Increased Expression and Activity of the Transcription Factor FOXO1 in Nonalcoholic Steatohepatitis

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OBJECTIVE—Nonalcoholic fatty liver, affecting 34% of the U.S. population, is characterized by hepatic insulin resistance, which is more marked in the presence of steatohepatitis, and frequently precedes hyperglycemia. The molecular mechanisms underlying the relationship between fatty liver and insulin resistance are still undergoing definition and have not been evaluated in humans. Our aim was to evaluate the relationship between insulin resistance and the expression and regulation of forkhead box–containing protein O subfamily-1 (FOXO1), a transcription factor that mediates the effect of insulin on the gluconeogenic genes PEPCK and glucose-6-phosphatase catalytic subunit (G6PC).

RESEARCH DESIGN AND METHODS—FOXO1, PEPCK, and G6PC mRNA levels were evaluated in 84 subjects: 26 with steatohepatitis, 28 with steatosis alone, 14 with normal liver histology without metabolic alterations, and 16 with hepatitis C virus chronic hepatitis, of whom 8 were with and 8 were without steatosis. Protein expression and regulation of FOXO1 and upstream insulin signaling were analyzed in a subset.

RESULTS—Expression of PEPCK was higher in steatohepatitis compared with steatosis alone and normal liver, and it was correlated with the homeostasis model assessment of insulin resistance (HOMA-IR) index. FOXO1 mRNA levels were higher in steatohepatitis, correlated with PEPCK and G6PC mRNA and with HOMA-IR. FOXO1 upregulation was confirmed at protein levels in steatohepatitis and, in the presence of oxidative stress, was associated with decreased Ser^{256} phosphorylation, decreased Akt1, and increased Jun NH_2-terminal kinase-1 activity. Consistently, immunohistochemistry showed increased FOXO1 expression and nuclear localization in steatohepatitis. FOXO1 mRNA levels correlated with nonalcoholic steatohepatitis activity score and were modulated by drugs counteracting hepatic lipogenesis.

CONCLUSIONS—FOXO1 expression and activity are increased in patients with steatohepatitis, and mRNA levels are correlated with hepatic insulin resistance. ***Diabetes*** **57:1355–1362, 2008**

Nonalcoholic fatty liver disease (NAFLD), affecting ~34% of the U.S. population (1), is considered the hepatic manifestation of the metabolic syndrome. NAFLD is characterized by hepatic insulin resistance, i.e., the inability of insulin to shut down hepatic glucose output (2), proatherogenic dyslipidemia, and early vascular damage (3). In about a third of NAFLD cases, fatty liver is complicated by nonalcoholic steatohepatitis (NASH), which is thought to be provoked by lipid peroxidation and mitochondrial dysfunction determining oxidative stress and cytokine release (4). The presence of NASH is associated with more severe metabolic derangement and insulin resistance, frequently evolving into glucose intolerance, and more advanced vascular damage (2,3). Alterations in liver enzymes reportedly precede the development of the metabolic syndrome and hyperglycemia (5,6), and the majority of NASH patients develop diabetes or glucose intolerance at long-term follow-up (7,8), thus suggesting a role of NASH in the debated pathogenic mechanism of metabolic syndrome (9). In addition, fatty liver is frequently observed in chronic hepatitis C virus (HCV) hepatitis and is associated with increased inflammation and heightened risk of type 2 diabetes (10).

Experimental models indicate that fatty liver may directly induce hepatic insulin resistance (11), and recent evidence suggests that deregulation of the insulin signaling pathway plays a role in this process (12). However, data concerning regulation of the insulin signaling and gluconeogenic genes in patients with NAFLD are still unavailable. Hepatic glucose output is regulated by glucose-6-phosphatase catalytic subunit (G6PC) and PEPCK, rate limiting enzymes for gluconeogenesis and glucose release. Although several transcription factors have been shown to regulate gluconeogenesis, evidence is accumulating that in vivo shutdown of hepatic glucose output by insulin involves Akt-dependent phosphorylation and nuclear exclusion of forkhead box–containing protein O subfamily-1 (FOXO1), a transcription factor controlling the expression of G6PC and PEPCK. In mice, FOXO1 haploinsufficiency reduced hepatic glucose output, whereas FOXO1 overexpression increased gluconeogenesis (13). FOXO1 binds to, and is required for activation of gluconeogenesis by, peroxisome proliferator–activated receptor-γ (PPAR-γ) coactivator 1α (PGC-1α) (14,15), CAAAT/enhancer-binding protein-α (C/EBPα), and possibly C/EBPβ (16), whose transcriptional activity is also downregulated by insulin signaling by induction of the inhibitory isoform LIP (liver-enriched transcriptional inhibitory protein) (17). Hepatic

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C/EBPα, CAAAT/enhancer-binding protein-α; CREB, cAMP response element–binding protein; FOXO, forkhead box–containing protein O subfamily-1; G6PC, glucose-6-phosphatase catalytic subunit; HCV, hepatitis C virus; HNF, hepatocyte nuclear factor; HOMA-IR, homeostasis model assessment of insulin resistance; JNK, Jun NH_2-terminal kinase; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PGC-1α, peroxisome proliferator–activated receptor-γ coactivator 1α; PPAR, peroxisome proliferator–activated receptor; SOD, superoxide dismutase.

TABLE 1
Demographic and clinical features of 84 subjects included in the study

	HCV hepatitis	Normal histology	Steatosis alone	NASH	*P*
n	16	14	28	26	—
Age (years)	56.1 ± 11*	44.5 ± 12	42.9 ± 9*	49.7 ± 10	0.003
Sex (F)	6 (33)	4 (29)	10 (36)	11 (42)	NS
BMI (kg/m^2)	23.8 ± 2*†	26.7 ± 7*†	33.2 ± 10	35.4 ± 8	<0.0001
LDL (mg/100 ml)	98 ± 30*†	127 ± 27	132 ± 30	141 ± 37	0.02
Triglyceride/HDL	0.9 ± 0.4*†	1.8 ± 1*†	2.9 ± 1.6	2.9 ± 1.1	0.0007
HOMA-IR	3.7 ± 1.4*	2.3 ± 1.3*	3.3 ± 1.5*	6.7 ± 3.6	<0.0001
IFG/IGT	4 (22)	0*	5 (18)*	13 (50)	0.003
Metabolic syndrome	0*	0*	15 (54)*	22 (85)	<0.0001
Percentage of steatotic hepatocytes	8 (50)*†	0*†	24 ± 16*	43 ± 22	<0.0001
Alanine aminotransferase (IU/ml)	59 ± 40	28 ± 13*†	48 ± 27	52 ± 28	0.02

Data are the means ± SD or *n* (%). For percentage of steatotic hepatocytes as detected by histology, values are means ± SEM, except for viral hepatitis, where the number and percentage of affected subjects is indicated. Metabolic syndrome is defined according to Adult Treatment Panel III criteria (24). *P* was determined by ANOVA. *$P \leq 0.05$ vs. NASH, †$P \leq 0.05$ vs. NAFLD. IFG, impaired fasting glucose; IGT, impaired glucose tolerance.

FOXO1 ablation impairs fasting- and cAMP-induced gluconeogenesis and curtails excessive hepatic glucose output caused by generalized ablation of the insulin receptor, providing a unifying mechanism for the regulation of hepatic glucose output by hormonal cues (14). Furthermore, downregulation of FOXO1 by antisense oligonucleotides reduced hepatic insulin resistance in a model of diet-induced obesity and fatty liver (18). Although NAFLD is characterized by hyperinsulinemia, under oxidative stress conditions, as in those observed in NASH (4), FOXO1 becomes unresponsive to insulin because of interaction with the deacetylase sirtuin 1, resulting in induction of glucogenetic genes (19,20). Therefore, deregulation of hepatic FOXO1 and the associated transcriptional machinery in response to steatosis and oxidative stress may play a role in the metabolic derangement of subjects with NAFLD by inducing hepatic glucose output. To test this hypothesis in humans, we sought to demonstrate hepatic insulin resistance at the gene expression level in patients with NAFLD and to analyze the relationship between insulin resistance, FOXO1 expression/regulation, and gluconeogenesis.

RESEARCH DESIGN AND METHODS

We considered 84 subjects who underwent liver biopsy at the Department of Internal Medicine ($n = 57$) or Surgery ($n = 27$) at the Ospedale Policlinico, Mangiagalli e Regina Elena, Fondazione IRCCS (Istituto di Ricovero e Cura a Carattere Scientifico) of Milan. We included 26 patients with NASH, 28 with steatosis alone, and 14 with normal liver biopsy. This latter group of control subjects was defined by *1*) the absence of viral hepatitis, metabolic alterations, and histological abnormalities, including steatosis, and *2*) the presence of normal alanine aminotransferase levels at the time of biopsy, which was performed for increased γ-glutamyltransferase/ferritin levels, evaluation for living donor liver donation, or routinely in patients undergoing bariatric surgery. We also considered 16 patients with chronic HCV-related hepatitis, 8 of whom were with and 8 without steatosis. Patients with diabetes, hypothyroidism, alcohol intake >40 g/week, and severe hepatic fibrosis (bridging fibrosis and cirrhosis) were excluded; five patients with NASH had glucose intolerance. Eight patients with NAFLD were on therapy with drugs acting on hepatic insulin resistance and lipogenesis (henceforth defined as "treated") (21,22). The sample part unnecessary for histological diagnosis was processed only for RNA extraction. Surgical samples were partly processed for histological evaluation and partly for RNA and/or protein extraction. We collected suitable material to perform both analyses in 15 cases.

The study was approved by the institutional review board of the Ospedale Policlinico, Mangiagalli e Regina Elena, Fondazione IRCCS (Istituto di Ricovero e Cura a Carattere Scientifico) of Milan, and each participating subject gave written informed consent.

Study protocol and measurement of insulin resistance. Subjects were advised to maintain a standardized normoglucidic diet (50% carbohydrates, 35% fat, and 15% protein) for 48 h before the procedure. Compliance was confirmed by at least one family member or by a friend. Liver biopsies and blood tests were performed after overnight fasting (10 h), and liver samples were routinely processed for histology or immediately frozen in liquid nitrogen and stored at −80°C in RNAlater (Invitrogen, Carlsbad, CA) for RNA, or they were lysed in radioimmunoprecipitation assay buffer for proteins. Insulin was measured by radioimmunoassay (Calbiochem, Bologna, Italy), and biochemistry was performed by standard methods. Insulin resistance was evaluated by homeostasis model assessment of insulin resistance (HOMA-IR) index, which mainly reflects hepatic insulin resistance (23) in subjects followed at the Department of Medicine. Because there is a linear relationship between insulin levels and hepatic glucose output, and insulin acts by downregulating the expression of PEPCK and G6PC, we also arbitrarily estimated insulin resistance at the gene expression level by adjusting PEPCK and G6PC mRNA levels per insulin levels. Metabolic syndrome was defined according to NCEP (National Cholesterol Education Program) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) criteria (24).

Histological analysis. A single expert pathologist unaware of gene expression data evaluated all biopsies, which were staged according to Kleiner et al. (25), based on the determination of the NASH activity score, the sum of steatosis severity (0–3), intralobular inflammation (0–3), and hepatocyte ballooning (0–2). The percentage of hepatocytes with steatosis was the mean value of at least 10 hepatic lobules per patient. Patients with probable (NASH activity score 3–4) and definite NASH (NASH activity score ≥5) were grouped together for analyses unless otherwise specified.

FOXO1 cellular distribution and intracellular localization were detected by immunohistochemistry on paraffin-embedded tissue samples using the antibody (Cell Signaling, Danvers, MA) in 15 representative subjects. Briefly, tissue samples were deparaffinized and hydrated, treated with EDTA (1 mmol/l, pH 8) to unmask antigens, and treated with 3% H_2O_2 to block endogenous peroxidase. Samples were then incubated with the primary antibody at 1:50 dilution and washed. The reaction was performed using the EnVision Dako system (Dako, Milan, Italy) and developed by diaminobenzidine. Mild contrast was provided by hematoxylin and Meyer stainings. Antibody specificity and the ability to recognize FOXO1 intracellular localization under normal growth conditions, starving, and oxidative stress was confirmed in HepG2 by immunocytochemistry (not shown).

Determination of mRNA levels. RNA was isolated by the RNeasy micro kit (Qiagen, Milan, Italy) and digested with DNaseI, and quality was evaluated by measuring the 260/280-nm absorbance ratio (≥1.8) and by electrophoresis. First-strand cDNA was synthesized using equal amounts (0.5 μg) of total RNA with the SuperScript first-strand synthesis system (Invitrogen, Carlsbad, CA). The mRNA levels were analyzed by qRT-PCR. The PCR mix contained TaqMan Universal Master Mix (1×) plus the assays specific for the genes of interest (MyScience; Applied Biosystems, Foster City, CA). All reactions were performed in triplicate with an ABI Prism 7700 analyzer in 25 μl final volume. Primers are available online at https://products.appliedbiosystems.com (assay ID: Hs 00231106 m1, Hs 00356436 m1, Hs 00609178 m1, Hs 99999903 m1, Hs 00173304 m1, Hs 00232764 m1, Hs 00230853 m1). Results were normalized for β-actin.

Protein analysis. Tissues (10 mg) were lysed in radioimmunoprecipitation assay buffer containing 1 mmol/l Na-orthovanadate, 200 mmol/l phenylmethylsulphonyl fluoride, and 0.02 μg/μl aprotinin. Equal amounts of proteins (25

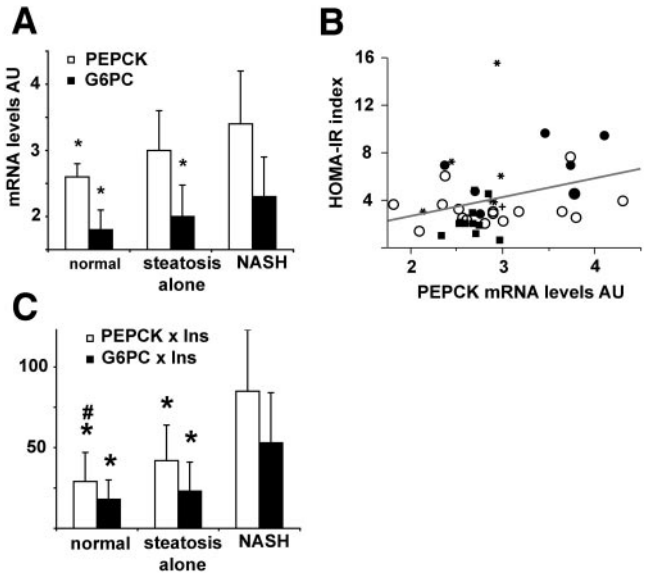

FIG. 1. Gluconeogenesis and insulin resistance. *A*: Expression of gluconeogenic genes evaluated by qRT-PCR according to the histological diagnosis. Data are shown after log transformation. $P = 0.05$ for PEPCK and G6PC expression among groups. AU, arbitrary units. *$P \leq 0.05$ vs. NASH. *B*: Correlation between PEPCK mRNA levels and HOMA-IR ($P = 0.04$). ■, normal biopsy; ○, steatosis alone; +, steatosis alone treated; ●, NASH; *, NASH treated. AU, arbitrary units. *C*: Expression of gluconeogenic genes adjusted for insulin levels according to the histological diagnosis ($P = 0.0006$ and $P = 0.002$ for PEPCK and G6PC, respectively). *$P \leq 0.05$ vs. NASH; #$P \leq 0.05$ vs. NAFLD.

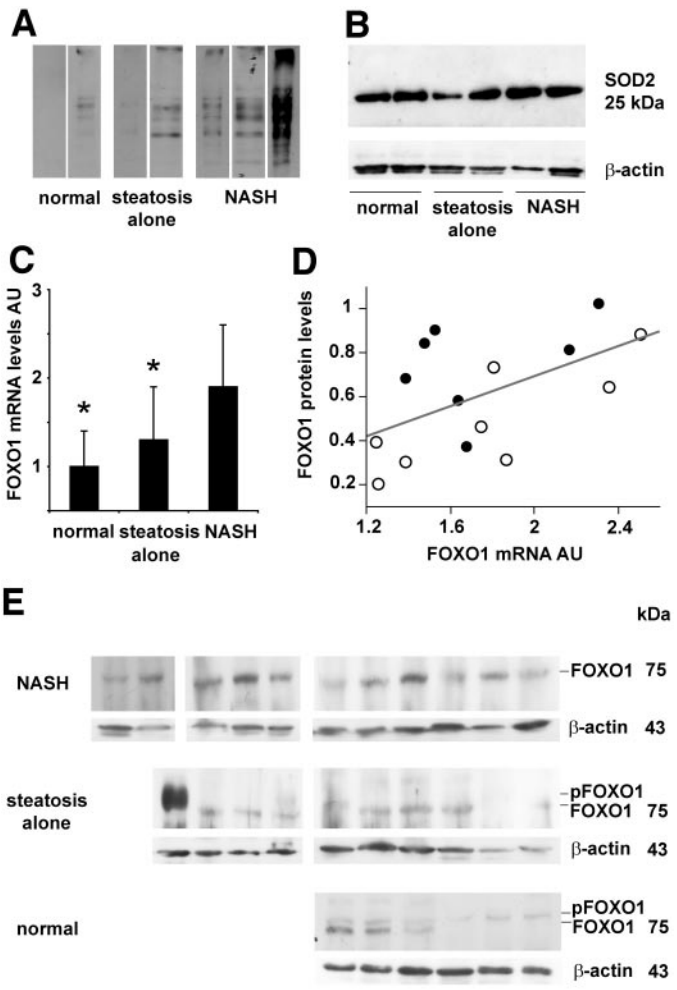

FIG. 2. Oxidative stress and FOXO1 expression. *A*: Oxidative stress levels as determined by Oxyblot in representative patients (two normal liver histology, two steatosis alone, three NASH). *B*: SOD2 expression in representative patients (one normal liver, two steatosis alone, two NASH). β-Actin was used to normalize results presented in Table 2, and HepG2 is shown as internal control. *C*: Expression of FOXO1 evaluated by qRT-PCR according to the histological diagnosis. $P = 0.0004$ for FOXO1 expression among groups. Data are shown after log transformation. AU, arbitrary units. *$P \leq 0.05$ vs. NASH. *D*: Correlation between FOXO1 mRNA and protein levels in 15 patients with NASH ($P = 0.04$). AU, arbitrary units. *E*: FOXO1 protein expression in patients with NASH ($n = 11$), steatosis alone ($n = 10$), and normal liver ($n = 6$). β-Actin is shown as a control.

μg) were separated by SDS-PAGE and transferred electrophoretically to a nitrocellulose membrane (Biorad, Hercules, CA). The blot was incubated with anti–insulin receptor-β, insulin receptor substrate-2, Akt1/2, FOXO1, PGC-1, Jun NH_2-terminal kinase (JNK), phosphorylated JNK, superoxide dismutase 2 (SOD2), C/EBPα, C/EBPβ, hepatocyte nuclear factor-4 (HNF-4), PPARα, cAMP response element–binding protein (CREB), β-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated Akt (Ser^{473}), phosphorylated FOXO1 (Ser^{256}) (Cell Signaling Technology, Danvers, MA), and anti–sirtuin 1 antibodies (Upstate, Chicago, IL). The gels were densitometrically scanned and images analyzed with ImageJ software (26). Results were normalized for β-actin. To normalize results between gels, we used a single pooled lysate from untreated HepG2 hepatocytes. Oxidative stress was measured by protein carbonylation and antioxidant response by protein levels of SOD2, a bona fide FOXO target involved in the scavenging of mitochondrial-derived superoxide (27). Protein carbonylation was measured by Oxyblot (Chemicon, Temecula, CA) (28) according to the manufacturer's instructions.

Statistical analysis. Due to nonnormal distribution, normalized mRNA levels were log-transformed before data analysis. Results, expressed as the means ± SD, were compared by ANOVA and post hoc analysis (Tukey-Kramer), and correlations were performed by Pearson's test. To analyze independent predictors of FOXO1 mRNA levels (above or below median levels) in patients without HCV chronic hepatitis, we performed nominal logistic regression analysis considering the severity of steatosis and necroinflammatory activity according to Kleiner et al. (25) and therapy with insulin-sensitizing drugs as independent variables. *P* was considered significant when <0.05 (two-tailed). Analyses were carried out with JMP 6.0 statistical analysis software (SAS Institute, Cary, NC).

RESULTS

Patient characteristics. Clinical features of patients included in this study are shown in Table 1. LDL cholesterol, BMI, triglycerides-to-HDL ratio, HOMA-IR index, the prevalence of metabolic syndrome and impaired fasting glucose/glucose tolerance, and the percentage of steatotic cells were significantly different among groups, with the highest levels in NASH. Subjects with normal liver had lower alanine aminotransferase levels compared with other groups. Patients with NASH were older than those with steatosis alone. The group of patients submitted to bariatric surgery had higher BMI values, younger age, and higher prevalence of female sex compared with those with suspected liver disease followed in the Internal Medicine Department ($P < 0.05$, not shown).

Insulin resistance and gluconeogenic genes. To test for the presence of hepatic insulin resistance at the gene expression level, we first analyzed the expression of gluconeogenic genes in patients without HCV. The expression of PEPCK and G6PC was significantly different among groups ($P = 0.05$) because of higher levels in NASH versus normal liver for PEPCK and higher levels in NASH versus normal liver and steatosis alone for G6PC (Fig. 1*A*). Mean PEPCK expression levels before log conversion were fourfold higher in NASH versus normal liver, and G6PC expression was 2.3- and 3.2-fold higher in NASH versus steatosis alone and normal liver, respectively. PEPCK, but not G6PC expression, was significantly correlated with the HOMA-IR index ($P = 0.04$) (Fig. 1*B*). We estimated insulin

TABLE 2
Oxidative stress markers; phosphorylation status (Ser^{256}) of FOXO1, sirtuin 1, and PGC-1α; and expression and activation status of Akt, determined by densitometric analysis of Western blottings, in obese subjects subdivided according to hepatic histological findings in normal liver histology, steatosis alone, and NASH

	Normal	Steatosis alone	NASH	*P*
n	6	10	11	—
Protein carbonylation	0.2 ± 0.2*	0.4 ± 0.2*	2.8 ± 2.2	<0.0001
SOD2	0.55 ± 0.2*	0.63 ± 0.2*	1.53 ± 0.2	<0.0001
Total FOXO1	0.27 ± 0.1*†	0.47 ± 0.2*	0.75 ± 0.1	<0.0001
Phosphorylated/total FOXO1	0.74 ± 0.1*†	0.33 ± 0.2	0.21 ± 0.1	<0.0001
Unphosphorylated FOXO1	0.09 ± 0.1*†	0.32 ± 0.1*	0.59 ± 0.1	<0.0001
PGC-1α‡	0.13 ± 0.01*†	0.076 ± 0.001	0.06 ± 0.007	0.01
Sirtuin 1‡	0.33 ± 0.3	0.15 ± 0.1	0.33 ± 0.1	NS
FOXO1-bound sirtuin 1‡	0.1 ± 0.1*†	0.61 ± 0.1*	1.04 ± 0.1	0.004
Akt	1.69 ± 0.1*†	1.06 ± 0.2	0.93 ± 0.1	<0.0001
Phosphorylated Akt	1.74 ± 0.1*	1.24 ± 0.3	1.05 ± 0.1	<0.0001
Phosphorylated/total Akt	0.97 ± 0.1	1.17 ± 0.1	1.13 ± 0.1	NS

Protein levels are normalized for β-actin. *P* was determined by ANOVA. *$P \leq 0.05$ vs. NASH; †$P \leq 0.05$ vs. NAFLD; ‡data are available for 2 subjects for each group.

resistance at the gene expression level by adjusting PEPCK and G6PC mRNA levels per insulin level in each patient. Both PEPCK × insulin ($P = 0.0006$) and G6PC × insulin ($P = 0.0005$) were significantly different among groups because of higher levels in NASH compared with the other groups (Fig. 1*C*). PEPCK × insulin was also higher in steatosis alone than in normal liver. These results suggest that gluconeogenic genes are not downregulated by hyperinsulinemia and that overexpression of PEPCK is likely involved in the pathogenesis of insulin resistance in NASH.

Oxidative stress levels. Oxidative stress levels were measured by protein carbonylation, as determined by Oxyblot, and the antioxidant response by detecting SOD2 protein levels. Results are shown in Fig. 2*A* and *B* and Table 2. Both protein carbonylation and SOD2 levels were significantly different among groups ($P < 0.0001$) because of higher levels in patients with NASH compared with those with simple steatosis and normal liver histology.

Hepatic expression of FOXO1. Preliminary experiments confirmed that FOXO1 was the most abundantly expressed FOXO in the liver in humans because mRNA levels were 14.5-fold higher than those of FOXO3A ($P < 0.0001$) independently of liver pathological findings, and FOXO4 was almost undetectable in all evaluated samples. We thus focused our study on FOXO1.

To investigate the molecular mechanisms underpinning PEPCK and G6PC upregulation, we analyzed the mRNA expression of FOXO1 and of its coactivators sirtuin 1 and PGC-1α. We observed significantly different FOXO1 expression among patients subdivided according to liver histopathological findings ($P = 0.0004$), with higher levels in NASH versus both steatosis alone and subjects with normal liver ($P < 0.005$ for both) (Fig. 2*C*), whereas no significant differences were observed for sirtuin 1 and PGC-1α mRNA levels (not shown). In patients with NASH, mean FOXO1 mRNA levels were 6.3-fold higher than in those with normal liver biopsy and 3.7-fold higher than in those with simple steatosis. FOXO1 expression was higher in NASH versus simple steatosis and normal liver both in patients with and in those without morbid obesity ($P < 0.05$ for both).

To determine whether increased FOXO1 mRNA levels translated into increased activity, we first analyzed the relationship between normalized mRNA and protein levels. We observed a significant correlation between FOXO1 mRNA and protein levels ($n = 15$, $R^2 = 0.3$, $P = 0.03$) (Fig. 2*D*). FOXO1 protein levels were higher in patients with NAFLD, in particular in those with NASH, than in those with normal liver (Fig. 2*E* and Table 2); moreover, upward shifting of FOXO1 in the majority of patients with normal liver and in some with simple steatosis suggested that FOXO1 is phosphorylated in normal liver but is unphosphorylated and thus transcriptionally active in NASH. In contrast, FOXO1 mRNA levels were not significantly influenced by the presence of fatty liver in patients with chronic HCV hepatitis (1.47 ± 0.3 vs. 1.51 ± 0.3 in patients without and with steatosis, P = NS).

FOXO1 regulation in the liver. We next analyzed FOXO1 posttranslational regulation by Western blotting and immunoprecipitation. In Fig. 3 we show the results obtained in two obese patients with normal liver histology, two patients with steatosis alone, and two patients with definite NASH (NASH activity score ≥5). Patients were representative of histological diagnoses, and results obtained in the overall series of patients are shown in Table 2. Despite significantly higher insulin levels (not shown in detail), confirming results presented in Fig. 2, FOXO1 was less phosphorylated at Ser^{256} in NASH compared with steatosis alone and control subjects (Fig. 3*A* and Table 2).

PGC-1α expression tended to decrease from subjects with normal liver histology to simple steatosis and NASH (Fig. 3*A* and Table 2). Sirtuin 1 protein levels did not differ in NASH versus simple steatosis and control subjects (Table 2), but coimmunoprecipitation experiments showed a higher amount of sirtuin 1 associated with FOXO1 in patients with NASH compared with those with simple steatosis and those with normal liver histology (Table 2 and Fig. 3*B*), suggesting increased interaction between FOXO1 and sirtuin 1 in NASH.

To determine whether the status of the upstream signaling pathways was also consistent with increased FOXO1 activity in NASH, we analyzed the expression of insulin receptor, Akt/phosphorylated Akt, and JNK1/phosphorylated JNK in patients with NAFLD and control subjects (Fig. 3*A* and Table 2). We observed a significant downregulation of Akt in patients with steatosis compared with control subjects, whereas phosphorylated Akt levels were decreased in NASH versus control subjects but was highly variable in simple steatosis (as shown in Table 2). Insulin

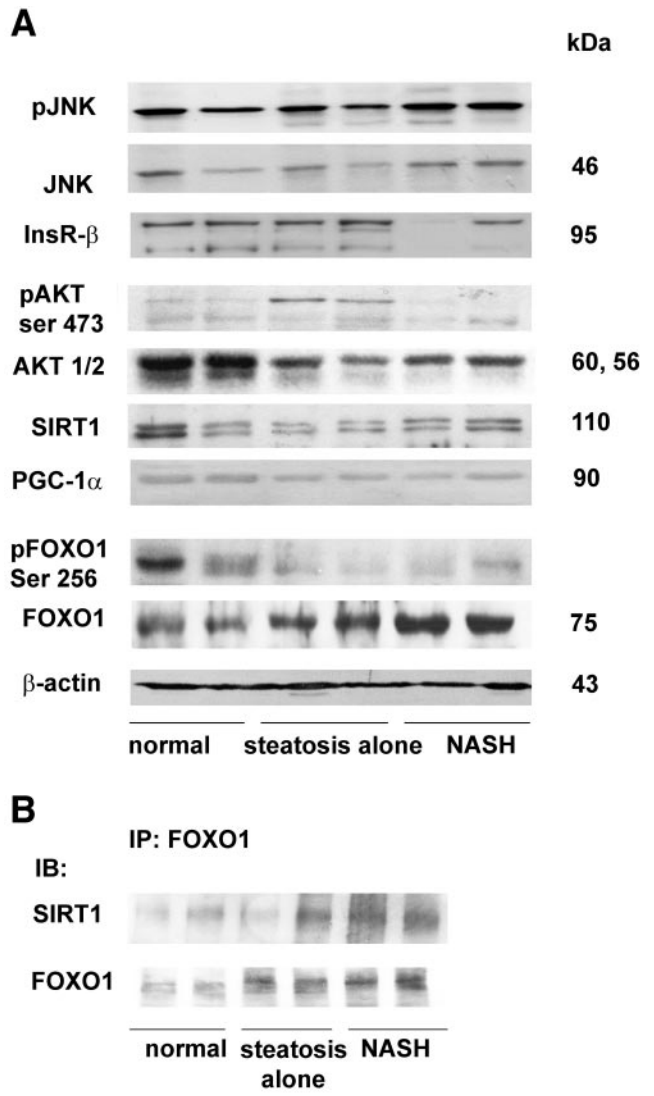

FIG. 3. FOXO1 regulation. We considered two subjects with liver biopsy within normal limits, two subjects with steatosis alone, and two with definite NASH. *A*: Protein expression and phosphorylation (Ser^{256}) of FOXO1, sirtuin 1 (SIRT1), PGC-1α, insulin receptor (InsR), Akt 1/2, phosphorylated Akt (pAKT), JNK1, and phosphorylated JNK (pJNK) are shown. β-Actin is shown as control. *B*: FOXO1 association with sirtuin 1 as detected by immunoprecipitation (IP). IB, immunoblotting.

receptor expression was decreased, whereas the expression and phosphorylation of the stress kinase JNK1 were increased in NASH versus other groups.

To determine the cellular localization of FOXO1, we performed immunohistochemical staining of liver samples. In patients with NASH (Fig. 4*A–C*), we observed diffuse hepatocellular nuclear staining and scattered positive nuclei of Kupffer cells, whereas FOXO1 was mainly cytoplasmic in subjects with simple steatosis (Fig. 4*D*), and FOXO1 staining was minimal in subjects with normal liver (Fig. 4*E* and *F*). Taken together, these data suggest that FOXO1 expression is influenced by histopathological findings and that FOXO1 activity is increased in hepatocytes in NASH.

Expression of other transcription factors involved in gluconeogenesis and lipid metabolism. The expression of other transcription factors involved in gluconeogenesis and lipid metabolism was evaluated in the same patients considered in Fig. 3*A*; results are shown in Fig. 5. Expression of C/EBPα, C/EBPβ, HNF-4, and PPAR-α was not higher in patients with NASH compared with that with simple steatosis and normal liver, whereas CREB protein levels were higher in subjects with fatty liver compared with those with normal histology.

FOXO1, severity of steatohepatitis, and insulin resistance. Because FOXO1 was increased in NASH, characterized by the presence of inflammation and hepatic insulin resistance, we next analyzed the relationship between FOXO1, the histological severity of the disease, and gluconeogenesis (Fig. 6). FOXO1 mRNA levels were correlated with HOMA-IR index ($R^2 = 0.2$, $P = 0.01$) (Fig. 6*A*) and NASH activity score ($R^2 = 0.2$, $P < 0.0001$) (Fig. 6*B*). FOXO1 was significantly correlated with both intralobular inflammation according to Kleiner et al. (25) ($R^2 = 0.15$, $P = 0.001$), and the percentage of steatotic hepatocytes ($R^2 = 0.13$, $P = 0.002$), but not with the severity of fibrosis. FOXO1 mRNA levels were correlated with the expression of both PEPCK ($R^2 = 0.2$, $P < 0.0001$) (Fig. 6*C*) and G6PC ($R^2 = 0.25$, $P < 0.0001$) (Fig. 6*D*). The correlation was even stronger when PEPCK and G6PC were adjusted for insulin levels ($R^2 = 0.4$ and 0.35, respectively, $P < 0.0001$ for both). No significant correlation was observed between FOXO1 and PGC-1α/sirtuin 1 mRNA levels. In patients with chronic HCV hepatitis, FOXO1 mRNA levels were significantly associated with HOMA-IR ($R^2 = 0.2$, $P = 0.05$), but not with PEPCK and G6PC.

Effect of therapy on FOXO1 mRNA levels. Finally, we analyzed the effect of pharmacological therapy on FOXO1 expression in patients with NAFLD. Of the 54 patients, 8 were taking drugs acting directly or indirectly on hepatic insulin resistance or hepatic lipid metabolism (1 PPAR-γ, 2 PPAR-α agonists, and 6 metformin). FOXO1 expression was significantly influenced by the presence of NASH and treatment with insulin sensitizers ($P < 0.0001$), being significantly higher in untreated patients with NASH compared with the other groups (NASH untreated 2.1 ± 0.6, simple steatosis untreated 1.4 ± 0.6, NASH treated 1.2 ± 0.8, simple steatosis treated 1.1 ± 0.7; $P < 0.05$ for untreated patients with NASH vs. all the other groups). At logistic regression analysis, considering steatosis, necroinflammatory activity, and therapy, high FOXO1 mRNA levels were independently associated with steatosis (odds ratio [OR] 2.62 per unit increase, 95% CI 1–7.7, $P = 0.05$), necroinflammatory activity (OR 2.65 per unit increase, 95% CI 1.2–6.8, $P = 0.02$), and therapy (OR 0.23, 95% CI 0.07–0.7, $P = 0.01$).

DISCUSSION

In this study, we measured insulin resistance at the gene expression level in patients with NAFLD, a widely prevalent condition associated with the features of metabolic syndrome (2,3,6), and investigated whether FOXO1 expression and activity were associated with insulin resistance. Our results confirm and extend findings in animal models, indicating that increased FOXO1 activity may play a role in the pathogenesis of hepatic insulin resistance associated with NAFLD.

Because NAFLD is characterized by impaired suppression of hepatic glucose output by insulin (2), we first determined mRNA expression of the rate-limiting gluconeogenic genes in patients and control subjects and found that both PEPCK and G6PC were not downregulated by hyperinsulinemia in NAFLD. In fact, PEPCK and G6PC levels were increased in patients with NASH,

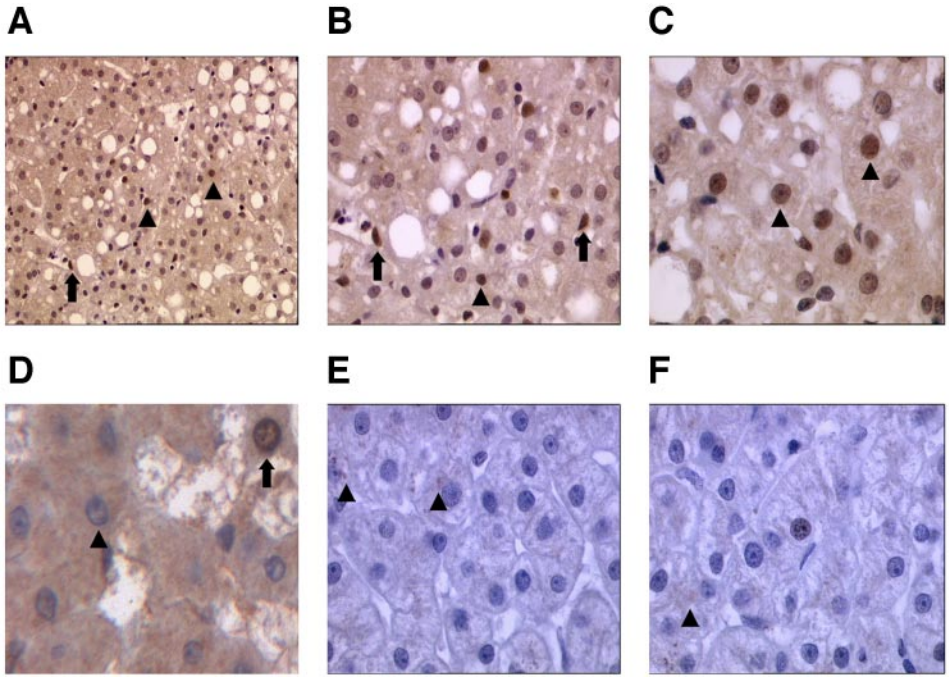

FIG. 4. FOXO1 localization. *A–C*: Patients with NASH. Diffuse hepatocellular nuclear staining (arrowheads), hepatocellular cytoplasmic staining, and scattered positive nuclei of Kupffer cells (arrows) are observed (200×, 400×, and 1,000×). *D*: Patient with simple steatosis: hepatocellular cytoplasmic staining, with negative nuclear staining (arrowhead), occasional positive hepatocellular nuclei with a dot-like pattern (1,000×). *E* and *F*: Patients with "normal" liver histology, showing minimal hepatocellular staining in scattered hepatocytes (arrowheads), and an isolated, weakly positive nucleus (1,000×). (A high-quality digital representation of this figure can be found at http://dx.doi.org/10.2337/db07-0714.)

characterized by severe hyperinsulinemia and a 50% prevalence of impaired glucose tolerance, and PEPCK expression correlated with the HOMA-IR index. Thus, we confirmed at the gene expression level the hepatic insulin resistance observed in clamp studies and animal models of NAFLD, and the increased fasting hepatic glucose output in those patients progressing toward hyperglycemia (2,11,29,30).

We next reasoned that deregulation of the forkhead transcription factor FOXO1, which has been demonstrated to

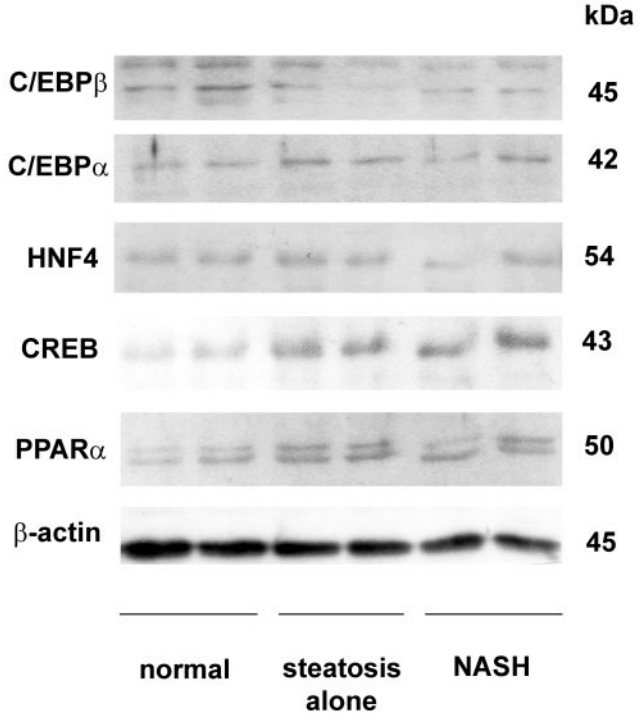

FIG. 5. Expression of other transcription factors C/EBPα, C/EBPβ, HNF4, CREB, and PPARα involved in the regulation of gluconeogenesis and lipid metabolism in representative patients with normal liver, simple steatosis, and NASH (as in Fig. 3*A*).

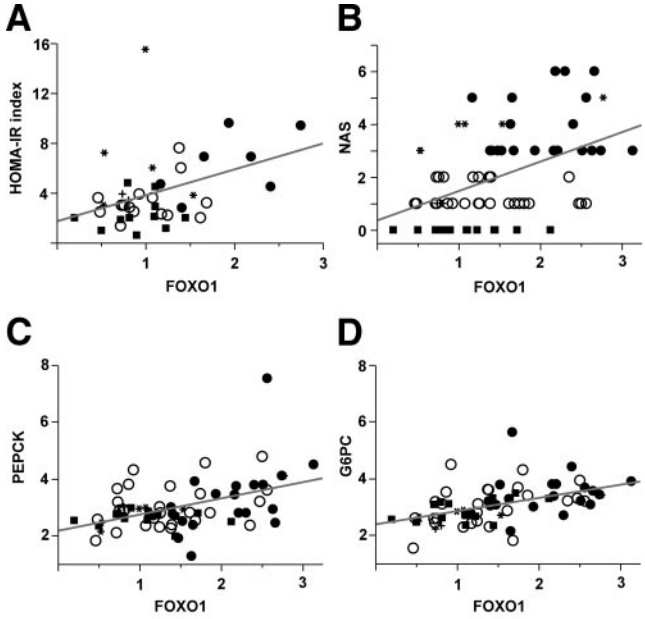

FIG. 6. FOXO1, NASH activity, and insulin resistance. Correlation between FOXO1 mRNA levels and HOMA-IR index ($P = 0.01$) (*A*), NASH activity score ($P < 0.0001$) (*B*), and mRNA levels of the glucogenetic genes PEPCK ($P < 0.0001$) (*C*) and G6PC ($P < 0.0001$) (*D*). ■, normal biopsy; ○, steatosis alone; +, steatosis alone treated; ●, NASH; *, NASH treated.

mediate the effect of insulin on gluconeogenesis in animal models (13), may play a role in this process. To check this hypothesis, we measured the expression of FOXO1 and interacting nuclear factors (20). We observed a progressive increase in FOXO1 mRNA levels from subjects with normal liver to those with steatosis alone and NASH, and a significant correlation between FOXO1 mRNA and protein levels. In addition, we detected increased interaction between FOXO1 and sirtuin 1 in patients with simple steatosis, more marked in NASH, which was associated with a relative decrease in Ser^{256} phosphorylation, findings consistent with increased transcriptional activity (19). Decreased PGC-1α expression suggests that increased recruitment of this coactivator at the promoter of gluconeogenic genes, because of interaction with FOXO1 and regulation by insulin signaling and sirtuin 1 (14,31,32), rather than transcriptional induction, supports gluconeogenesis in NASH.

Interestingly, increased FOXO1 mRNA and protein levels have been observed in the liver in mouse models of diet-induced insulin resistance (18,33). The biological plausibility of transcriptional regulation of FOXO1 is reinforced by the finding of a positive correlation with the expression of its bona fide transcriptional targets PEPCK and G6PC and the HOMA-IR index. Moreover, FOXO1 mRNA levels were independently associated with the degree of steatosis and necroinflammatory activity, suggesting that steatosis and inflammation are involved in FOXO1 regulation, possibly by inducing oxidative stress (28,34), and negatively associated with treatment with insulin-sensitizing drugs. Nevertheless, the low number of patients and the lack of prospective evaluation suggest caution in interpreting these latter results.

The activation status of the upstream insulin signaling pathway also favored increased FOXO1 activity. Indeed, we observed a significant decrease in Akt levels in patients with simple steatosis and NASH compared with control subjects. Decreased insulin receptor expression in NASH and the mechanisms underpinning Akt downregulation deserve further evaluation. At the same time, JNK1 and phosphorylated JNK were increased in NASH compared with normal liver and simple steatosis, in agreement with that observed in rodent models of this disease (35). Interestingly, JNK has previously been shown to induce downregulation of insulin signaling through insulin receptor substrate-1, which selectively regulates glucose metabolism in the liver (36), and to directly interact with FOXO1, favoring nuclear translocation, transcriptional activation, and gluconeogenesis (37), suggesting that increased JNK may be implicated in further activation of FOXO1 in NASH.

Thus, it appears that different mechanisms may be recruited to increase FOXO1 activity in NASH. The first one involves progressive upregulation of mRNA levels secondary to steatosis and inflammation. The second encompasses sequential alterations in intracellular signaling pathways, i.e., decreased Akt activity associated with steatosis and increased JNK in patients progressing to NASH. The third is related to increased interaction with sirtuin 1 and other nuclear factors at gene promoters.

Immunohistochemical evaluation supports these conclusions by showing increased FOXO1 hepatocellular staining in patients with fatty liver as well as prominent nuclear localization in patients with NASH. The functional significance of FOXO1 expression in hepatic nonparenchymal cells is still undergoing definition: FOXO1 inhibits proliferation of hepatic stellate cells and fibrogenesis, but the activation status was not evaluated in NASH (38), and no data are available for Kupffer cells.

Importantly, our results do not implicate that FOXO1 is the only culprit of increased hepatic glucose output in NASH, and they in fact suggest that upregulation of CREB, regulated by cAMP and insulin signaling through TORC2 (transducer of regulated CREB activity 2) (39), may play a role, suggesting again an integrated hormonal control of gluconeogenesis by CREB/FOXO1 (14).

The study suffers from limitations related to the variability of human biological samples because of environmental and genetic confounders, but it also has strengths: we were able to test in humans molecular mechanisms of disease so far demonstrated only in animal studies providing complementary findings, including also patients affected by the metabolic syndrome with clinical and demographic features reflecting those of the general population. In addition, we could analyze the relationship between liver inflammation related to metabolic disease, which was deeply connected with insulin resistance in previous studies (4,40,41), and metabolic gene expression.

In conclusion, we provide evidence that in humans *1*) fatty liver and steatohepatitis are associated with a progressive increase in the expression of gluconeogenic genes; *2*) PEPCK expression correlates with HOMA-IR, reflecting whole-body insulin resistance; *3*) FOXO1 levels are increased in NASH because of increased mRNA levels; *4*) in the presence of oxidative stress, FOXO1 is hypophosphorylated at Ser^{256} and localized to the nucleus; *5*) FOXO1 mRNA levels correlate with that of its targets PEPCK and G6PC and with insulin resistance; and *6*) FOXO1 mRNA levels correlate with the severity of steatosis and necroinflammation and are possibly influenced by insulin-sensitizing drugs.

The current and other authors' results (14) suggest that increased FOXO1 activity is involved in insulin resistance in patients with NAFLD by inducing gluconeogenic genes. Whether upregulation of FOXO1 expression plays a causal role in the pathogenesis of the metabolic syndrome and is modulated by genetic factors (42) has to be evaluated in future studies.

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