Physiol. Res. 43: 219–221, 1994

Methylene Blue Inhibition of Oestradiol-Induced Increase of Ceruloplasmin Serum Levels in Rats

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Received August 2, 1993
Accepted January 26, 1994

Summary

The administration of oestrogens increases the hepatic synthesis and plasma level of ceruloplasmin both in man and laboratory animals. Methylene blue, an oxidizing agent and inhibitor of soluble guanylate cyclase, is widely used to block the effects of endothelium-derived relaxing factor (nitric oxide). We describe the inhibitory effect of methylene blue on the increase of ceruloplasmin plasma level in rats during oestradiol treatment.

Key words

Ceruloplasmin – Copper – Oestrogens – Methylene blue

Introduction

The liver is an oestrogen-responsive organ. Both cytosolic and nuclear receptors for oestrogens were discovered in hepatocytes (Clemente *et al.* 1992). The administration of oestrogens both in man and laboratory animals increases the hepatic synthesis of many proteins including ceruloplasmin (Schreiber and Přibyl 1977). The physiological role of this effect is not clear, but some evidence about the participation of ceruloplasmin in angiogenesis may indicate that ceruloplasmin takes part in the endometrial cycle.

From this point of view, the aim of our study was to evaluate the possible part of nitrenergic mechanism involving soluble guanylate cyclase and cyclic guanosine monophosphate in the oestrogen-mediated increase of ceruloplasmin levels in an animal model.

Methylene blue (MB) is an oxidizing agent utilizable as inhibitor of cytosolic guanylate cyclase (Johnstone *et al.* 1993). MB prevents the activation of this enzyme by the endothelium-derived relaxing factor (nitric oxide, NO), calcitonin gene-related peptide, and some other endogenous mediators through generating oxygen radicals. The mediator of this response is the hydroxyl radical (Kontos and Wei 1993, Mayhan 1993). MB has minor effects on prostaglandin synthesis and sympathetic function too. The activation of cytosolic guanylate cyclase (by NO, atrial natriuretic peptide and other mediators) increases the levels of the second messenger cyclic guanosine monophosphate (cGMP) in vascular smooth muscle, leading to vasorelaxation (Johnstone *et al.* 1993).

MB is currently used in medicine for the treatment of cyanide poisoning, methaemoglobinaemia, and nitrite poisoning. The bactericidal effect of MB may be effective in the treatment of stomatitis (Fletcher and Wilson 1993) and urinary tract tuberculosis. MB as an inhibitor of superoxide generation by xantine oxidase (Salaris *et al.* 1991) is a potential new drug for the attenuation of ischaemia/reperfusion injury.

Material and Methods

Forty male rats (Wistar strain descendants, Velaz, Prague) kept at 22±2 °C in a 12 h light/12 h darkness regimen and fed on a standard laboratory diet (Larsen diet, Velaz, Prague) were divided into four groups: (1) controls (C), (2) those who were administered oestradiol benzoate (E) in a microcrystalline suspension 1 mg/rat, twice a week, i.m. (Agofollin depot, SPOFA, Prague), (3) those given 0.5 % methylene blue (MB) (SERVA, Heidelberg), and (4) those receiving both oestradiol and methylene blue (E+MB). After 25 days of treatment the animals were killed by decapitation and the blood was collected in EDTA glass test tubes.

For the estimation of plasma ceruloplasmin levels the enzymatic assay based on its polyphenoloxidase activity (Přibyl 1976) (Přibyl's modification of Ravin's original method - Ravin 1961) was used. Each serum was put into 2 tubes (0.1 ml for "blank" and 0.1 ml for "sample"). Three millilitres of the substrate p-phenylendiamindichloride in an acetate buffer (pH 5.2) were added to both "blanks" and "samples" and incubated at 37 °C. The enzymatic process was blocked by 0.1 ml of NaN3 in "blanks" and after 20 min in the "samples". The absorbance was read at 530 nm. Because the calibration curve constructed from standard human serum without an original rat standard is not representative for the rat model, the concentrations of ceruloplasmin were given in relative units of absorbance.

The copper plasma levels were determined by the LACHEMA^{R} test. After deproteinizing 1 ml of plasma with 1 ml trichloracetic acid (0.6 mol/l) and hydrochloric acid (2 mol/l) and centrifugation at 3000 rpm, the supernatant (1 ml) was added to 1 ml of bathocuproin (sodium 2,9-dimethyl-4,7-diphenyl-1,10-phenanthrolin-3,6-disulphonicum). Absorbance was read at 480 nm. The reagent blank and standard were substracted from 0.9 % NaCl solution, or a standard copper solution (30 μmol/l) undergoing the same procedure as the samples. The calibration curve was linear in the measured range.

Statistical parameters were evaluated by the analysis of variance and multiple F-test.

Table 1
The ceruloplasmin and copper plasma levels after oestradiol and/or methylene blue treatment

Groups	Controls	Oestradiol	Methylene blue	Oestradiol + Methylene blue
No. of rats	10	10	10	10
Ceruloplasmin (relative units)	0.378 ±0.047	1.017 ±0.202	0.546 ±0.052	0.574 ±0.071
Copper (μmol/l)	17.8 ±3.7	24.3 ±3.1	18.2 ±4.1	17.6 ±4.0

Data are means ± S.D.

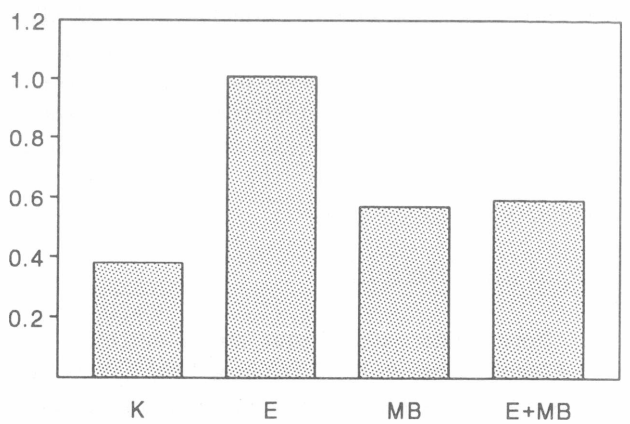

Fig. 1
Ceruloplasmin plasma levels in rats after oestradiol and/or methylene blue treatment.
C = controls, E = oestradiol, MB = methylene blue
The concentrations (mean ± S.D.) are given in relative units of absorbance.

Results

The ceruloplasmin and copper plasma levels are given in Table 1. The differences in ceruloplasmin levels among the groups are shown in Fig. 1. In agreement with other authors (Schreiber and Přibyl 1977), oestradiol treatment (group 2) increased plasmatic levels of ceruloplasmin ($p<0.01$) and copper ($p<0.05$). MB alone (group 3) also increased ceruloplasmin enzymatic activity with a limitation mentioned below, but this increase was less striking ($p<0.05$) than in group 2. The rats treated with the combination of oestradiol and MB (group 4) showed similar results as group 3. There were significant differences between group 1 and group 4 ($p<0.05$) and between group 2 and group 4 in ceruloplasmin plasma levels ($p<0.01$).

The plasma copper levels were significantly higher in group 2 than in all other groups ($p<0.05$), but there were no differences between groups 1, 3, and 4.

We found that the addition of MB in concentrations 10^{-2} to 10^{-5} g/l to rat sera *in vitro* after 1 h incubation at 37 °C increased the absorbance values obtained by the enzymatic method for

ceruloplasmin assessment as compared with the original sera. The difference of absorbances ΔA was 0.09 for the addition of 10^{-4} g/l MB to the sera, higher concentrations of MB did not have a more intensive effect. Although we did not estimate the actual concentrations of MB *in vivo*, we assume that the increase of enzymatic activity of ceruloplasmin in group 3 compared with group 1 is at least partially given by interference of MB with this method.

Discussion

Experimental evidence indicates that MB, inhibitor of cytosolic guanylate cyclase, also acts as a specific direct inhibitor of NO synthase. This direct effect is more significant than inhibition of guanylate cyclase (Mayer *et al.* 1993). It is likely that MB inhibits the potential effect of NO as a neurotransmitter of the nonadrenergic/noncholinergic neurones (Rajfer *et al.* 1992). This NO-guanylate cyclase-cGMP pathway is involved physiologically in mediating vascular smooth muscle relaxation (Braner *et al.* 1993) and pathologically in hypotension accompanying the liver failure (Midgley *et al.* 1991).

The effect of MB was recently explained by an increase of blood thyroxine levels after MB treatment (Schreiber *et al.* 1993). The mechanism of this effect is not clear, yet. The inhibitory effect of MB on the oestradiol-stimulated increase of ceruloplasmin plasma level supports the possibility that a mechanism involving cytoplasmic guanylate cyclase and cGMP participates in the liver response to oestradiol. Recently, we described a similar inhibitory effect of MB to oestradiol action at the pituitary level. MB partially inhibited the growth response of the anterior pituitary to oestradiol (Schreiber *et al.* 1993). The oestradiol treatment increased cGMP content in the pituitary and this mechanism is probably also involved in the liver. The results of Braner *et al.* (1993) suggest that the majority of endogenous cGMP is generated by the release of endothelium-derived relaxing factor. However, the exact mechanism by which MB alters the oestradiol effect in the liver and other tissues remains to be elucidated.

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Reprint Requests

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