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Effect of cholesterol and its autooxidation derivatives on endocytosis and dipeptidyl peptidases of aortic endothelial cells

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Summary. The effects of cholesterol (CHO) and cholesterol autooxidation derivatives (CAD) on the endocytosis of cationized ferritin (CF) by endothelial cells have been investigated. The effect of both substances on the activity of lysosomal enzymes dipeptidyl peptidase I (DPP I) and dipeptidyl peptidase II (DPP II) was also studied. Treatment of rats with CAD induced striking alterations in the ultrastructure of endothelial cells and makes it impossible to analyze the effect of this toxin on endocytosis processes. In contrast, CHO-treated cells displayed a good ultrastructural preservation and showed an increased ability to endocyte ferritin, as compared with controls. Both DPPI and DPP II activities increased after 3 weeks of CAD or CHO treatment. Our results indicate that although CHO damage endothelial cells, the most important effects could be attributed to CAD which usually accompanies CHO-supplemented diets.

Key words: Cationized ferritin, Endocytosis, Cholesterol oxides, Proteases, Aorta

Introduction

It has been proposed that CAD rather than CHO are responsible for arterial injury in diet-induced atherosclerosis (Cook and McDougall, 1967). CHO is rapidly and easily oxidized in the air and in cooking procedures and CAD are absorbed by the gastrointestinal wall (Combe et al., 1981; Fornas et al., 1984). Previous work in our laboratory has shown that primary alterations in rats fed with a CAD-supplemented diet include loss of endothelial cells in large areas of the aorta as well as changes in the production of prostacyclins (Martínez-Sales et al., 1983; Fornas et al., 1987). This effect appears to be related to the presence of CAD in the diet.

As the atherosclerotic process is usually related to an increase in collagen synthesis and changes in the type of collagen synthetized, DPP I and DPP II increase may be an indication that the aortic atherosclerotic regression process as a defence mechanism has been initiated (Lojda, 1985).

Attempts to relate changes in the activities of membrane-bound and cytosol proteases to the early changes in the development of the atherosclerotic process have not been successfull (Lojda, 1985), but lysosomal proteases DPP I and DPP II have a positive correlation with diet - induced atherosclerosis (Lojda et al., 1984) and human atherosclerotic lesions (Berberian and Fowler, 1979).

Plasma membrane of endothelial cells plays an important role in the molecular sieving properties of vascular endothelium (Gil, 1983; Schneeberger, 1983). On the other hand, it has been reported that increasing membrane viscosity induced «in vitro» by chemical effectors such as CHO resulted in the slowing down of endocytosis in lymphoid cells (Elguindi et al., 1985). Therefore, it appears of interest to analyze the effect *«in vivo»* of CAD and CHO on endocytosis in aortic endothelial cells and to relate the early changes induced by these diets to the changes in the activity of the lysosomal proteases DPP I and DPP II, which should indicate that the aortic regenerative processes have been initiated.

In the present work we have analyzed, using stereological procedures and CF, a widely-used marker in endocytosis studies (Forquhar, 1983), the effect of CAD- and CHO-supplemented diets on endocytosis of endothelial aortic cells. Moreover, the effect of these diets on the lysosomal proteases DPP I and DPP II has also been investigated.

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Materials and methods

Animals and diets

Three groups of 5 Wistar rats, with body weight between 190 and 200 g were fed for a period of 5 weeks with: 1) standard chow (Montagut Standard Chow) for the control group; 2) standard chow supplemented with 0.2% of CAD; 3) standard chow supplemented with 0.2% CHO (USP grade). The rats had free access to the chow and water. The chow for the experimental diet was prepared immediately before use. It was impregnated with the corresponding amount of CAD or CHO dissolved in ethanol, and the solvent was evaporated in an air current. The standard chow for the control group was impregnated in the same way with pure ethanol.

Tracer experiments

The rats were kept under pentobarbital anesthesia and the thoracic aorta of each rat was removed and immediately immersed in saline bubbled with 5% CO_2 + 95% O_2. It was then cut into 5 segments, which were incubated for 60 min at 37° C in Ham F-12 media containing CF (Sigma) at 0.3 mg/ml. During the incubation the medium was continuously gassed with a mixture of 5% CO_2 and 95% O_2.

Fixation

After incubation, the segments of aorta were quickly washed in saline solution and fixed for 2 hours in cold 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. The segments were then washed in cacodylate buffer, postfixed in 2% OsO_4 + 0.8% ferrocyanide for 2 hours and washed in water. Segments were then dehydrated in a graded series of acetone (from 50 to 100%), 5 min each and embedded in Epon 812.

Quantitative analysis

Ultrathin sections were analyzed in an electron microscope and micrographs at x50,000 were randomly obtained for the stereological analysis. On the micrographs of the endothelial cells a division line equidistant to the luminal and basal membranes was drawn to divide the cell in apical and basal areas. For the study of the intravesicular distribution of the ferritin, CF density of particles (XCF) and CF dispersion coefficient (DCCF) (Schwartz and Benditt, 1973), 5 endocytic vesicles (EV) were taken of each area, apical and basal. In order to eliminate possible bias in the selection of the vesicles taken for the statistical study, they were selected closest to a line perpendicular to the line that separated the apical and basal areas. The number of vesicles taken for the study of the different stereological parameters is indicated in the results, but the minimum was determined by the progressive mean technique (confidence limit = ± 5%).

Stereological analysis of aortas at 60 min incubation in CF was performed using point counting procedures. The parameters considered were: EV volume and numerical density (VvEV and NvEV, respectively) (Weibel et al., 1973) and EV absolute volume (VEV) (Lindberg and Vorwerk, 1970, 1972).

The quantitative comparison of the CF bound to cell membranes was made by measuring the depth of the CF layer (DCF) in the electron microscope micrographs (x 50,000). A total of 30 measurements or each experimental group, CHO and control, were performed, and 3 measurements per cell with a fixed distance between them to eliminate the introduction of any bias in the measurements.

The results are expressed as mean ± SD. Statistical analysis ($P < 0.05$) was carried out using the Student's test for the VvEV, NvEV, VEV and DCF, and the Mann and Whitney test for XCF and DCCF (Schwartz, 1963; Schwartz and Benditt, 1973).

DPP I and DPP II activities

The rats were kept under pentobarbital anesthesia and the thoracic aorta of each rat was removed and immediately immersed in saline bubbled with 5% CO_2 + 95% O_2. The adventitia was carefully taken off by stripping and the aorta was weighed and homogenized in a Potter-Elvehjem with distilled water with cooling to give 2% homogenates.

The enzymatic activities of the homogenates were determined as described by Lojda et al. (1984). The substrate solution used for measuring DPP I was: H-Gly-Arg-4-Methoxy-ß-naphtylamide. HCl (Novabiochem, dissolved in a few drops of 0.5 mM dimethyl sulphoxide), 3.0 mM dithiotreitol (Sigma) and 40.0 mM in NaCl in cacodylate buffer (pH 5.5). For DPP II the substrate solution was prepared in the same way, using the substrate H-Lys-Ala-4-Methoxy-ß-naphtylamide HCl (Novabiochem).

The following procedure was used: 0.15 ml homogenate (0.15 ml distilled water for the controls) was added to 1 ml of substrate solution, incubated for 3 hours at 20° C and then an azo-coupling was performed for 5 min at 20° C by adding 1ml with Fast Blue B (Sigma), 1 mg/ml in 0.1M cacodylate buffer, (pH 5.5). The protein was then precepitated 2 ml of 10% trichloroacetic acid and then colourant was extracted with 3 ml of ethyl acetate, shaking and centrifuging at 100 g for 5 min, and then the colour of the ethyl acetate was measured at 525 nm. Optical values have not been converted into µmoles, as they were used just to compare the activities of DPP I and DPP II in the different groups of animals.

Results

Analysis of thin and ultrathin sections revealed that endothelial cells corresponding to arteries of both control and CHO rats displayed a well-preserved ultrastructure.

In the samples obtained from CAD animals,

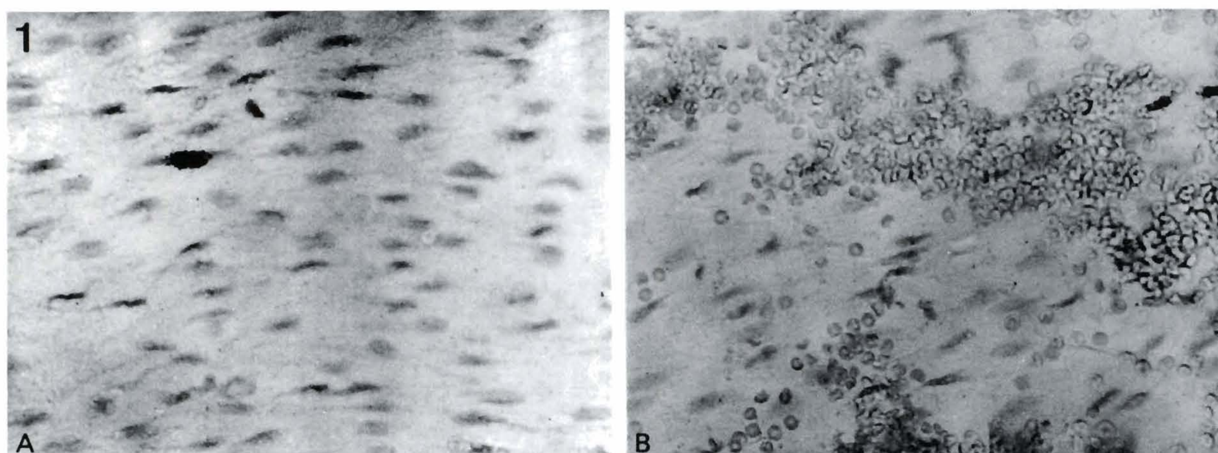


Fig. 1A. Aortic endothelial Hautchen preparation of a rat fed standard chow. Endothelial cells are intact. **B.** Aortic endothelial Hautchen preparation of a rat fed the CAD - supplemented diet. Endothelial cells are absent in many areas of the aorta and erythrocyte clusters can be seen adhering to the intima. (^{3}H) thymidine-labelled nuclei can be seen in both photographs. Preparations were weakly methylene-blue stained. A and B. × 500

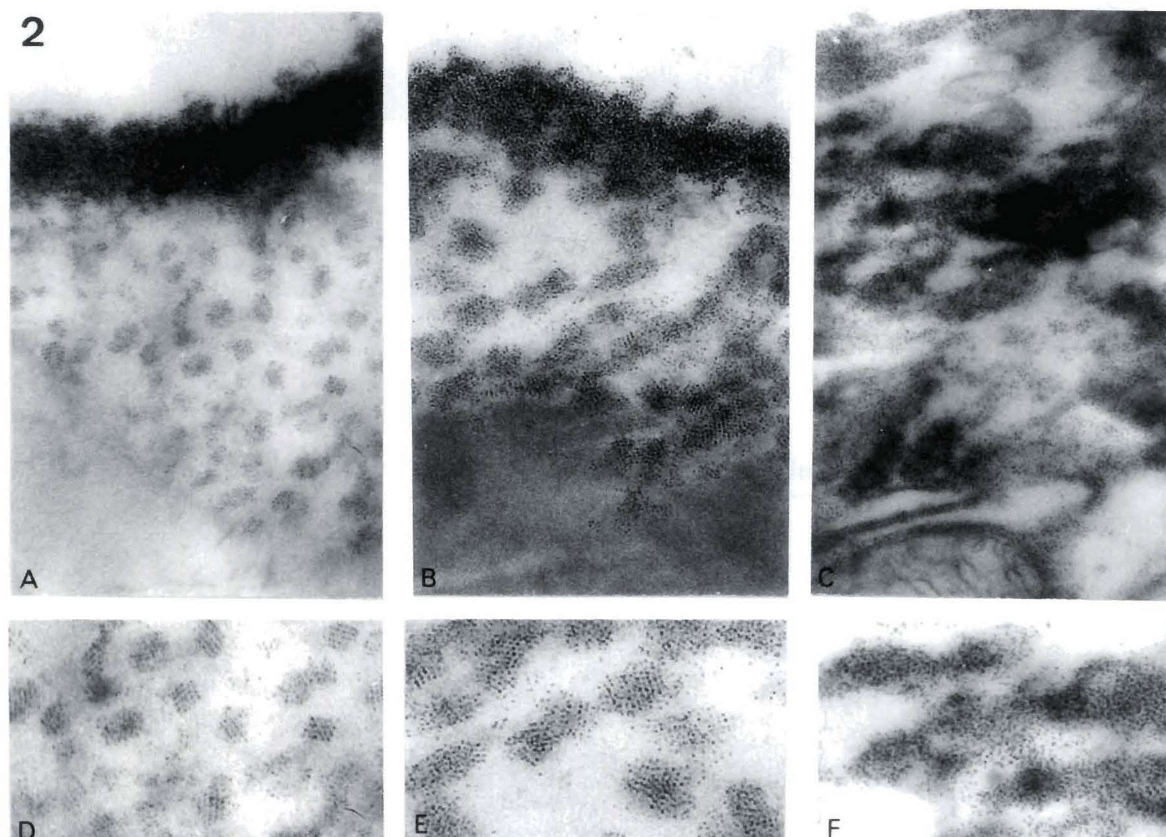


Fig. 2. Arterial endothelial cells, 60 min incubation in a CF containing media: A,D) Control animals. B,E) Animals fed a 0.2% CHO supplemented diet. C,F) Animals fed a 0.2% CAD supplemented diet. A, B and C x47,500; D, E and F x66,500

however, the cells showed striking alterations and, in many cases, the endothelium appeared almost destroyed. In a previous work we also observed clusters of erythrocytes adhering to the intima, although in the areas where the endothelium remained, the cell turnover was not altered (Fornas et al., 1987). To study the endothelial cell kinetics, we developed a new Hautchen preparation method (Fornas and Fortea, 1987) with a very weak staining to facilitate the counting of the nuclei labelled with

Fig. 3. Ferritin parameters in the EV in the groups fed for 5 weeks a 0.2% CHO-supplemented diet and standard chow. Tracer experiments with CF. Parameters: density of particles XCF (μm^{-2}); DCCF; and DCF (μm). Standard deviations are indicated by the brackets.

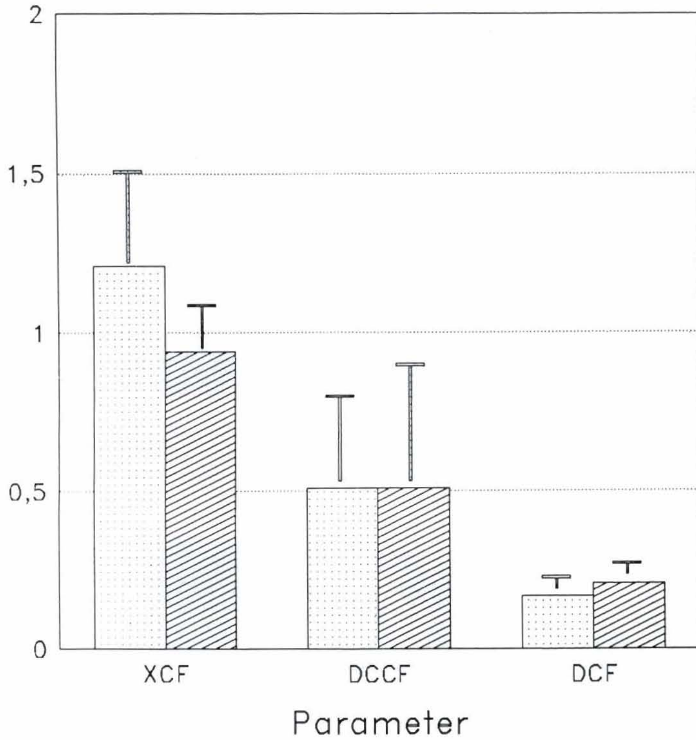
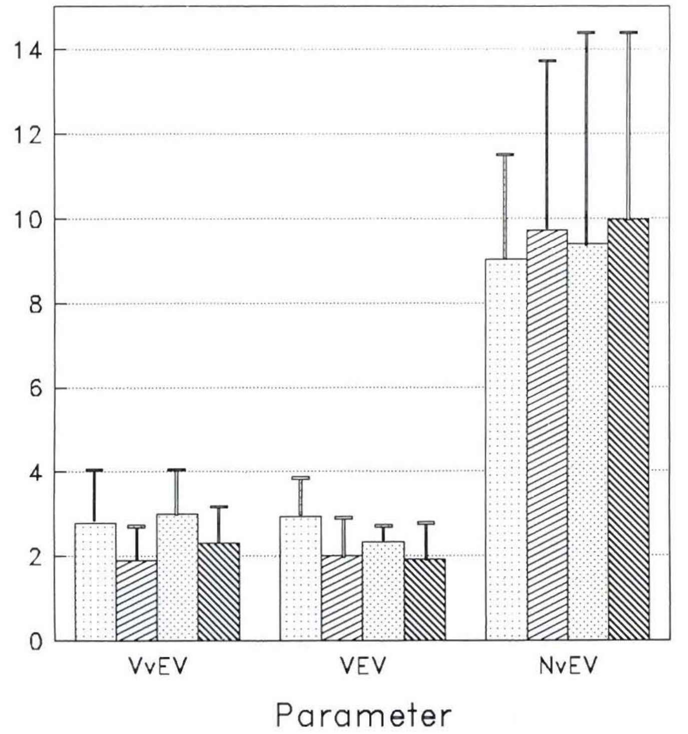


Fig. 4. Stereological data (mean ± SD) of EV in the groups fed a 0.2% CHO-supplemented diet and a standard chow for 5 weeks. Tracer experiments with CF. On the Y these parameters are represented: volume density VvEV × 10; absolute volume VEV × 10^4 (μm^3); and numerical density NvEV × 10^{-2} (μm^{-3}). Standard deviations are indicated by the brackets.



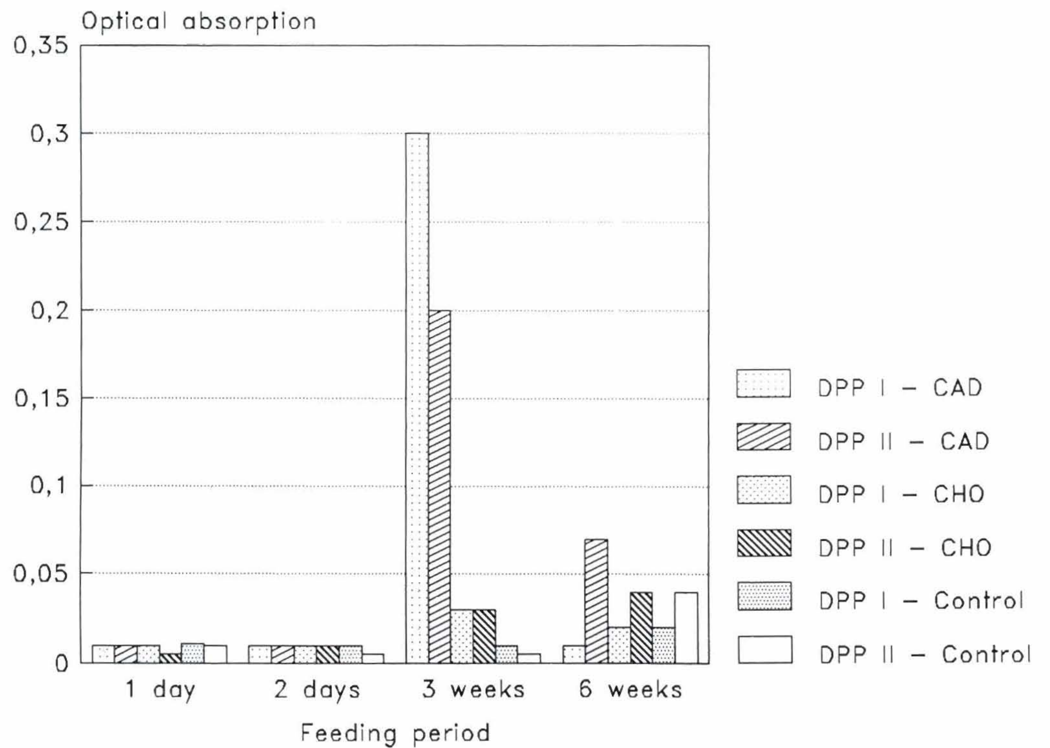


Fig. 5. Optical absorptions at 525 nm showing the relative DPP I and DPP II enzymatic activities in aorta homogenates. The rats were fed with the experimental diets supplemented with CHO and CAD for periods ranging from 1 day to 6 weeks.

(^{3}H)thymidine. Photographs from autoradiographies of rat aortic endothelial Hautchen preparations with some labelled nuclei can be seen in Fig. 1. The analysis of EV was not, therefore, possible in the CAD animals and the stereological study was restricted to studying the effect of CHO-supplemented diets on the EV compartment.

In both control and CHO endothelial cells, tracer experiments showed that, after 60 min of incubation, CF particles were concentrated in or close to nascent EV and in clear vacuoles, propably endosomes. Neither the cisternae of the Golgi apparatus nor the tubules and vesicles of the GERL contained CF. However, tracer particles were also absent in lysosomes and multivesicular bodies. Interestingly, two different patterns of particle distribution in EV were observed. In some cases, the CF particles appeared to be distributed at random, whereas in others they were clearly ordered (Fig. 2).

When the DCF on the plasma membrane was determined, it was found that CHO-treated animals showed a decrease in this parameter, as compared with controls (Fig. 3). This reduction was about 20%. Assuming that the XCF in this layer was similar in both cell groups, the results indicate a disminution in the number of CF particles bound to the plasma membrane of treated cells.

Stereological results are summarized in Fig. 4. Analysis of these data indicated significant differences between EV of both cell populations. Thus, whereas the VvEV and the VEV of EV were greater in both the basal and apical areas of CHO animals than in controls, no differences in the relative number of EV were found. Moreover, these differences were more apparent in the basal area (Fig. 4). Interestingly, the relative number of CF particles endocyted was also increased in treated animals as compared with controls, indicating that this treatment increases non-specific endocytosis.

Nevertheless, the DCCF of the CF inside the EV showed the same low value in both experimental groups, indicating a regular distribution of the CF within the vesicles of both groups of animals.

As shown in Fig. 5, the DPP I and DPP II enzymatic activities of the aorta homogenates were much higher in the animals fed a CAD-supplemented diet than in the other groups. Furthermore it seems that the enzymatic activities in both enzymes tend to their normal values when the feeding period is extended to 6 weeks. On the other hand, no differences were found between the activities of the enzymes measured in the animals fed the CHO-supplemented diet and the ones fed the standard diet.

Discussion

The 2% CHO-supplemented diet has been the most commonly used to induce atherosclerosis in experimental animals (Howard, 1974). However CHO is easily autooxidized in air, and the amount of CAD produced in these conditions and, therefore, present in the CHO diets, has not been controlled. We have found that about 10% CHO may be converted into CAD in a few days (data not shown). Because the CHO-induced toxicity could be due to CAD rather than CHO, in the present work we have used a 0.2% CAD-supplemented diet. This amount of CAD would correspond to that present in 2% CHO-supplemented diets, after air autooxidation of CHO. To compare the toxic effect of CAD with that of CHO, a 0.2% CHO-supplemented diet was prepared immediately before use. In these conditions the CAD diet induced striking alterations in endothelial cells indicating that this concentration was very toxic to these cells. These alterations could be correlated with an increase in the activities of both DPP I and DPP II, which showed a maximum in the third week of experimental diet in both enzymes, probably as a trigger mechanism to produce the regression of the atherosclerosis. This mechanism could include degradation of the proteins in the atherosclerotic lesions and therefore elimination of the materials resulting from cell destruction (Lojda, 1985). The proteolitic activities measured decreased almost to the normal values in the sixth week, being observed at the same time that some aortic endothelial areas were being regenerated (Fornas et al., 1987).

In rats fed with a CHO diet, there was a decrease in the CF surface binding. This agrees with previous studies that used this and other tracers, and reported a progressive decrease of the number of surface anionic sites correlating with the concentration of CHO used (Pino, 1989). However, we have found that CHO increases the endocytosis of CF, as demonstrated by an increase of the VEV as well as the XCF. However, CHO treatment does not increase the NvEV. These results are in contrast with previous ones showing that CHO induces a decrease in endocytosis of membrane immunoglobulin in lymphoid cells (Elguindi, 1985). These discrepancies could be due to the notable differences in the location and chemical composition of cell surface moities and in permeability properties not only between different cell types but also between morphologically similar endothelia. The decrease in the density of CF over the surface of treated cells could be due to the CHO-induced changes in the physiochemical properties of plasma membrane glycoproteins, including their electric charge. The CHO-induced increase of CF endocytosis suggests a mechanism to eliminate plasmatic molecules through the vacuolar system of the cell. However, we have not found an increase in the activity of DPP enzymes or in the elements of this system; i.e. lysosomes and multivesicular bodies. Therefore it appears that this increase could be due to a mechanism to compesate for the lost of cell surface anionic cells including transport of blood molecules (Gil, 1983; Schneeberger, 1983). Another question which remains open to discussion is whether the effects described in CHO-treated rats are due to

this toxin or correspond to the CAD present in this diet. Studies to clarify this point are in progress.

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