

Protective effect of the flavonoid silybin dihemisuccinate on the toxicity of phenylhydrazine on rat liver

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Phenylhydrazine is a hemolytic agent whose mechanism of action is related with the formation of free radicals and the induction of lipid peroxidation. The flavonoid silybin dihemisuccinate is an antihepatotoxic principle used in the treatment of many liver diseases; its mechanism of action has been ascribed to its antioxidant properties. This work demonstrates, using a hemoglobin-free perfusion system, the protective effect of the *in vivo* treatment of the rat with silybin dihemisuccinate on the hepatic glutathione depletion and lipid peroxidation induced by the infusion of phenylhydrazine into the perfusion buffer.

Phenylhydrazine Flavonoid Lipid peroxidation Glutathione depletion Hepatic damage

1. INTRODUCTION

Hydrazine derivatives are widely used in industry as fuels, pesticides and explosives as well as in medicine in the treatment of tuberculosis and hypertension. However, despite their industrial and therapeutic use, hydrazines are toxic and can cause irreversible cellular damage [1]. Hydrazines are formed during the hepatic metabolism of several therapeutic agents such as phenelzine, isoniazid, iproniazid and mebanazine [2,3].

Phenylhydrazine has long been known as a hemolytic agent [4] and was used in the past as a therapeutic agent in the treatment of polycythemia vera. The mechanism of action of this hydrazine has been extensively studied [5,6]. The interaction of phenylhydrazine with hemoglobin generates hydrogen peroxide and destroys the pigment through the formation of oxidized derivatives and free radicals of the hydrazine [7]. Phenylhydrazine is capable of inhibiting the function of liver microsomal cytochrome P-450 through the formation of phenyldiazene free radicals [8]. The same intermediates have been demonstrated in the microsomal metabolism of isopropylhydrazine (the metabolite of iproniazid) and acetylhydrazine

(the metabolite of isoniazid) where a covalent binding of its metabolites to the microsomal protein is produced [2]. The formation of free radicals during the microsomal oxidation of hydrazines leads to the hypothesis that such free radicals are involved in the hepatotoxicity of hydrazine derivatives [9].

Silybin dihemisuccinate is a water-soluble form of one of the structural isomers of the flavonoid silymarin extracted from the milk thistle *Silybum marianum* [10]. Silybin dihemisuccinate and silymarin have been proved to be effective protective agents against the hepatotoxicity of phalloidin, α -amanitin, praseodymium, carbon tetrachloride and ethionine [11]. Although the protective mechanism of silymarin (and silybin) has not been elucidated, it is generally accepted that the flavonoid exerts a membrane-stabilizing action preventing or inhibiting lipid peroxidation [12]. Recently it has been demonstrated that silymarin can prevent the liver glutathione (GSH) depletion and lipid peroxidation induced by an acute intoxication with ethanol in the rat [13]. GSH is considered the most important biomolecule against chemically induced cytotoxicity [14].

This study was undertaken to investigate the ef-

fect of a single intraperitoneal dose of silybin dihemisuccinate on the hepatic toxicity of phenylhydrazine using a hemoglobin-free perfusion system. The effect of the flavonoid was assessed by the determination of lipid peroxidation of the liver and of the perfusate, by the oxygen consumption of the liver and the levels of reduced and oxidized hepatic glutathione (GSH and GSSG).

2. EXPERIMENTAL

Male Wistar rats (Instituto de Nutrición y Tecnología de los Alimentos) weighing 200–250 g were injected intraperitoneally with silybin dihemisuccinate (50 mg/kg). Controls received only saline. Animals were fed with a standard laboratory chow and water ad libitum and after 16 h of treatment were anesthetized prior to surgery with sodium pentobarbital (10 mg/100 g body wt). Livers were perfused in situ employing a hemoglobin-free nonrecirculating perfusion system and a flow rate of $30 \text{ ml} \cdot \text{min}^{-1}$. The perfusion medium was a Krebs-Henseleit bicarbonate buffer [15] (pH 7.4) saturated with a mixture of oxygen-carbon dioxide (95%:0.5%) maintained at 37°C . Phenylhydrazine was infused into the perfusion system as indicated by the horizontal bars in the figures. Oxygen consumption was monitored with a Clark type oxygen electrode placed immediately following the liver in the perfusion system and recorded with a YSI monitor. Lipid peroxidation of the liver and of the perfusate was assessed by evaluation of the malondialdehyde thiobarbituric complex (MDA) according to Wilbur et al. [16]. MDA concentration was expressed as $\mu\text{mol MDA/ml}$ perfusate or nmol MDA/g hepatic protein using an E value of $1.56 \times 10^3 \text{ l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$. The determination of GSH and GSSG was carried out enzymatically according to Bernt and Bergmeyer [17]. This technique was applied to supernatant fractions from 2% (w/v) liver homogenates as in [18]. Proteins were measured according to Lowry et al. [19]. Silybin dihemisuccinate was a gift from Dr Madaus and Co. (FRG). Phenylhydrazine and other chemicals were obtained from Sigma (St. Louis, USA). Results are expressed as means \pm SE and the significance of the differences between mean values was assessed by Student's t -test for unpaired results.

3. RESULTS AND DISCUSSION

3.1. Effect of phenylhydrazine perfusion on oxygen consumption and malondialdehyde release of the liver

The infusion of phenylhydrazine (2.5 mM) into the perfusate resulted in a rapid increase in oxygen consumption of the liver from control animals (fig.1A). As can be observed in this figure, high rates of oxygen consumption, in a period over 10 min, are produced even when the infusion of the hydrazine was stopped. Livers from animals treated with silybin dihemisuccinate respond with a rather low increase in oxygen consumption, which decreases and returns to basal values during the final 10 min of phenylhydrazine perfusion.

MDA release of the liver from control animals (fig.1B) increases 5 or 6 min after phenylhydrazine perfusion was initiated, reaching high values which are maintained after suspension of the hydrazine intoxication. Livers from rats treated with the flavonoid evidence only a slight increase in MDA values of the perfusate compared with those obtained from liver not perfused with phenylhydrazine.

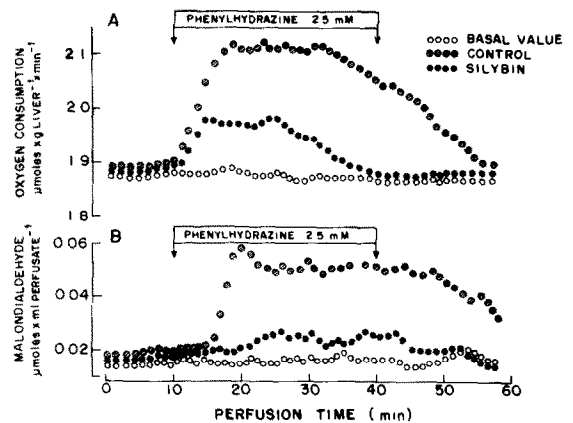


Fig.1. Effect of phenylhydrazine perfusion on oxygen consumption (A) and MDA release (B) of the liver from rats treated with silybin dihemisuccinate (50 mg/kg body wt). The liver was infused for 30 min with phenylhydrazine (2.5 mM) as indicated by the horizontal bar. Fractions of 2.5 ml of the perfusate were collected for MDA determination. Each point represents the mean of 5 experiments. Other conditions are described in section 2.

3.2. Time course of malondialdehyde formation during perfusion of the liver with phenylhydrazine

The MDA content of the liver increases after the infusion of phenylhydrazine into the perfusion buffer (fig.2) concomitantly with the rise in the oxygen consumption (fig.1). Livers obtained from untreated animals show a net increase in MDA values. However, livers obtained from rats treated with the flavonoid respond with a low increase in MDA formation which declines when the hydrazine infusion is concluded. The low MDA release into the perfusate observed in those livers may reflect the capacity of the organ to metabolize the lipid peroxidative products. This is not the case for livers from untreated animals where high MDA production is accompanied by high MDA values in the perfusate.

3.3. Effect of phenylhydrazine perfusion on the glutathione content of the liver

The increase in oxygen consumption and MDA formation in control livers may be a consequence of uncontrolled lipid peroxidation induced by phenylhydrazine. These effects are accompanied by a 42% depletion of the GSH and total glutathione (GSH_T) of the liver after hydrazine perfusion (fig.3C) compared with the values ob-

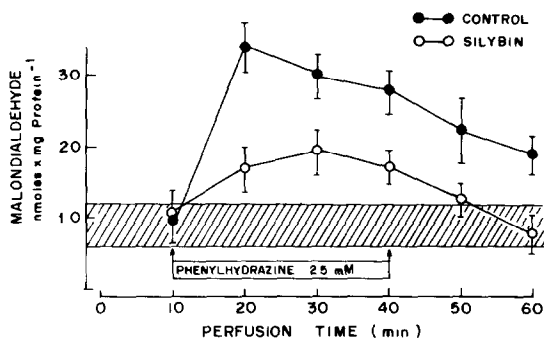


Fig.2. Time course of hepatic malondialdehyde formation after phenylhydrazine perfusion of the liver from rats treated with silybin dihemisuccinate (50 mg/kg body wt). The liver was infused for 30 min with phenylhydrazine (2.5 mM) as indicated by the horizontal bar. The shaded zone corresponds to the basal values of MDA obtained from the liver of untreated animals. Each point represents the mean of 4 experiments \pm SE. Other conditions are described in section 2.

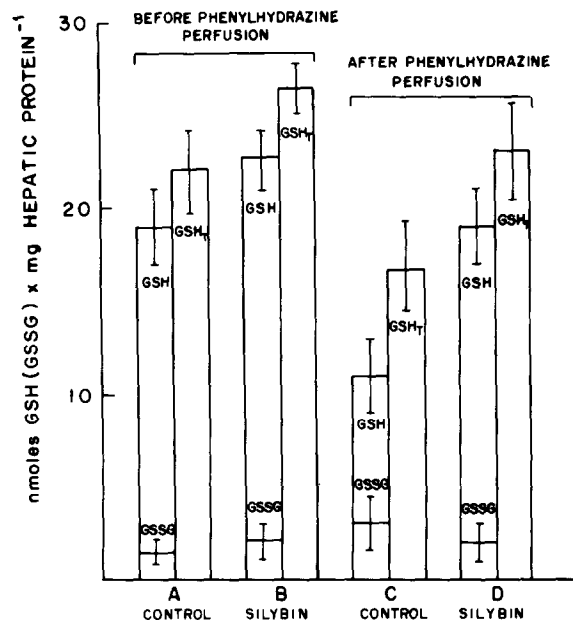


Fig.3. Effect of phenylhydrazine (2.5 mM) perfusion on glutathione levels of the liver from rats treated with silybin dihemisuccinate (50 mg/kg body wt). Data represent the mean \pm SE for 8 animals. Total GSH equivalents (GSH_T) were calculated according to the relation $GSH_T = GSH + 2GSSG$. Significance studies: GSH = A vs B vs C ($p < 0.005$); A vs D (N.S.); B vs C ($p < 0.005$); B vs D ($p < 0.05$). GSSG = A vs B and D (N.S.); A vs C ($p < 0.005$); B vs C ($p < 0.05$); B vs D (N.S.). GSH_T = A vs B vs C ($p < 0.005$); A vs D (N.S.); B vs C ($p < 0.005$); B vs D (N.S.). N.S., not significant.

tained before perfusion (fig.3A). The increase in GSSG content observed after phenylhydrazine treatment may be interpreted as being the result of oxidative stress imposed by phenylhydrazine on the liver. However, the net decrease in total glutathione values indicates release of the tripeptide from the liver into the perfusate (as GSH and/or GSSG) which may be a consequence of the structural and functional damage produced by the hydrazine or its metabolites (as the phenyldiazene free radical) [8].

Livers from animals treated with the flavonoid show an increase in the content of GSH and total glutathione (fig.3B) before phenylhydrazine perfusion compared with control animals (fig.3A). The mechanism of the increase in liver glutathione after silybin dihemisuccinate or silymarin [13] treatment is still unknown and is currently being studied.

Phenylhydrazine perfusion produces a decrease in glutathione content of the liver from rats treated with the flavonoid (fig.2D). However, due to the high values of this parameter observed before perfusion with the hydrazine, the glutathione status of these livers after perfusion resembles control values before intoxication.

It has been proposed that the increase in liver glutathione content obtained after silymarin [13] or silybin dihemisuccinate treatment would afford a better protection of the tissue against an oxidative stress. The low increase in oxygen consumption, MDA formation and MDA release into the perfusate observed in the liver from rats treated with the flavonoid may be the result of an increased biodegradability of the tripeptide. GSH would be utilized in the liver principally as a conjugation agent, a cofactor of the enzyme glutathione peroxidase in the metabolism of organic peroxides and as a direct free radical scavenger [14]. This latter role of GSH would be reinforced by the antioxidant properties of silybin dihemisuccinate which, acting as a free radical scavenger, may inhibit or suppress lipid peroxidation induced by the hydrazine. This protective effect of silybin dihemisuccinate may extend to other xenobiotics whose hepatotoxicity is related with glutathione depletion and/or lipid peroxidation.

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REFERENCES

- [1] Back, K.C. and Thomas, A.A. (1970) *Annu. Rev. Pharmacol.* 10, 395-412.
- [2] Nelson, S.D., Mitchell, J.R., Snodgrass, W.R. and Timbrell, J.A. (1978) *J. Pharmacol. Exp. Ther.* 206, 574-585.
- [3] Kato, R., Takanaka, A. and Shoji, H. (1969) *Jap. J. Pharmacol.* 44, 315-322.
- [4] Janol, J.H., Engle, J.K. and Allen, D.W. (1960) *J. Clin. Invest.* 39, 1818-1836.
- [5] Jain, S.K. and Subrahmanyam, D. (1978) *Biochem. Biophys. Res. Commun.* 82, 1320-1324.
- [6] Valenzuela, A., Ríos, H. and Neiman, G. (1977) *Experientia* 33, 912-963.
- [7] Misra, H.P. and Fridovich, J. (1976) *Biochemistry* 15, 681-687.
- [8] Jonek, H.G., Werringloer, J., Prough, R.A. and Estabrook, R. (1982) *J. Biol. Chem.* 257, 4404-4411.
- [9] Ohara, A., Ortiz de Montellano, P.R. and Quintanilha, A. (1981) *Biochem. Biophys. Res. Commun.* 101, 1324-1330.
- [10] Lewis, G.P. and Piper, P.S. (1975) *Nature* 259, 308-311.
- [11] Lecomte, S. (1975) *Rev. Med. Liege* 30, 110-114.
- [12] Greimel, A. and Koch, H. (1977) *Experientia* 33, 1417-1418.
- [13] Valenzuela, A., Lagos, C., Schmidt, K. and Videla, L.A. (1984) *Biochem. Pharmacol.*, in press.
- [14] Videla, L.A. and Valenzuela, A. (1982) *Life Sci.* 31, 2395-2407.
- [15] Krebs, H.A. and Henseleit, K. (1932) *Z. Physiol. Chem.* 210, 33-36.
- [16] Wilbur, K.M., Bernhein, F. and Shapiro, O.W. (1949) *Arch. Biochem. Biophys.* 29, 305-308.
- [17] Bernt, E. and Bergmeyer, H.V. (1979) in: *Methods of Enzymatic Analysis* (Bergmeyer, H.U. ed.) vol.4, pp.1643-1647, Academic Press, New York.
- [18] Videla, L.A., Fernández, V., Fernández, N. and Valenzuela, A. (1981) *Substance and Alcohol Actions/Misuse* 2, 160-193.
- [19] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.* 193, 265-275.