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Comparison of bipyridyl, maltol and kojic acid action as organic vanadium ligands on activity of galactosyltransferase (EC 2.4.1.38), some physiological parameters and ultrastructure of Golgi complexes in rat hepatocytes

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Abstract: The biochemical activity and morphology of control and streptozotocin-diabetic rat liver Golgi complexes were previously investigated by us under influence of some vanadium [V(IV)] compounds. The effectiveness of these derivatives depends on the kind of complexing ligands. This paper presents the investigation of the effect of bipyridyl, the ligand of a new vanadium compound, tested by us with maltol and kojic acid (two ligands studied by the present and other authors). The three ligands alone action was tested under the same experimental conditions as in the case of whole compounds with vanadium and applied to liver Golgi complexes of control rats. A preliminary study for maltol and kojic acid had been previously carried out by us parallel with tests of whole vanadium complexes, but valuable differences in biological action found in our condition of experiments suggested the extension of studies to include the two above-mentioned ligands and to compare the effects of the three investigated ligands. The supplementary part of the experiment focused mainly on the ultrastructure of Golgi complexes in hepatocytes. Four groups of animals were used: C - control rats, C + M (maltol), C + $(ka)_2$ (kojic acid) and C + $(bpy)_2$ (bipyridyl). The control rats received 0.09M NaCl as drinking liquid; all the other animals were given 3.6 mmol/L of appropriate ligand solution in 0.09M NaCl during 7 days. All the animals survived the experiments. Only in group C + $(bpy)_2$ did the authors observe statistically significant differences as compared with the controls (group C). The differences were detected in physiological studies and manifested as body weight decreased by approximately 20% during the experiment, lower liquid ($p<0.001$) and food ($p<0.01$) intake and increase of free blood sugar level ($p<0.01$). The yield of Golgi membrane isolation decreased in this group ($p<0.01$). The main investigated biochemical parameter, *i.e.* the activity of liver Golgi marker enzyme - galactosyltransferase - was not statistically significantly changed in comparison with the controls in all the investigated groups of rats; a similar dispersion of individual results were found in the four groups. In the three experimental groups, ultrastructural observations demonstrated a predominance of cylindrical Golgi structures, which were haphazardly twisted in the majority of cases. Typically shaped structures were encountered sporadically. The ligands alone evoked numerous subcellular changes in hepatocytes; these alterations most frequently involved the mitochondria and endoplasmic reticulum. No such changes had been seen, or else they had been less advanced when complex vanadium compounds were employed in our earlier experiments. As it follows, the ligands alone were demonstrated to be much more toxic to morphology of control liver Golgi apparatus as compared to complex compounds, which showed the ability of the former to normalize Golgi complexes of diabetic animals.

Key words: Golgi complex - Maltol - Bipyridyl - Kojic acid - Pathomorphology

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Introduction

In the previous study of experimental diabetes induced in rats by single intraperitoneal injections of streptozotocin (STZ), we found characteristic changes of the biochemical activities and morphology of rat liver Golgi complexes [1-3]. These changes were so characteristic for the model of this disease, that we used them in subsequent investigations as a convenient test of cytoprotective [4,5] or normalizing [6-9] properties of various drugs tested. Within the past years, we were especially interested in the effect of bis(oxalate)oxovanadium(IV) [8,9], bis(maltolato)oxovanadium(IV) [BMOV] [6,10], bis(kojato)oxovanadium(IV) [$VO(ka)_2$] [7,11] and bis(2,2'-bipyridine)oxovanadium(IV) [$VO(bpy)_2$] [12,13] as anti-diabetic drugs. The best properties in normalizing diabetic symptoms in our model diabetes were shown by BMOV [6,10]. This result was in agreement with data provided by other authors, who carried out various physiological and biochemical studies under experimental conditions other than those employed by us and found the effect of BMOV to be superior to that of vanadium kojate or vanadyl sulfate [14,15]. Two vanadium(IV) complexes: bis(oxalate)- and bis(2,2'bipyridine)oxovanadium(IV) constituted new, hitherto not tested vanadium complexes; however, in our experiments, they were also proven to be less effective than BMOV. In diabetic animals, they caused an "improving" effect only in the morphology of Golgi complexes structure. Since along with diabetic groups, parallel investigations of control rats were always carried out under identical experimental conditions (the same batch of rats, the same time and the same concentrations of vanadium compound solution), we found that the three investigated vanadium(IV) complexes acted in a different way on liver Golgi apparatus in healthy animals. The fact prompted an investigation of the influence of three ligands (which we previously used to obtain whole complexes with vanadium) acting alone under our experimental conditions. This paper presents the results obtained by application of bipyridyl [$(bpy)_2$] and compares them with preliminary result of maltol (M) [6] or kojic acid [$(ka)_2$] [11] acting alone, the latter being employed in control rat liver Golgi apparatus over the same time and at the same concentrations as in the case of the whole complex with vanadium(IV).

Materials and Methods

Animals. Four groups of approximately 6-month old of female inbred Wistar rats, weighing 210-240 g were used in experiments by permission of the Cracow Ethics Commission for Animal Experiments. The rats were fed with pelleted food and tap water prior to experiments. During experiments, the animals received 0.09 M NaCl or one of the three ligand solutions dissolved in 0.09 M NaCl (because in complexes with vanadium the solution was previously used to diminish their toxicity) as described below. Concentrations of the ligands were the same as in complexes with vanadium. The solutions were freshly prepared every two days.

Group C - control animals, receiving 0.09 M NaCl as drinking solutions for 7 days. This group consisted of all the control rats, investigated in subgroups of 5 to 7 animals parallel with the rats from the other three experimental groups; for this reason, the number of animals was higher than in the other groups (20 rats).

Group [C+M] - The rats received 3.6 mmol/L maltol solution in 0.09 M NaCl as drinking liquid for 7 days (9 rats).

Group [C+ $(bpy)_2$] - The rats received 3.6 mmol/L bipyridyl solution in 0.09 M NaCl as drinking solution for 7 days (11 rats).

Group [C+ $(ka)_2$] - The rats treated with 3.6 mmol/L kojic acid in 0.09 M NaCl as drinking solution for 7 days (9 rats).

All the animals survived the experiments. The rats were not fasted before sacrificing to eliminate factors other than ligands that might have influenced the animals. The rats were weighed each day, and the amounts of drinking liquid and food consumed by the animals were measured. On the last day of the experiment, liver samples for morphological analysis were taken under general anesthesia, and the livers immediately used for isolation of Golgi membranes followed by estimation of GalT activity [16]. Morbital (pentobarbitalum) administered intraperitoneally at the dose of 1 ml/100 g of body weight was used as an anesthetic drug. The blood was collected from the hepatic vein with a heparinized syringe.

Analytical methods. The free blood sugar level was estimated according to Somogyi and Nelson [17]. Protein was estimated by the method of Lowry *et al.* [18] with crystalline serum bovine albumin as the standard. The Golgi-rich membrane fraction was isolated and galactosyltransferase (GalT) activity estimated according to the Fleischer method [16].

Ultrastructural examination. For electron microscopy, two to four biopsy specimens from each group were fixed in formaldehyde-glutaraldehyde fixative overnight at 4°C with the method of Karnovsky [19]. The tissue was subsequently postfixed in 1% osmium tetroxide. After dehydration in graded concentrations of ethyl alcohol and propylene oxide, the tissue was embedded in Spurr medium. Samples were sectioned with an ultramicrotome Reichert Ultracut S using a diamond knife. Semi-thin sections were stained with methylene blue and ultra-thin sections, with 8% uranyl acetate dissolved in 50% methanol and then in lead citrate according to Venable and Coggeshal [20]. All studies were performed under an electron microscope (Zeiss EM 900) operating at 80kV. Electronograms had primary magnification of 20 000×.

Statistical analysis. All the results expressed as mean ± SD were tested for statistical significance by Student's t-test. Statistically significant values ($p<0.05$) are marked below the Table 1 or in the text.

Reagents. Sodium cacodylate, serum bovine albumin, TRIS and β-mercaptoethanol came from Koch-Light Lab., UDP-Gal, Triton X-100, streptozotocin, maltol, bipyridine, kojic acid and Dowex 2x8 (200-400 mesh) were obtained from Sigma Chemical Co., ^{14}C UDP-Gal with specific activity 292 mCi/mmol was obtained from Radiochemical Centre Amersham, morbital (pentobarbitalum) came from Biowet (Puławy, Poland). All other reagents were purchased in analytical grades from Polish Chemical Reagents POChem Gliwice. For electron microscopy, uranyl acetate and lead citrate were obtained from Sigma Chemical Co., Spurr epoxy resin from Pelco Co., and formaldehyde, glutaraldehyde and sodium tetroxide were purchased from Polysciences Inc.

Table 1. Some physiological and biochemical characteristics of investigated rats and isolated rat liver Golgi-rich membrane preparations.

Characteristics	Group of rats (number of animals)			
	C (n=20)	C+M (n=9)	C+$(bpy)_2$ (n=11)	C+$(ka)_2$ (n=9)
Final body weight [g]	234.5 ± 13.4	213.5 ± 18.0	217.9 ± 21.0[a]	233.9 ± 15.1
Change of body weight [%]	↑ 6,3%	0%	↓ 20.3%	↑ 3.6%
Liquid intake [ml/rat/day]	24.1 ± 2.9	19.8 ± 2.7	10.8 ± 4.9[b]	23.1 ± 4.2
Food intake [g/rat/day]	19.2 ± 1.7	19.2 ± 2.5	15.8 ± 2.5[c]	18.6 ± 1.4
Weight of liver [g]	8.2 ± 1.0	7.3 ± 0.8	8.3 ± 1.5	7.9 ± 1.3
Free blood sugar level [mg/100 ml]	115 ± 21	94 ± 20	202 ± 74[d]	117 ±46
Yield of Golgi membrane isolation [mg protein/g of liver]	0.600 ± 0.235	0.564 ± 0.294	0.367 ±0.102[e]	0.581 ± 0.228
Specific activity of GalT [nmoles of Gal transferred/h, mg of protein]	137.2 ± 87.3	184.5 ± 114.5	196.2 ± 82.6	111.4 ± 86.8
Total activity of GalT [nmoles of Gal transferred/h, whole liver]	557.8 ± 225.2	685.0 ± 326.4	619.6 ± 300.7	437.0 ± 258.0

n = number of animals, GalT = galactosyltransferase,
The data are represented as the mean value ± SD. In comparison with group C, the t and p values were: [a] t=2.141, 0.02<p<0.05, [b] t=7.001, p<0.001, t=3.645, [c] 0.001<p<0.01, [d] t=3.571, 0.001<p<0.01, [e] t=3.094, 0.001<p<0.01,
Dispersion of individual results of activity of GalT calculated as nmoles Gal transferred per h and per g of liver is presented in Fig 1.

Results

Table 1 summarizes some physiological and biochemical parameters in the four investigated groups of rats. In the C+M group, the biochemical results similar to control values were obtained. Total galactosyltransferase (GalT) activity in this group expressed in nmoles Gal transferred per h and per whole liver (which express the liver glycosylation potency) was the highest among all the investigated groups (Table 1), but statistically non-significant, similarly as the enzyme activity calculated per 1 g of liver (Fig 1). This result was in agreement with our preliminary morphological study [6], which showed some stimulation of secretory activity.

The treatment of the control rats with bipyridyl [group C+$(bpy)_2$] caused the greatest alterations of the investigated parameters both in physiological and biochemical studies (except GalT activity), especially pronounced in an approximately 20% decrease in body weight and statistically significant diminished liquid ($p<0.001$) and food ($p<0.01$) intake during the experiment. In this group, the free blood sugar level was ele-

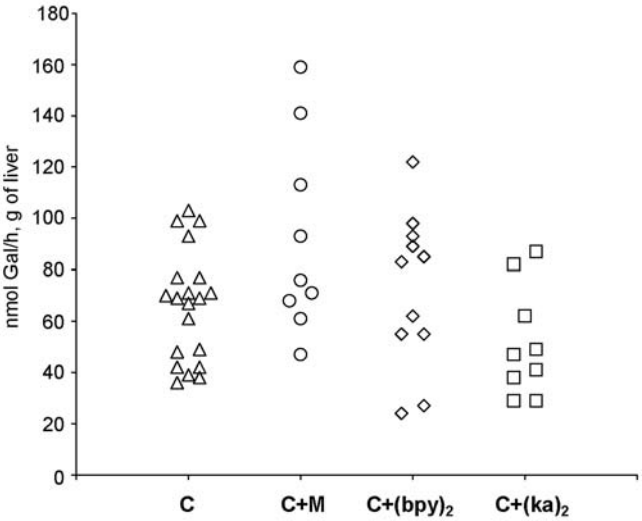

Fig. 1. Dispersion of results of galactosyltransferase activity (expressed as nmoles Gal transferred per 1 hour and per g of liver). In all the groups, values and dispersion of results were similar to the controls. The results of specific and total activities of this enzyme are given in Table 1.

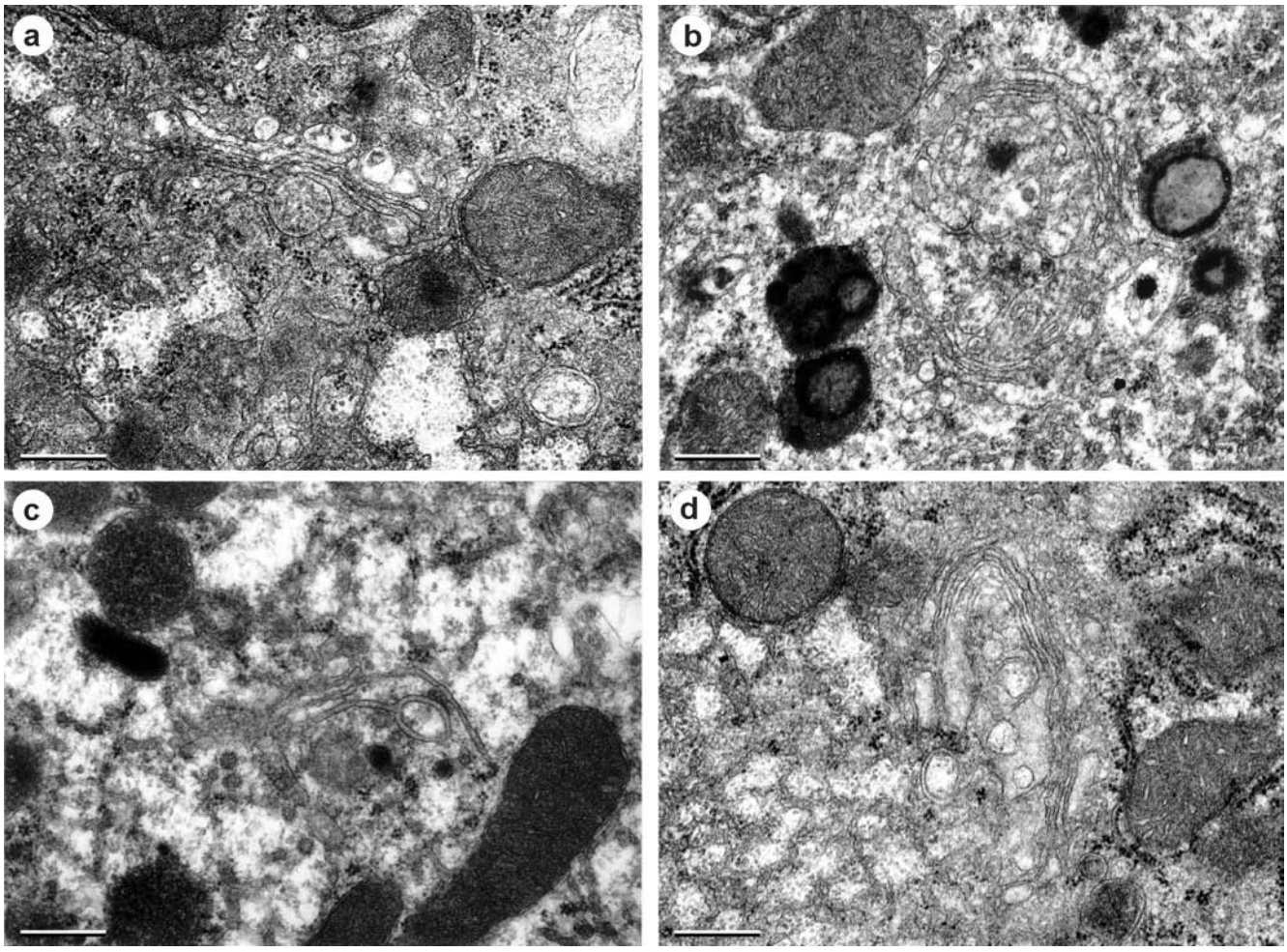

Fig. 2. The region of Golgi complexes in the rat hepatocytes from: **a**. Control rats. On the trans side - close to Golgi stack - a large vacuole is visible. The bar represents 0.5μm (magnification ×20 000). **b**. Maltol treated rats. A large, round, irregularly shaped Golgi complex is surrounded by lysosome-like structures. The bar represents 0.5μm (magnification ×20 000). **c**. Kojic acid treated rats. A small sized, irregularly shaped Golgi complex. In the vicinity, small coated and dense-core vesicles are seen. The bar represents 0.5μm (magnification ×20 000). **d**. Bipyridyl treated rats. Aggregates of electron-lucent vacuoles surrounded by narrow cisterns of round Golgi apparatus. The bar represents 0.5μm (magnification ×20 000).

vated as compared to the controls ($p<0.01$), what had never been observed in either of the investigated groups treated with a whole vanadium with ligands compound [6-13,21,22] or ligands alone. The biochemical study showed that the yield of isolation of Golgi-rich membrane fraction was the lowest in group C+$(bpy)_2$, and - in comparison to group C - statistically significant ($p<0.01$). The changes in the activity of the Golgi marker enzyme, *i.e.* galactosyltransferase (GalT), were statistically non-significant in all the experimental groups as compared to the controls, irrespectively of the employed calculation methods: as specific (expressed as nmoles Gal transferred per h and per mg of protein) or total activity (in nmoles Gal transferred per h and total liver) Table 1. The dispersion of results in individual rats (in nmoles Gal transferred per h and per g of liver) was similar in the four investigated groups as presented in Fig. 1.

The animals treated with kojic acid [group C+$(ka)_2$] did not show alterations in physiological and biochemical parameters, the mean value of total GalT activity was the lowest of the four investigated groups, but the difference was statistically non-significant.

Figures 2a, b, c, d present electron microscopic examinations of Golgi complex regions in rat hepatocytes. Fig. 2 a shows a control rat liver, untreated with drugs. The morphological investigation carried out in the maltol-treated group showed considerable ultrastructural abnormalities of Golgi complexes. In the majority of cases, the cisternae were irregularly twisted, leading to chaotic images seen in the cross-sections (Fig. 2b). In the group of rats that received kojic acid, the hepatocytes demonstrated the presence of small Golgi structures with irregularly bent, narrow, bifurcated cisternae. In the vicinity of such structures, small, coated vesicles were seen, along with dense-

core type vesicles (Fig. 2c). This group showed the lowest prevalence of vacuoles enclosed in a double membrane. In the bipyridyl group, electron microscopy showed changes of rat liver Golgi structures. The predominating type of Golgi complexes was markedly arched or oval in shape (Fig. 2d).

Discussion

Vanadium shows various biological, pharmacological and therapeutic activities, such as insulin-mimetic, cytotoxic, genotoxic, anti- or pro-carcinogenic; it affects the activity of many enzymes, lowers cholesterol, triglycerides and glucose levels, exerts diuretic and natriuretic effects, as well as enhances oxygen-affinity of hemoglobin and myoglobin [23-26]. In addition to such variables as the strain of experimental animals or cells, as well as the method of application and duration of experiments, the kind of ligands used to obtain appropriate vanadium complexes is of a great importance, because the efficacy and toxic side effects of such complexes depend on the ligands themselves.

Thompson and Orvig [27] review the most important organic vanadium ligands that have been already employed in various investigations as potential "anti-diabetic agents". The key problem here is the incorporation of results obtained while investigating animal models to therapeutic management of diabetes in humans. Inasmuch as the physico-chemical properties of these compounds and their pharmacology and toxicity are fairly well known, studies on the effect of ligands as such on physiological, biochemical and pharmacological parameters investigated by various authors in various models are relatively obscure. This is particularly true in the case of experiments carried out at the subcellular level. In our model, we investigated the behavior of Golgi complexes, an organelle that is responsible for protein and lipid glycosylation (the metabolism of which is altered in diabetes) in hepatocytes. Some authors [15,27-29] emphasize the fact that in the case of organic vanadium derivatives, a decisive factor that determines the effectiveness of the activity may be the ligand itself and this is why they advocate the employment of non-toxic ligands, with maltol or ethyl maltol considered prime examples.

The anti-hyperglycemic effect of various cations, such as vanadium, tungsten, molybdenum, cobalt, zinc, chromium or copper, on some metabolic alterations in diabetes were described [30,31]. The effectiveness of these complexes as anti-diabetic drugs depends, rather than on the kind of cations, on ligands used in these cations chelating [14,15,24]. In our experimental model, *i.e.* the study of the rat liver Golgi complexes as an indicator of alterations caused by STZ-diabetes, we found diversified effects of V(IV) and V(V) vanadium complexes on the control and STZ-diabetic rats [4-7,9-15,21,22]. These effects depended on the kind of ligands used, such well known [2-4,7,8,13-16,28,31], as in new vanadium derivatives [8,9,12,13]. We decided to investigate the effect of three organic ligands alone on biochemical activity and morphology of Golgi complexes in control rat livers. The ligands were used in the same concentration and under the same experimental conditions as in the case of experiments with whole vanadium derivatives.

In physiological investigations, during the experiment only bipyridyl did cause a statistically significant decrease of weight ($p<0.05$) and liquid ($p<0.001$) and food ($p<0.01$) intake as compared to the controls. As it follows from our experiments, bipyridyl - contrary to kojic acid and maltol - changes physiological parameters without affecting galactosyltransferase activity. One may thus surmise that the glycosylation potential (total GalT activity) has been preserved.

Morphological investigations showed changes in rat liver Golgi structures. Rats receiving one of the ligands alone, demonstrated intermediate subcellular changes of the Golgi apparatus region. The most astonishing fact was the presence of oval Golgi apparatus cisternae in more than 85% of the cases: triple, double or single narrow cisternae surrounded multiple and large vacuoles, either electron-lucent or filled with flocculent material. In the vicinity, lysosome-like structures and vesicles were seen. It seems that ligand alone was also not as effective as a corresponding complex compound in normalizing biochemical and morphological parameters. In addition, it had cytotoxic properties, leading to damage of other cellular organelles.

In conclusion, it seems that the new compound $VO(bpy)_2$, although it does normalize the investigated selected parameters, in view of its toxicity [12,13] and the effect of bipyridyl itself, will be of a limited use and most likely will not be commonly employed as a "drug" in humans, the more so that -as it has been emphasized by Thompson and Orvig [27] to date, the best compounds include BMOV (maltol derivatives) and BEOV (ethyl maltol compounds), in which the very ligands are non-toxic to man.

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