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Comparative therapeutic effects of metformin and vitamin E in a model of non-alcoholic steatohepatitis in the young rat

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ABSTRACT

Only in the last few years has non-alcoholic steatohepatitis been recognized as an important and relatively common liver disease. To date, the therapeutic options are limited, vitamin E and metformin have been proposed for the treatment of this condition, although their mechanisms are not completely clarified as yet. The aim of this study was to investigate the anti-inflammatory and anti-oxidative mechanisms of these drugs in an experimental model of non-alcoholic steatohepatitis in the young rat. Male rats, just after weaning, were divided into four groups: a control group that received a standard diet; a high fat diet group; two high fat diet fed groups treated with vitamin E or metformin, respectively. After 4 weeks, we evaluated in the liver the modification of lipid peroxidation, assessed by malondialdehyde, TNF-α levels, S-nitrosylated protein, inducible nitric oxide sinthase (iNOS), and peroxisome proliferators-activated receptors (PPAR) expression and metalloproteinase activity. High fat diet increased malondialdehyde, nitrotyrosilated proteins, and TNF-α tissue content. Moreover, a decrease of PPAR-α and an increase of PPAR-γ expression were observed. An increase of metalloproteinase activity was also shown. Among drug treatments, metformin reduced body weight gain and fat mass, metalloproteinase activity, and TNF-α tissue content, while it restored PPAR-α expression and downregulated PPAR-γ expression. Vitamin E reduced the oxidative damage, protein nitrotyrosilation, and tissue TNF-α levels. Moreover a decrease of PPAR-γ expression was also shown. These findings confirm the efficacy of both drugs as therapeutic tools in preventing the early onset of liver damage and non-alcoholic fatty liver disease progression.

1. Introduction

The hallmark of non-alcoholic steatohepatitis is the accumulation of large-droplet fat in hepatocytes, including fat alone as well as fat with non-specific inflammation. Although several predisposing factors, such as obesity, insulin resistance and type 2 diabetes, are related to the pathogenesis of non-alcoholic steatohepatitis, the exact mechanism of its progression to fibrosis and chronic liver disease is still unclear (Farrell and Larter, 2006). Simple steatosis and non-alcoholic steatohepatitis are similar in terms of excessive accumulation of fatty acids in the hepatocytes, that protect themselves by binding, transforming, catabolizing, and exporting excess of free fatty acids. The β-mitochondrial oxidation of these fatty acids is a source of free radicals and produces hydrogen peroxide and reactive oxygen species. Lipid peroxidation, together with cytokines, and other pro-inflammatory compounds are believed to play a critical role in the transition from steatosis to non-alcoholic steatohepatitis (Farrell and Larter, 2006; McCullough, 2006). The ligand-activated transcription factors belonging to the peroxisome proliferators-activated receptor (PPAR) family are involved in the regulation of energy homeostasis and inflammation. In particular, PPAR-α is prominently expressed in the liver, where its activation results in increased uptake and oxidation of free fatty acids (Reddy, 2001). Increased expression of PPARγ has been reported in high-fat diet-induced liver steatosis (Inoue et al., 2005). Overexpression of *PPARγ1* in liver of *PPARα* null mice induced the expression of lipogenesis-related genes, leading to hepatic steatosis (Yu et al., 2003). *PPARγ2* has also been reported to induce lipid accumulation in hepatocytes (Schadinger et al., 2005). Weight reduction through a healthy diet and a regular medium-intensity exercise is the mainstay of the current treatment, especially in children.

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Current therapeutic agents are focused on several targets at different steps in the pathogenesis of development of hepatic steatosis or progression to steatohepatitis in children (Kerkar, 2004; Roberts and Yap, 2006). Vitamin E (alpha-tocopherol) is a potent antioxidant particularly effective against membrane lipid peroxidation, therefore it is considered a cytoprotective agent. Besides, it has already been claimed as a possible therapeutic approach of non-alcoholic steatohepatitis in pediatric patients, in that it shows an improvement of serum aminotransferases (Lavine, 2000; Vajro et al., 2004).

Metformin is an insulin-sensitizing agent reported to reverse hepathomegaly, steatosis and liver tests abnormalities in a model of fatty liver (Lin et al., 2000). Clinical data reported a controversial metformin effect on liver damage markers (Marchesini et al., 2001; Nair et al., 2004; Bugianesi et al., 2005; Schwimmer et al., 2005). A recent meta-analysis about drugs improving insulin resistance in non-alcoholic steatohepatitis suggests that further clinical studies are needed to either support or refute the use of these drugs, which are however rated as having a favourable role (Angelico et al., 2007).

The aim of the present study was to comparatively evaluate the therapeutic effect of vitamin E and metformin in an experimental model of non-alcoholic steatohepatitis induced in the young rat.

For this purpose, young male rats, just after weaning, were allowed free access to high-fat diet, to induce the typical pathological features of steatosis (Lieber et al., 2004a,b). This animal model of steatosis, differently from a genetically modified animal (e.g. ob/ob mouse), might better mirror what happens in the human non-alcoholic steatohepatitis due to fatty diets.

In particular, 4 weeks after drug treatments, we investigated the modifications of liver lipid peroxidation, evaluated as malonyldialdehyde and tissue TNF-α content, hepatic protein nitrotyrosilation, inducible nitric oxide (iNOS), and PPAR-α and PPAR-γ expression. Moreover, we evaluated the activity of metalloproteinases, in particular metalloproteinase-2 and metalloproteinase-9.

2. Materials and methods

2.1. Drugs and reagents

High-fat liquid diet was purchased from Research Diets Inc. (New Brunswick, NJ, USA). Standard diet (Global diet 2018) was purchased from Harlan Italy (San Pietro al Natisone, Udine, Italy). Vitamin E and metformin (1,1-dimethylbiguanide hydrochloride) were purchased from Sigma Chemicals (Milan, Italy).

2.2. Animals and treatments

Young male Sprague-Dawley rats (average body weight 115.3 ± 3.1 g) were purchased from Charles River Laboratories (Wilmington, MA, USA) and randomly divided into four groups (n=6 animals for each group) as following: 1) a control group receiving the standard diet, 2) a high fat diet fed group; 3) high fat diet fed animals treated by gavage with vitamin E (30 UI/kg/daily), and 4) high fat diet fed animals treated by gavage with metformin (250 mg/kg/daily). Rats from the high fat diet and control groups were gavaged water as vehicle. After weaning, the animals were allowed free access to a standard diet containing 15% of energy derived from fat, 22% from protein, and 63% from carbohydrate or to a high fat diet that contained 71% of energy derived from fat, 11% from carbohydrates, and 18% from protein. This diet rich in unsaturated fat and low in carbohydrates developed the pathologic changes, key features of non-alcoholic steatohepatitis, already at 3 weeks, as previously reported (Lieber et al., 2004a,b). The pharmacological treatment started together with the high fat diet and continued for 4 weeks. All procedures involving the animals were carried out in accordance with the Institutional Guidelines and complied with the Italian D.L. no.116 of January 27, 1992 and associated guidelines in the European Communities Council Directive of November 24, 1986 (86/609/ECC).

2.3. Animal body weight, fat mass and tissue collection

All rats were weighed after weaning and before sacrifice, in order to calculate body weight modifications as obtained during the experimental period, elicited by different diets and treatments. Throughout the treatment period food intake was monitored once a week. Bioelectrical impedance analysis was applied to assess body composition at 4 weeks using a BIA 101 analyzer, modified for rat (Akern, Florence, Italy). Fat-free mass was calculated using the bioelectrical impedance analysis (50 kHz) prediction equation of Ilagan et al. (1993), and fat mass content was determined as the difference between body weight and fat-free mass. After 4 weeks treatment, all animals were anesthetized by enflurane and sacrificed by cervical dislocation. The livers were excised weighed and immediately frozen in liquid nitrogen and used for later experimental procedures. Blood was collected by cardiac puncture and centrifuged at 1500 ×g at 4 °C for 15 min and sera were stored at −70 °C for later measurements of aspartate amino transferase (AST), and alanine amino transferase (ALT) by standard automated procedures. In order to evaluate the effect of pharmacological treatment on steatosis, hepatic triglyceride content was also measured by using commercially available kit (Wako Pure Chemical Industries, Japan).

2.4. Liver Malonyldialdehyde and TNF-α level in livers

Malonyldialdehyde levels in the livers were determined as an indicator of lipid peroxidation. Tissues were homogenized in 1.15% KCl solution. An aliquot (200 µl) of the homogenate was added to a reaction mixture containing 200 µl of 8.1% SDS, 1.5 ml of 20% acetic acid (pH 3.5), 1.5 ml of 0.8% thiobarbituric acid, and 600 µl of distilled water. Samples were then boiled for 1 h at 95 °C and centrifuged at 3000 ×g for 10 min. The supernatant absorbance was measured by spectrophotometry, and malonyldialdehyde values were calculated by comparison with OD_{550} of standard solutions of malonyldialdehyde bis (dymethyl acetal) 1,1,3,3-tetramethoxypropan 99% (Sigma, Milan, Italy).

Portions of liver tissues were homogenized and tissue TNF-α levels were evaluated, using a colorimetric, commercial kit (Calbiochem-Novabiochem Corporation, USA) according to the manufacturer instructions. All TNF-α determinations were performed in duplicate serial dilutions.

2.5. Nitrotyrosine detection, iNOS, PPAR-α and PPAR-γ expression by Western blotting

Liver tissue (0.1 g) was disrupted by homogenization on ice in a lysis buffer. Protein lysates were subjected to SDS-PAGE (8% polyacrylamide). The blot was performed by transferring proteins from a slab gel to a nitrocellulose membrane (Protran nitrocellulose transfer membrane; Whatman Schleicher and Schuell, Dassel, Germany) and probed with specific antibodies against PPAR-α (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1:500), PPAR-γ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, 1:100), or anti-3-nitrotyrosine antibody (Upstate Biotechnology, Lake Placid, NY; 1:5000). The secondary antibody (antirabbit IgG peroxidase conjugate) was incubated for 1 h at room temperature. Western blot for β-actin protein (Sigma; 1:10 000) was performed to ensure equal sample loading. The protein bands of iNOS (~130 kDa), PPAR-α (~55 kDa), and PPAR-γ (~55 kDa) on x-Omat film (Eastman Kodak, Rochester, NY) were scanned and densitometrically analyzed with a model GS-700 imaging densitometer (Bio-Rad Laboratories, Hercules, CA).

2.6. MMPs activity

Gelatin-zymography was performed to determine the level of the active and pro-enzyme forms of MMPs, as previously described (Okada et al., 2001). Briefly, aliquots of liver standardised homogenates were subjected to electrophoresis in (2 mg/ml) gelatin-containing polyacrylamide gels in the presence of SDS under non-reducing conditions. After

Table 1
Modification of liver weight and transaminases in control (CON) or high fat diet fed rats (HFD) treated with vitamin E (HFD+VIT E) or metformin (HFD+MET) for 4 weeks

	CON	HFD	VIT E	MET
Liver weight (g/100 g)	3.82±0.07	4.42±0.08[a]	4.12±0.18	4.11±0.18
Liver triglyceride (mg/g)	100.3±4.2	274.3±14.5[b]	203.3±23.0[c]	178.0±15.3[d]
ALT (U/l)	45.25±2.02	60.25±2.29[a]	45.00±1.78[c]	56.00±4.32
AST (U/l)	161.80±4.53	236.50±16.54[a]	204.30±13.94	154.50±18.67[c]

Values are means±S.E.M. of 4 rats.
[a] $P<0.05$ vs CON.
[b] $P<0.001$ vs CON.
[c] $P<0.05$ vs HFD.
[d] $P<0.01$ vs HFD.

electrophoresis and washes, the gel slabs were then incubated at 37 °C overnight in 0.1 M Tris–HCl gelatinase-activation buffer (pH 7.4) containing 10 mM $CaCl_2$ and subsequently stained with 0.5% Coomassie Blue. After intensive destaining, proteolysis areas appeared as clear bands against a blue background.

MMPs were identified by their molecular weight compared with standards. 1,10-Phenanthroline, a general inhibitor of metalloproteinases, completely blocks both the inactive and active forms, thus confirming the specificity of these molecules (data not shown). To measure the activities of the detected enzymes, zymograms were read using a ScanJet 3c scanner (Hewlett-Packard, Boise, ID). The intensities of the separate bands were analyzed using Sigma Gel measurement software (Jandel, San Rafael, CA). Quantitative evaluations of both surface and intensity of lysis bands, on the basis of grey levels, were compared relative to non-treated control wells and expressed as 'relative expression' of gelatinolytic activity.

2.7. Statistical analysis

All data were presented as mean±S.E.M. All analysis were conducted using Graph-Pad Prism (Graph-Pad software Inc., San Diego, CA). Statistical analysis was performed by ANOVA test for multiple comparisons followed by Bonferroni's test. Statistical significance was set at $P<0.05$.

3. Results

3.1. Effect of drugs treatment on body and liver parameters

In rats receiving the high fat diet a significant increase in liver weight, hepatic triglyceride content and AST and ALT serum levels was observed as compared with rats receiving control diet (Table 1). Despite vitamin E

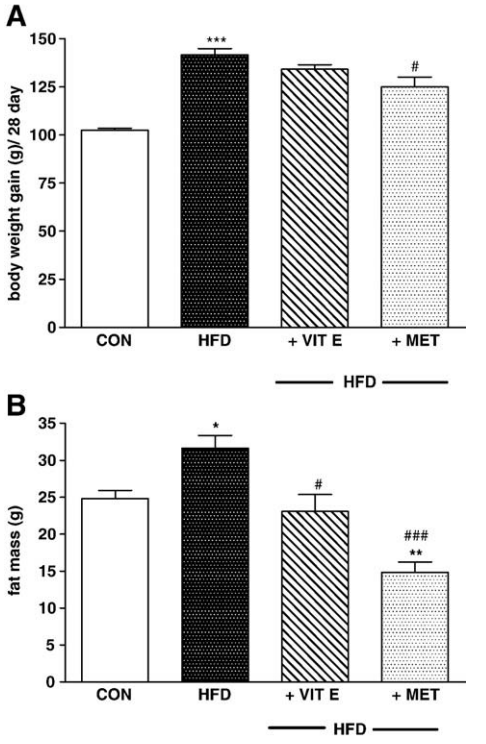

Fig. 1. Body weight gain (A) and fat mass (B) in rats from control diet (CON), high-fat diet (HFD) or high fat diet-fed groups treated with vitamin E (HFD+VIT E) or metformin (HFD+MET) for 4 weeks. Body weight gain was expressed in g/28 days. Values are given as means±S.E.M. of 6 rats.*$P<0.05$, **$P<0.01$, and ***$P<0.001$ vs CON; #$P<0.05$, ###$P<0.001$ vs HFD.

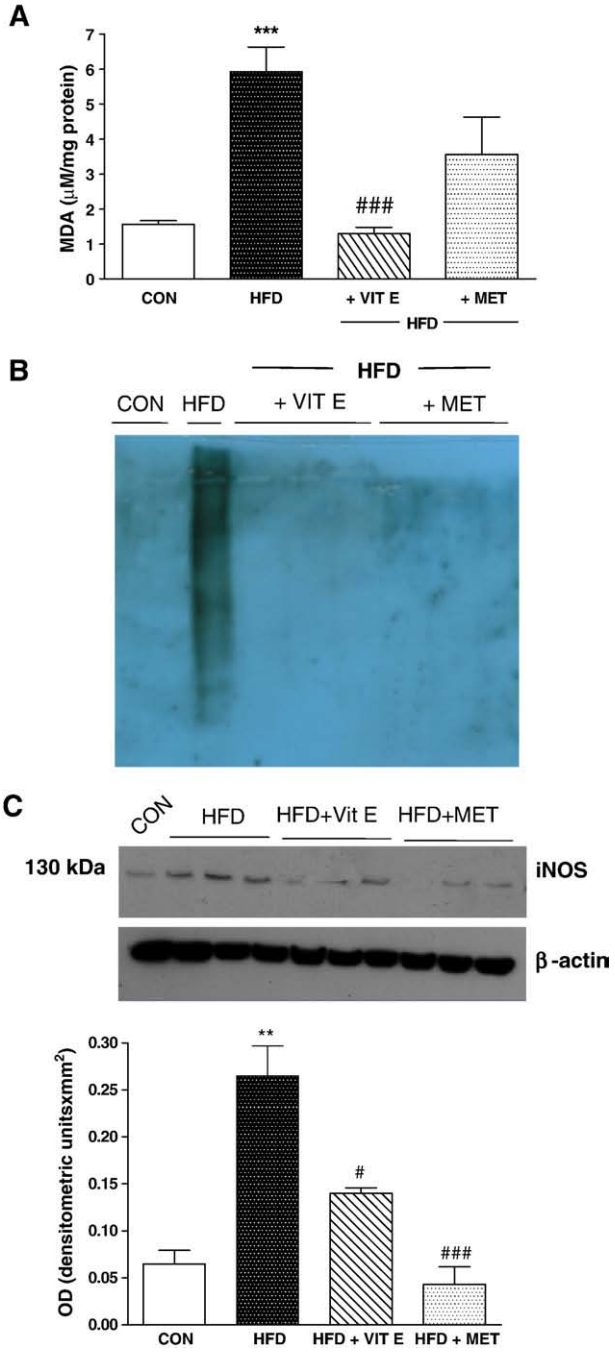

Fig. 2. Malonyldialdehyde (MDA) content (A), protein nitrotyrosilation levels (B), and iNOS expression (C) in liver from control diet (CON), high-fat diet (HFD) or high-fat diet -fed groups treated with vitamin E (HFD+VIT E) or metformin (HFD+MET) for 4 weeks. Malonyldialdehyde values, expressed as μM/mg protein, are mean±S.E.M. of 5 animals. The data in panel C represent relative density normalized to β-actin. **$P<0.01$, ***$P<0.001$ vs CON; #$P<0.05$, ###$P<0.001$ vs HFD.

and metformin treatment showed a weak reduction of liver weight, they were able to decrease ALT or AST ($P<0.05$), respectively, which were altered in the high fat diet fed group. Both drugs significantly reduced liver triglyceride content and therefore steatosis extent.

Moreover, in rats receiving high fat diet a significant increase in body weight gain and fat mass was observed with respect to the control diet group (Fig. 1A). The treatment with metformin significantly inhibited body weight gain and fat mass induced by high fat diet. Vitamin E treatment, while not significantly modifying body weight gain, conversely presented a significant decrease in fat mass content compared to that of high fat diet fed rats (Fig. 1B). No variation in food intake was shown between the high fat diet fed group and metformin or vitamin E treated high fat diet fed animals (192.0±7.20 vs 183.8±3.57 and 189.6±9.74 ml/4 weeks, respectively).

3.2. Effect of drugs on increased lipid peroxidation and S-nitrosylation induced by high fat diet in liver

Malonyldialdehyde and other malonyldialdehyde-like aldehydes and ketones are generated by phospholipids peroxidation. In particular malonyldialdehyde is the major product reacting with thiobarbituric acid. High fat diet induced a marked increase in the amount of malonyldialdehyde (Fig. 2A; $P<0.001$ vs control), and vitamin E treatment significantly reversed this effect ($P<0.001$ vs high fat diet fed group). No significant decreasing trend of lipid peroxidation was evident ($t=2.576$) in metformin treated animals.

We have also determined the levels of nitrotyrosines (downstream reaction products of peroxynitrite) of hepatic proteins and iNOS expression by Western blot analysis. The increase of these parameters may result in inflammation and liver damage. Nitrosylation of hepatic protein of high fat diet fed animals (Fig. 2B) was coupled to a parallel iNOS induction (Fig. 2C) compared with control rats and all pharmacological treatments reversed significantly these modifications to control levels.

3.3. TNF-α modulation by pharmacological treatments in liver from high fat diet fed rats

As reported in Fig. 3, a significant increase in tissue TNF-α was found in liver from high fat diet fed animals ($P<0.01$). This effect was significantly reverted by vitamin E or metformin ($P<0.001$ and $P<0.05$, respectively), contributing to their anti-inflammatory activity.

3.4. Effect of pharmacological treatments on PPAR-α and PPAR-γ expression

High fat diet induced a significant decrease of PPAR-α in liver lysates ($P<0.05$; Fig. 4A) and only metformin treatment significantly prevented the high fat diet-induced PPAR-α degradation ($P<0.05$). On the contrary, PPAR-γ expression was upregulated in liver from high fat diet fed animals, and both treatments prevented its increase (Fig. 4B).

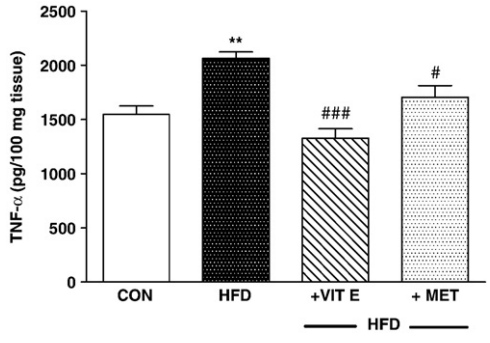

Fig. 3. Liver TNF-α levels, expressed as pg/100 mg of tissue, were obtained from control diet (CON), high-fat diet (HFD) or high-fat diet-fed groups treated with vitamin E (HFD+VIT E), or metformin (HFD+MET) for 4 weeks. Values are given as means±S.E.M. of 6 rats. *$P<0.05$, and **$P<0.01$ vs CON; #$P<0.05$, ##$P<0.01$, and ###$P<0.001$ vs HFD.

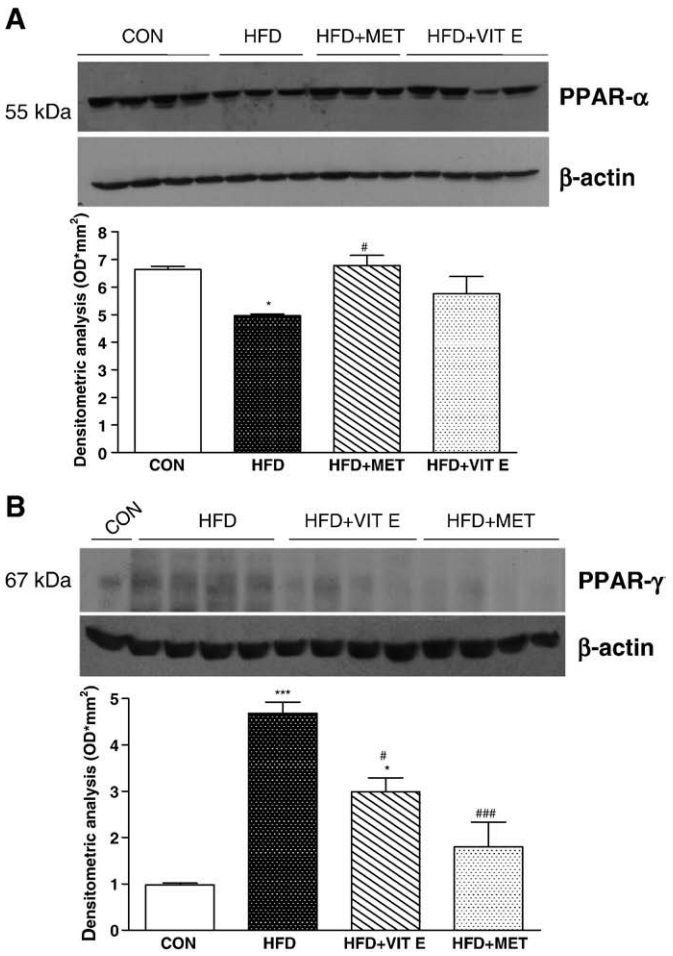

Fig. 4. Representative Western blot of PPAR-α (A) and PPAR-γ (B) expression in tissue lysates of liver from control diet (CON), high-fat diet (HFD) or high-fat diet-fed groups treated with vitamin E (HFD+VIT E), or metformin (HFD+MET) for 4 weeks is shown. PPAR-α and PPAR-γ modulation was revealed by densitometric analysis of all protein bands. The data represent relative density normalized to β-actin. Values are given as means±S.E.M. of 6 rats. *$P<0.05$ vs CON; #$P<0.05$ vs HFD.

3.5. Evaluation of hepatic proMMP-2 and proMMP-9 activity

MMPs plays an important role in the pathogenesis and evolution of inflammatory diseases. We determined the activity of gelatinase A and B (MMP-2 and pro MMP-9, respectively) in livers from all groups, since they are principally involved in liver damage. As reported in Fig. 5, both pro-MMP-2 and pro MMP-9 were significantly up-regulated in liver from HFD animals (Fig. 5). Even if a trend to decrease for both proMMP-2 and proMMP-9 was revealed, vitamin E reduced significantly the active form of MMP-2. Conversely metformin treatment significantly reduced all MMPs activity. The lack of active MMP-9 in the zymogram could be due to its high level of instability and to the removal of active enzyme during the washing of specimens, as reported above (Deleve et al., 2003).

4. Discussion

Non-alcoholic steatohepatitis is an increasingly recognized condition that may eventually progress to an end stage liver disease. A net retention of lipids within hepatocytes is a prerequisite for the development of the disease. Increased intrahepatic levels of fatty acids provide a source of oxidative stress which may in large part be

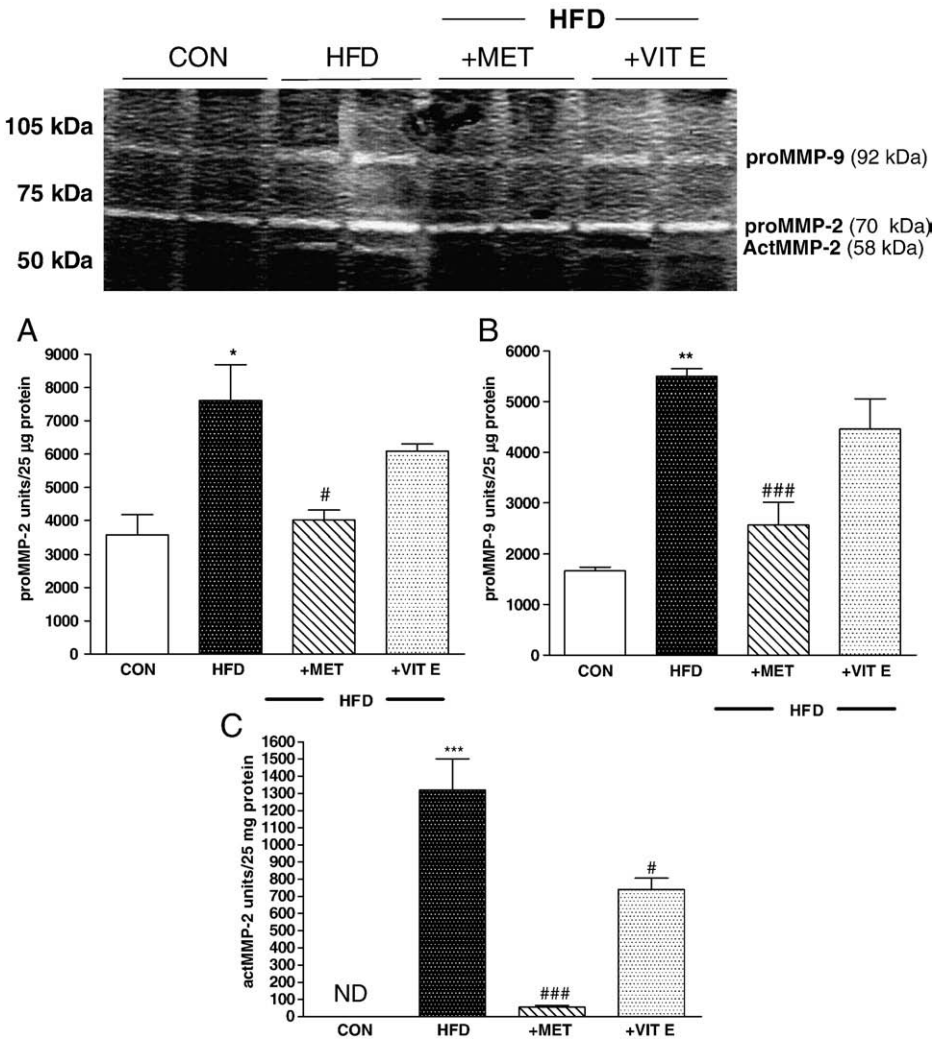

Fig. 5. Active and proMMP-2 and proMMP-9 activity in liver lysates from control diet (CON), high-fat diet (HFD) or high-fat diet-fed groups treated with metformin (HFD + MET) or vitamin E(HFD + VIT E) for 4 weeks was shown by gelatin-zymography. Densitometric analysis of proMMP-2 (A), proMMP-9 (B) and active MMP-2 (C) bands was also reported. Values are given as means ± S.E.M. of 6 rats. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ vs CON; #$P<0.05$, and ##$P<0.01$ vs HFD.

responsible for the progression from steatosis, to steatohepatitis, to cirrhosis.

Pathophysiology of this condition still has not been so far clarified. The presumed factors initiating the progression of non-alcoholic steatohepatitis are insulin resistance (Bugianesi et al., 2005; Larter and Farrell, 2006), an increased oxidative status with subsequent lipid peroxidation, pro-inflammatory cytokines (principally TNF-α), and hormones derived from adipose tissue (adipocytokines) (Albano et al., 2005; Duvnjak et al., 2007; Kojima et al., 2007).

As already reported by Lieber et al. (2004a,b), a 3 weeks feeding with high fat diet induced pronounced hepatic steatosis and abundant mononuclear inflammatory cells evidenced by histopathological analysis. In our experimental conditions, we prolonged to 4 weeks the high fat diet assumption and we reported a crucial and significant alteration of liver parameters (liver weight, transaminases, trygliceride content), induced by high fat diet. Moreover, we demonstrated that in young rat this diet induced a significant increase in the malonyldialdehyde content in the liver. Malonyldialdehyde and other substances, such as 4-hydroxynonenal, 8-isoprostane, and 3-nitrotyrosine are produced by lipid peroxidation and the level in the plasma of several oxidative stress markers was found to have increased in non-alcoholic steatohepatitis patients (Loguercio et al., 2001; Sumida et al., 2003). In our experimental conditions, the high oxidative damage induced by 4-week high fat diet was confirmed by the increase of liver protein *S*-nitrosylation, that was accompanied with an increase of iNOS.

Moreover, we found a significant decrease in PPAR-α expression in liver from the high fat diet fed group, most likely related to the progression of steatosis. Conversely, an upregulation of PPAR-γ was shown. As known, PPAR-α activation reduces or even reverses steatohepatitis induced by a methionine- and choline-deficient diets and a loss of expression in PPAR-α gene in mice results in hepatic steatosis under conditions of an increased fatty acid metabolism in the liver, such as in fasting or a high fat diet (Kallwitz et al., 2008). The anti-inflammatory role of PPAR-α in the development of steatohepatitis is further supported by the evidence that animals lacking PPAR-α developed steatohepatitis accompanied by an increased number in infiltrated lymphocytes and macrophages (Kashireddy and Rao, 2004). A role for hepatic PPAR-γ in liver trygliceride accumulation has been also suggested, indeed PPAR-γ deficient mice are protected against development of steatosis. Conversely, even if this receptor isoform is able to protect other tissues from triglyceride accumulation and insulin resistance, it contributes to hepatic steatosis, regulating triglyceride homeostasis (Gavrilova et al., 2003). Moreover, a high fat diet increased PPAR-γ expression together with cAMP-response element-binding protein, an upstream molecule of PPAR-γ signalling, in the nuclei of hepatocytes in mice (Inoue et al., 2005).

Here, it has also been demonstrated that hepatic gelatinase activity of prometalloproteinase-2 and prometalloproteinase-9 in the high fat diet fed group was significantly higher than that of animals fed with control diet, thus showing the involvement of prometalloproteinases in the evolution of the liver inflammatory process induced by high fat diet. These prometalloproteinases degrade the basement membrane and extracellular matrix, facilitate leukocyte migration and the release of TNF-α from its membrane-bound form (McGeehan et al., 1994).

In this model we used two therapeutic tools, i.e., metformin, as insulin sensitizers and vitamin E, as cytoprotective agent.

Metformin was reported to reverse hepathomegaly, steatosis and liver tests abnormalities in a model of fatty liver (Lin et al., 2000). Several clinical studies confirm the improvement in liver tests, insulin sensitivity, and loss of body weight (Marchesini et al., 2001; Angulo and Lindor, 2002; Schwimmer et al., 2005) induced by metformin treatment, even if some other results showed no effect (Nair et al., 2004). Although an amelioration of aminotransferase level and liver histology was evidenced, on the other hand, no statistically significant change in the severity of liver inflammation or fibrosis after metformin treatment was reported (Uygun et al., 2004).

In our experimental conditions, as expected, this drug reduced body weight gain and fat mass in comparison to high fat diet fed animals. It did not significantly reduce liver weight, but ameliorates transaminase profile and reduced significantly liver trygliceride content. Although having a slight effect on lipid peroxidation (malonyldialdehyde content) metformin was found markedly reduce nitrotyrosylation of hepatic proteins and to strongly inhibit the induction of iNOS. This anti-inflammatory effect was confirmed by inhibition of TNF-α content in liver tissue and of MMPs activity. Metformin effect on lipid metabolism was also evidenced by the normalization of liver PPAR-α and PPAR-γ expression, resulting in an inhibition of steatosis progression.

On the other hand, vitamin E treatment did not significantly modify body weight gain and liver weight, but slightly reduced fat mass and ameliorated transaminase profile. It strongly reduced hepatic tryglyceride content, the oxidative damage, protein nitrotyrosilation, and iNOS expression. The cytoprotective effect of vitamin E was confirmed by inhibition of TNF-α content in liver tissue, while no modification of MMPs activity was evidenced. Differently from metformin, vitamin E did not modify PPAR-α expression, showing a lean effect on lipid metabolism; conversely it normalizes liver PPAR-γ expression, linked to the partial reduction of liver inflammatory damage.

Together with metformin, the second generation thioglitazones are also able to improve insulin sensitivity and considered a candidate for non-alcoholic steatohepatitis treatment, as they stimulate the storage of free fatty acids in subcutaneous adipocytes as opposed to liver and omental fat. They show anti-inflammatory properties, downregulating NF-kB activity and increasing adiponectin levels (Mohanty et al., 2004; Lutchman et al., 2006). Anyway, their use in paediatric patients has to be taken with care, since even if safer than first generation drugs, they are contraindicated in the presence of active liver disease or of elevated ALT level. Moreover, the treatment with these agents may need to be life-long as evidenced by recourse to steatosis after drug cessation (Lutchman et al., 2007). Larger multi-year clinical trials are necessary to determine their efficacy and safety, since cardiovascular risks and weight gain are associated to their use (Nathan, 2007; Promrat et al., 2004). Due to all these limits, the use of these thioglitazones in children is not advisable.

To avoid liver disease progression, prevention of liver damage is particularly urgent ever since the early age. Therefore, to date among the major potential therapeutic options, insulin sensitizers, such as metformin, and antioxidant vitamin E (Bugianesi et al., 2006; Mishra and Younossi, 2007) are surely efficacious and well-tolerated, but their long-term clinical efficacy either alone or in combination has not yet been confirmed. Although experimental and clinical studies suggest for these drugs a favourable role in improving non-alcoholic fatty liver disease, further investigations and large randomized trials will be needed.

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