ORIGINAL ARTICLE

HEME-OXYGENASE-1 UPREGULATED BY S-ADENOSYLMETHIONINE. POTENTIAL PROTECTION AGAINST NON-ALCOHOLIC FATTY LIVER INDUCED BY HIGH FRUCTOSE DIET

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Abstract

Excessive dietary fructose intake may have an important role in the current epidemics of fatty liver disease, obesity and diabetes- features of metabolic syndrome. We evaluated the relationship between lipid peroxidation and other oxidative stress biomarkers with changes in expression of heme oxygenase-1 (HO-1) in rat fatty liver, induced by high fructose diet (HFD) and the effect of S-adenosylmethionine (SAMe). Twenty-one male rats were randomly assigned to three groups of seven animals each: HFD (35% fructose in drinking water for 16 weeks) group, HFD + SAMe (20 mg/kg b.w. in drinking water for 16 weeks) group and control group. HO-1 expression, malonyl dialdehyde (MDA) (a marker of lipid peroxidation), triglycerides (TG), SH group levels and histological studies were performed on hepatic tissue. HFD group showed microvesicular steatosis without inflammation and fibrosis. In HFD+SAM group microvesicular steatosis was not established. The HO-1 expression was significantly increased in HFD rats. SEMe augmented the increase in expression of HO-1. The levels of MDA and TG were elevated in HFD group. In HFD rats with lower levels of SH exhibited higher expression of HO-1. SAMe inhibited the increase in lipid peroxidation and TG levels and prevented the decrease in SH levels. In conclusion, SAMe has an important hepatoprotective effect and its protection is most probably exerted by increasing the expression of the antioxidant enzyme HO-1, in order to prevent the development of fatty liver.

Rezumat

Aportul excesiv de fructoză în dietă, joacă un rol important în instalarea steatozei hepatice, obezității și diabetului - caracteristicile cheie ale sindromului metabolic. A fost evaluată legătura dintre peroxidarea lipidelor și altor biomarkeri de stres oxidativ, cu modificarea expresiei hem oxigenazei-1 (HO-1) în steatoza hepatică la șobolan, indusă prin dietă bogată în fructoză (HFD). De asemenea a fost evaluat efectul protector al S-adenozilmetioninei (SAMe). Douăzeci și unu de șobolani masculi au fost împărțiți în trei grupuri de câte șapte animale fiecare: grupul HFD (35% fructoză în apa de băut timp de 16 săptămâni), grupul HFD + SAMe (20 mg/kg corp în apa de băut timp de 16 săptămâni) și un grup control. Au fost determinate nivelele hepatice ale HO-1, al malonil dialdehidei (MDA) (marker al peroxidării lipidelor), trigliceridelor (TG), tiolilor (grupările SH). De asemenea au fost efectuate studii histologice. În grupul HFD au fost constatate steatoza microveziculară fără inflamație și fibroză, exprimarea semnificativă a HO-1, creșteri semnificative ale nivelurilor de MDA și TG, precum și niveluri mai scăzute ale grupărilor SH, comparativ cu cu grupul control. În grupul HFD+SAMe s-a regăsit o expresie crescută a HO-1, fără steatoză microveziculară, niveluri semnificativ mai mari ale grupărilor SH și mai mici ale MDA și TG în comparație cu grupul HFD.

Keywords: high fructose diet, non-alcoholic fatty liver, heme-oxygenase-1, S-adenosylmethionine

Introduction

Excessive fructose intake may play an important role in the current epidemics of metabolic syndrome, including fatty liver disease, obesity and diabetes [5, 12, 15, 19, 24]. Fructose promotes both *de novo lipogenesis* and intrahepatic lipid inhibition of long-chain fatty acid mitochondrial beta-oxidation, triglyceride formation and steatosis followed by non-alcoholic fatty liver disease (NAFLD) [27, 38]. High fructose diet (HFD) increases protein fructosylation, and formation of reactive oxygen species that require quenching by hepatic antioxidants [1]. Increased production of reactive oxygen species (ROS) and the imbalance between oxidant and antioxidant species play an important role in the pathogenesis of NAFLD. Various nutraceuticals along with drugs such as carnosine, R/S-α-lipoic acid, rutin, quercetin, resveratrol/melatonin combination, vitamin E etc. given to animals on high fructose diet protect liver against oxidative damage and prevent from steatosis [11, 14, 21, 28, 32, 37].

The dietary supplement S-Adenosylethionine (SAMe) has been reported to have, both cytoprotective and antioxidant properties [9]. A variety of clinical trials indicate that treatment with SAMe is beneficial in the case of chronic liver diseases [1, 6, 16, 17, 25, 30]. SAMe is the biological methyl donor. As a precursor of glutathione and being one of the important antioxidants, it exerts a key function in liver, plays a critical role in oxidative stress defence and is involved in redox balance homeostasis [4]. SAMe has been demonstrated to provide protection against liver injury caused by alcohol and other hepatotoxins in animal models by preventing depletion of glutathione and oxidative stress [10, 39]. SAMe has antioxidant and cytoprotective properties demonstrated in human liver injuries related with its depletion [13, 18]. However, the pathogenetic mechanism of SAMe effect on fructose-induced hepatic damage has not been yet fully investigated.

Heme oxygenase-1 (HO-1) is a stress-protein responsible for the break-down of heme into carbon monoxide (CO) and bilirubin and free iron [33]. Bilirubin is a potent free radical scavenger and acts as a strong antioxidant. CO has been shown to provide vasodilatory, antiapoptotic and antiinflammatory actions and support the cytoprotective effects of bilirubin [36]. The HO-1-dependent release of free iron during heme catabolism results in the increase of a secondary antioxidant protein, ferritin [9]. Recent studies suggest that activation of HO-1 is frequently detected in various pathological states under conditions of cellular oxidative stress [25]. Upregulation of HO-1 plays a key role in maintaining antioxidant/ oxidant homeostasis in case of cellular injury [40]. Activation of HO-1 plays a protective role in models of hepatic damage, induced by alcohol and other hepatotoxins [2, 22, 33].

On the basis of these finding we examined the relationship between lipid peroxidation and other oxidative stress biomarkers with changes in expression of HO-1 in rat fatty liver, induced by HFD and the effect of SAMe.

Materials and Methods

Animal models

Male albino Wistar rats were housed at 20 ± 2ºC room temperature and with a standard 12 h light/dark cycle. All animals received a standard diet and water *ad libitum*. The standard diet was composed of starch – 50%, protein – 20%, fat – 4.5%, 5% cellulose, standard vitamins and mineral mix. At the beginning of the experiment the body weight of rats was 140 - 180 g. After acclimatization (two weeks) animals were randomly assigned to three groups of seven animals each: HFD (35% fructose in drinking water for 16 weeks) group, HFD+SAMe (20 mg/kg b.w. in drinking water for 16 weeks) group and a control group (water drinking). Fructose (Amilym, Bulgaria) and SAMe (NOW Foods, USA) were used.

At the end of the experiment, rats were killed using a lethal dose of thiopental. All procedures were performed at 4 - 8ºC. Analysis was performed immediately after thawing of the samples. The experimental procedures were approved by the Home Office for Care and Use of Laboratory Animals and performed with a strong consideration for ethics of animal experimentation according to the International Guiding Principles for Animal Research approved in Bulgaria.

Tissue collection

Under general anaesthesia with thiopental 30 mg/kg b.w. (rapid intravenous) a laparotomy was conducted. The weight of both the body and the liver of the rats were registered. Tissue extracts were prepared by homogenisation with phosphate buffer saline (PBS) = 50 mM, pH = 7.4 (1 g tissue, 9 mL of buffer) on ice using a teflon glass homogenizer (2,000 rpm/3 min). Homogenates were centrifuged in a refrigerated centrifuge (4,000 rpm/10 min). The supernatant was used for further analysis, after the quantification of proteins by usual biochemical methods.

Biochemical assays

Membrane lipid peroxidation was assayed by malonyl dialdehyde (MDA) measured by its thiobarbituric acid (TBA) (Merk, Germany) reactivity in hepatic homogenates using the method detailed by Porter *et al.* [29]. Results were expressed in nmol MDA per g tissue and were determined using the extinction coefficient of MDA–TBA complex at 532 nm = 1.56 x 10^{-5} cm^{-1} M^{-1} solution.

Hepatic SH groups were determined by the method of Hu [5], based on the absorption of the colour complex between thiol groups and 5,5'-dithiobis-(2-nitrobenzoic acid (DTNB) (Merk, Germany) at 412 nm. Standard solutions of reduced glutathione were used to calculate the concentration of thiol groups.

The serum concentrations of TGs were determined using a commercially available kit (Merk, Germany) and the automatic analyser Olympus AU640.

Histological study

For the histological examination, there were used liver specimens with dimensions 10 mm length, 10 mm wide and 5 mm thick, fixed with 10% formalin (pH = 7.0). The material processing included de-hydratation with an ascending series of alcohols and embedded in paraffin. Sections (5 µm thick) were stained with hemalaun and eosin (H&E) and histologically evaluated in order to assess hepatic changes.

Immunohistochemistry

Rat liver specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. The sections (5 µm thick) were treated with 1% hydrogen peroxide for peroxidase activity inhibition for 5 min. Then they were rinsed in 0.1 M phosphate buffered

saline (PBS) (pH = 7.4) and treated in normal goat serum for 20 min. Subsequently, the sections were incubated with the polyclonal primary antibody for 24 h at room temperature. Rabbit anti-HO-1 antibody (Santa Cruz, USA) was used. After rinsing with PBS the sections were incubated for 20 min in goat anti-rabbit immunoglobulins at room temperature. Then they were rinsed in PBS again, treated with rabbit peroxidase antiperoxidase complex for 20 min at room temperature and then rinsed in PBS. Finally, the peroxidase activity was estimated by the diamino-benzydine-tetrachloride H_2O_2 method. Negative controls were incubated with non-immune sera instead of the primary antibody.

Statistical analyses. All results were expressed as means ± SEM as indicated in the figures and table. Statistical significance of the studied parameters was performed by Student's t test. p-values less than 0.05 were considered as statistically significant.

Results and Discussion

Biochemical assays

The levels of MDA (with 25.3%; $p < 0.005$) (Figure 1) and TGs (with 63.3%; $p < 0.05$) (Figure 2) were elevated in HFG group compared to control group.

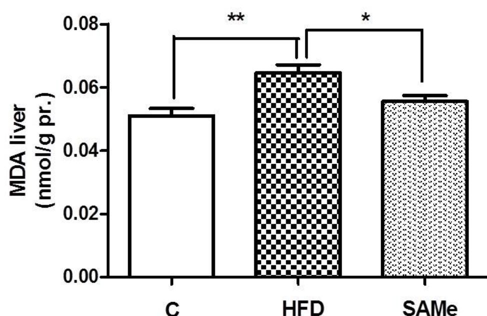

Figure 1.

Effect of SAMe on HFD – induced changes in hepatic MDA in rats

Mean levels ± SEM; n = 7; C - control group rats; HFD - fructose-drinking rats; SAMe - fructose- drinking rats treated with SAMe; * $p < 0.05$ - SAMe *vs.* HFD group; ** $p < 0.005$ - HFD *vs.* control group

SAMe decreased the levels of lipid peroxidation product (by 14%; $p < 0.05$) and TGs levels (by 56%; $p < 0.005$), which reached levels close to the control group.

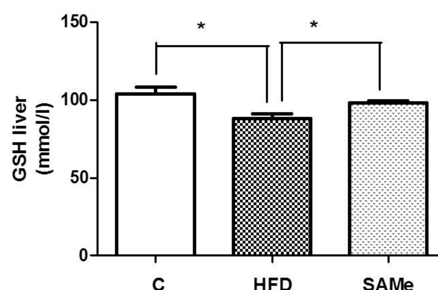

Figure 2.

Effect of SAMe on HFD – induced changes in hepatic GSH levels in rats

$^{*}p < 0.05$ HFD *vs.* control group; $^{*}p < 0.05$ SAMe *vs.* HFD group

The hepatic SH levels were decreased (by 15.2%; $p < 0.05$) in HFG group compared to control group, while in SAMe group these levels were elevated (by 11.2%; $p < 0.05$) compared to HFD group and reached levels close to the control group (Figure 3).

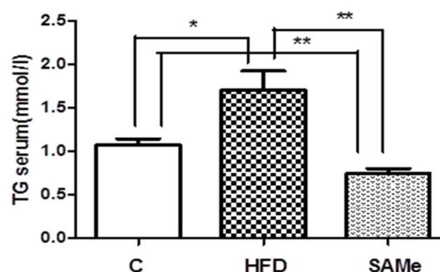

Figure 3.

Effect of SAMe on HFD – induced changes in TG serum in rats

$^{*}p < 0.005$ - HFD *vs.* control group; $^{**}p < 0.05$ - SAMe *vs.* HFD group; $^{**}p < 0.05$ - SAMe *vs.* control group

Histological study

Liver cells in HFD group showed microvesicular steatosis over 10% of the hepatocytes (Figure 4). The changes were localized in centrilobular areas of the liver lobules while under treatment with SAMe microvesicular steatosis was not established.

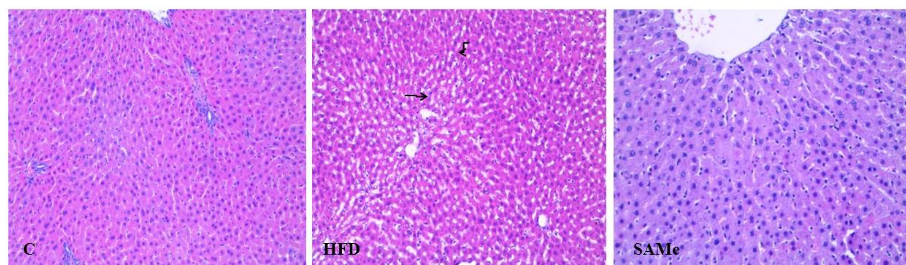

Figure 4.

Effect of SAMe on HFD – induced histological changes in rats liver

Mean levels ± SEM; n = 7; C - control group rats; HFD - fructose-drinking rats; SAMe - fructose- drinking rats treated with SAMe. H & E staining, original magnification, 200x, 400x

Immunohistochemical study

The control group (C) showed a weak expression of HO-1 established in sinusoidal cells (Figure 5) with no expression detected in liver cells. HFD group demonstrated unevenly dyed liver cells with mainly moderate intensity of staining. Both the HFD and SAMe group showed alike HO 1 staining intensity in sinusoidal cells. In SAMe group the number of positive for HO-1 liver cells was increased as compared to HFD group. HO-1 intensity in liver cells varied from moderate to high.

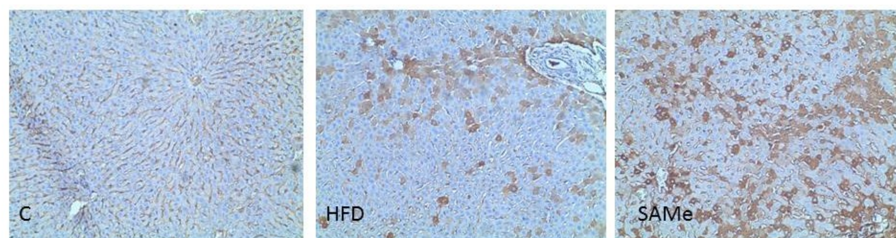

Figure 5.

Effect of SAMe on HO-1 expression in rats liver; (immunohistochemistry, 400x).

Immunohistochemically detection of heme-oxygenase-1 (HO-1) in liver showed that it is localised in SECs of the liver in the control (C) group. The staining intensity of HO-1-positive cells was weak. In HFD group, HO-1 positivity was observed in the sinusoidal cells. It was moderate to strong in the individual cells. In the HFD treated with SAMe, HO-1 positivity was observed principally in the sinusoidal cells. The number of HO-1 positive cells was higher than in the burn non-treated group.

In this study, we showed out that the protective effect of SAMe on the liver cells in an experimental model of HFD, induced liver damage carried out by an increased expression of the antioxidant enzyme HO-1. There were no such changes in sinusoidal cells.

We established that keeping rats on a high-fructose diet causes TG levels increase, fat accumulation in the liver and liver steatosis. In case of fatty liver the surplus of fatty acid and the excessive oxidation leads to ROS production accompanied by oxidative stress [22, 36]. Our data demonstrated enhanced oxidative stress that corresponds with the histological changes in rats kept on high-fructose diet. Compared to the control group, the lipid peroxidation process was raised in the HFD group. The morphological changes in the liver observed in the study were in accordance with the increased MDA in liver homogenates. Unlike the increased level of MDA, the SH levels are considerably decreased. Glutathione, as a principal non-protein SH pool plays a crucial role in redox balance maintaining and oxidative stress defence [35]. It is known that glutathione in the liver decreases due to its depletion in oxidative stress conditions. Our results and those of other authors, suggest that oxidative stress and altered redox balance play a crucial role in the pathogenesis of steatosis [38]. We showed that SAMe avoids SH depletion, increases antioxidant defence, restricts elevation of lipid peroxidation and TG level and inhibits steatosis. SAMe provides a source of cysteine for the production of GSH, the main source of non-protein SH fraction [7, 16, 17]. The antisteatotic effect of SAMe may be due to its role as a modulator of the reduced glutathione liver content. The beneficial effect of SAMe on gluthatione and oxidative stress are also observed in ethanol induced fatty liver [17].

Recently, attention has been focused on HO-1 because of its broad bioactivity [2, 18, 29, 31]. HO-1 catalyses the conversion of heme into CO, bilirubin and ferritin [4]. Bilirubin converts from biliverdin and carbon monoxide (CO) exhibits antioxidant activity, vasodilation and inhibition of platelet aggregation, respectively [8]. Therefore, HO-1 induction might also be important in the antioxidant defence and the anti-inflammatory response [26, 33, 34].

In this study the expression of HO-1 was found significantly increased in hepatocytes. There is an evidence that HO-1 induction can increase resistant capacity against oxidative stress and related liver damages [23, 42]. We suggest that the induction of HO-1 in rat hepatocytes may be an adaptive response to fructose-induced oxidative stress, but this is not enough to protect against cellular stress in fatty liver induced by HFD.

In agreement with other authors, we found that the use of the SAMe protects against liver damage [9]. On the other hand the induction of HO-1 in hepatocytes is higher in the group SAMe as opposed to HFD. The increased expression of the HO-1 suggests that at least part the hepato-protective effect of SAMe in HFD may be due to HO-1. Previous data show that activation of HO-1 by antioxidants may provide a cytoprotection against oxidative stress and the redox unbalance in liver damage-induced by ethanol and other hepato-toxins [3, 38].

We suggest that SAMe acts as a redox modulator, which activates HO-1 and its antioxidant and cytoprotective effects are related in part with the increased expression of this enzyme. Recent studies show that activation of HO-1 by SAMe plays a protective role in models of hepatic damage, induced by alcohol indicating that the activation of this enzyme may be an endogenous defence mechanism to reduce tissue injury [9]. These results suggest that the up-regulation of HO-1 expression attenuates oxidative stress and inhibits progression of liver injury [41, 42].

Conclusions

In conclusion, the present study shows that SAMe attenuates HFD-induced oxidative stress and steatosis through HO-1 upregulation. Increased levels of HO-1 and its enzymatic products by SAMe may stimulate the antioxidative capacity against the HFD-induced oxidative stress and hepatic damage.

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