Effects of indole-3-carbinol on metabolic parameters and on lipogenesis and lipolysis in adipocytes

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ABSTRACT: Indole-3-carbinol (I3C) was found to have possible anticarcinogenic, antioxidant and anti-atherogenic effects on the organism. So far, its influence on metabolic pathways has been unknown. This work was the first attempt to determine the carbohydrate and lipid metabolism changes *in vivo* after administration of 150 mg/kg b.wt./day I3C to male rats. Additionally, the aim of this trial was to evaluate the direct effect of I3C on basal and hormone-induced lipogenesis and lipolysis in isolated rat adipocytes at concentrations 1, 10, 100 μM *in vitro*. We can corroborate that adipocytes are susceptible to the direct action of I3C. The incubation of adipocytes with I3C at the three above-mentioned concentrations resulted in its influence on restriction of glucose entry into adipocytes in the basal as well as insulin-stimulated conditions. However, it was observed that I3C at these concentrations strongly intensified basic and epinephrine-stimulated lipolysis. I3C also has a significant influence on metabolism *in vivo*. Its administration to rats caused a significant increase in the content of triglycerides and a decrease in glycogen in the liver. The considerable augmentation of glucose, triglycerides, cholesterol in high-density lipoprotein and insulin with a concomitant decrease in FFA concentrations was noted in the blood serum. I3C did not alter phospholipids, total, free, esterified cholesterol in the serum and the liver cholesterol. The results obtained *in vivo* and *in vitro* indicate that the effect of I3C is adverse for the majority of metabolic parameters which were investigated. The most important finding in this study is the effect of I3C on liver steatosis and that the observed lower lipogenesis at higher lipolysis in fat cells may be involved in the mechanism.

Keywords: indole-3-carbinol; lipid metabolism; carbohydrate metabolism; rat

Indole-3-carbinol (I3C) is a major derivative of glucobrassicin (3-indolylmethyl glucosinolate), a plant product common to vegetables of the class Cruciferae (McDanell et al., 1986). I3C is both an anti-initiator and a promoter of carcinogenesis depending on the timing and dose of administration. It has been found to inhibit the development of tumours in forestomach, glandular stomach, mammary gland, prostate, uterus, tongue, and liver of rodents, as well as in the trout liver, when administered prior to or during carcinogen exposure by gavage or in the diet (Kim et al., 1997). The consumption of I3C by humans and rodents can lead to marked increases in activities of cytochrome P-450-dependent monooxygenases and in a variety of phase II drug metabolizing enzymes like glutathione S-transferase (GST) resulting in the increased hepatic metabolic capacity towards chemical carcinogens (Bjeldanes et al., 1991). The induction of four different P450 forms (P450, 1A1, 1A2, 2B1 and 3A) was described in the liver of rats fed I3C for as short a period as 2 days (Wortelboer

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et al., 1992; Manson et al., 1997). Activated P450 enzymes are involved in carcinogen activation, increasing the production of reactive oxygen species (ROS). The induction of CYP1A1 by indole-3-carbinol has been associated with an increase in oxidative DNA damage in a cell culture, which suggests that the induction can lead to a leak of oxygen radicals (Park et al., 1996). These properties of I3C are considered to contribute to the known ambiguous property (anti- and pro-oxidative) of this compound.

Early studies demonstrated that I3C was effective only when given orally and not when given i.p. or i.v. Namely, under low pH conditions in stomach, several condensation reactions of I3C occur which result in the formation of 3, 3'-diindolylmethane (DIM) and indolylcarbazole (ICZ). I3C is an active inducer of CYP 4501A1 increasing 2-hydroxylation of oestrogens. Having a sustained oestrogen modifying effect I3C is a good candidate for clinical trials in women at an increased risk of developing breast cancer (Brignall, 2001). In addition to these indirect effects as a result of altered oestrogen metabolism, indole-3-carbinol as well as DIM has been shown to have direct effects on apoptosis and cyclin D, resulting in blockage of the cell cycle (Bradlow et al., 1999). Because it facilitates the inactivation of oestrogen, it is possible that I3C might tend to promote osteoporosis in postmenopausal women and could interfere with oestrogen therapies (birth control pills and hormone replacement therapy).

The mechanism of I3C action in the anticarcinogenic activity has been intensively investigated, and now it is quite well understood on the contrary to its influence on carbohydrate and lipid metabolism. It is known that the biologically active products of degradation of glucosinolate precursors can have an influence on metabolism in rats (Okulicz et al., 2005). Therefore, the purpose of our experiments was to ascertain the effect of I3C at a dose of 150 mg/kg body weight/day on some metabolic parameters and blood insulin concentration under physiological conditions in normal rats. In addition, the aim of our work was to investigate the direct effect of I3C on lipogenesis and lipolysis in isolated rat adipocytes not caused by hormonal and metabolic changes. It is worth noting that the alterations in the whole body lipid metabolism result also, at least in great part, from changes in adipocytes (Szkudelska et al., 2002).

MATERIAL AND METHODS

In vitro experiment

Preparation of adipocytes

After 7 days of adaptation the male rats weighing 160 ± 5 g were sacrificed by decapitation and the epididymal adipose tissue was taken. Adipocytes were isolated according to the method of Rodbell (1964) with minor modifications (Szkudelska et al., 2000). The fat tissue removed from 10 rats was polled, rinsed with saline, cut with scissors and placed in a plastic flask containing Krebs-Ringer buffer (118mM NaCl, 4.8mM KCl, 1.3mM $CaCl_2$, 1.2mM KH_2PO_4, 1.2mM $MgSO_4$, 24.8mM $NaHCO_3$) with 3mM glucose, 3% bovine serum albumin (BSA, fraction V), 10mM HEPES and 2 mg/ml collagenase (EC 3.4.24.3. from *Clostridium histolyticum*, type II). Incubation was performed for 90 min by shaking at 37°C. Isolated adipocytes after incubation were rinsed four times with the warm (37°C) Krebs-Ringer buffer without collagenase, filtered through nylon mesh and counted under the microscope with Bürker-Türk counting chamber. Cell viability was about 95% as determined by trypan blue exclusion.

Lipolysis

Fat cell suspensions (10^6 cells/ml) were incubated in plastic tubes at 37°C for 90 min with the Krebs-Ringer buffer containing 3mM glucose, 10mM HEPES and 3% BSA in the absence or the presence of 1 µM epinephrine. The examined I3C was dissolved in DMSO and 10 µl of this solution (or DMSO only in the case of control tubes) was added to 990 µl of the buffer with adipocytes. The final concentrations of I3C were 1 µM, 10 µM, 100 µM. Each experiment was performed three times with five replications. Glycerol released from adipocytes, reflecting the intensity of lipolysis, was measured using the method of Foster and Dunn (1973).

Lipogenesis

Adipocytes (10^6 cells/ml) were incubated in plastic tubes at 37°C with Krebs-Ringer buffer, pH 7.4, containing 3% BSA, 10mM HEPES, 0.5 µCi of (U- ^{14}C)glucose (specific activity 9.80 GBq/mmol,

New England Nuclear Research Products), 5.56mM unlabelled glucose in the absence or presence of 1nM insulin. Similarly like in the case of lipolysis, I3C was dissolved in DMSO and was added to the buffer with adipocytes to the final volume 1 ml. The final concentrations of I3C were 1μM, 10μM, 100μM. Each experiment was performed three times with five replications. Incubations were carried out with shaking for 90 min at 37°C. The reaction was stopped by adding 5 ml of Dole's extraction, containing isopropanol-heptane-1 N H_2SO_4 (40:10:1). Tubes were shaken and then 2 ml of H_2O and 3 ml of heptane were added for lipid extraction. After shaking, samples of the upper phase were transferred into counting vials containing a scintillation cocktail (OptiPhase 'Hi Safe' Wallac) and total lipid radioactivity was determined.

In vivo experiment

Feeding experiment

Rats were administered I3C at an estimated dose of 150 mg/kg b.wt./day. This dose had no effect on food consumption, rat weight, or general appearance and behaviour and was comparable to those used by other researchers (Bradlow et al., 1991; LeBlanc et al., 1994; Stresser et al., 1995).

Male Wistar rats, initially weighing 120 ± 5 g, were used in the experiment. Rats were kept in cages in standard conditions with a constant temperature of 21 ± 1°C, 12-h dark-light cycle, and were fed a laboratory diet *ad libitum* (Labofeed, Kcynia, Poland). They were randomly divided into two groups consisting of eight rats each. During the experiment lasting 14 days, the rats in both groups drank tap water *ad libitum*. Rats in one group were treated with 150 mg/kg b.wt. I3C. This compound was dissolved in maize oil and was administered intragastrically once a day for 14 days at the volume of 0.5 ml/100 g b.wt. The rats from the control group received the same volume of sole maize oil as a vehicle. 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, free fatty acids (FFA), phospholipids, triglycerides (TG), total, free and esterified cholesterol, total cholesterol in high-density lipoproteins (HDL) and insulin.

In the liver cholesterol, triglycerides and glycogen were determined.

Glucose was assayed colorimetrically by the enzymatic method with glucose oxidase, peroxidase and o-dianisidine (Hugget and Nixon, 1957). Free fatty acids were determined according to Duncombe (1964) and triglycerides were assayed by the method of Foster and Dunn (1973). Total, free, and esterified cholesterol levels and cholesterol in HDL were measured by the enzymatic method of Richmond (1973). High-density lipoproteins were separated from the blood serum using polyethylene glycol 6 000 according to Demacker et al. (1980). Phospholipids were determined enzymatically with a kit provided by BioMerieux (France).

Insulin was assayed radioimmunologically using the kit specific to rat hormone (Linco Research, St. Charles, Missouri, USA).

Liver cholesterol was assayed after extraction of lipids (Folch et al., 1975) and evaporation of the

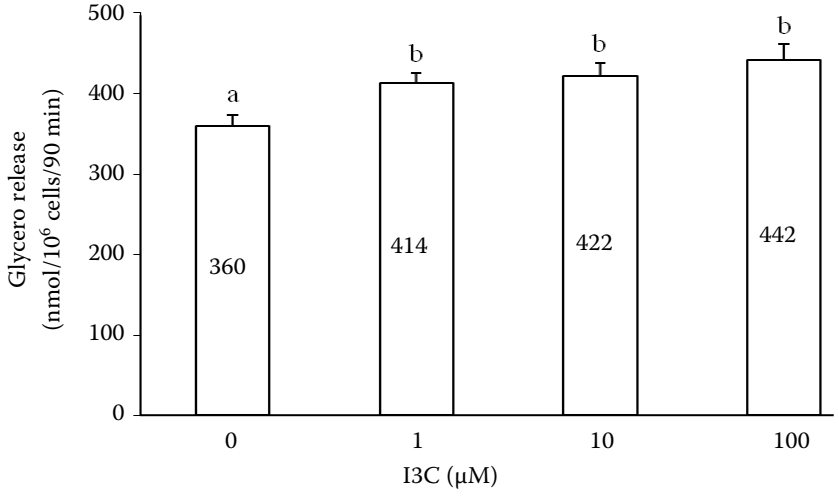

Figure 1. The effect of indole-3-carbinol on basal lipolysis in isolated rat adipocytes. Each column represents the mean ± SEM for five replications (n = 5). Mean values marked by different letter superscripts differ statistically ($P \leq 0.05$)

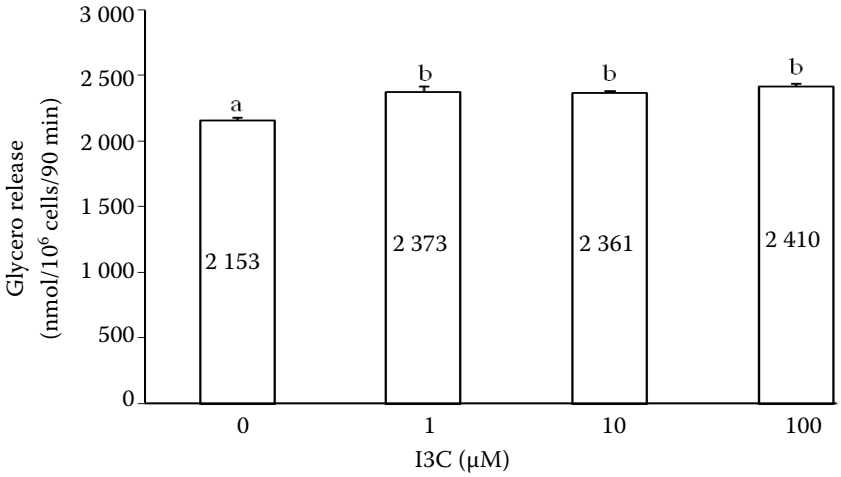

Figure 2. The effect of indole-3-carbinol on lipolysis stimulated by epinephrine (1μM) in isolated rat adipocytes. Each column represents the mean ± SEM for five replications (n = 5). Mean values marked by different letter superscripts differ statistically ($P \leq 0.05$)

extract (Richmond, 1973). The amount of liver glycogen was determined after its extraction in 30% KOH and hydrolysis with amyloglucosidase. Liver TG were assayed after extraction (Folch et al., 1975).

The results were evaluated statistically using one-way analysis of variance (ANOVA) and Duncan's multiple range test at $P \leq 0.05$.

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

RESULTS

In vitro

Effect of I3C on lipolysis

I3C affected lipolysis significantly. Figures 1 and 2 show glycerol release from adipocytes exposed to I3C (1 μM, 10 μM, 100 μM) in the absence and in the presence of epinephrine. I3C did not show a dose-dependent significant enhanced effect on basal lipolysis. We noted 15.0%, 17.2%, 22.8% increased basal lipolysis in comparison with the control, respectively (Figure 1). Epinephrine-stimulated lipolysis was also influenced by I3C at all concentrations. We noted 10.2%, 10.0%, 11.9% increased epinephrine-stimulated lipolysis (Figure 2).

Effect of I3C on lipogenesis

Figures 3 and 4 show (U-^{14}C)glucose conversion to lipids in adipocytes exposed to three doses of I3C (1 μM, 10 μM, 100 μM) in the absence and in the presence of insulin. We noted that I3C clearly inhibited basal lipogenesis at three different concentrations. The lipogenesis activity after the treatment with I3C in comparison with the control was 67.6%, 73.3% and 69.3%, respectively (Figure 3). Insulin-stimulated lipogenesis was also strongly reduced by I3C

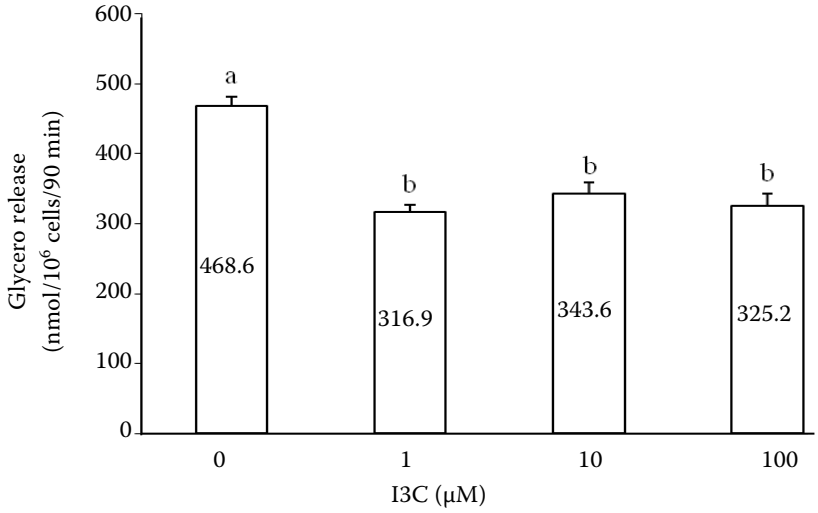

Figure 3. The effect of indole-3-carbinol on basal lipogenesis from glucose in isolated rat adipocytes. Each column represents the mean ± SEM for five replications (n = 5). Mean values marked by different letter superscripts differ statistically ($P \leq 0.05$)

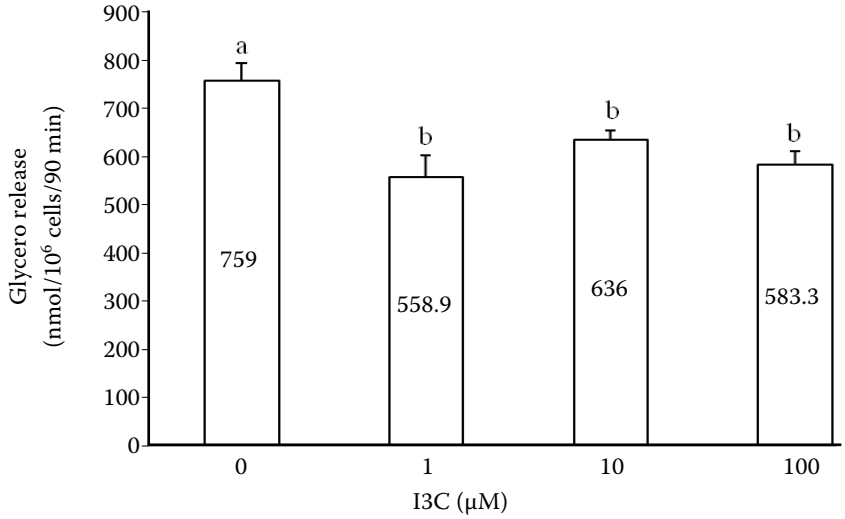

Figure 4. The effect of indole-3-carbinol on insulin-stimulated (1nM) lipogenesis from glucose in isolated rat adipocytes. Each column represents the mean ± SEM for five replications (n = 5). Mean values marked by different letter superscripts differ statistically ($P \leq 0.05$)

at all tested concentrations, in no dose-dependent manner. The insulin-stimulated lipogenesis activity was 73.6%, 83.8% and 76.9% in comparison with the control, respectively (Figure 4).

In vivo

The results obtained in the experiment *in vivo* are presented in Table 1. The administration of I3C at the dose of 150 mg/kg b.wt./day caused a significant increase in the content of triglycerides (60.9%) and a decrease in glycogen (23.8%) in the liver. A considerable increase in glucose (36.0%), triglycerides (32.2%), cholesterol in high-density lipoprotein (21.3%) and insulin (53.6%) with a concomitant decrease in FFA (65.4%) concentrations was noted in the serum.

I3C did not alter phospholipids, total, free, esterified cholesterol in the serum and liver cholesterol.

Table 1. The influence of oral administration of I3C on blood insulin and metabolic parameters in rats

Hormonal and metabolic parameters	Maize oil	Maize oil + I3C
Blood serum		
Insulin (ng/ml)	0.56 ± 0.07[a]	0.86 ± 0.08[b]
Glucose (mmol/l)	5.41 ± 0.22[a]	7.36 ± 0.21[b]
Free fatty acids (mmol/l)	0.26 ± 0.02[a]	0.09 ± 0.01[b]
Phospholipids (mmol/l)	1.21 ± 0.17	1.36 ± 0.15
Triglycerides (mmol/l)	2.70 ± 0.11[a]	3.57 ± 0.11[b]
Total cholesterol (mmol/l)	1.43 ± 0.07	1.48 ± 0.11
Free cholesterol (mmol/l)	0.49 ± 0.03	0.50 ± 0.03
Esterified cholesterol (mmol/l)	0.50 ± 0.05	0.49 ± 0.08
HDL-cholesterol (mmol/l)	0.47 ± 0.02[a]	0.57 ± 0.04[b]
Liver		
Cholesterol (mg/g WT)	2.12 ± 0.10	2.19 ± 0.09
Triglycerides (mg/g WT)	8.03 ± 0.65[a]	12.92 ± 0.86[b]
Glycogen (mg/g WT)	36.63 ± 1.78[a]	27.92 ± 2.48[b]

WT = wet tissue

I3C was administered intragastrically at the dose of 150 mg/kg body weight/day for 14 days. Values are given as mean ± SEM for eight animals. Mean values in rows marked by different letter superscripts differ statistically ($P \leq 0.05$)

DISCUSSION

Adipose tissue lipolysis is the major regulator of the body's supply of lipid energy because it controls the release of fatty acids into the plasma, where they circulate as FFA complexes to albumin. Hormone sensitive lipase of adipose tissue (HSL, EC 3.1.1.3) is the proximal and determinant enzyme for whole-body lipid fuel availability. We can corroborate that adipocytes are susceptible to the direct action of I3C (Figures 1, 2, 3, 4). It was demonstrated in the present report that I3C significantly induced basal as well as epinephrine-stimulated (1 μM) lipolysis in isolated rat adipocytes at all used concentrations of I3C (1, 10, 100 μM) (Figures 1, 2). The intensity of these processes was not significantly dependent on the I3C concentration. Basal lipolysis (triglyceride decomposition) is a hormone-independent process providing glycerol and fatty acids continuously. The ability of indole-3-carbinol to stimulate basal lipolysis indicates that the action of this compound involves changes in further steps of the lipolytic cascade, i.e. adenylate cyclase, cAMP content, protein kinase A (PKA), hormone sensitive lipase activity or translocation of this enzyme from the cytosol to the lipid droplet (Szkudelski and Szkudelska, 2002). The exact mechanism of I3C in this activation is difficult to ascertain at this stage. Whereas the noted intensified lipolysis in adipocytes in the presence of epinephrine indicates the stimulatory influence of I3C on the epinephrine action. The incubation of adipocytes with I3C at the three above-mentioned concentrations resulted in its influence on the restriction of glucose entry into adipocytes in the basal as well as insulin-stimulated conditions (Figures 3, 4). Glucose conversion to lipids is preceded by glucose transport into cells and involves its metabolism to acetyl-CoA and, finally, malonyl-CoA. Then, long-chain fatty acids are formed and esterified to triglycerides. The inhibitory effect of I3C on basal glucose metabolism to lipids indicates that it restricts predominantly insulin independent steps of lipogenesis, without influencing the insulin receptor. In basal lipogenesis glucose is transported predominantly via GLUT1 and this transport is insulin-independent. Therefore, it is possible that tested I3C attenuates lipogenesis via disturbance of the hexose transporter GLUT1. However, we noticed also the anti-lipogenetic action of I3C in insulin-stimulated conditions (Figure 4). It is quite possible that tested I3C, additionally, diminished the insulin-induced uptake of glucose into adipocytes. I3C significantly attenuated glucose conversion to lipids, probably by disturbance of the function of insulin due to decreased glucose transporter GLUT4 translocation to the cell surface. The inhibitory effect of I3C on glucose transport may be accompanied by a substantial reduction in phosphatidylinositol 3-kinase (PI 3-K) activity. This enzyme regulates the insulin-induced translocation of glucose transporting protein-4 (GLUT4) and thereby enables the transport of glucose into fat cells (Szkudelska et al., 2007).

In the performed trial a significant influence of I3C on metabolism was also shown (Table 1). According to LeBlanc et al. (1994) the hepatic microsomal total cholesterol levels were not significantly altered following the treatment with 100 and 240 mg I3C/kg/day for 1 week in mice. It means that the pivotal activity of hepatic cholesterol 7α-hydroxylase, which enhances cholesterol conversion to bile acids, and hepatic enzymes cholesterol ester hydrolase were not altered by I3C. Such a result was also obtained in our trial, at the used dosage of 150 mg/kg b.wt./day of I3C in rats. However, according to these authors I3C decreased serum cholesterol levels, which we did not observe in our experiment. The unchanged level of total, free, esterified cholesterol in the serum in our trial could suggest limited generation of acid condensation products from the used I3C dosage because the results of many studies demonstrated that not I3C per se, but several acid condensation products of I3C inhibit acyl-CoA:cholesterol acyltransferase (ACAT) activity at micromolar concentrations (Dunn and LeBlanc, 1994). It is worth underlining that the inhibition of intestinal/hepatic ACAT plays pivotal roles in the impediment of cholesterol uptake from the intestinal cells or accumulation in the liver.

I3C itself is found by many researchers to be a highly enzyme-inducing cytochrome. It is known that several drugs inducing the microsomal cytochrome P-450-dependent enzyme system in the liver and the intestine may increase plasma HDL concentrations (Nanjee et al., 1996). These authors do not exclude the possibility that some phytochemicals may also have such effect. Such hypothesis was confirmed in our trial, where I3C caused an increase in the plasma HDL concentration. Since in our experiment the drop in liver glycogen was accompanied by simultaneous augmentation in the blood insulin and glucose concentration, it

can be postulated that lower liver glycogen storage results from the rise of glycogenolysis in I3C treated rats. The high concentration of serum glucose could be elevated, additionally, by disturbance of hexose transporters GLUT1, GLUT4, which was confirmed in our *in vitro* experiment. Therefore, the indirect I3C effect on an increase in the insulin level in blood serum seems to be more likely, by its enhancing glycogenolysis and disturbance of hexose transporters GLUT1 and GLUT4.

The symbiotic relationship between adipose tissue and hepatic TG mobilisation is clearly disturbed by I3C in our research. This impediment was confirmed by the elevated concentration of TG in serum and in liver and lowered concentration of serum FFA *in vivo* with lower lipogenesis and higher lipolysis in adipocytes *in vitro*.

Under normal conditions *de novo* TG synthesis represents only 5% of liver TG, therefore the majority of liver TG is from serum or intestinal fatty acids. The serum FFA are derived mainly from adipose tissue. Fatty acids released by adipocytes are the major substrates for the hepatic very low-density lipoprotein (VLDL) production, and in turn, VLDL TG fatty acids are returned to adipose tissue by the action of lipoprotein lipase (LPL, EC 3.1.1.34) (Gibbons et al., 2000). The excess lipolysis may induce hypertriglyceridaemia via the increased production of very low-density lipoproteins (Coppack et al., 1994). Therefore the intensified lipolysis *in vitro* observed in our research may explain hypertriglyceridaemia in the liver *in vivo*. Because we observed the simultaneous liver and serum TG enhancement, it is highly probable that the observed TG increase in the serum was not from an excessive flux of VLDL out of liver but mainly from the influence of I3C per se on the decreasing clearance of triglyceride-rich lipoproteins in serum derived from liver and intestine. The enzyme responsible for the hydrolysis of triacylglycerols from plasma lipoproteins, mainly chylomicrons and very low-density lipoproteins, is the above-mentioned lipoprotein lipase. Its activity is influenced by the nutritional and hormonal status and by environmental conditions. Therefore the putative I3C inhibition action on LP seems to be very likely. This hypothesis could additionally be confirmed by FFA depletion in serum despite intensified lipolysis, which was observed *in vitro*. The FFA drop in serum resulted from the limited accumulation of TG stored within adipocyte cytosolic lipid droplets by the inhibition of FFA release from VLDL TG. So I3C is hypothesized to attenuate not only lipogenesis *de novo* (by restriction of glucose entry into adipocytes) but also by inhibition of lipogenesis from FFA by LPL inactivation.

I3C showed multifaceted activities, influencing metabolic pathways during the long-term action *in vivo* and metabolism of isolated rat adipocytes *in vitro*. The elevated concentration of TG (in serum and liver), serum glucose, serum insulin and lowered concentration of serum FFA after I3C consumption show an adverse effect of this substance. HDL-cholesterol was moderately elevated after I3C administration, which is positive. To conclude, the obtained results indicate that the global effect of 150 mg/kg b.wt./day on lipid and carbohydrate metabolism is negative in rats.

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