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ORIGINAL ARTICLE

Cholesterol overload in hepatocytes affects nicotinamide adenine dinucleotide phosphate oxidase (NADPH) activity abrogating hepatocyte growth factor (HGF) induced cellular protection

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Abstract

The increment in the prevalence of obesity incidence in Mexico is leading to the increase in many chronic maladies, including liver diseases. It is well known that lipid-induced liver sensitization is related to the kind of lipid rather than the amount of them in the organ. Cholesterol overload in the liver aggravates the toxic effects of canonical liver insults. However, the status on the repair and survival response elicited by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and the hepatocyte growth factor (HGF) is not completely understood. In the present, work we aimed to figure out the HGF/NADPH oxidase-induced cellular protection in the hepatocyte with a cholesterol overload. Our results show that a hypercholesterolemic diet induced liver damage and steatosis in mice. The hepatocytes isolated from these animals exhibited an increase in basal NADPH oxidase activity, although transcriptional levels of some of its components were decreased. No effect on the oxidase activity was observed in HGF treatments. The protective effect of HGF was abrogated as a result of cholesterol cellular overload, calculated by a survival assay. In conclusion, the cholesterol overload in hepatocytes impairs the HGF/NADPH oxidase-induced cellular protection. (Gac Med Mex. 2015;151:428-35)

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Introduction

Obesity represents the main public health problem in our society, since, just as diabetes and metabolic syndrome, it is considered a pandemic disorder in developed countries[1].

Non-alcoholic fatty liver disease (NAFLD) is considered the hepatic manifestation of metabolic syndrome; however, this disorder can occur independently of this condition, since it is part of the natural history of many liver diseases[2].

Although fatty liver is assumed to be the result of trl-glyceride (TG) accumulation, the toxic potential of other lipids, such as cholesterol, has been poorly studied. Over the past few years, some experimental studies have reported that perhaps cholesterol overload represents the main toxic mediator in NAFLD or steatohepatitis[3,4]. In humans, free cholesterol has been found to be

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increased in patients with non-alcoholic steatohepatitis (NASH) and was correlated with sterol regulatory element-binding protein (SREBP-2) activation, a transcriptional factor that drives the expression of enzymes involved in the synthesis of cholesterol[5].

Cholesterol is a lipid with an indisputable biological importance; it is an essential component of biologic membranes and is precursor of many hormones and biliary salts; however, loss of balance in this lipid generates problems that impact on health, either due to lack or to excess of it[6].

Recently, we published that a high-cholesterol (HC) diet (2% cholesterol and 0.5% sodium cholate) disrupts the HGF-mediated repair process in the liver[4].

HGF represents one of the main liver protection mediators when it interacts with its receptor, the C-Met proto-oncogene; a signaling cascade is initiated, mediated by systems such as PI3K/Akt, Stat3 or Erk, which activate systems conferring survival, mitogenesis, motogenesis and antioxidant protection[7-9]. Our reserch group has been characterizing the latter aspect in recent years. We have reported that HGF is able to induce expression of antioxidant enzymes such as catalase and superoxide dismutase (SOD), which confer protection against toxic effects induced by the metabolism of ethanol[8] or by antifimic drugs rifampicin and isionazid[10]. On the other hand, we have reported that the absence of c-Met signaling determines the seriousness of fibrogenic[11] and carcinogenic[12,13] processes in studies caried out in mice with c-Met conditional elimination in the liver.

Recently, we published that HGF/c-Met regulates pro-oxidative systems, such as NADPH oxidase, and HGF was found to exert a biphasic effect on the system, thus stimulating the production of reactive oxygen species (ROS) in short periods and transcriptionally suppressing them in periods longer than 12 h of treatment with the growth factor. This biphasic regulation was shown to elicit hepatocyte protecting and survival responses in both cases[14]. As a matter of fact, global transcriptome analyses or microarrays carried out by our team have shown that the simple elimination of the c-Met receptor increases NADPH oxidase activity and oxidative stress due to the loss of c-Met control on oxidase[12].

NADPH oxidase is a multi-component system formed by membrane sub-units such as catalitic NOX1-5 and p22; on the other hand, there are regulating sub-units found in cytosol, such as p47 and p67, among others, which are recruited by NOX and p22 to start the production of ROS, particularly superoxide anion[15]. One of the best preserved and distributed systems in practically all cell types is the NOX2 system, which requires p47 activation for an efficient function. p47 is known to be a central regulator in the activity of the enzyme and that it is activated by kinases such as protein kinase C, which in turn is activated by HGF/c-Met[14].

Knowing that all different forms of NADPH oxidase are found as integral protein membranes and that a cholesterol overload can markedly affect cell membranes, in the present work we have focused on the study of the regulating effect exerted by HGF on the NADPH oxidase system in hepatocytes with cellular cholesterol overload.

Materials and methods

Animals

In the present studio, 8-12-week old male mice of the C57BL6 strain were used. The animals were kept in the animal care facility of the Instituto Nacional de Rehabilitación and their handling was carried out according to the NOM-062-ZOO-1999 standard and the NIH Guidelines for the Care and Use of Laboratory Animals. The animals were maintained under controlled temperature conditions (22 °C), light-dark cycle of 12:12 h and a food and water free-access regimen. Two groups were formed, with 10 animals each. Prior to the start of the diet, the mice were fasted for 12 h. The first group was fed for 48 h with a standard balanced diet (Chow), and the second group was fed a hypercholesterolemic diet consisting of the standard Chow diet supplemented with 2% of cholesterol and 0.5% of sodium cholate.

Experimental design

Once the treatment was finished, whole blood was drawn from the animals in both groups and serum was separated to perform liver function biochemical tests, as well as to determine the lipid profile. Hepatic tissue was sectioned to be fixed in neutral paraformaldehyde at 4% and to be embedded in paraffin for histological analyses; other part was frozen at -80 °C until its utilization.

Histology

7-µm sections of the paraffin embedded tissues were obtained and routine staining with hematoxylin and eosin was carried out.

Biochemical and liver function tests

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), cholesterol and TG measurements were analyzed with an automated method using the Reflotron system (Roche, Inc.).

Hepatocyte isolation and primary culture

Other group of animals was used for hepatocyte isolation, which were obtained using the double perfusion method with collagenase, following the protocol previously reported by Gómez-Quiroz, et al. in 2008[16]. The hepatocytes were cultured in Petri dishes at a density of 250,000 cells/cm^2 in Williams E medium supplemented at 10% with fetal bovine serum (FBS) and incubated in a controlled athmosphere (37 °C, 5% CO_2). The adhered hepatocyte monolayer was washed with phosphate saline buffer and the medium was replaced with another free of FBS.

The cultured hepatocytes were treated with 50 ng/ml of HGF (Peprotech, USA) at different times (0.5, 1, 3, 6 and 12 h). After the HGF treatment, the cells were used to determine the lipid content or were lysated to extract total protein for Westrn blot. Other group of cells was used to determine the NADPH oxidase activity.

Determination of free cholesterol with filipin

The cholesterol content in hepatocytes was determined using filipin in hepatocytes isolated from both groups following the protocol reported by Marí, et al[3].

Determination of ROS in hepatocytes

The determination of ROS, particularly of superoxide anion, was performed according to a previous report[10].

Western blot

The protein content analysis was carried out by Western blot, according to the previously reported protocol[17]. Antibodies against NOX2, p47 and Nrf2 were used, all of them obtained from Santa Cruz Biotechnology. Load normalization was carried out with anti-actin (NeoMarkers, Fremont, CA).

Histological determination of neutral lipids

The determination of neutral lipids was performed using the oil red O technique, as previously reported[4].

NADPH oxidase activity

Enzyme activity was established by superoxide anion determination in a system with NADPH oxidase inhibitors, as reported by Pescatore, et al. in 2012[18].

Viability assay

Viability was determined using the CCK-8 commercial kit (Dojindo, Inc) following the manufacturer's instructions. 5,000 cells/well were seeded in 96-well plates; subsequently, the medium was removed and were supplemented with a serum-free fresh medium.

The Chow and HC cells were treated with antimicyn A (AA) (15 µM) for 12 h, in the presence or not of HGF (50 ng/ml). Additionally, another group of cells was pre-treated with diphenyl iodonium (DPI), a NADPH oxidase inhibitor (10 µg/ml) 30 min prior to the treatment with HGF; subsequently, AA was added.

Protein quantification

Protein quantification was performed using the commercially available BCA Protein Assay Kit (Pierce-Thermo Scientific, Inc.), based on the bicinchoninic acid technique following the manufacturer's instructions.

Statistical analysis

Data are represented as the mean ± standard error in at least three independent experiments. The comparison between groups was performed using a one-way analysis of variance test, with a *post hoc* Bonferroni test using the GraphPad Prism 5 software for Mac OS X. Differences were considered significant with a p-value < 0.05.

Results

After two days of treatment, the animals did not show significant changes with regard to their behavior, total weight and the liver (data not shown). When the liver was examined, a pale coloration was noticed in the liver of animals fed with the HC diet, just as we had reported previously[4].

In order to determine the steatotic phenotype in the HC diet-fed animals, routine hematoxylin-eosin staining

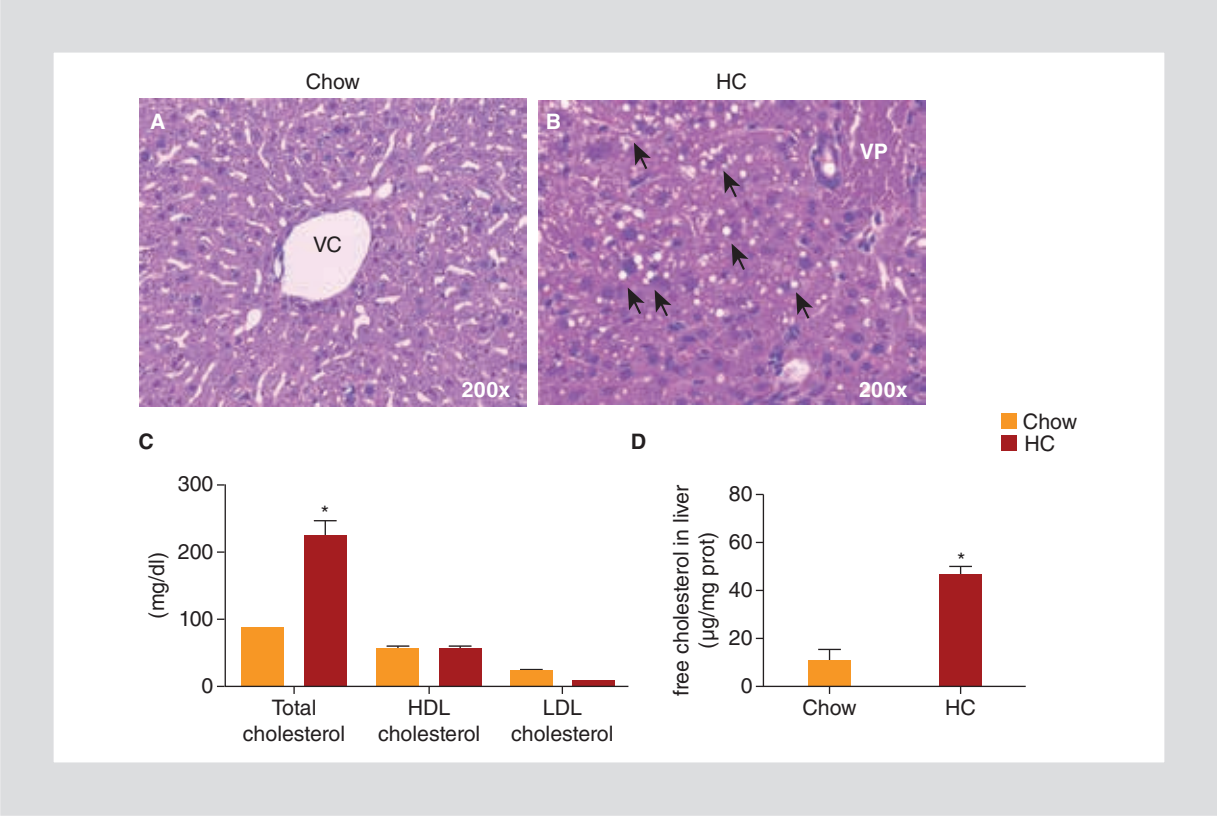

Figure 1. *The HC diet induces steatosis. The livers of animals fed with regular Chow or HC diet for 2 days were extracted and prepared for analysis with hematoxylin and eosin.* **A:** *Chow liver section.* **B:** *HC liver section. Arrows indicate lipid droplets. Representative images of at least 3 animals. Original augmentation: 200X.* **C:** *lipid profile.* **D:** *free cholesterol determined by automated methods. Each bar represents the mean ± SEM. *p < 0.05 versus Chow.*

was performed. Figure 1 B clearly shows a steatosis, particularly microvesicular compared with balanced Chow diet-fed animals (Fig. 1 A).

An analysis of the animals' serum lipid profile was conducted, where total cholesterol (CL) and triglyceride levels were found to be elevated (Fig. 1 C), with no significant changes in HDL and LDL. Similarly, CL was found to be elevated in HC animals liver tissues (Fig. 1 D) with regard to the Chow animals.

Liver damage was evidenced by a significant increase in AST, ALT and LDH enzymes activity in HC animals with regard to Chow animals.

Subsequently, hepatocytes were isolated and maintained as a primary culture. In order to verify that the isolation and culture process did not affect both cholesterol and neutral lipids overload, detection of free cholesterol was performed by microscopy using filipin, a natural antibiotic that recognizes the free cholesterol hydroxyl group by fluorescence emission, and oil red O for neutral lipids. Figure 2 B clearly shows that HC mice hepatocytes exhibit more fluorescence with regard to Chow mice; the fluorometric analysis is shown in figure 2 C. Very similarly, HC hepatocytes showed a significant increase in the content of neutral lipids in comparison with Chow hepatocytes (Figs. 2 E and F).

Previously, we reported that, in primary hepatocytes, HGF exerts a biphasic effect on NADPH oxidase, by increasing its activity at short periods and decreasing it at long periods of treatment[14]. In order to assess if cholesterol overload modifies this behavior, we performed an assay on the enzyme's activity by determining superoxide anion generation in presence or absence of DPI, an oxidase inhibitor, and SOD, a superoxide dismutating enzyme; figure 3 shows that HC hepatocytes exhibit a significant increase in baseline activity (HC NT) with regard to non-treated Chow cells; however, HGF was unable to modify in any way the activity of the enzyme.

A Western blot analysis on the content of the main catalitic (NOX2) and regulator (p47) components of the NADPH oxidase system, as well as of the transcription factor that responds to NADPH oxidase (Nrf2), were analyzed by Western blot. Figure 4 shows the densitometric analysis of these proteins and representative

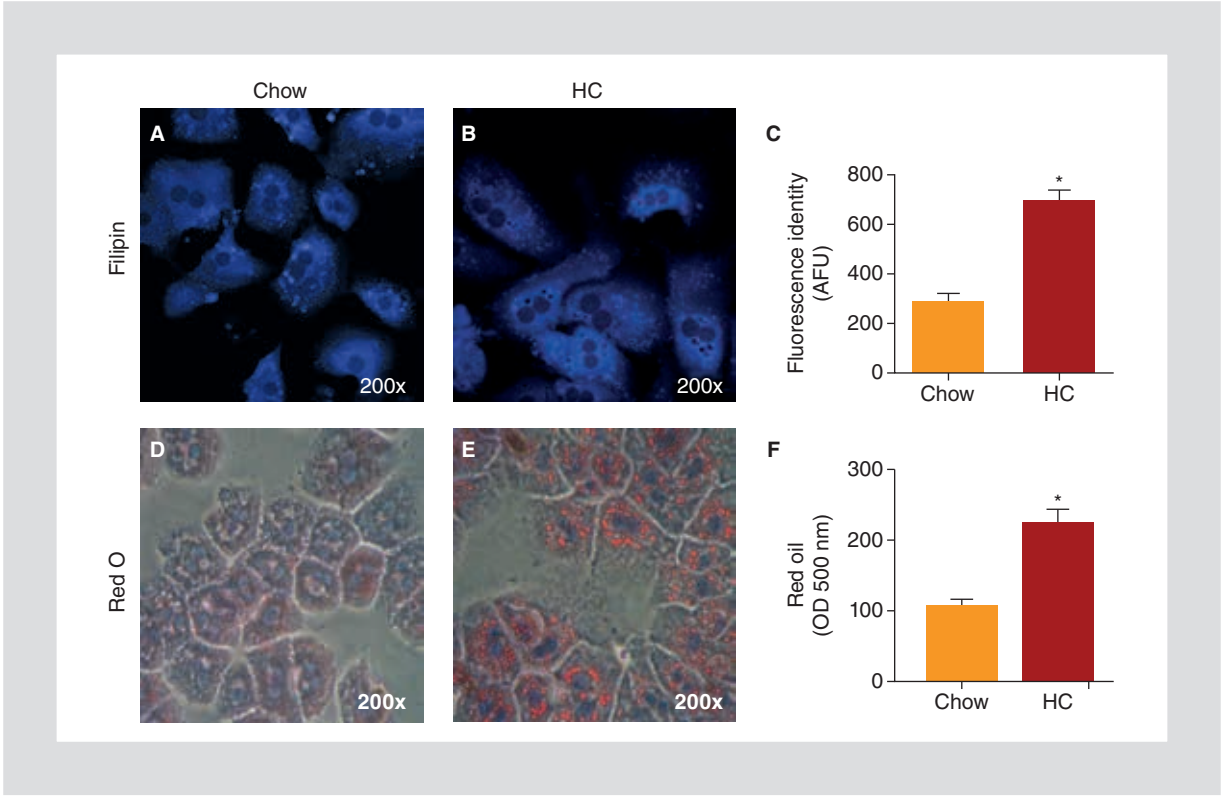

Figure 2. *Cholesterol and lipid contents in isolated and cultured hepatocytes. Hepatocytes were isolated using the double perfusion method with collagenase and were cultured in the conditions specified in the material and methods section. The presence of cholesterol was determined with filipin in Chow* **(A)** *and HC hepatocytes* **(B)**. *Quantification of filipin intensity* **(C)** *was performed with the Image J. software. Neutral lipids were detected with the oil red O test in Chow* **(D)** *and HC hepatocytes* **(E)**. *Subsequently, the red oil was extracted and quantified by spectrophotometry at 500 nm (F). Photographs are shown at the 200X original augmentation. Each bar represents the mean ± SEM. FAU: fluorescence arbitrary unit. *p < 0.05 versus Chow.*

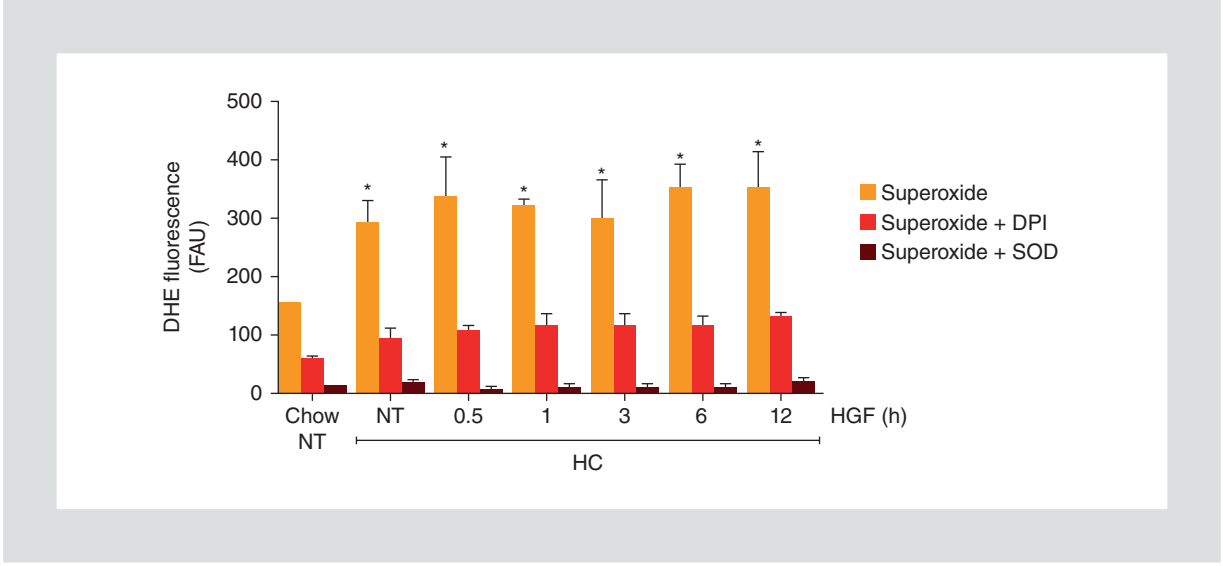

Figure 3. *NADPH oxidase activity. Hepatocytes were isolated from Chow and HC mice, cultured and treated or not with 50 mg/ml of HGF for the indicated times. The production of superoxide anion was quantified according to the technique specified in the "Materials and methods" section in the presence or not of the NADPH oxidase inhibitor DPI or the SOD enzyme. Each bar represents the FAUs mean ± SEM. *p < 0.05 versus Chow.*

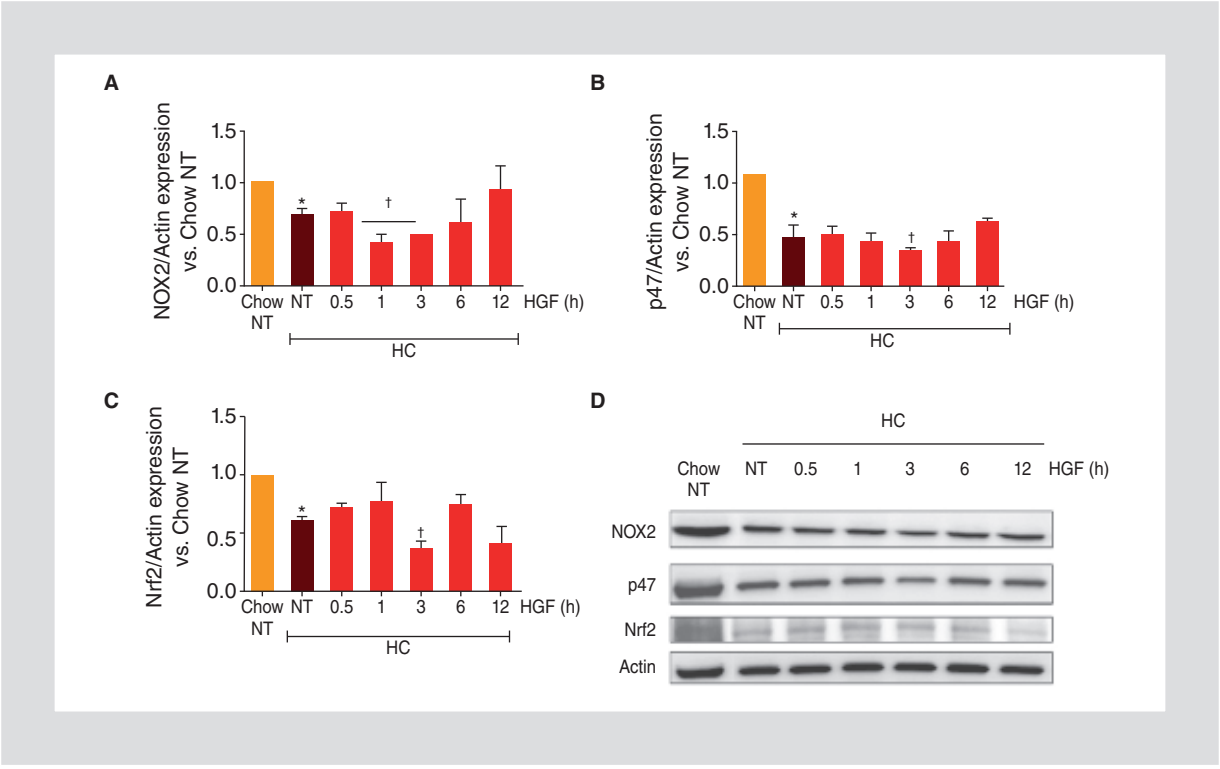

Figure 4. *Expression levels by Western blot. Total protein was isolated and the Western blot assay was carried as specified in the "Materials and methods" section; at least 3 independent tests were run. The densitometric analysis graph is reported.* **A:** *NOX2.* **B:** *p47.* **C:** *Nrf2.* **D:** *representative gels. Actin was used as loading control. Each bar represents the mean ± SEM. *p < 0.05 versus Chow NT. †< 0.05 versus HC NT.*

gels (Fig. 4 D). The data show that far from having a transcriptional increase, a significant decrease was observed in HC NT cells with regard to Chow NT. Transcriptionally, HGF induced a decerase at 1 and 3 h of treatment in NOX2, at 3 h in p47 and at 3 h in Nrf2 with regard to HC NT.

Finally, to determine the protecting state that HGF has in HC hepatocytes, a viability study was conducted with CCK-9. Figure 5 shows that HGF was able to protect against AA pro-oxidative effects, whereas the HC cells did not show any HGF-mediated protecting effect. The NADPH oxidase inhibitor DPI abrogated HGF's protecting response observed in Chow cells with no effect on HC cells.

Discussion

The serious problem existing in Mexico with obesity, especially in children, has been widely documented[19], and it is affecting human beings not only in the cardiocirculatory system or the metabolism, but also at the hepatic level, where its impact on disorders such as NAFLD and NASH has been documented.

The World Health Organization has made it clear that the three main risk factors for chronic diseases are smoking, hypertension and elevated cholesterol plasma levels[20]. Some studies have shown, particularly in Mexican adolescents and children, that cholesterol levels are too high, which positions them as persons at high risk for suffering chronic diseases in adulthood[21-23].

A high cholesterol diet has been reported to generate an increase in the content of this lipid in experimental animals' hepatocytes, showing that the liver is more susceptible to cytotoxic stimuli mediated, for example, by the tumor necrosis factor α. This sensitization exerted by accumulated lipids occurred exclusively in animals with cholesterol overload and not in those receiving a diet that favored TG, but not cholesterol, overload, thus confirming that it is the type of lipids, not the quantity, what determines liver damage[3].

The HC diet fed to the animals was shown to exert a clear steatogenic effect; histologies shown in figure 1 reveal liver tissue with steatosis, particularly microvesicular, whereas free cholesterol serum levels are significantly higher with regard to the animals on the Chow

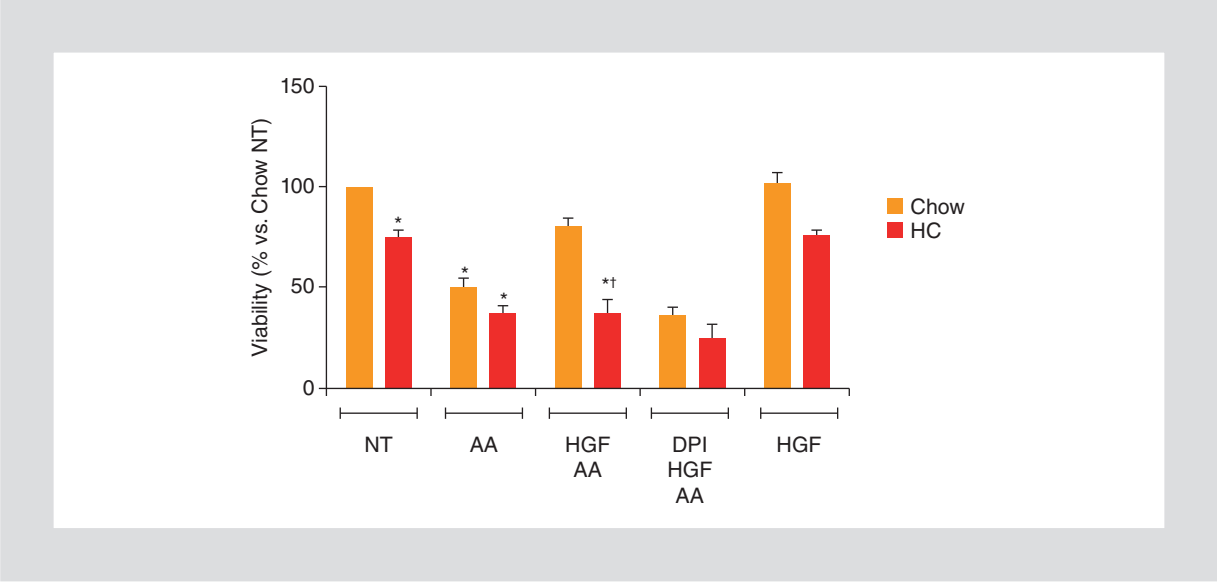

Figure 5.

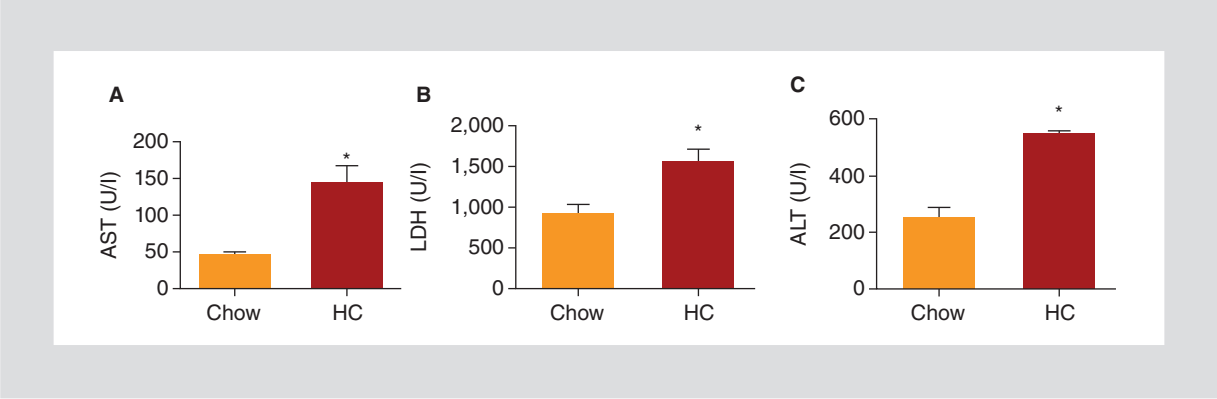

Figure 6. *Liver function tests.* **A:** *AST.* **B:** *LDH.* **C:** *ALT. The determination was carried out using automated methods as referred in the "Materials and methods" section. Each bar represents the mean ± SEM. *p < 0.05 versus Chow.*

diet, with no HDL and LDL changes (Fig. 1 C). Studies conducted in patients with steatosis and NASH revealed that the free cholesterol content is higher in NASH than in steatosis, and this was correlated with transcription factor SREBP-2 induction[5].

Liver damage was demonstrated by an increase in liver function tests values (Fig. 6), which were significantly elevated in animals fed with the HC diet, which is consistent with previously reported observations by Marí, et al[3], thus underscoring again that the cholesterol content is related to the severity of liver damage.

In order to directly know the effect on the protecting mechanism driven by HGF and NADPH oxidase in hepatocytes, we isolated hepatocytes from animals of both groups and analyzed the lipid contents. Figure 2 shows that even in cultures, hepatocytes retain both cholesterol (Fig. 2 B) and neutral lipid (Fig. 2 E) overload.

We determined the NADPH oxidase activity in HC hepatocytes. Figure 3 shows that non-treated HC cells exhibit a significant increase in NADPH oxidase activity with regard to non-treated Chow cells; however, unlike previous findings in Chow hepatocytes[14], HGF did not show any effect on this activity in HC hepatocytes. Clavijo-Cornejo, et al.[14] previously reported an activity increase from 5 min on, with a peak reached at 30 min of treatment and activity dropping at levels lower than those in the control at 24 h. Both effects

were shown to be esential to the protection driven by HGF. Early increase tended to activate Nrf2, a transcription factor in charge of the expression of antioxidant and phase II and III detoxification proteins[24-26], whereas HGF-mediated transcriptional repression prevented the use of NADPH oxidase by cytotoxic factors such as transforming growth factor β. The results obtained in the present research show that cholesterol overload abrogated NADPH oxidase activation by HGF. Furthermore, although diet itself induced transcriptional repression of proteins of the system such as NOX2, NOX4 and p47, HGF did not influence on this negative effect (Fig. 4).

Interestingly, data show a compensating result in NADPH oxidase, since although the HC diet reduced its expression, its activity is elevated, suggesting a clear compensatory effect.

Our group has characterized the effects of HGF as a liver-protecting factor. We know that the lack of its signaling causes oxidative, apoptotic, fibrotic and carcinogenic damages[11-23,16], clearly indicating how essential HGF response is to liver protection. In the present work we showed that cholesterol overload blocks HGF protecting response, leading to oxidative stress (Fig. 5).

These results strongly suggest that, in hypercholesterolemic states, HGF is unable to exert its protecting response, thus leaving the liver vulnerable, particularly against pro-oxidative challenges, as hepatitis B or C infections, alcohol or systemic inflammatory processes can be. Free cholesterol hepatic levels control is positioning as a new target for therapeutic intervention.

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