HMGB1 Is Increased by CFTR Loss of Function, Is Lowered by Insulin, and Increases In Vivo at Onset of CFRD

Luisa Montanini, Francesca Cirillo, Arianna Smerieri, Giovanna Pisi, Ida Giardino, Maria d'Apolito, Cinzia Spaggiari, Sergio Bernasconi, Sergio Amarri, and Maria Elisabeth Street

Department of Pediatrics (L.M., A.S., G.P., C.S., S.B.), University Hospital of Parma, 43126 Parma, Italy; Department of Pediatrics (F.C., S.A., M.E.S.), Istituto di Ricovero e Cura a Carattere Scientifico-Arcispedale Santa Maria Nuova, 42123 Reggio Emilia, Italy; and Department of Clinical and Experimental Medicine (I.G, M.d.), University of Foggia, University Hospital of Foggia, 71122, Foggia, Italy

Context: Cystic fibrosis-related diabetes (CFRD) is associated with worsening of inflammation and infections, and the beginning of insulin treatment is debated.

Objectives: To verify high-mobility group box 1 protein (HMGB1) levels in CF patients according to glucose tolerance state, and analyze relationships with insulin secretion and resistance. To verify, in an in vitro model, whether HMGB1 gene expression and protein content were affected by insulin administration and whether these changes were dependent on CF transmembrane conductance regulator (CFTR) loss of function.

Patients and Methods: Forty-three patients in stable clinical conditions and 35 age- and sex-matched controls were enrolled. Glucose tolerance was established in patients based on a 5 point oral glucose tolerance test (OGTT). Fasting glucose to insulin ratio (FGIR), HOMA-IR index, whole-body insulin sensitivity index (WIBISI), and the areas under the curve for glucose (AUCG) and insulin (AUCI) were calculated. HMGB1 was assayed in serum, in cell lysates and conditioned media using a specific ELISA kit. For the in vitro study we used CFBE41o− cells, homozygous for the F508del mutation, and 16HBE14o− as non-CF control. HMGB1 gene expression was studied by real-time RT-PCR. Cells were stimulated with insulin at 2.5 and 5 ng/mL. The CFTR inhibitor 172 and CFTR gene silencing were used to induce CFTR loss of function in 16HBE14o− cells.

Results: HMGB1 levels were increased at onset of CFRD (5.04 $\pm$ 1.2 vs 2.7 $\pm$ 0.3 ng/mL in controls; $P < .05$) and correlated with FGIR ($R = +0.43$; $P = .038$), and AUCI ($R = +0.43$; $P = .013$). CFTR loss of function in the 16HBE14o− cells increased HMGB1 and was lowered by insulin.

Conclusion: HMGB1 was increased in CF patients with deranging glucose metabolism and showed relationships with indexes of glucose metabolism. The increase in HMGB1 was related to CFTR loss of function, and insulin lowered HMGB1. Further research is required to verify whether HMGB1 could potentially be a candidate marker of onset of CFRD and to establish when to start insulin treatment. **(*J Clin Endocrinol Metab* 101: 1274–1281, 2016)**

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in USA

Received October 20, 2015. Accepted January 8, 2016.
First Published Online January 13, 2016

Abbreviations: AUCG, area under the curve for glucose concentration; AUCI, area under the curve for insulin; BMI, body mass index; CFRD, cystic fibrosis-related diabetes; CFTR, CF transmembrane conductance regulator; CV, coefficient of variation; FEV1, forced expiratory volume in the first second; FGIR, fasting glucose to insulin ratio; HMGB1, high-mobility group box 1 protein; HOMA-IR, homeostasis model assessment index—insulin resistance; IGT, impaired glucose tolerance; NGT, normal glucose tolerance; OGTT, oral glucose tolerance test; SDS, SD score; siRNA, short interfering RNA; TLR, Toll-like receptor; WBISI, whole body insulin sensitivity index.

Cystic fibrosis-related diabetes (CFRD) is the most frequent and severe comorbidity in CF and has been shown to be related with both insulin deficiency, and insulin resistance (1–3). CFRD increases with age and is more frequent in F508del homozygote patients; however, the presentation and severity are quite variable.

Currently, the diagnosis relies on the criteria of the American Diabetes Association (4). CFRD onset is well known to be associated with worsening of inflammation and clinical conditions (5).

CF is the most frequent life-threatening recessive genetic disorder in Caucasians (6), caused by mutations in a gene that encodes for the CF transmembrane conductance regulator (CFTR) protein. CF is a condition well known to be characterized by chronic inflammation (7, 8), and patients are subject to infections. In particular, *Pseudomonas aeruginosa* and *Burkholderia cenocepacia* infections are associated with worsening of the disease and progression to end-stage disease (9). The recognition of these pathogens is mediated in part by Toll-like receptors (TLRs) activation of an intracellular signaling cascade leading to increased cytokine synthesis (10). Bacterial lipopolysaccharide is recognized by TLR4 and is expressed by the above mentioned pathogens (9). TLR activation has been shown also to be the a cause of increased ERK1/ERK2 activation in CF lung disease (10, 11). The receptor for advanced-glycation-end-products (RAGE) regulates immune responses and inflammation, and recent evidence suggests a role of this receptor in contributing to inflammation in CF and to a greater extent in CFRD (12). Receptor for advanced-glycation-end-products (RAGE) activation, activates the nuclear factor kappa light chain enhancer of activated B cells (Nf-κB) pathway, besides the MAPK pathway promoting cytokine production (13). Interestingly, all these receptors are activated by the high-mobility group box 1 protein (HMGB1) (13). HMGB1 is a chromatin-linked nonhistonic protein with cytokine activity that has nuclear, cytosolic and extracellular actions. In the nucleus, HMGB1 binds chromosomal DNA contributing to nucleosomal structure, regulation of gene expression, DNA recombination, repair, and replication, and interacts with many proteins such as transcrptional factors (14). In the cytosol, HMGB1 promotes autophagy and acts as a cell-membrane form. In the presence of inflammation, HMGB1 is actively secreted in the extracellular space and is responsible for autocrine and paracrine effects as activation of nuclear factor kappa light chain enhancer of activated B cells (Nf-κB), diffuse endothelial activation, systemic activation of inflammatory cells, and other effects besides presenting bactericidal activity (14). Studies in (CFTR)(−/−) mice have shown that neutralizing HMGB1 using specific monoclonal antibodies protected against *P. aeruginosa*-induced neutrophil recruitment, lung injury, and bacterial infection (15).

Interestingly, in recent years HMGB1 has been shown to be altered in other chronic inflammatory conditions as those present in obesity and insulin resistance, and it has been shown to be a stimulatory factor of insulin secretion of β-pancreatic cells (16).

During islet tranplantation for the treatment of type 1 diabetes mellitus (DM), an HMGB1-mediated inflammatory response has been shown to be able to cause early islet loss through TLR2 and TLR4 (17, 18).

Furthermore, HMGB1 serum concentrations were described to be increased in obese children and to be associated with signs of the metabolic syndrome (19). In CF, HMGB1 in sputum is higher than in healthy subjects, and in particular in those who present a faster decline of lung function (20).

In a rat model with endotoxin-induced acute lung injury and induced hyperglycemia, HMGB1 was shown to increase significantly and to be associated with enhanced lung damage. However, insulin treatment for hyperglycemia lowered both blood glucose and serum HMGB1 levels, reducing lung damage (21). These findings could share common aspects that are seen in CF patients at onset of CFRD.

The aim of this study was first, to investigate HMGB1 serum concentrations in CF patients with normal, impaired glucose tolerance (IGT), and at the onset of CFRD. Second, to correlate HMGB1 serum levels with the glucose tolerance state, and indexes of insulin secretion and resistance. Furthermore, in a CF in vitro model, we verified whether HMGB1 gene expression and protein content were affected by insulin administration and whether these changes were dependent on CFTR loss of function.

Patients and Methods

Patients

Forty-three patients with CF and 35 controls from a previous study (22) were subdivided into prepubertal, pubertal and adults. The 2 groups were comparable for chronological age, sex, pubertal stage, and body mass index (BMI). Control subjects were enrolled from young physicians training at our medical school and children attending a growth clinic who had no evidence of endocrine dysfunction, chronic illness, or dysmorphic features, and healthy at the time of the study.

Pubertal development was assessed according to the criteria of Marshall and Tanner (23, 24). The adult condition was defined by the attainment of final height and Tanner stage 5. Height and weight were measured and BMI (kg/m^2) calculated. BMI was expressed as SD scores (SDSs) using Italian reference data (25). Clinical details of patients and controls are presented in Table 1.

The diagnosis of CF had been previously confirmed in all patients by genetic analysis and a sweat test. All patients were in

Table 1. Clinical Features of Patients and Control Subjects

CF Patients	n (M/F)	C.A. (y)	BMI SDS	FEV1
Prepubertal	14 (5/9)	8.8 ± 0.6	1.4 ± 0.2	85.2 ± 3.2
Pubertal	12 (7/5)	14.3 ± 1.0	−0.09 ± 0.4	83.4 ± 4.8
Adults	17 (7/10)	23.0 ± 1.5	0.15 ± 0.21	78.5 ± 2.7
Controls				
Prepubertal	13 (8/5)	9.8 ± 1.2	−0.1 ± 0.55	
Pubertal	12 (7/5)	12.8 ± 1.2	0.6 ± 0.3	
Adults	20 (8/12)	23.8 ± 1.3	−0.08 ± 0.24	

n, number; M, males; F, females; C.A., chronological age.

functional classes I and II. All had exogenous pancreatic insufficiency that required enzymatic supplementation and were treated with ongoing antibiotic therapy. None of the patients had been taking steroids for at least 3 months, none was receiving azitromycin at the time of the study, and none was positive for *P. aeruginosa* and *Burkholderia cepacia* infections. The Shwachman-Kulcyski score in all subjects was over 41, and all were in stable clinical conditions at the time of assessment (22).

Thyroid and liver function tests and serum electrolytes were found to be normal in all patients.

For each patient pulmonary function tests assessed by spirometry, measuring forced expiratory volume in the first second (FEV1) on a VMax 20 Spirometer, expressed as the percentage of predicted values for age, sex, ethnical background, weight, and height, based on the standards of the European Community for Coal and Steel (26). Nutritional status was defined according to classification of European standards (27). All patients were in good nutritional condition as confirmed by the BMI SDSs.

Assessment of glucose tolerance

Fasting blood glucose level and insulin were measured in all patients at enrolment. All patients underwent an oral glucose tolerance test (OGTT) after an overnight fast, in the morning (1.75-g/kg body weight, maximum 75 g of glucose, as 50% glucose solution). Glucose and insulin values were measured at baseline, 30, 60, 90, and 120 minutes. The patients were classified into 3 groups according to American Diabetes Association diagnostic criteria (4): normal glucose tolerance (NGT) (glucose levels at T120 < 7.7 mmol), IGT (glucose levels at T120 > 7.7 < 11.1 mmol), or diabetes mellitus (DM) (glucose levels at T120 > 11.1 mmol) (Table 2).

The whole-body insulin sensitivity index (WBISI) was calculated based on insulin (IU/mL) and glucose (mg/dL) concentrations obtained during the OGTT and the corresponding fasting values, as originally described by Matsuda and DeFronzo (28). The area under the curve for glucose (AUCG) and area under the curve for insulin (AUCI) were calculated using trapezoidal integration (29).

Assessment of fasting indices of insulin sensitivity

From fasting glucose and insulin the homeostasis model assessment index—insulin resistance (HOMA-IR), and the fasting glucose to insulin ratio (FGIR) were calculated according to standard formulas (30). For the HOMA-IR index, glucose was expressed in mmol/L and insulin in mIU/mL.

Biochemical assays

Peripheral blood was obtained by venepuncture and then coagulated at room temperature for 0.5–2 hours. Serum was collected by centrifugation at 2000*g* for 5 minutes and then at 12 000*g* for 10 minutes to completely remove cell debris. Serum was stored at −80°C for HMGB1 detection.

Plasma glucose concentrations were assayed using a polarographic method (Synchron CX Systems).

Insulin was measured using a chemiluminescence method by Diagnostic Products Corp for reading by Immulite2000. The intraassay coefficient of variation (CV) was 6.5 and the interassay CV 7.1%. Light intensity was read using BN2 and BN Nephelometers (normal range, 0–5 mg/L).

HMGB1 was assayed using a specific research ELISA kit (IBL International GmbH). The intraassay CV was 5.4%, and the

Table 2. Clinical Features of Patients Subdivided According to Glucose Tolerance and Genotype Classes

CF Patients	N (M/F)	C.A. (y)	BMI SDS	FEV1	Genotype (n)
NGT	19 (8/11)	14.5 ± 1.2	0.4 ± 0.3	88.7 ± 2.8	n = 9 homozygote F508del n = 6 heterozygotes N = 4 other
IGT	15 (7/8)	15.6 ± 2.7	−0.4 ± 0.4	72.8 ± 6.1	n = 8 homozygote F508del n = 4 heterozygotes N = 3 other
CFRD at onset	9 (3/6)	21.5 ± 2.2	0.1 ± 0.5	86.8 ± 10.1	n = 4 homozygote F508del n = 3 heterozygotes n = 2 other

n, number; M, male; F, female; C.A., chronological age. Homozygote F508del, homozygotes for the F508del mutation; heterozygotes, heterozygotes for the F508del mutation; other, other mutations in the CFTR gene: G553X, N1303K, 2789 + 5G>A, 4016 insT, L1065P, 3849 + 10kbc-T, E585X, 1898 + 3A>G, R1066H, D1152H, R553X, W57G, 4382delA, G85E, G542X, T338I, W1282X, Q652X, MiV, 4022insT, 1717–1GA, R1006C, and 2183AA-G.

interassay CV was 8.2%. The sensitivity of the method was less than 0.15 ng/mL.

Ethical approval

Informed consent was obtained from the subjects and/or the parents as appropriate. The study was approved by the Ethical Committee at the University of Parma.

Cell lines and cultures

Two airway epithelial cell lines, CFBE41o−, homozygous for the F508del mutation (derived from a bronchial isolate of a CF patient homozygous for the F508del CFTR mutation), and 16HBE14o− (derived from normal bronchus) as non-CF control, were used (31).

Cells were grown as previously described (11) and were tested for mycoplasma contamination.

Both cell lines were treated with insulin (ProSpec-Tany TechnoGene Ltd) at 2.5 and 5 ng/mL for 24 hours. Cell lysates for RNA extraction were collected after a starvation in serum-free medium for 24 hours.

CFTR inhibition and gene silencing

Only normal cells (16HBE14o− cells) were treated with the CFTR inhibitor (CFTR inh 172) to recreate the CF condition (32, 33). Cells were treated with either Dimethyl Sulfoxide (DMSO) (Sigma) as vehicle control or 20mM CFTR inh 172 (Sigma) prepared in DMSO and diluted from a 1:1000 stock.

No toxicity from the treatment with the CFTR inhibitor was observed using trypan blue exclusion assay. Moreover, after CFTR inhibition, insulin stimulation was performed as previously described.

All experiments were repeated at least 3 times in triplicate for each cell line.

To reduce the amount of endogenous CFTR, 16HBE14o− cells were transfected with short interfering RNA (siRNA) against human CFTR (FlexiTube GeneSolution GS1080 for CFTR). All reagents for silencing were obtained from QIAGEN. We used a pooled solution of 4 siRNA (target sequence: 5′-TCG ATATATTACTGTCCACAA-3′, 5′-ATCGCGATTTATCTAGGCATA-3′, 5′-CTCGAAAGTTATGATTATTGA-3′, and 5′-ATGGCCAACTCTCGAAAGTTA-3′). Transfection was carried out by using HiPerFect transfection reagent and 10nM siRNA per well, according to the manufacturer's instructions. A negative control siRNA was used as a control. Silencing was monitored at the mRNA level using QuantiFast Probe assay double probe kits (β-actin housekeeping gene) for 1-step real-time RT-PCR. Gene silencing was approximately 55% (data not shown).

Total protein content

Lysates were obtained using modified radio immunoprecipitation assay (RIPA) buffer (50mM Tris-HCL [pH 7.4]; 1% Nonidet P40 (NP-40); 0.25% Na-deoxycolate; 150mM NaCl; 1mM EDTA; and 1mM phenyl sulfonyl fluoride (PMSF); 1-μg/mL aprotinin, leupeptin, and pepstatin; 1mM Na_3VO_4; and 1mM NaF) and stored at −80°C until assayed. The total protein content was determined using the "microassay Bio-Rad" protocol (Bio-Rad Lab). Briefly, a standard curve was prepared using bovine albumin (Bio-Rad Lab). The dye was added to each sample and standard, and the absorbance was read at 595 nm using VICTOR X4 multilablel reader (PerkinElmer).

Protein assays in lysates and conditioned media

HMGB1 content was assayed using the same research kit as for serum and as specified above.

Concentrations were normalized per mg of total protein content in the lysates and conditioned media.

RNA isolation and characterization

Total RNA was extracted from cell lysates using RNeasy Mini kit (QIAGEN) according to the manufacturer's protocol including Deoxyribonuclease (DNase) step. The RNA concentration of all samples was quantified by NanoDrop 1000 (Nanodrop). All RNA samples were stored at [minus 80°C until RT-PCR analysis.

Real-time RT-PCR

The mRNA from airway epithelial cell lines was reverse transcribed by SuperScript III First Strand Synthesis System (Life Technology).

Quantitative real time polymerase chain reaction (qRT-PCR) was performed using Power SYBR Green (Applied Biosystems) with the following primer pairs: HMGB1fwd, 5′-GGGCCCTAATTCGGTCATGT-3′ and HMGB1rev, 5′-TTGGGTCAGGCGGTTCATAC-3′ for the amplification of HMGB1 (GenBank accession number NM_002015) target gene; β-actfwd, 5′-CCAACCGCGAGAAGATGA-3′ and β-actrev, 5′-CCAGAGGCGTACAGGGATAG-3′ for the amplification of β-actin (GenBank accession number XM_006715764) as the endogenous control.

The mRNA was reverse transcribed by SuperScript III First Strand Synthesis System (Life Technology). Experiments were performed in triplicate in optical 96-well reaction plates on an iCycler iQ Multicolor Real-Time PCR Detector (Bio-Rad) with iQ SYBR green supermix (Bio-Rad).

Expression levels of HMGB1 mRNA were normalized to β-actin levels in the same sample. Melting curves were analyzed to ensure that fluorescence signals solely reflected specific amplicons.

Statistical analysis

Standard statistical analysis was performed using the statistical package SPSS 21.0.0 for Windows (Copyright IBM Corp 1989, 2012) as appropriate. The normality of the data was assessed using the Smirnov-Kolmogorov test. We did not find significant differences between the data from males and females, thus the data were pooled. Statistical analysis was performed using a one-way ANOVA test followed by Scheffé's test to study differences among groups. Correlation analysis was performed using Pearson's regression analyses to investigate relationships between HMGB1 and fasting glucose, fasting insulin, FGIR, HOMA-IR index, AUCG, AUCI, and WBISI as specified above. Only significant data are reported in the text. Data were presented as mean ± SEM. $P < .05$ was considered statistically significant.

Results

HMGB1 serum concentrations in patients and controls

HMGB1 serum concentrations were similar in control subjects and in CF patients with NGT (2.7 ± 0.3 and 2.8 ±

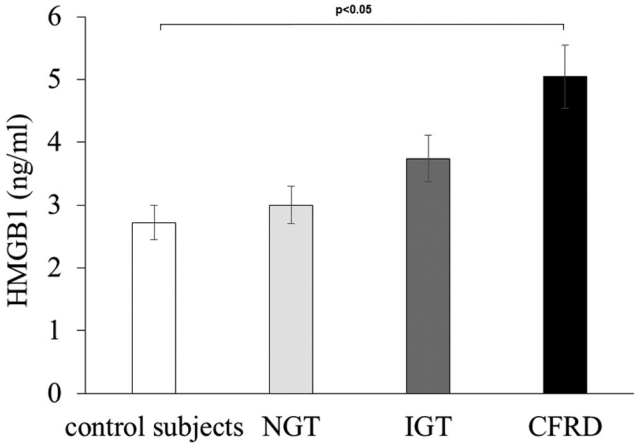

Figure 1. HMGB1 concentrations in serum from healthy controls and in CF patients having NGT and IGT and at onset of CFRD. Levels were significantly increased at onset of CFRD with respect to comparable age-matched controls; *, $P < .05$.

0.3 ng/mL, respectively), whereas HMGB1 levels were slightly increased in CF patients having IGT (3.96 ± 0.96 ng/mL) and significantly higher at onset of CFRD (5.04 ± 1.2 ng/mL) with respect to healthy controls ($P < .05$) (Figure 1).

Correlation analysis

In the CF patients, HMGB1 serum concentrations were found to be positively correlated with both the fasting glucose concentrations (R = +0.39; $P = .014$) and with the FGIR (R = +0.43; $P = .038$).

HMGB1 levels were also correlated with the AUCI (R = +0.43; $P = .013$) (data not shown).

Increased HMGB1 is a feature of CF

To determine whether the increase of HMGB1 levels was a feature of CF, we measured HMGB1 expression and protein concentrations in CF-affected and in normal cell lines. Under baseline conditions, HMBG1 relative gene expression was significantly higher in the CFBE41o− cells than in the 16HBE14o− cells (0.2 ± 0.03 vs 0.002 ± 0.002; $P < .05$) (Figure 2A), whereas the HMGB1 protein content within the cell lysates was 2.45-fold higher in the CFBE41o− cells than in their normal counterpart (178 ± 5.8 vs 73 ± 4.7 ng/mL; $P < .05$) (Figure 2B).

In conditioned media, HMGB1 protein content was always found to be significantly lower in the CFBE41o− cells compared with the normal cell line (Figure 3).

Insulin reduces HMGB1 expression and protein content in normal and CF cell lines

Because insulin treatment has been shown to decrease serum HMGB1 concentration (21), HMGB1 gene expression and protein levels were measured in CFBE41o and in the 16HBE14o− cells line after 24 hours of incubation with different amounts of insulin. Insulin treatment reduced HMGB1 gene expression (Figure 2A) and HMGB1 protein content within the cell lysates (Figure 2B) in both cell types with a maximum effect, in the CF-affected cell line, at 2.5 ng/mL.

In conditioned media, no change in HMGB1 levels was seen after stimulation with insulin in both cell lines (Figure 3) after a 24-hour incubation period.

HMGB1 expression is related to CFTR function

16HBE14o− cells were treated with CFTR inhibitor 172 in order to assess whether changes in HMGB1 gene expression between the 2 cell lines were dependent on CFTR function.

In baseline conditions, CFTR inhibitor determined a significant increase in HMGB1 relative gene expression (2.49 ± 1.22 vs 1.02 ± 0.1; $P < .05$) (Figure 4A). The effect of CFTR loss of function on HMGB1 expression was confirmed by silencing the CFTR gene in the 16HBE14o− cells, as described in the method section. The CFTR silencing caused a 2.75-fold increase of HMGB1 relative gene expression (0.36 ± 0.03 vs 0.99 ± 0.09; $P < .05$) that was significantly reduced by insulin treatment (Figure 4B).

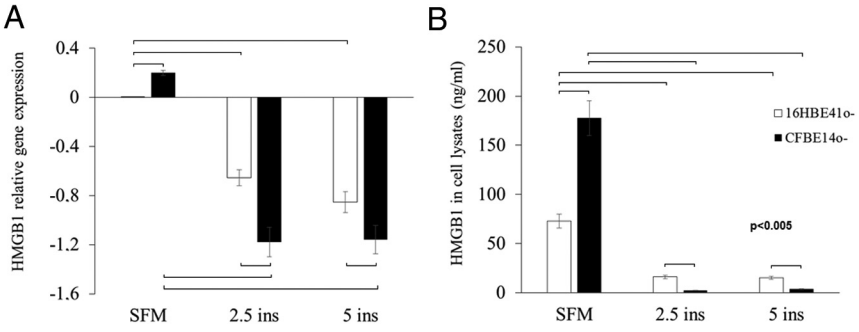

Figure 2. HMGB1 relative gene expression in CF-affected cells and unaffected cells (A), and corresponding protein content in the corresponding cell lysates (B). Gene expression was significantly higher in the CF cell line and was lowered by insulin stimulation with 2,5 and 5 ng/mL for 24 hours. Protein content was also significantly higher in the CF cell line. In both cell types, insulin lowered HMGB1 protein content. Data were normalized with respect to the β-actin gene expression levels. □, 16HBE140−; ■, CFBE410−; SFM; serum-free medium; ins 2.5 and 5, insulin stimulation at 2.5 and 5 ng/mL for 24 hours; *, $P < .005$ vs 16HBE14o− cells.

Discussion

This study showed that HMGB1 serum concentrations was slightly increased in CF patients with IGT, and significantly elevated at the onset of CFRD, compared with CF individuals with normal glucose metabolism and controls.

HMGB1 was found to correlate with blood glucose concentrations,

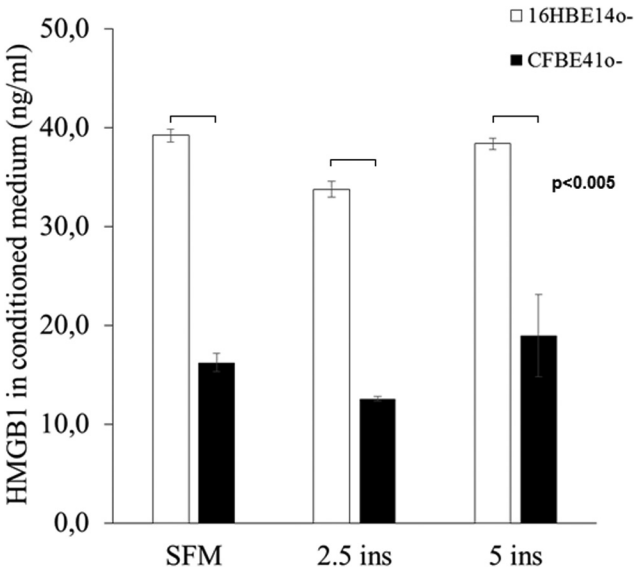

Figure 3. HMGB1 content in conditioned media of CF-affected and unaffected cells. Concentrations were normalized per mg of total protein content and were lower in the CF-affected cells under all conditions. □, 16HBE140−; ■, CFBE410−; SFM, serum-free medium; ins 2.5 and 5, insulin stimulation at 2.5 and 5 ng/mL for 24 hours; *, $P < .005$ vs 16HBE14o− cells.

FGIR, and with the amount of secreted insulin (AUCI). The in vitro study confirmed that increased HMGB1 gene expression and protein content in lysates was a feature of CF and was dependent on CFTR loss of function. Furthermore, we observed that insulin was able to lower HMGB1 in CF-affected cells.

Overall, these data confirmed that HMGB1 has a close relationships with the control of glucose metabolism besides of inflammation, as suggested by other recent findings (12) and provides a possible link among susceptibility to infections, inflammation, worsening of clinical conditions and CFRD.

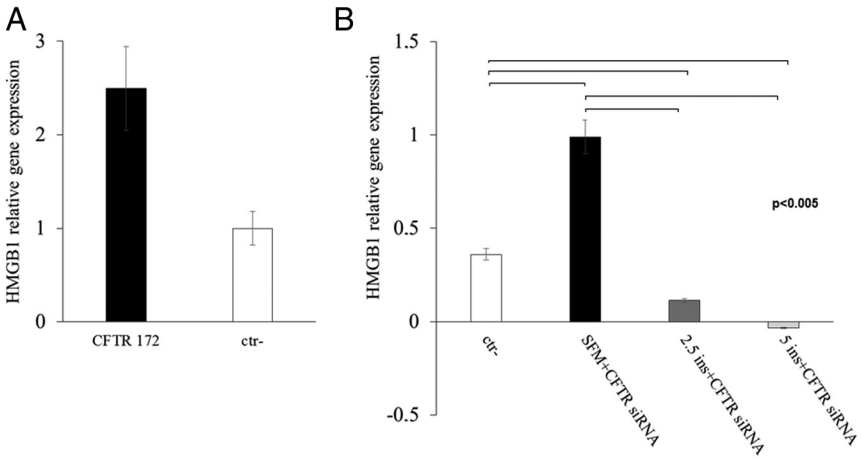

Figure 4. HMGB1 relative gene expression in 16HBE140− treated with CFTR inhibitor 172 (A), and after CFTR gene silencing and stimulation with insulin at 2.5 and 5 ng/mL. CFTR loss of function induced an increase in HMGB1 in both conditions. In B, insulin lowered significantly HMGB1 relative gene expression. Data were normalized with respect to the β-actin gene expression levels. □, 16HBE140−; ■, CFBE410−; SFM, serum-free medium; ins 2.5 and 5, insulin stimulation at 2.5 and 5 ng/mL for 24 hours; *, $P < .005$.

The data also reinforce the association of HMGB1with insulin resistance, the first underlining cause of the metabolic syndrome (19), and possibly with coronary artery disease as well (34). Insulin resistance has been now proven also on a molecular basis in CF, and the changes in the insulin-signaling cascade have been shown to be related to CFTR function (11), as the increase in HMGB1 expression that we describe in this study.

The positive correlation of HMGB1 with fasting blood glucose is compatible with the increase in glucose and HMGB1 observed in IGT and at onset of diabetes as well as the relationship with the FGIR (as glucose increases and insulin lowers, HMGB1 increases).

Furthermore, the positive correlation with the AUCI suggests a close relationship of HMGB1 with the amount of insulin being secreted, which might have a possible protective effect until the onset of diabetes, as suggested by a recent study (16). These authors described HMGB1 as a stimulatory factor of insulin secretion of β-pancreatic cells (16). Furthermore, HMGB1 has been described to be increased at onset of type 2 diabetes and to be more elevated in the obese patients (35).

Pulmonary function could be a possible additional factor influencing HMGB1 concentrations (20, 21); however, the lack of any significant difference in FEV1 among the CF subgroups considered (Table 2) does not suggest any significant effect in this setting and possibly can suggest that the decline in lung function is in part subsequent to the onset of diabetes.

Our in vitro findings showed that the increase in HMGB1 was dependent on CFTR loss of function. HMGB1 was found to be increased in the cell lysates, reflecting nuclear and cytosolic actions of HMGB1, and thus mainly affecting transcriptional factors, matrix metalloproteinases 2 and 9, viral components, and the plasminogen activation system in the nucleus and autophagy in the cytosol (14). In baseline conditions, HMGB1 in conditioned media was lower in the CF-affected cells, and we did not detect any changes after insulin stimulation. This could be due to the short incubation period but could also represent a compartmentalization of HMGB1 towards the functions as described above. There are no previous studies, in cells, to our knowledge, we can compare with.

Insulin was capable of lowering HMGB1 in the in vitro model we used. This is very important, because

it would suggest that humans behave as the mouse model described by Hagiwara et al (21) and that insulin is able to lower glucose, inflammation, and reduce lung damage. It has already been shown that inhibition of HMGB1 protects against *P. aeruginosa* infection (15) and that susceptibility in CF to this infection is related to increased glucose concentrations in airway surface liquid (36, 37).

This could be a strong point in favor of early insulin treatment in CF patients. Since a few years, it has been observed that early identification of CFRD and treatment with insulin was associated with improved survival (38); however, it is yet unclear whether this is due to direct pulmonary effects of insulin or to lower glucose. HMGB1 offers to be a candidate target operating on both. Clinical evidence, in humans, suggests that early insulin treatment does help reduce lung function deterioration (39, 40).

Furthermore, insulin infusion has been shown to suppress TLRs and HMGB1 in mononuclear cells from type 1 diabetic patients (41).

Concluding, HMGB1 was increased in the serum of CF patients with deranging glucose metabolism and showed clear relationships with indexes of glucose metabolism. The in vitro data showed that the increase in HMGB1 was due to CFTR loss of function and that insulin stimulation lowered HMGB1.

Further studies are needed to verify whether HMGB1 could potentially be a candidate marker of onset of CFRD, to establish when to start insulin treatment, and for monitoring treatment in patients with CFRD.

Acknowledgments

We thank Maria Angela Ziveri for her help with the HMGB1 assays and the patients and their families who accepted to participate in the study. We also thank the CF association in Parma for their support over the years.

Address all correspondence and requests for reprints to: Maria Elisabeth Street, MD, PhD, IRCCS-Arcispedale Santa Maria Nuova, Viale Risorgimento 80, 42123 Reggio Emilia, Italy. E-mail: mariaelisabeth.street@asmn.re.it.

This work was supported by the Emilia Romagna Cystic Fibrosis Association and by funds of the Department of Pediatrics, University of Parma.

Disclosure Summary: The authors have nothing to disclose.

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