Institut de Chimie des Substances Naturelles CNRS
Gif-sur-Yvette 91190
France
L. SZABADOS
M. MESTER
L. MESTER*

K. G. Medical College
Department of Pharmacology
Lucknow 226 003
India
K. P. BHARGAVA

University Paris XI
Laboratory of Neurophysiology
Orsay 91405
France
S. PARVEZ
H. PARVEZ

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* To whom enquiries should be directed.

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Effect of cycloheximide on the incorporation of [^{14}C]glucosamine into UDP-*N*-acetylglucosamine, cell free and protein-bound *N*-acetylneuraminic acid, and plasma membrane glycoproteins of chicken liver

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There are extensive reports dealing with the effect of cycloheximide on various macromolecular biosyntheses. A transient inhibition of protein synthesis in rat liver, followed by a recovery period was established with non-lethal doses of cycloheximide [1–4]. This antibiotic was used for investigating the secretory pathway of rat serum glycoproteins, and the localization of newly formed proteins within the endoplasmic reticulum [5]. The effect of the inhibitor was followed by glycosphingolipid biosynthesis in phytohemagglutinin stimulated human lymphocytes [6].

There is no information whether the glycosylation of nascent polypeptides is coupled with a continuous protein synthesis and whether the blocking in protein synthesis causes some alterations in the content and biosynthesis of nucleotide sugars, the direct carbohydrate donors in the glycoprotein, and glycolipid formation.

The present study was undertaken in an effort to investigate the effect of cycloheximide (CHI) on the incorporation rate of [^{14}C]glucosamine into UDP-*N*-acetylglucosamine, cell free *N*-acetylneuraminic acid (*N*-acetylneuraminic acid + CMP-*N*-acetylneuraminic acid), and protein-bound *N*-acetylneuraminic acid, into plasma membrane glycoproteins from chicken liver.

All studies were performed using 50–70 g white leghorn chickens. Cycloheximide at a dose of 2 mg/kg body wt [7] was administered intraperitoneally 30 min before the labelling *in vivo* (3 μCi/100 g body wt) with [^{14}C]glucosamine (sp. act. 3.4 mCi/mmole; Radiochemical Centre, Amersham, UK) or with [^{14}C]leucine (sp. act. 300 mCi/mmole; Radiochemical Centre) in a dose of 20 μCi/100 g body wt. The animals were killed by decapitation 2 hr after the isotope injection. This time point was chosen because our preliminary experiments with [^{14}C]leucine [8] have demonstrated that at the given dose CHI inhibits the incorporation of the label into chicken liver plasma membrane proteins more than 90%. In the present study the radioactive proteins were prepared for counting by earlier methods [9]. The removal of livers, the isolation of plasma membranes and of radiochemically pure UDP-*N*-acetylglucosamine, were described previously [10]. The separation and purification of cell free and protein-bound *N*-acetylneuraminic acid (NANA) was achieved according to the procedure described elsewhere [11]. For measuring of the radioactivity associated with the acid-insoluble plasma membrane fraction (plasma membrane glycoproteins) the procedure [12] was applied. The membrane preparations were treated with 20% (w/v) trichloracetic acid (5 ml for 3–4 mg membrane protein). After centrifugation, the sediment was washed with 10% trichloracetic acid, once with methanol/ether/chloroform (1:1:2 by vol.), twice with 95% (v/v) ethanol and dried overnight at 37°. Five milligrams of the dried sample was dissolved in 0.2 ml of NCS solubilizer at 50°, then 10 ml of 0.6% 2,5-diphenyloxazol in toluene was added. To the samples of UDP-*N*-acetylglucosamine, cell free and protein-bound NANA, 5 ml of a mixture containing 1 vol. of Triton X-100 and 2 vol. of toluene PPO/dimethyl POPOP phosphor were added. The radioactivity was determined in a Packard Tricarb 3330 scintilation spectrometer.

Analysis of the effect of CHI on the incorporation of

Table 1. Content and specific activity of liver UDP-*N*-acetylglucosamine of normal and cycloheximide treated chickens

Animals	Content (μmole/g body wt)	Specific activity (cpm/μmole)	Total incorporation (cpm/g tissue)
Control	0.133 ± 0.017	21,430 ± 1870	2850 ± 163
Treated	0.418 ± 0.038	271,781 ± 20,200	113,604 ± 1029

Each value is the average of 8 experiments. The data are presented as mean ± S.E.M.

Table 2. Effect of cycloheximide on the incorporation of [^{14}C]glucosamine into cell free and protein-bound *N*-acetylneuraminic acid and into plasma membrane glycoproteins

Animals	Free *N*-acetylneuraminic acid (cpm/nmole)	Protein-bound *N*-acetylneuraminic acid (cpm/nmole)	Plasma membrane glycoproteins (cpm/mg protein)
Control	481 ± 143	351 ± 50	182 ± 19
Treated	4862 ± 460	189 ± 27	46 ± 6

Each value is the average of 6 experimental animals. The data are given as mean ± S.E.M.

[^{14}C]leucine into total cell proteins showed a 94% inhibition. The treatment of chickens with this inhibitor provokes an increase in the content and specific activity of liver UDP-*N*-acetylglucosamine (Table 1). Since the concentration of UDP-*N*-acetylglucosamine is considerably increased in the CHI treated animals, the incorporation rate of the labelled precursor was expressed also as a total incorporation. It is seen from the table that the total incorporation of [^{14}C]glucosamine into the nucleotide-sugar under investigation is also higher, compared to that from normal liver.

The data presented in Table 2 show the influence of the drug on the incorporation of [^{14}C]glucosamine into cell free and protein-bound NANA, and into plasma membrane glycoproteins. It is apparent that the specific activity of free NANA is increased nearly ten times, while that of protein-bound NANA and that of the plasma membrane glycoproteins is significantly decreased.

The liver is the major biosynthetic site of many secretory and structural glycoproteins. [^{14}C]Glucosamine has proved to be a useful label in the study of glycoprotein synthesis by liver because its metabolism is well known [13]. As shown, it is efficiently incorporated into protein-bound glucosamine and NANA [14].

Glycosylation is a post-translational process occurring once the acceptor polypeptide chain has been produced and requiring glycosyltransferases and nucleotide sugars as carbohydrate donors.

According to recent data [14, 15] glucosamine occupies two different positions in the carbohydrate chain of glycoproteins. Some glucosamine is incorporated in the 'core region' of the glycoproteins, while the newly synthesized polypeptides are still attached to the ribosomes. Further glucosamine residues are incorporated as the polypeptides traverse the channels of the endoplasmic reticulum and in the Golgi membrane fraction. Part of the radioactivity incorporated from radioactive glucosamine is present as NANA, which becomes attached predominantly in the Golgi apparatus, as a terminal residue of the carbohydrate chain.

From the data presented in this work one can conclude that the effect of CHI is not exclusively to block the protein synthesis. One can point out, that the drug treatment provokes, as a side effect, an elevation in the quantity of UDP-*N*-acetylglucosamine and an increased rate of labelling of this nucleotide and of cell free NANA. The inhibited protein synthesis leads to both an impairment of the plasma membrane protein glycosylation and a blockage of the sialylation of liver cell proteins. The lower rate of [^{14}C]glucosamine incorporation into protein-bound NANA and into plasma membrane glycoproteins is not due to a lack of the corresponding nucleotide sugar donor. The net accumulation of UDP-*N*-acetylglucosamine under the conditions of arrested protein synthesis is consistent with the suggestion that its utilization is considerably restricted.

It has been shown recently [16], that an inhibition of protein synthesis in canine kidney cells inhibits the formation of lipid-linked oligosaccharides, which are the immediate precursors in the synthesis of the 'core region' of glycoproteins. This finding supports the suggestion of decreased utilization of UDP-*N*-acetylglucosamine as a consequence of protein synthesis inhibition.

It is possible the effect of CHI on the glycosylation is caused either by the lack of available polypeptide acceptors, or by an inhibition of the carbohydrate residue transfer from nucleotide sugars to lipid or protein acceptors.

Institute of General and Comparative Pathology
Bulgarian Academy of Sciences
Sofia 1113
Bulgaria

H. CHELIBONOVA-LORER
S. IVANOV
E. GAVAZOVA

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Potentiating effects of clofibrate on prostaglandin-dependent and -independent pathways of human platelet activation: evidence for involvement of cyclic AMP

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Plasma lipids and blood platelets both play major roles in atherogenesis. A relationship between elevated plasma lipids and an increased platelet sensitivity to aggregatory agents in hyperlipidemic patients has been reported [1]. It has been shown that clofibrate, which lowers plasma lipids, also decreases the sensitivity of platelets to ADP, collagen and epinephrine in Type II_B hyperlipoproteinemic patients [1]. These dual hypolipidemic and antithrombotic actions make clofibrate a potentially effective and unique drug for use in the treatment of thromboembolic and coronary artery diseases.

Biochemical mechanisms of the hypolipidemic action of clofibrate have been linked to an inhibition of adenylate cyclase activity and a lowering of cAMP levels in various tissues [2, 3]. However, the antiaggregatory action of clofibrate appears to be unrelated to this biochemical effect since cAMP lowering in platelets is associated with an induction of aggregation, and not with an inhibition of platelet function [4, 5]. In earlier reports, we have examined the antiaggregatory effects of clofibrate on human platelets and have shown that this drug inhibits aggregation, secretion of serotonin and β-glucuronidase, platelet factor 3 activity, incorporation of [^{14}C]acetate into platelet phospholipids, release of arachidonic acid from platelet phospholipids, and prostaglandin biosynthesis [6–8]. Our recent findings suggest that clofibrate inhibits platelet activation by blocking prostaglandin biosynthesis in platelets [8].

By contrast, we have observed that the aggregation response to arachidonic acid in human platelets is potentiated in the presence of clofibrate [6, 8]. In another report, we have shown that phospholipase C (PLC) causes platelet aggregation and secretion of serotonin by a mechanism independent of prostaglandin biosynthesis and that clofibrate also enhances platelet activation by PLC [9]. These studies show that clofibrate exhibits divergent antiaggregatory and proaggregatory effects on human platelet function. Whereas the antiaggregatory action of clofibrate may be related to the inhibition of prostaglandin biosynthesis [8], the stimulatory effect of this drug remains to be explained. An elucidation of the mechanism of proaggregatory action by clofibrate may be helpful in assessing the overall effect of this drug on platelet function, *in vivo*.

These studies were initiated to better understand the mechanism of this proaggregatory action of clofibrate on arachidonic acid- and PLC-induced pathways of platelet activation. The latter pathway, using low concentrations of PLC as an inducer of platelet aggregation and serotonin secretion, is independent of prostaglandin biosynthesis [10, 11]. The concentration-dependent actions of clofibrate were examined on (a) arachidonic acid-induced aggregation and oxygen consumption in washed platelets, (b) platelet aggregation and secretion of [^{14}C]serotonin ([^{14}C]-5HT) by PLC, and (c) PGE_1-elevated cAMP levels in platelets. The data in this report suggest that clofibrate achieves its stimulatory effect on platelet function by lowering cAMP levels.

Methods and reagents used for collection of human blood, preparation of platelet-rich plasma, washed platelets, and platelet aggregation and secretion of serotonin ([^{14}C]-5HT) are identical to those published recently in this journal [8]. The sodium salt of clofibrate [2-(4′-chlorophenoxy)-2-methylpropionic acid] was provided by Ayerst Laboratories, Inc., New York, NY. Phospholipase C from *Clostridium perfringens* and arachidonic acid were obtained from the Sigma Chemical Co. (St. Louis, MO).

Oxygen consumption by platelets was measured by the method of Pickett and Cohen [12]. Incubations were done in a reaction vessel fitted with a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH), in a total volume of 3 ml. The platelet count was adjusted to 5×10^8/ml. Arachidonic acid (final concentration, 200 μM) was added in a volume of 20 μl.

Platelet cyclic AMP levels were estimated by the radioimmunoassay of Brooker *et al.* [13]. At the end of the incubation of platelets with various agonists and antagonists, 1/10 volume of trichloroacetic acid (100%) was added to lyse platelets [14], and samples were centrifuged at 12,000 *g* for 1 min. Supernatant fractions (1 ml) were extracted three times with 3 ml of water-saturated diethyl ether. After evaporation of the ether phase, 100 μl of 1 M acetate buffer was added to bring the pH to 6.2. Aliquots (100 μl) of samples and standard solutions of cAMP (0.312 to 10,000 fmoles) were acetylated by mixing with 10 μl of triethylamine–acetic anhydride (5 : 2, v/v). All samples were incubated for 16 hr at 4° after addition of antiserum for cAMP and of [^{125}I]cAMP-tyrosine methyl ester containing bovine γ-globulin. After 16 hr, 2 ml of 60% ammonium sulfate was added to each sample, which was then centrifuged at 1500 *g* for 15 min at 4°. The supernatant fractions from sample tubes were discarded, and the tubes were counted in a Beckman model 8000 gamma scintillation counter (Irvine, CA).