IN VIVO METABOLIC AND ANTIOXIDATIVE EFFECTS OF SULPHORAPHANE DERIVED FROM BROCCOLI IN WATER- AND ETHANOL-DRINKING RATS

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Key words: antioxidative effects, broccoli, ethanol, metabolism, sulphoraphane

Sulphoraphane (SF) is isothiocyanate that is present naturally in widely consumed vegetables and has been shown to block the formation of many kinds of tumors. The effect of sulphoraphane, administered intragastrically (10 mg/kg) once a day for 2 weeks, on some metabolic and antioxidative parameters in water- and ethanol-drinking (10% ethanol solution) rats was investigated. Its protective effect was previously observed, but its influence on metabolic changes has not been yet characterised. Our trial confirmed antioxidative properties of sulphoraphane by limited generation of lipid peroxidation and enhanced functional capacity of detoxifying enzyme glutathione S-transferase (GST). SF in water-drinking rats caused a decrease in malondialdehyde (TBARS) and glutathione peroxidase (GPx) activity, whereas glutathione S-transferase (GST) activity was augmented significantly. However, we observed unfavourable interaction between sulphoraphane and ethanol, where antioxidative properties of SF were abolished in ethanol-drinking rats.

The present trial was the first attempt to determine the carbohydrate and lipid metabolism changes after SF administration in both aforementioned conditions. We found that SF per se caused insulinaemia, steatosis as well as beneficial HDL-cholesterolaemia and serum triacylglycerols drop.

Under ethanol treatment we noted the following disturbances: increased triacylglycerols and drop of glycogen and TBARS content in the liver. Such characteristics unfavourably elevated liver level of triacylglycerols was not mitigated by sulphoraphane. The other ethanol disturbances were normalized by SF.

In our trial we noted clear interaction between sulphoraphane and ethanol. Liver glycogen decrease caused by ethanol and serum insulin increment due to sulphoraphane ingestion was normalized by their interaction. Serum triacylglycerols and HDL-cholesterol changes caused by SF per se were maintained also after sulphoraphane treatment in the ethanol-receiving rats. However, after their interaction, the content of triacylglycerols in the liver did not increase strongly in spite of significant increment during their individual action.

The results obtained demonstrate that SF exerts an influence on insulin level as well as lipid metabolism. Only insulin or glycogen disturbance caused by sole SF or ethanol was normalized during their interaction. SF does not attenuate ethanol steatosis. Additionally, our trial showed that the confirmed antioxidative properties of SF were abolished in the presence of ethanol.

ABBREVIATIONS

FFAs – free fatty acids; GPx – glutathione peroxidase; GST – glutathione S-transferase; HDLs – high-density lipoproteins; ITCs – isothiocyanates; MDA – malondialdehyde; TBARS – thiobarbituric acid reactive substances; QR – quinone reductase; ROS – reactive oxygen species; SF – sulphoraphane; TGS – triacylglycerols.

INTRODUCTION

Broccoli is known as the “Crown Jewel of nutrition” for its vitamin-rich, high-fiber, and low-calorie properties. It has multiple cancer-fighting compounds including vitamin C, beta carotene, fiber and phytochemicals (glucosinolates). The predominant glucosinolate in broccoli is glucoraphanin [Matusheski & Jeffery, 2001], which is hydrolysed to 1-isothiocyanato-4-(methylsulfinyl)-butane referred to as sulphoraphane (SF) [Kushad et al., 1999]. SF shows antioxidative properties preventing from multiple diseases, including several types of cancer, high blood pressure, macular degeneration and stomach ulcers [Zhang, 2000; Gao et al., 2001]. It is a very potent inducer of phase II enzymes like QR and GST activities in vitro as well as in vivo [Zhang & T alalay, 1998]. In rats and mice increased phase II enzyme activities were recorded in the liver, lung, mammary gland, pancreas, stomach, small intestine, and colon [Keck et al., 2002; Matusheski & Jeffery, 2001]. In spite of anti-cancer effects of sulphoraphane as mentioned above, the mode of its action on carbohydrate and lipid metabolism and blood insulin is poorly elucidated. It seems worthwhile to complete the recent findings, in particular, that ITCs as biologically active compounds can exert an influence on metabolism in rats [Okulicz et al., 2005]. Therefore, the purpose of our experiment was to examine the effect of SF on some metabolic parameters, blood insulin and antioxidative effects under physiological conditions in normal rats. The experiment was also performed on ethanol-drinking rats to determine the effect of SF under patho...
logical conditions in order to assess the potential interaction between SF and ethanol.

MATERIALS AND METHODS

SF was purchased from ICN Biomedicals Inc. (1263 South Chillicothe Road, Aurora, Ohio 44202). All other chemicals were purchased from Sigma Chemical (St Louis, MO, USA).

Male Wistar rats, initially weighing 120±5 g were used in the experiment. Rats were maintained in cages at standard conditions with a constant temperature of 21±1°C, a 12-h dark-light cycle and were fed ad libitum a laboratory diet (Labofeed, Kcynia, Poland). They were randomly divided into four groups consisting of eight rats each. During the 14 days experiment, the rats in two groups were provided tap water, whereas the others – 10% ethanol solution (on volume basis) as the sole drinking fluid ad libitum in order to induce pathological changes appearing as a result of ethanol intake. At the end of the study, the animals did not drink more 10% ethanol solution. Rats in one group of water-drinking and one group of ethanol-drinking animals were treated with 10 mg SF/kg/b.w. This compound was dissolved in 0.85% NaCl and was administered intragastrically once a day for 14 days at the volume of 0.5 mL/100 g b.w. Rats from the two remaining groups received the same volume of vehicle in order to maintain the same way of treatment in all experimental groups. Rats were decapitated 12-14 h after the last intragastric treatment and their blood serum and liver samples were collected and stored at –80°C until analysis.

The serum was used for the determination of blood glucose, FFAs, phospholipids, TGs, total, free and esterified cholesterol, total cholesterol in HDLs and insulin.

Liver samples were determined for cholesterol, triacylglycerols, glycogen, TBARS, sulphydryl group contents and the activity of GPx and GST.

Glucose was assayed colorimetrically by the enzymatic method with glucose oxidase, peroxidase and o-dianisidine (Hugget & Nixon, 1957). FFAs were determined according to Duncombe [1964] and TGs were assayed by the method of Foster & Dunn [1973]. Total, free, and esterified cholesterol levels and HDL-cholesterol were measured by the enzymatic method of Richmond [1973]. HDLs were separated from blood serum using polyethylene glycol 6000 according to Demacker et al. [1980]. Phospholipids were determined enzymatically with a kit provided by BioMerieux (France). Insulin was assayed radioimmunologically by using the kit specific for rat hormone (Linco Research, St. Charles, Missouri, USA).

Liver cholesterol was assayed after extraction of lipids [Folch et al., 1975] and evaporation of the extract [Richmond, 1973]. The level of liver glycogen was determined after its extraction in 30% KOH and hydrolysis with amyloglucosidase 12 U/mL (6000 U/g; SIGMA). Liver TGs were assayed after extraction [Folch et al., 1975]. The activity of GPx was assayed by the method described by Rice-Evans et al. [1991] with hydrogen peroxide as a substrate and that of GST with 1-chloro-2, 4-dinitrobenzene as a substrate. The protein content in 1% liver homogenate was determined according to Lowry et al. [1951]. The concentration of TBA reactive substances in the liver was determined according to Buege & Aust [1978]. The concentration of sulphydryl groups was assayed according to Sedlak & Lindsay [1968].

The results were evaluated statistically using two-way analysis of variance (ANOVA) and Holm-Sidak’s multiple range test at p≤0.05.

The experiment was performed according to rules accepted by Local Ethical Commission for Investigation on Animals.

RESULTS

Metabolic and hormonal changes

The metabolic change caused by sulphoraphane in normal rats was an increase in blood insulin concentration (76%), HDL-cholesterol (29%), triacylglycerols content in the liver (16%) and serum triacylglycerols drop (13%) in comparison to control (Table 1).

No changes were observed in carbohydrate and other lipid parameters in serum nor in liver.

One of the characteristic metabolic pathological changes under ethanol ingestion is depression in body weight (b.w.) gain, which is accompanied by some hormonal and metabolic changes [Szkudelska et al., 2007]. The ethanol-drinking animals did not show dependence on ethanol during the experiment. The mean volume of water drunk by the rats was 18.12 mL/100 g b.w./day, whereas the mean amount of alcohol solution was 17.94 mL/100 g b.w./day, i.e. 1.79 mL of pure ethanol/100 g b.w./day. The only metabolic change caused by ethanol was an increase in triacylglycerols (27%) and drop of glycogen (33%) in the liver.

During the interaction between SF and ethanol we noted a significant drop of blood serum triacylglycerols (14%) and an increase in HDL-cholesterol (29%) and liver triacylglycerols (23%) in comparison to control (water-drinking rats).

TBARS and GPx, GST activity changes

Changes in TBA reactive substances, GPx and GST activities in liver were observed in the water-drinking rats after SF administration (Table 2). SF caused decrease in content of TBARS (23%) and GPx activity (15%), whereas GST activity was augmented significantly (21%).

A distinct decrease concerning the content of TBA reactive substances (16%) was observed only in the ethanol-drinking rats, whereas GST and GPx activities were unaffected.

Our investigation demonstrated the lack of effect on liver sulphydryl groups content in water- and ethanol-drinking rats in the presence or lack of SF.

Discussion

The dose of SF used in the performed experiment constituted common molecular weight glucoraphanin in broccoli [Abercrombie et al., 2005], which consumption was estimated at 25 g/kg b.w. of rat per day (approximately 10% food intake per day).

The main part of our work was to investigate for the first time the influence of SF administration on lipid and carbohydrate metabolism and insulin in rats under normal as well as pathological conditions (caused by 10% ethanol intake). SF clearly exerts an influence on lipid metabolism. We noticed an increase in the concentration of HDL-cholesterol caused
TABLE 1. The influence of oral administration of sulphoraphane on blood insulin and metabolic parameters in water- and ethanol-drinking rats.

<table>
<thead>
<tr>
<th>Hormonal and metabolic parameter</th>
<th>Water</th>
<th>Water + sulphoraphane</th>
<th>Ethanol</th>
<th>Ethanol + sulphoraphane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (ng/mL)</td>
<td>1.06±0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.87±0.13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.70±0.13&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.90±0.17&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.61±0.14</td>
<td>5.86±0.21</td>
<td>5.30±0.15</td>
<td>5.38±0.18</td>
</tr>
<tr>
<td>Free fatty acids (mmol/L)</td>
<td>0.21±0.02</td>
<td>0.19±0.01</td>
<td>0.25±0.02</td>
<td>0.25±0.02</td>
</tr>
<tr>
<td>Phospholipids (mmol/L)</td>
<td>1.63±0.07</td>
<td>1.64±0.07</td>
<td>1.62±0.08</td>
<td>1.87±0.12</td>
</tr>
<tr>
<td>Triacylglycerols (mmol/L)</td>
<td>3.63±0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.17±0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.52±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.13±0.17&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>1.71±0.04</td>
<td>1.77±0.05</td>
<td>1.69±0.06</td>
<td>1.79±0.08</td>
</tr>
<tr>
<td>Free cholesterol (mmol/L)</td>
<td>0.20±0.02</td>
<td>0.13±0.02</td>
<td>0.18±0.03</td>
<td>0.25±0.02</td>
</tr>
<tr>
<td>Esterified cholesterol (mmol/L)</td>
<td>1.51±0.04</td>
<td>1.64±0.04</td>
<td>1.51±0.07</td>
<td>1.54±0.06</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/L)</td>
<td>0.34±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.44±0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.30±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.44±0.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

TABLE 2. The influence of oral administration of sulphoraphane on some antioxidative parameters in liver in water- and ethanol-drinking rats.

<table>
<thead>
<tr>
<th>Antioxidative parameter in the liver</th>
<th>Water</th>
<th>Water + sulphoraphane</th>
<th>Ethanol</th>
<th>Ethanol + sulphoraphane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione peroxidase (nmol/min/mg protein)</td>
<td>585.72±20.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>497.99±29.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>565.66±26.60&lt;sup&gt;b&lt;/sup&gt;</td>
<td>605.55±17.67&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glutathione S-transferase (nmol/min/mg protein)</td>
<td>555.15±18.73&lt;sup&gt;a&lt;/sup&gt;</td>
<td>672.53±14.89&lt;sup&gt;a&lt;/sup&gt;</td>
<td>655.50±43.50&lt;sup&gt;b&lt;/sup&gt;</td>
<td>595.55±33.71&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>TBARS (nmol/g WT)</td>
<td>48.61±1.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37.58±1.54&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40.98±1.69&lt;sup&gt;a&lt;/sup&gt;</td>
<td>46.29±1.48&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total SH groups (mmol/100 g WT)</td>
<td>1.47±0.07</td>
<td>1.50±0.03</td>
<td>1.53±0.03</td>
<td>1.56±0.07</td>
</tr>
<tr>
<td>Non-protein SH groups (mmol/100 g WT)</td>
<td>0.09±0.01</td>
<td>0.09±0.01</td>
<td>0.09±0.01</td>
<td>0.11±0.01</td>
</tr>
<tr>
<td>Protein-bound SH groups (mmol/100 g WT)</td>
<td>1.35±0.10</td>
<td>1.41±0.05</td>
<td>1.43±0.04</td>
<td>1.45±0.08</td>
</tr>
</tbody>
</table>

by SF in the water-drinking rats (Table 1). Our result is in good agreement with a pilot study conducted by Murashima et al. [2004] who found that among individuals who consumed 3½ ounces of broccoli sprouts a day, the total cholesterol level decreased, whereas HDL level increased. In the 14-day ethanol-drinking rats we did not observe any increase in HDL-cholesterol in spite of data that moderate alcohol intake is associated with augmentation of HDL cholesterol concentration [De Oliveira e Silva et al., 2000; McDonough, 2003]. According to Nanjee et al. [1996], high plasma concentration of HDL can be associated with an increased activity of microsomal cytochrome P450 in liver and intestine. In our trial the noted indirect lack of formation of reactive oxygen species (TBARS drop) via the ethanol-specific cytochrome P450 2E1 system after two weeks of alcohol drinking may explain no raise in plasma HDL-cholesterol concentration. Such an effect noted in water- and ethanol-drinking rats in the presence of SF was probably evoked by absolute SF per se, which can increase cytochrome activity in contradistinction to its bio-transformation products.

Chronic administration of ethanol is usually associated with an increased accumulation of hepatic TGs [Tijburg et al., 1988; Venkatesan et al., 1998]. Our results are consistent with this data, where increased liver content of TG stores was noted (Table 1). This augmentation is the consequence of NADH production during ethanol metabolism, which is used to synthesize sn-glycerols-3-phosphate [Yu & Cronholm, 1997]. Such liver lipid accumulation was also found in the SF-treated animals (Table 1), where this change seems to be evoked mainly by inhibited triacylglycerols transport from the liver with concomitant significant increase in the concentration of insulin. Insulin can have a positive effect on expression of lipogenic enzymes such as fatty acid synthase, mitochondrial glycerol-3-phosphate acyltransferase [Sul & Wang, 1998]. Additionally, the observed decreased concentration of TGs in serum after SF administration in both ethanol- and water-drinking rats confirmed restricted mobilisation of TGs from liver under normal and pathological conditions in its presence (Table 1). The characteristic ethanol-induced increase in liver TGs content was not disturbed by SF, moreover, this
high content of TGs was obtained to maintain in the ethanol-drinking rats treated with SF.

The observed increase in blood insulin caused by SF in normal rats was not accompanied by any change in blood glucose concentration. According to Conaway et al. [2000], the total isothiocyanate metabolites in plasma peaked between 0 and 12 h. The elimination half-life of SF itself is about 2.2 h [Hu et al., 2004] and 6.7 h for its biotransformation products [Cornblatt et al., 2007]. Taking into account the mentioned data and additionally, the time of insulin level measurement after the last administration (12 h), assumption of direct action on insulin secretion of rather not pure but the conjugated form of SF is quite possible. In the performed trial, a statistically insignificant decrease in insulin concentration was observed in the ethanol-drinking rats in comparison to the water-drinking rats. This result is in accordance with the observation of Szkudelski et al. [2004]. Lower blood insulin level with concomitant unchanged blood glucose concentration seems to result from the inhibition of its secretion.

It is noted that insulin secretion from isolated pancreas in alcohol-treated rats was decreased in vivo and in vitro [Samols & Stagner, 1980; Singh et al., 1980]. Disturbance in insulin level induced by SF was normalized during interaction between SF and ethanol. In the ethanol-drinking rats additionally liver glycogen content was reduced. It is widely known that ethanol ingestion reduces gluconeogenesis. Under such conditions, glycogenolysis is accelerated in order to prevent hypoglycaemia. Also this disturbance was normalized by their interaction.

In the second part of our investigation we displayed antioxidant properties of sulforaphane. Results obtained are in good agreement with recent reports by Gao et al. [2001] and Chuangphongpanich et al. [2006]. We noted an increase in GST and a decrease in GPx activity with a concomitant drop in the content of TBARS in the liver after SF administration in the water-drinking rats (Table 2). The conjugation with reduced glutathione to form a dithiocarbamate in the presence of GST is a major metabolic route of ITCs in rodents which can be an additional cause of an increase in GST activity (Table 2). A considerable decrease in the number of liver microsomes CYP2E1, 2B1, 1A1 and 2 in rat or human liver microsomes [Conaway et al., 1996; Jiao et al., 1996]. Moreover, Conaway et al. [2001] have confirmed that also thiol conjugates of isothiocyanates are transient inhibitors of P450 enzymes involved in carcinogen activation, reducing generation of reactive oxygen species. Biotransformation product of SF has been shown to inhibit at least one cytochrome P-450 CYP2E1 [Barcelo et al., 1996; Faulkner et al., 1998]. On the other side, this cytochrome P-450 CYP 2E1 is induced by chronic alcohol use. The showed favourable SF protection was disturbed exactly in rats drinking ethanol, which resulted in the observed decrease in the content of TBARS in liver with concomitant tendency for a decrease in GST and an increase in GPx activity (Table 2). Surprisingly, our study showed that 14-day sole ethanol drinking did not evoke lipid peroxidation in spite of knowledge that ethanol metabolism leads to the formation of reactive oxygen species via the ethanol-specific cytochrome P450-2E1 system. In our trial ethanol consumption evoked even a significant decrease in the content of TBARS. These inconsistent properties seem to depend on alcohol dose and time of treatment. It has been reported that radical formation was significantly increased by 1 week of alcohol feeding (consistent with the time for CYP2E1 to increase) and decreased to control levels from weeks 2 through 8 (probably time for the liver to enhance its capacity to neutralize radicals) [Nava- sumrit et al., 2000]. The evidence of limited antioxidant potential in ethanol-drinking rats in the presence of SF suggests the unfavourable interaction between these both compounds in terms of ability to generate reactive oxygen species.

Conclusion
The results obtained indicate that SF significantly influences lipid metabolic pathways and these metabolic changes are partially beneficial. The augmentation of cholesterol concentration in plasma HDL and drop in TG induced by SF tends towards its possibility to decrease the risk of coronary heart disease. These metabolic changes remained unaltered in the presence of ethanol. The hormonal status concerning the high concentration of blood insulin observed in the presence of biotransformation products of SF was attenuated in the ethanol-drinking rats. Additionally, we confirmed antioxidative properties of SF, but our performed trial reveals unfavourable interaction between sulforaphane and ethanol, which abolishes the antioxidative properties of SF (drop GST and enhanced MDA).

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