PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 179, 128–131 (1985)

RAPID COMMUNICATIONS

INHIBITION OF ASCORBIC ACID UPTAKE BY ENDOTOXIN: EVIDENCE OF MEDIATION BY SERUM FACTOR(S)

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Abstract: The effect of bacterial endotoxin on the ascorbic acid uptake by 3T6 fibroblasts was studied. Endotoxin inhibited ascorbic acid uptake by fibroblasts in a dose dependent manner. The inhibition by endotoxin takes place only in the presence of unheated serum; decomplementing serum by heat inactivation at 56°C for 30 minutes eliminates endotoxin's inhibitory effect on ascorbic acid uptake. The effect of endotoxin appears to be instantaneous since the inhibition seen in the cells without any preexposure was similar to the cells preexposed to endotoxin for up to 6 hours. Polymyxin B sulfate which is known to bind the lipid A portion of endotoxin did not reverse the inhibition of ascorbic acid uptake caused by endotoxin.

Bacterial endotoxin, lipopolysaccharides obtained from Gram-negative bacteria, are known to influence the metabolism of a number of different tissues and cells (reviewed in 1-3); fibroblasts are among the most susceptible cells for endotoxin's deleterious effects(4). Earlier it was observed in this laboratory that endotoxin, an etiologic agent in periodontal disease accumulates on the root surface of periodontally-involved teeth (5,6). In an attempt to understand the influence of endotoxin on the metabolism of the surrounding tissues, particularly collagen synthesis by fibroblasts, we studied the effect of endotoxin on ascorbic acid uptake by 3T6 fibroblasts. In this paper, evidence is presented that endotoxin activates heat-labile factor(s) in serum which appear to regulate cellular ascorbic acid uptake. These data help to identify the plasma factors alluded to in another study which were suggested to regulate ascorbic acid uptake by leukocytes (7).

Material and Methods. Cells: Mouse 3T6 fibroblasts (ATCC CCL96) were obtained from American Type Culture Collection, Rockville, Maryland. Unless specified otherwise the cells were grown in NUNC tissue culture flasks, with a surface area of 25 cm^2 in Dulbecco-Vogt modification of Eagle's medium, supplemented with 20% unheated fetal bovine serum. The cells were grown at 37°C in an atmosphere of 5% CO_2; the growth medium was changed every 48 hours.

Endotoxin: From Escherichia coli, 0127: B8, butanol extract, obtained from Sigma.

Ascorbic Acid Uptake: When the cells were grown to near confluency (72-96 hrs), they were incubated with fresh medium containing indicated amounts of endotoxin for a specified time at the end of which ascorbic acid uptake was carried out as follows.

Ascorbic acid uptake was carried out in growth medium containing respective amounts of endotoxin or polymyxin B sulfate as indicated, and (1-^{14}C) ascorbic acid (2.75 μM, sp. act 7.6 mCi/mmol., from New England Nuclear). After 1 hour of incubation at 37°C, the ascorbic acid uptake was terminated by removing the radioactive medium followed by washing the cell-sheet 4 times with 5ml of ice cold phosphate buffered saline. The washed cell-sheet was dissolved overnight in 2.5ml of 0.4 N NaOH and two ml of the dissolved cells were placed in Aquasol (NEN) and counted for radioactivity. A portion of dissolved cells was used for protein estimation by the method of Bradford (8). In order to assure the radioactivity taken up by the cells was indeed in ascorbic acid, unlabelled ascorbic

0037-9727/85 $1.50

TABLE I. EFFECT OF ENDOTOXIN ON THE ASCORBIC ACID UPTAKE

Endotoxin μg/ml	Unheated Serum Ascorbic Acid uptake pmoles/mg/hr	% of control	Heat Inactivated Serum Ascorbic Acid uptake pmoles/mg/hr	% of control
00 (control)	237 ± 4	100	266 ± 12	100
10	-- --	--	265 ± 2	100
25	222 ± 3	94	271 ± 20	102
50	191 ± 6	81	269 ± 17	101
100	177 ± 4	75	282 ± 6	106
200	125 ± 12	53	272 ± 22	102
500	-- --	--	285 ± 6	107

Cells were grown in Dulbecco-Vogt modification of Eagle's medium supplemented with heat-inactivated or unheated serum. When near confluency (48-72 hrs), the medium from the flasks was replaced with fresh medium containing an indicated amount of endotoxin and the flasks were incubated for 4 hrs. Ascorbic acid uptake was then carried out as described in 'Material and Methods'.

acid added to the tissue culture medium inhibited the uptake of labelled ascorbic acid. Furthermore, >95% of meta-phosphoric acid extracted radioactive material from the cells comigrated with an ascorbic acid standard using thin layer chromatography. Under all the experimental conditions described, cells remained firmly attached to the flask surface with no apparent cell damage as determined by Trypan Blue exclusion. Endotoxin and/or polymyxin B sulfate were present during uptake studies as indicated. Values presented are mean ± standard deviation of at least triplicate determinations.

Results: The effect of endotoxin on ascorbic acid uptake is shown in Table I. In the presence of unheated serum, bacterial endotoxin inhibits ascorbic acid uptake in a dose dependent manner. Heat inactivation of the serum at 56°C for 30 minutes eliminates endotoxin's inhibitory effect on ascorbic acid uptake.

The time required for endotoxin to inhibit ascorbic acid uptake seems to be minimal. Where endotoxin and (1-^{14}C) ascorbic acid are added simultaneously to the cells (0 hour preexposure) the inhibition of ascorbic acid uptake by endotoxin was very similar to the cells which received up to 6 hours of preexposure to endotoxin (Table II). This suggests that prior exposure to endotoxin is not required to activate the heat-labile factors in serum which apparently are involved in the regulation of ascorbic acid transport.

Polymyxin B sulfate is known to bind to the lipid A moiety of endotoxin and thereby prevent the effects of endotoxin which are due to the lipid A portion. We investigated whether polymyxin B sulfate could reverse the inhibitory effect of endotoxin on the ascorbic acid uptake. As can be seen from Table III, polymyxin B sulfate did not reverse the inhibitory effect of endotoxin, suggesting that the carbohydrate and not the lipid A moiety of endotoxin is involved in the inhibition of ascorbic acid uptake.

Discussion: An in vitro study in this laboratory has shown that endotoxin inhibi-

TABLE II. EFFECT OF TIME OF ENDOTOXIN PRETREATMENT OF 3T6 CELLS ON THE INHIBITION OF ASCORBIC ACID UPTAKE

Time of Endotoxin Pretreatment	Ascorbic Acid Uptake pmoles/mg/hour	% Inhibition
Control	247 ± 11	---
0 hr	137 ± 7	44
2 hr	141 ± 3	44
4 hr	138 ± 2	44
6 hr	145 ± 2	41

Cells were grown in a medium containing unheated serum. When near confluency, cells were exposed to endotoxin (200 μg/ml) for indicated times at the end of which ascorbic acid uptake was carried out as outlined in 'Materials and Methods'.

ted the uptake of ascorbic acid in endotoxin-challenged fibroblasts in a dose dependent manner only in the presence of unheated serum. Furthermore, polymyxin B sulfate which is known to neutralize the endotoxicity of endotoxin by binding the lipid A portion of the molecule (3), did not reverse the inhibition of ascorbic acid uptake caused by endotoxin suggesting that the carbohydrate moiety of endotoxin is active in this situation.

The implication of these findings are several and far reaching. First, it appears that endotoxin reacts with heat labile factors in serum, probably protein in nature, and that the result of this interaction produces an inhibition of cellular uptake of ascorbic acid. In an earlier study (7), it was shown that plasma from different individuals varied the uptake of ascorbic acid by leukocytes, in vitro, also suggesting the presence of plasma factors regulating the uptake of ascorbic acid; these factors have never been identified.

It is well known that endotoxin can activate both the classical and alternate

TABLE III. EFFECT OF POLYMYXIN B SULFATE AND ENDOTOXIN ON THE ASCORBIC ACID UPTAKE BY 3T6 CELLS

Addition	Ascorbic Acid Uptake pmoles/mg/hr	% Inhibition
None	222 ± 4	control
Endotoxin, 200 μg/ml	139 ± 2	37
Polymyxin B, 50 μg/ml	182 ± 2	18
Polymyxin B, 200 μg/ml	163 ± 5	26
Endotoxin, 200 μg/ml + polymyxin B, 50 μg/ml	133 ± 5	40
Endotoxin, 200 μg/ml + polymyxin B, 200 μg/ml	102 ± 3	54

Cells were grown to near confluency in a medium containing unheated serum. Cells were exposed to either endotoxin or polymyxin B or both for 4 hours, at the end of which ascorbic acid uptake was carried out as described in 'Materials and Methods'. In cases where both endotoxin and polymyxin B were added, the mixture of endotoxin and polymyxin B were preincubated at 37°C for 15 min before adding it to cells.

pathways of complement (9). The extent of activation of the two pathways appears to be different depending upon the source and isolation of the endotoxin (10). The inability of polymyxin B sulfate to prevent the inhibition of ascorbic acid uptake by endotoxin suggests that the alternate pathway of complement is involved. In another study (11), it was shown that the lipid A part of endotoxin initiates the classical pathway activation, whereas the activation of complement through the alternate pathway is initiated by the polysaccharide portion.

This study has demonstrated the loss of activity of endotoxin's inhibitory effect on cellular ascorbic acid uptake in the presence of serum decomplemented by heat-inactivation (56°C for 30 min). Since endotoxin and many diseases and disease states are known to activate the complement system in serum, and heat-inactivation of serum destroys complement, the mechanism of ascorbic acid uptake, and perhaps transport, may be regulated by the activation or inactivation of the serum complement system or other heat-labile serum constituents. Ascorbic acid is not synthezised by humans, it must be ingested from external sources. It is intriguing to speculate that although intake levels of the ascorbic acid may be adequate, the ultimate level in tissues may be regulated by factors which alter the serum complement system.

Acknowledgement: The authors wish to acknowledge financial support from the National Institutes of Health Grant DE 05766.

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Received January 21, 1985.
P.S.E.B.M. 1985, Vol. 179.
Accepted March 7, 1985.