 254†,‡

* $P < 0.05$ with respect to the corresponding value with 1 mM.
† $P < 0.05$ with respect to the corresponding control group (none).
‡ $P < 0.05$ with respect to 10 min of incubation.
¶ $P < 0.05$ with respect to 30 min of incubation.
§ $P < 0.05$ with respect to 60 min of incubation.

LPS. However, both kinds of cells (mainly macrophages), produce ROS in high amounts during this state[2,5,6,8] showing that lymphocytes can mainly accumulate AA like a protective mechanism against peroxidation death, while macrophages use it to avoid the damage caused by ROS. We have observed in previous studies[38] that, following stimulation of phagocytosis in mice, there is a rapid consumption of intracellular ascorbate in the presence or absence of peritoneal lymphocytes, while in the guinea pig an animal that does not synthesize AA, peritoneal lymphocytes maintained the intracellular AA of macrophages during phagocytosis.

Liver synthesizes AA that can be transported across cellular membranes by two distinct mechanisms. One is by a sodium-dependent saturable transporter,[39] and the other as dehydroascorbic acid (DHA), since AA present outside cells can be oxidized to this compound.[40] Once it is inside the cells, DHA is immediately reduced to AA by both chemical and protein mediated processes. Since we have observed in the present work that the AA level changes in macrophages and lymphocytes as well as in the liver after LPS injections, we measured at different incubation times the incorporation capacity of AA *in vitro* using 0.01, 0.1 and 1 mM concentrations, which had been shown effective on different functions of macrophages and lymphocytes in previous studies, specially at 1 mM.[2,8] We observed that the two different types of immune cells accumulate AA, but that lymphocytes do it in higher amount than macrophages. This result may seem paradoxical since macrophages produce ROS in higher amounts than lymphocytes. Thus AA seems to play an important role in the physiological function of lymphocytes, which are markers of the general tissue reserves of this antioxidant.[37] Lymphocytes accumulate AA

TABLE III Intracellular levels of AA in the peritoneal macrophages from mice at different times after LPS injection and in the presence of several AA concentrations (the results are the mean ± S.D. of eight values corresponding to eight animals, with each value being the mean of duplicate assays.)

Time after LPS injection (h)	AA (mM)	Time of incubation (min)			
		10	30	60	180
0	None	558 ± 74	469 ± 61	621 ± 65	684 ± 70
	0.01	671 ± 61*	857 ± 78*,†	926 ± 83*,†,‡	867 ± 79*,†
	0.1	765 ± 70*,†	962 ± 93*,†	1093 ± 120*,†,‡	1052 ± 101*,†,‡
	1	1007 ± 169†	2404 ± 236†,‡	3608 ± 371†,‡,¶	2796 ± 253†,‡,§
2	None	362 ± 41	317 ± 45	292 ± 25	327 ± 35
	0.01	433 ± 31*,†	507 ± 46*,†	616 ± 45*,†,‡	667 ± 79*,†,‡
	0.1	515 ± 43*,†	602 ± 53*,†	712 ± 67*,†,‡	649 ± 51*,†
	1	1468 ± 67†	1438 ± 210†	4054 ± 276†,‡,¶	2489 ± 249†,‡,¶,§
4	None	251 ± 54	206 ± 24	183 ± 14	185 ± 11
	0.01	414 ± 41*,†	397 ± 36*,†	346 ± 31*,†	337 ± 29*,†
	0.1	567 ± 48*,†	532 ± 48†	512 ± 47†	479 ± 43*,†
	1	881 ± 147†	632 ± 72†,‡	555 ± 104†,‡	570 ± 115†,‡
12	None	326 ± 24	312 ± 37	410 ± 49	394 ± 49
	0.01	614 ± 53*,†	497 ± 52*,†,‡	476 ± 41*,‡	397 ± 30*,‡,¶
	0.1	817 ± 71*,†	882 ± 76†	772 ± 67†	669 ± 60†,‡,¶
	1	2360 ± 152†	733 ± 64†,‡	871 ± 110†,‡,¶	640 ± 70†,‡,¶,§
24	None	289 ± 38	257 ± 24	299 ± 44	251 ± 41
	0.01	274 ± 23	291 ± 32*	276 ± 41*	265 ± 30*
	0.1	307 ± 30	302 ± 26*	272 ± 25*	259 ± 22*
	1	373 ± 64†	513 ± 81†,‡	574 ± 72†	779 ± 72†,‡,¶,§

* $P < 0.05$ with respect to the corresponding value with 1 mM.
† $P < 0.05$ with respect to the corresponding control group (none).
‡ $P < 0.05$ with respect to 10 min of incubation.
¶ $P < 0.05$ with respect to 30 min of incubation.
§ $P < 0.05$ with respect to 60 min of incubation.

mainly at 30 min, but at the end of the experiment, at 24 h after LPS injection, when the hepatic AA levels are practically unmeasurable, AA accumulates at the start of incubation (10 min). By contrast, macrophages accumulate AA at 60 min of incubation for the controls and after 2 h of LPS injection, while after this time the intake capacity decrease. It is probable that the high ROS production by macrophages in this endotoxic shock model[1–3] causes membrane damage in these cells which cannot incorporate AA from the medium, and for this reason at 24 h after LPS injection the intake capacity was decreased. However, in control conditions, macrophages can incorporate AA from the medium easily.[38]

In conclusion, these studies point out that AA is a key antioxidant during situations of oxidative stress, like endotoxic shock, in which lymphocytes accumulate AA while macrophages use it, because the incorporation capacity is higher in lymphocytes than in macrophages. These preliminary studies appear to be necessary before the introduction of possible therapies that rely on the use of scavengers of free radicals, i.e. compounds that can neutralize those radicals, such as AA and other antioxidants.

Acknowledgements

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