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THE EFFECT OF DIPHTHERIA TOXIN ON THE CELLULAR UPTAKE AND EFFLUX OF L-CARNITINE

EVIDENCE FOR A PROTECTIVE EFFECT OF PREDNISOLONE

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Summary

Diphtheria toxin added to the incubation medium reduced the rate of uptake and increased the efflux of L-[^{3}H]carnitine in an established cell line from human heart (CCL 27, Girardi human heart cells). This resulted in a decrease in the level of intracellular carnitine to about 55% of control after exposure to 10^{-8} mol/l diphtheria toxin for 24 h. As expected, a decrease in protein synthesis was found to be caused by the toxin, and this inhibition seemed to a large extent to antedate the alterations in the transport processes. Measurement of the kinetic parameters for the mechanism of uptake of L-[^{3}H]-carnitine revealed a reduction in V with unaltered K_m after exposure to the toxin. We therefore suggest that diphtheria toxin imposes its effect on carnitine transport by inhibiting the synthesis of carriers.

Prednisolone in the medium along with the toxin opposed its effect both on the uptake and efflux mechanism, but not on the inhibition of protein synthesis. Still, the decline in the intracellular level of carnitine was prevented by the corticoid hormone. Such a decline, accompanied by accumulation of triacyglycerols, occurs during the course of a diphtheric myocarditis. It is possible that prednisolone, by counteracting the effects of diphtheria toxin on the carnitine transport processes, could be beneficial in the treatment of this condition.

Introduction

In 1964, Wittels and Bressler [1] reported that in hearts from guinea-pigs, diphtheria toxin caused a substantial decrease in the intracellular concentration

of carnitine. This was accompanied by an excessive accumulation of triacylglycerols and a marked depression in the ability to oxidize long-chain fatty acids, which however, could be improved by exogenous carnitine [1,2]. Carnitine administration to animals exposed to the toxin was shown to have a protective effect, doubling the LD_{50} and improving the hemodynamic performance of the left ventricle [3,4].

The ADP-ribosylation of elongation factor 2 (EF-2) is now well established as the principal mode of action of diphtheria toxin, resulting in the inhibition of protein synthesis [5,6]. Other events can be described as secondary to this inhibition. The protective effect of carnitine, however, indicates that secondary changes in carnitine metabolism must be of major importance for cellular viability.

The reduced intracellular concentration of carnitine in the heart after exposure to diphtheria toxin can be due to a reduced uptake or an increased efflux across the plasma membrane. We have previously described the uptake and efflux mechanism for L-carnitine in an established cell line from human heart (CCL 27, Girardi human heart cells) [7—11]. The aim of the present study was to investigate the effect of diphtheria toxin on the transmembrane transport of L-carnitine, and if possible to reveal a mechanism that could oppose the action of the toxin.

Materials and Methods

The Girardi human heart cells were obtained from Flow Laboratories Ltd., Irvine, U.K., and treated as previously described [7—11]. The diphtheria toxin was provided by The Norwegian Institute for Public Health (SIFF), and contained about 100 flocculation units/ml of stock solution. Diphtheria antitoxin was obtained from Statens Seruminstitutt, Department of Biological Standardization, World Health Organisation International Laboratory for Biological Standards, Copenhagen. Thymidine 5′-monophospho-*p*-nitrophenyl ester was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Other reagents were obtained as earlier reported [7—11].

The methods used in measuring the uptake and efflux of L-[*Me*-^{3}H]carnitine have previously been described [7,8,11]. They allow separate evaluation of influx and efflux, since under these experimental conditions variations in the opposing flux have negligible impact on the one being measured [7,11]. The separation of intracellular free carnitine from esterified forms was achieved by thin-layer chromatography as described by Christiansen and Bremer [12]. Viability of the cells was assessed by the trypan blue dye exclusion test [13]. Free L-carnitine and phosphodiesterase I (EC 3.1.4.1) were assayed as described previously [14,15]. Protein synthesis was assessed by measuring the incorporation of radioactive amino acids (L-amino acid mixture [U-^{14}C], New England Nuclear, Boston, MA, U.S.A.) into protein precipitated by 0.5 M $HClO_4$. The method of Lowry et al. [16] was used to quantitate protein.

The kinetic analysis of the uptake of L-carnitine was performed using a direct linear plot as described by Eisenthal and Cornish-Bowden [17,18]. The kinetic parameters are given as the median of the sample population with 95% confidence intervals.

Results

A significant decrease in the rate of uptake of L-[^{3}H]carnitine was noted after exposure of the cells to diphtheria toxin. This inhibition increased with the time of exposure and concentrations of the toxin above $5 \cdot 10^{-10}$ mol/l (Fig. 1). Incubating the cells with the toxin for shorter periods of time than depicted in the figure revealed no immediate effect on the uptake of L-carnitine (not shown).

Kinetic analysis of the uptake was performed before and after the exposure of the cells to a 10^{-8} mol/l concentration of the toxin for 24 h, and based on 16 separate observations for each parameter. The V value of the transport decreased from 17.0 pmol/μg DNA per h (95% confidence interval: 15.0—22.0 pmol/μg DNA per h) to 11.5 pmol/μg DNA per h (9.4—14.6 pmol/μg DNA per h), while the corresponding results for K_m were 8.0 μmol/l (95% confidence interval: 6.0—10.8 μmol/l) and 7.9 μmol/l (4.8—12.0 μmol/l). The reduction in V is highly significant ($P < 0.001$, Wilcoxon's rank sum test) while K_m seemed unaltered ($P = 0.281$).

The reduction in the rate of uptake was reversed by adding diphtheria antitoxin to the medium (Table I). Thus, the inhibition seems to be a specific effect of diphtheria toxin.

The toxin also induced a moderate increase in the efflux rate constants for L-carnitine (Table II and Fig. 2). Accelerated exchange diffusion, induced by

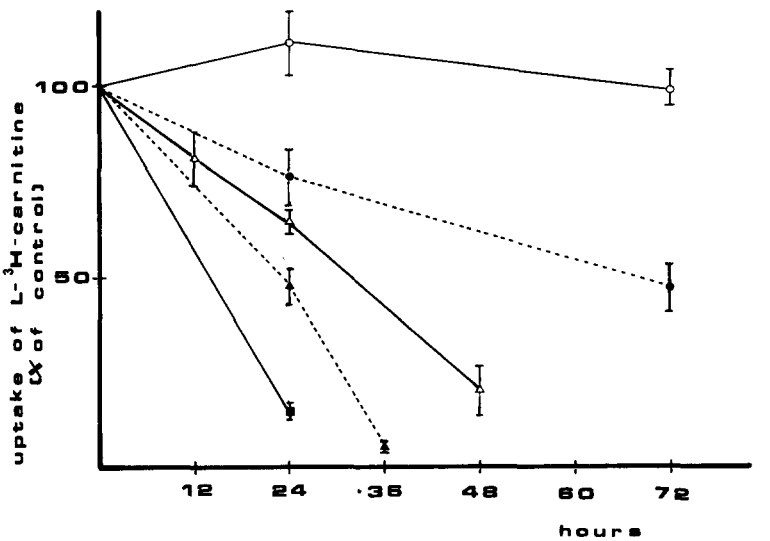

Fig. 1. The effect of diphtheria toxin on the uptake of L-[^{3}H]carnitine. The monolayers were incubated at the indicated period of time with different concentrations of the toxin. At the end of the incubations the cells were washed three times with 3 ml fresh medium before the rate of uptake of L-[^{3}H]carnitine was measured during a 2 h incubation. The results are given in percent of control (100% = mean of four control incubations), and represent the means of three to nine individual incubations. The vertical bars indicate the standard error of the mean. The observations are compiled from nine different experiments. ○———○, $5 \cdot 10^{-10}$ mol/l of the toxin; ●- - - - -●, $2.5 \cdot 10^{-9}$ mol/l; △———△, $5 \cdot 10^{-9}$ mol/l; ▲- - - - -▲, 10^{-8} mol/l; and ■———■, $5 \cdot 10^{-8}$ mol/l.

TABLE I

THE EFFECT OF DIPHTHERIA ANTITOXIN ON THE INHIBITION OF UPTAKE OF L-[^{3}H]CARNITINE BY DIPHTHERIA TOXIN

The monolayers were incubated with diphtheria toxin in the medium at the indicated concentrations for 48 h. 1 I.U. diphtheria antitoxin was added to the same medium 6 and 24 h after the addition of the toxin. The cells were washed three times with 3 ml fresh medium, before the rate of uptake of L-[^{3}H]-carnitine was measured during a 2 h incubation at 37°C. The results are means ±S.D. of four separate incubations that contained 38—391 μg DNA (325—3350 μg protein). The time of incubation is given in parentheses.

Incubation medium	% uptake
Control (48 h)	100 ± 5
$5 \cdot 10^{-9}$ mol/l toxin (48 h)	46 ± 13
1 I.U. antitoxin (48 h)	95 ± 10 *
$5 \cdot 10^{-9}$ mol/l toxin (48 h) and 1 I.U. antitoxin (42 h)	95 ± 5 *
$5 \cdot 10^{-9}$ mol/l toxin (48 h) and 1 I.U. antitoxin (24 h)	61 ± 8 *

* Significantly different from incubations with toxin alone ($P < 0.01$, Wilcoxon's rank sum test).

unlabeled L-carnitine in the efflux medium, could no longer be elicited after the cells had been exposed to the toxin (Table II).

A concentration of 10^{-8} mol/l of the toxin for 24 h in the medium reduced the intracellular concentration of free L-carnitine from (mean ±S.D.) 53.0 ± 9.2 to 30.2 ± 1.0 nmol/mg protein (significant difference, $P = 0.01$, Wilcoxon's rank sum test).

TABLE II

RATE CONSTANTS FOR THE EFFLUX OF L-[^{3}H]CARNITINE

The efflux of L-[^{3}H]carnitine was measured during a 4 h incubation after the cells had been loaded with 4 μmol/l L-[^{3}H]carnitine for 24 h. The efflux rate constant (k) was computed from the following equation: ln % intracellular L-[^{3}H]carnitine = ln 100 — $k \cdot t$ (t, time in h), as previously reported [11]. Linear regression with the least-squares method was used, pooling the data from three to five separate incubations. The correlation coefficient of the results to the equation was above 0.97 in all cases. Unless otherwise stated, the efflux took place into medium without any addition. The time of incubation is given in parentheses.

Experiment number	Incubation medium	k
I	Control	0.0591
	$5 \cdot 10^{-9}$ mol/l toxin (24 h)	0.0733 *
II	Control	0.0607
	$5 \cdot 10^{-9}$ mol/l toxin (48 h)	0.0768 *
III	Control	0.0396
	$5 \cdot 10^{-9}$ mol/l toxin (24 h)	0.0568 *
	$5 \cdot 10^{-9}$ mol/l toxin and 10^{-6} mol/l prednisolone (24 h)	0.0411
IV	Control	0.0621
	Control with 10^{-4} mol/l L-carnitine in efflux medium	0.0967 *
	$5 \cdot 10^{-8}$ mol/l toxin (24 h)	0.1579 *
	$5 \cdot 10^{-8}$ mol/l toxin (24 h) and 10^{-4} mol/l L-carnitine in efflux medium	0.1584 *

* Significantly different from control ($P < 0.01$, Wilcoxon's signed rank test).

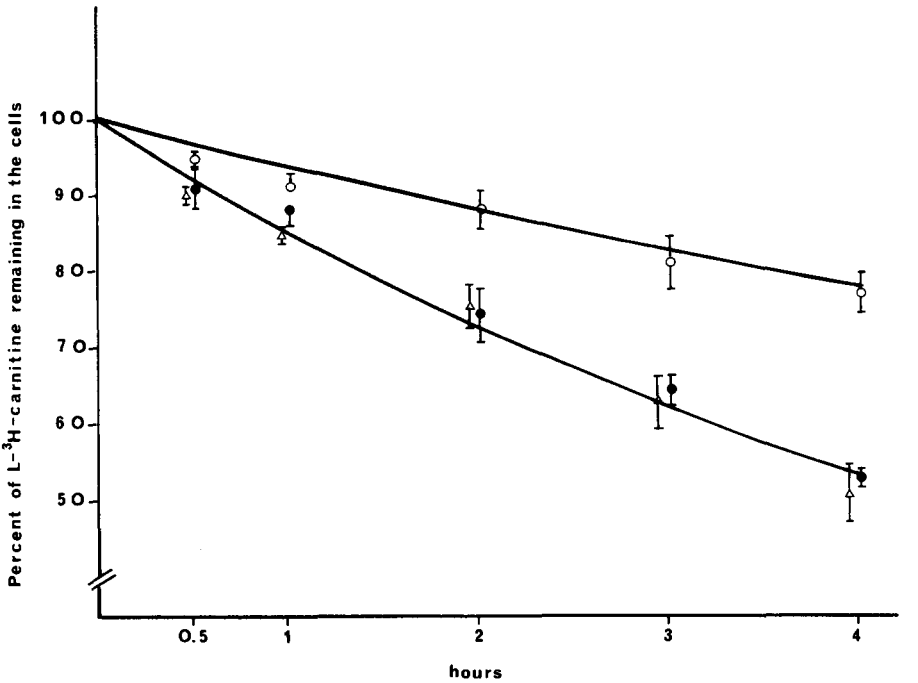

Fig. 2. The effect of diphtheria toxin on the efflux of L-[^{3}H]carnitine. The monolayers were incubated with or without $5 \cdot 10^{-8}$ mol/l diphtheria toxin and loaded with 4 μmol/l L-[^{3}H]carnitine for 24 h. The cells were washed six times before the efflux of L-[^{3}H]carnitine into fresh medium was measured during a 4 h incubation. The results (mean ±S.D.) are given as the decline in the amount of intracellular L-[^{3}H]-carnitine with time, producing the efflux rate constants stated in Table II. The curves are drawn on the basis of linear regression (least-squares method) from three to five separate incubations. ○———○, control incubations; ●———●, $5 \cdot 10^{-8}$ mol/l toxin, both without unlabeled L-carnitine in the efflux medium; △, $5 \cdot 10^{-8}$ mol/l toxin and 100 μmol/l L-carnitine in the efflux medium.

TABLE III

THE EFFECT OF DIPHTHERIA TOXIN ON THE INCORPORATION OF AMINO ACIDS INTO PROTEIN

The monolayers were incubated with diphtheria toxin and prednisolone at the indicated time and concentrations. The cells were then washed three times with 3 ml medium, and subsequently incubated 2 h with medium containing ^{14}C-labeled amino acids. Thereafter, the cells were trypsinized, and protein precipitated with 0.5 M $HClO_4$. The precipitate was washed twice in 2 ml of 0.5 M $HClO_4$, and finally dissolved in 2 ml of 1 M NaOH for determination of radioactivity and amount of protein. The results are means ±S.D. of three to four separate incubations that contained 150—1522 μg protein. The time of incubation is given in parentheses.

Experiment number	Incubation medium	cpm/mg protein	% of control
I	Control	721 ± 73	100 ± 10
	$5 \cdot 10^{-8}$ mol/l toxin (3 h)	644 ± 115	89 ± 16
	$5 \cdot 10^{-8}$ mol/l toxin and 10^{-4} mol/l prednisolone (3 h)	618 ± 129	86 ± 16
II	Control	1269 ± 311	100 ± 25
	$5 \cdot 10^{-8}$ mol/l toxin (12 h)	421 ± 166	33 ± 13 *
	$5 \cdot 10^{-8}$ mol/l toxin and 10^{-4} mol/l prednisolone (12 h)	181 ± 152	14 ± 12 *

* Significantly different from control ($P = 0.01$, Wilcoxon's rank sum test).

The toxin also imposed a significant decrease in the incorporation of amino acids into protein (Table III). $5 \cdot 10^{-8}$ mol/l of the toxin reduced the activity of the synthesis to about 30% of control already after 12 h. The toxin did not seem to change the intracellular distribution of radioactivity between free carnitine, acetylcarnitine and acylcarnitine (not shown). Neither was there any change in the activity of phosphodiesterase I, an enzyme chiefly localized in the plasma membrane [19], after exposure to 10^{-8} mol/l of the toxin for 24 h (not shown). The trypan blue dye exclusion test revealed that practically 100% of the cells used in the experiment retained their ability to exclude the dye after being exposed to the toxin in the present concentrations.

Prednisolone in the medium counteracted the effect of diphtheria toxin on the uptake mechanism. The previously reported increase in rate of uptake induced by the hormone [10] could still be elicited in the presence of the toxin (Table IV). Prednisolone also abolished the increase in the efflux rate constants caused by the toxin (Table II). An increase in the activity of phosphodiesterase I can be observed after treating this cell line with prednisolone. This increase, however, was inhibited by diphtheria toxin (not shown). The intracellular content of free L-carnitine (mean ±S.D.: 53.0 ± 9.2 nmol/mg protein) remained unaffected (57.7 ± 7.4 nmol/mg protein) when 10^{-5} mol/l prednisolone was added to the medium concomitant with 10^{-8} mol/l of the toxin for 24 h. Thus, prednisolone prevented the fall in the cellular content of L-carnitine, although the hormone did not seem to counteract the inhibition of protein synthesis imposed by the toxin (Table III).

Increasing the amount of L-carnitine in the growth medium results in an increase in the rate of uptake of L-carnitine in this cell line [9]. However, no effect of additional L-carnitine in the medium was noted on the action of diphtheria toxin, either on the uptake or on the efflux (not shown).

TABLE IV

THE EFFECT OF PREDNISOLONE ON THE UPTAKE OF L-[^{3}H]CARNITINE IN THE PRESENCE OF DIPHTHERIA TOXIN

The monolayers were incubated with diphtheria toxin and prednisolone at the indicated time and concentrations. The cells were washed three times with 3 ml fresh medium before the uptake of L-[^{3}H]carnitine was measured during a 2 h incubation. The results are means ±S.D. of three to five separate incubations that contained 22—131 μg DNA (190—2750 μg protein).

Experiment number	Incubation medium	% uptake of control
I	Control	100 ± 25
	$7.5 \cdot 10^{-10}$ mol/l toxin (48 h)	69 ± 16
	$7.5 \cdot 10^{-10}$ mol/l toxin and $5 \cdot 10^{-4}$ mol/l prednisolone (48 h)	206 ± 27 *
II	Control	100 ± 10
	$2.5 \cdot 10^{-9}$ mol/l toxin (24 h)	63 ± 26
	$2.5 \cdot 10^{-9}$ mol/l toxin and 10^{-5} mol/l prednisolone (24 h)	128 ± 23 *

* Significantly different from results with the toxin alone ($P \leq 0.05$, Wilcoxon's rank sum test).

Discussion

Diphtheric myocarditis is characterized by an excessive accumulation of intracellular triacylglycerols [20]. This is probably induced by the decreased level of carnitine in the cytosol [1,2]. In the present study, diphtheria toxin did cause such a reduction in the cellular content of carnitine. Thus, this system seemed suitable for the investigation of the changes in carnitine metabolism imposed by the toxin.

As expected, the inhibition of protein synthesis was observed to be an early event (Table III), and this principal mode of action of diphtheria toxin [6] seemed to a large extent to antedate the alteration in the transport processes. It therefore seems reasonable to explain the observed changes on the basis of this prime inhibition of protein synthesis.

Both the uptake and efflux of carnitine are probably carrier-mediated processes [7,11]. Assuming the carriers are of protein nature, the observed reduction in rate of uptake is most readily accounted for by a decrease in the synthesis of new carriers caused by the inhibited protein synthesis. The reduction in V with unaltered K_m of the transport process supports this assumption. The mechanism for the increase in efflux is less evident. At least part of it may consist of a non-carrier-mediated leakage, since accelerated exchange diffusion no longer could be elicited after exposure of the cells to the toxin (Table II).

The activity of phosphodiesterase I, reflecting the state of the plasma membrane, remained constant even after the transport processes for carnitine had been considerably modified. Thus, alteration in carnitine transport occurs as an early event in the membrane, thereby probably indicating its importance in pathogenesis.

Prednisolone and increased amounts of L-carnitine in the growth medium increased the rate of uptake of L-carnitine in this cell line by an increase in the number of carriers [9,10]. This probably occurred by different mechanisms, since an additive effect was noted by combining the two principles [10]. Cycloheximide inhibited the increase in the rate of uptake induced by additional L-carnitine in the medium [9], indicating the dependence of intact protein synthesis. In accordance with this, increasing the amount of L-carnitine in the medium did not modify the effect of diphtheria toxin on the transport processes (not shown). Prednisolone, however, still induced an increase in the rate of uptake in the presence of the toxin (Table IV). The hormone did not seem to counteract the inhibition of protein synthesis induced by the toxin (Table III). Thus, the effect of prednisolone is probably due to the ability to decrease the rate of degradation of the carriers that mediate the uptake of L-carnitine. Such an assumed preservation of membrane proteins might also account for the opposing effect of the steroid on the increase in efflux of L-carnitine induced by the toxin (Table II).

Our observations do not indicate that exogenous L-carnitine interferes with the action of diphtheria toxin. Thus, no specific explanation is disclosed for the reported protective effect of L-carnitine in guinea-pigs [3]. Prednisolone, on the other hand, seems to oppose the effects of the toxin, and could therefore probably be of benefit in the treatment of a myocarditis caused by

this toxin. A combination with exogenous L-carnitine might enhance the efficiency of this therapy.

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