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The flavonoid monoHER promotes the adaption to oxidative stress during the onset of NAFLD

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease. An evidence-based pharmacological treatment for NAFLD is still lacking, but flavonoids have shown therapeutic potential. The present study was designed to investigate the effect of the flavonoid monoHER on the onset of NAFLD in *Ldlr*$^{-/-}$ mice on a high-fat and high-cholesterol diet. The focus was put on the effect on oxidative stress as well as the adaptive response. Wild type mice served as a control and the effect of monoHER was compared to that of a placebo.

In the *Ldlr*$^{-/-}$ group, monoHER provided only a mild protection against oxidative stress. In the placebo *Ldlr*$^{-/-}$ group an adaptive response elicited by the NRF2 antioxidant defense system was observed, evidenced by a higher *HO-1* and *Gpx3* gene expression, as well as an increased redox status, evidenced by the higher GSH/GSSG ratio. In the monoHER treated *Ldlr*$^{-/-}$ group both the adaptive response as well as the increase in redox status tended to be higher, although this did not reach significance on a group level. Unexpectedly, a strong within animal relationship was found that links a high adaptive response to a low redox status in the monoHER *Ldlr*$^{-/-}$ group. This correlation was absent in the placebo and wild type group.

The concept that emerges is that a thiol-reactive oxidation product of monoHER, formed during oxidative stress, selectively induces the NRF2 pathway and enforces the endogenous antioxidant shield, to provide protection against NAFLD.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in Western countries affecting 20–30% of the general population, and this percentage is still on the rise. NAFLD includes a spectrum of liver disorders ranging from steatosis to non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis and hepatocellular carcinoma [1]. Up to date, no evidence-based pharmacotherapy is available for NAFLD. To find a way to prevent NAFLD, the molecular processes involved in the onset of the disease should be controlled.

One of the hallmarks in the etiology of NAFLD is oxidative stress [2,3]. During oxidative stress, the unbalanced production of reactive oxygen species (ROS) results in damage to virtually any cellular component, which explains the prominent role of ROS in NAFLD. However, ROS also destruct KEAP1, the inhibitor protein of NRF2. NRF2 serves as a master redox switch which turns on the expression of endogenous antioxidant genes e.g. *HO-1* and *Gpx3* [4] and ultimately increases the cellular redox potential. Consequently, cells adapt to ROS through this potent feedback mechanism which enforces the protection against ROS. This fits

Abbreviations: NAFLD, non-alcoholic fatty liver disease; *Ldlr*$^{-/-}$, low-density lipoprotein receptor knockout; monoHER, 7-mono-O-(β-hydroxyethyl)-rutoside; NRF2, nuclear factor erythroid 2-related factor 2; *HO-1*, heme oxygenase-1; *Gpx3*, glutathione peroxidase 3; GSH, reduced glutathione; GSSG, glutathione disulfide; NASH, Non-alcoholic steatohepatitis; ROS, reactive oxygen species; WT, wild type; MDA, malondialdehyde; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); TNB, 5-thio-2-nitrobenzoate; p, placebo.

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in the current concept that health is a dynamic process characterized by the ability to adapt to challenges on a delicate homeostasis [5].

In this concept of health, drugs are bioactive compounds that help to restore and enforce homeostasis. Numerous studies have demonstrated the beneficial effect of flavonoids – a group of antioxidant bioactives commonly found in our diet – in animal models of NAFLD [6]. Among the flavonoids, 7-mono-O-(β-hydroxyethyl)-rutoside (monoHER) appears to be one of the most promising ones for treatment of NAFLD [6]. This flavonoid is the main ingredient of Venoruton, a drug that has been used for several decades in the treatment of venous insufficiency. MonoHER displays excellent antioxidant activity [7,8], has a relatively high bioavailability and is relatively safe [6]. Based on these favorable characteristics monoHER was selected for this study.

To evaluate its efficacy, the effect of monoHER on oxidative stress and the adaptive response during the onset of NAFLD was determined in an animal model. Previous studies have shown that *Ldlr*$^{-/-}$ mice on a high-fat and high-cholesterol diet are a suitable model to study the development of NAFLD [9]. Wild type mice served as control and the effect of monoHER was compared to that of a placebo. The effect was evaluated on three levels, namely the direct damage by ROS, the NRF2-induced gene expression and the adaptive response on the cellular redox status.

2. Materials and methods

2.1. Animals and treatment

Female C57BL/6J wild type mice (WT mice) were obtained from Jackson laboratories (Bar Harbor, Maine, USA) and bred at the breeding facility of Maastricht University. Female LDL-receptor knockout mice on a C57BL/6J background (*Ldlr*$^{-/-}$ mice) were bred at the breeding facility of Maastricht University. Mice were maintained in a temperature- and light-controlled facility and were permitted ad libitum consumption of water and chow. At the age of 13 weeks, all mice started a high-fat and high-cholesterol diet (HFD) (D11012302) (Research Diets, New Brunswick, USA) with 0.2% cholesterol and fat derived from palm oil, which provided adequate levels of vitamins (Vitamin Mix V10001, Research Diets, New Brunswick, USA). The mice were divided in four experimental groups: WT mice treated with placebo ($n = 12$), WT mice treated with MonoHER ($n = 12$), *Ldlr*$^{-/-}$ mice treated with placebo ($n = 12$) and *Ldlr*$^{-/-}$ mice treated with MonoHER ($n = 12$). MonoHER was administered daily subcutaneously at a dosage of 500 mg/kg of body weight (25 μl/g of body weight). With this dose, monoHER provided complete protection against doxorubicin-induced cardiotoxicity in mice [10]. Daily subcutaneous injection of a physiological saline solution (25 μl/g of body weight) was used as placebo. Mice were weighed every three days and food intake was recorded for every 2 mice housed together. Diet and treatment were continued for 3 weeks, after which mice were anaesthetized with 0.05 mg/kg buprenorphine and isoflurane 0.4 l/min and sacrificed by exsanguination from the vena cava. The liver was removed and divided in different parts for further investigation. Part of the large left lobe was used for RNA isolation.

2.2. Direct damage by ROS

To determine the direct damage by ROS, oxidative damage to poly-unsaturated fatty acids in the liver was determined by measuring malondialdehyde (MDA) formation. Liver homogenate (250 mg liver/ml) was prepared in 145 mM potassium phosphate buffer (pH 7.4). MDA was determined by reaction of MDA with thiobarbituric acid [11] and quantified by HPLC [12].

2.3. Cellular redox status

The cellular redox status was assessed by quantifying the GSH/GSSG ratio. Total glutathione content and glutathione disulfide (GSSG) were measured according to the enzymatic recycling method [13] quantifying the conversion of DTNB into TNB spectrophotometrically at 412 nm and 37 °C. The GSH/GSSG-ratio was calculated from the content of GSSG and total glutathione.

2.4. Activation of the Nrf2 antioxidant defense system

An adaptive response by the NRF2 antioxidant defense system was evaluated by measuring *HO-1* and *Gpx3* gene expression in the liver. Liver parts that were stored in RNAlater® at −80 °C were used for RNA isolation using miRNeasy Mini Kit with DNase treatment (Qiagen, Venlo, The Netherlands) according to protocol. Isolated RNA was converted into cyclic DNA (cDNA) using iScript cDNA synthesis kit (Bio-rad, Veenendaal, The Netherlands). Two step qRT-PCR was performed using 12.5 μl SensiMix SYBR & Fluorescein Kit (Bioline, London, UK) with 5 μl cDNA, and 300 nM of each primer (*HO-1*: forward: 5′-GAGCCTGAATCGAGCAGAAC-3′, reverse: 5′-CCTTCAAGGCCTCAGACAAA-3′, *Gpx3*: forward: 5′-CATCCTGCCTTCTGTCCCT-3′, reverse: 5′-ATGGTACCACTCATACCGCC-3′) in a 25 μl reaction mixture. PCR was conducted as follows: denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 45 s. Following PCR, a melting curve (60–95 °C) was produced for product identification and purity check. *Gapdh* (Glyceraldehyde-3-phosphate dehydrogenase) and *Ywhaz* (Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein, Zeta Polypeptide) (Eurofins, Breda, The Netherlands) were used as housekeeping genes [14]. An average value of the expression of the house keeping genes *Gapdh* (forward: 5′-TTGATGGCAACAATCTCCAC-3′, reverse: 5′-CGTCCCGTAGACAAAATGGT-3′) and *Ywhaz* (forward: 5′-CAGCAGATGGCTCGAGAATA-3′, reverse: 5′-GAAGCATTGGGGATCAAGAA-3′) was used for the calculation of the ΔCt-value. The validity was confirmed by the high within-animal correlation between the values of *Gapdh* and *Ywhaz* ($R^2 = 0.8041$). Primer sequences are listed in Table 1. Data were analyzed using the MyIQ software system (BioRad, Veenendaal, The Netherlands) and were expressed as relative gene expression (fold change) using the $2^{\Delta\Delta Ct}$ method.

2.5. Statistics

Data were analyzed with GraphPad Prism 5 (Graphpad software, CA, USA). Values presented in the study are expressed as mean ± standard deviation (SD). The relationships of the GSH/GSSG ratio with *HO-1* and *Gpx3* gene expression were evaluated using linear correlation. Differences between groups were analyzed using the Kruskal–Wallis test. Post-hoc testing was performed using the Mann–Whitney *U*-test with Bonferroni correction. A $P < 0.05$ was considered to be statistically significant, $P < 0.1$ and >0.05 as indicating a trend.

Table 1
Activation of the NRF2 antioxidant defense system. *HO-1* and *Gpx3* gene expression was measured to evaluate the activation of the NRF2 antioxidant defense system in liver tissue of WT and *Ldlr*$^{-/-}$ mice treated with placebo (p) or monoHER. Data represent fold change compared to WT + p group. Mean ± SD is shown.

Gene	WT + p	WT + monoHER	*Ldlr*$^{-/-}$ + p	*Ldlr*$^{-/-}$ + monoHER
HO-1	1.00 ± 0.40	0.98 ± 0.29	2.27 ± 1.48*	2.66 ± 1.25**
Gpx3	1.00 ± 0.57	1.43 ± 0.51	2.21 ± 1.01*	3.02 ± 1.09**

* $P < 0.05$ compared to WT + p group.
** $P < 0.05$ compared to WT + monoHER group.

3. Results and discussion

There were no significant differences in food intake between the groups (Supplemental data). Initial and also final body weight and liver weight did not differ between the groups. In the placebo treated *Ldlr*$^{-/-}$ mice only a trend towards increased liver/total body weight-ratio compared to placebo treated WT mice was observed, confirming that in the relatively short period of 3 weeks only the onset of NAFLD was studied.

A trend was seen towards a higher average level of lipid peroxidation (MDA) in *Ldlr*$^{-/-}$ mice compared to WT mice (Fig. 1A), although this difference failed to reach significance. No pronounced effect of monoHER against ROS damage was seen. This could be due to the relatively large variation in the results. An explanation for the relatively large variation is that the onset of the disease is examined. At the onset of the disease, the effects are relatively small compared to the biological variation. Moreover, the time course of the gradual progression into a clear manifestation of the disease will vary between the mice studied. The effect was only examined at a single point in time, and this also contributes to a relatively large inter-animal variation. The relatively large variation might explain that only a mild effect was found on a group level.

The general protective effect of monoHER against oxidative stress can be explained by its ability to scavenge ROS [7]. However, scavenging might block the activation of the innate NRF2 pathway by ROS. In this case, the paradoxical consequence is that no protective adaptation is elicited because antioxidant administration prevents the endogenous defensive shield to be raised. This has fueled the vexed dispute on the use of antioxidants in the prevention and treatment of diseases [15]. However, in the present study it was found that monoHER did not block the adaptive response; in contrast, a trend towards further increased levels of *HO-1* and *Gpx3* gene expression was found with monoHER (Table 1).

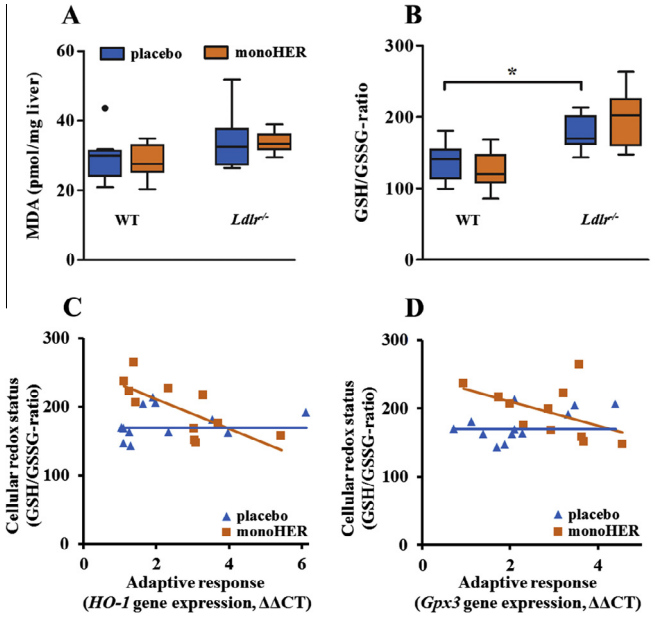

Fig. 1. Direct damage by ROS (determined as MDA formation) and cellular redox status (determined as GSH/GSSG ratio). In panel A and B the Tukey Box-plot is shown of MDA and GSH/GSSG ratio, measured in liver tissue of WT and *Ldlr*$^{-/-}$ mice treated with placebo or monoHER. *$P < 0.01$. The outlier is included in the statistical evaluation. Panel C and D show the within-animal linear correlation between the cellular redox status and the NRF2-dependent adaptive response during the onset of NAFLD in the liver of *Ldlr*$^{-/-}$ mice. In the group treated with monoHER, the adaptive response, expressed as *HO-1* (C) and *Gpx3* (D) gene expression, was relatively high at a low cellular redox status, indicated by a low GSH/GSSG ratio ($P = 0.012$ and $P = 0.046$ respectively). This relationship is lacking in the placebo group.

The higher NRF2 induced gene expression in *Ldlr*$^{-/-}$ mice (Table 1) was corroborated by the adaptive response on the cellular redox status (Fig. 1B). In *Ldlr*$^{-/-}$ mice monoHER also tended to further increase the GSH/GSSG-ratio in *Ldlr*$^{-/-}$ mice (Fig. 1B).

That monoHER does not inhibit the adaptive response, can be explained by the formation of a thiol reactive oxidation product of monoHER when it scavenges radicals. This oxidation product of monoHER selectively adducts a cysteine residue in KEAP1 and this adduction of KEAP1 induces the adaptive response [8]. In fact, the rather unspecific reactivity of ROS that does not only destroy KEAP1 but also vital cellular components such as other proteins and DNA, is exchanged for the much more selective reactivity of the oxidized product of monoHER towards KEAP1 [8].

MonoHER administration did not induce an adaptive response in the WT animals. In this respect, it should be noted that monoHER itself lacks thiol reactivity. MonoHER first needs to be converted into a thiol-reactive oxidation product to induce an adaptive response [8]. This conversion happens when monoHER scavenges radicals. In the control animals the generation of the thiol-reactive oxidation product will be relatively low. Therefore, no adaptive response as a result of monoHER administration is expected in the control animals.

The formation of the thiol-reactive oxidation product, exclusively during oxidative stress can be seen as a form of targeting. It results in an adaptive enforcement of the antioxidant system when this is needed. Interestingly, in the monoHER treated *Ldlr*$^{-/-}$ mice also evidence for targeting was seen. We expected that monoHER would activate NRF2 which subsequently would results in a higher cellular redox status. This was indeed found, but to our surprise in the monoHER group an improved redox status was associated with a relatively low NRF2 activation. Apparently, monoHER administration results in a relatively potent adaptation. In the monoHER treated *Ldlr*$^{-/-}$ mice NRF2 activation was relatively high at a low GSH/GSSG ratio. This indicates that a relatively low redox status, i.e. a low GSH/GSSG ratio, tends to increase NRF2 activation in the monoHER treated *Ldlr*$^{-/-}$ group. Moreover, in the liver of animals that have a relatively high GSH/GSSG ratio, no evidence for further stimulation of NRF2 by monoHER was seen, indicating that in the animals that have adapted, no further adaptation is needed (Fig. 1C and D). In retrospect, this relationship is more logical than the anticipated high redox status when the adaptive response is high, i.e. that in the adapted animals more adaptation is needed, which of course is not necessary. No correlation was found in the corresponding placebo group and in both control groups with WT mice probably because there was no substantiated adaptation. The higher level of adaptation and the reported correlation with NRF2 activation in monoHER treated *Ldlr*$^{-/-}$ mice was absent in the other group. This indicates that monoHER has promoted the adaptive response.

In the present *in vivo* study, only a mild protective effect of monoHER against oxidative stress was seen. As mentioned above, this may be related to the relatively low level of oxidative stress and relatively large variation in the lipid peroxidation marker (Fig. 1A) because the onset of pathology was examined. An additional reason for this mild protection is that monoHER is administered once a day, whereas the half-life of monoHER is only half an hour [16]. Most of the time, monoHER will be practically absent [17], and for full protection by radical scavenging the compound has to be present continuously at a relatively high concentration. Scavenging of radicals at moments when concentrations of monoHER are relatively high, can explain the subtle effect of monoHER administration on oxidative stress. In addition, as mentioned above, the scavenging of radicals and the concurrent formation of the thiol-reactive oxidation product can selectively activate the

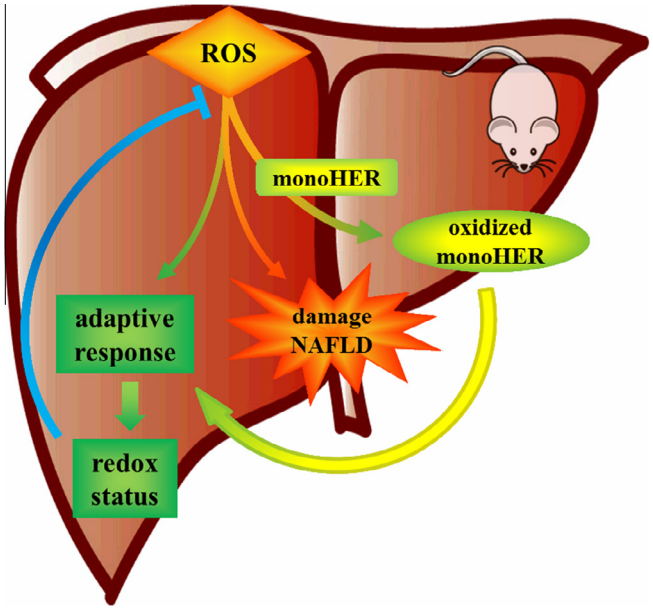

Fig. 2. Overview of the protective effect of monoHER in the onset of NAFLD in mice. The high and therefore non selective reactivity of ROS results in damage to virtually any cellular compound. This oxidative damage is a hallmark in the etiology of NAFLD. ROS also activate Nrf2 which leads to adaptation. By scavenging the ROS, oxidation products of monoHER are formed that promote the adaptation of the cell to oxidative stress. This helps to maintain homeostasis and prevents that relatively mild perturbations gradually progress into a severe pathology such as NAFLD.

innate NRF2 machinery. As demonstrated previously, a single and acute exposure to a thiol-alkylating agent can induce an adaptive response [18–20]. This would mean that in order for monoHER to induce an adaptive response, only part of the radicals needs to be scavenged since this already leads to the formation of the thiol-alkylating oxidation product of monoHER.

Flavonoids have therapeutic potential in NAFLD which relates to their protection against oxidative stress. Our study supports a modified mechanism of action of the antioxidant flavonoid monoHER, which might also apply for other free radical scavenging antioxidants, as illustrated in Fig. 2. The results show that the direct scavenging of radicals is only one aspect of protection by monoHER. The present study shows that activation of the antioxidant defense system (i.e. the activation of NRF2) by the oxidized flavonoid also plays a crucial role in the protective effect against oxidative stress (i.e. the improved GSH/GSSG ratio). This molecular mechanism does not only result in a very potent antioxidant effect, it also ensures that monoHER will act selectively in a condition of oxidative stress. In this way monoHER empowers the buffering capacity of the body to maintain homeostasis and to prevent that relatively mild but nevertheless insidious and persistent perturbations gradually progresses into a severe pathology, such as NAFLD.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.11.055.

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