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NF-κB mediates lipid-induced fetuin-A expression in hepatocytes that impairs adipocyte function effecting insulin resistance

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Fetuin-A, a hepatic secretory protein, has recently been implicated in insulin resistance and Type 2 diabetes. It is an endogenous inhibitor of insulin receptor tyrosine kinase. However, regulation of fetuin-A synthesis in relation to insulin resistance is unclear. In the present paper, we report that both non-esterified ('free') fatty acids and fetuin-A coexist at high levels in the serum of *db*/*db* mice, indicating an association between them. For an in-depth study, we incubated palmitate with HepG2 cells and rat primary hepatocytes, and found enhanced fetuin-A secretion to more than 4-fold over the control. Interestingly, cell lysates from these incubations showed overexpression and activity of NF-κB (nuclear factor κB). In NF-κB-knockout HepG2 cells, palmitate failed to increase fetuin-A secretion, whereas forced expression of NF-κB released fetuin-A massively in the absence of palmitate. Moreover, palmitate stimulated NF-κB binding to the fetuin-A promoter resulting in increased reporter activity. These results suggest NF-κB to be the mediator of the palmitate effect. Palmitate-induced robust expression of fetuin-A indicates the occurrence of additional targets, and we found that fetuin-A severely impaired adipocyte function leading to insulin resistance. Our results reveal a new dimension of lipid-induced insulin resistance and open another contemporary target for therapeutic intervention in Type 2 diabetes.

Key words: adipogenesis, fetuin-A, insulin resistance, non-esterified ('free') fatty acid, nuclear factor κB (NF-κB), Type 2 diabetes.

INTRODUCTION

Fetuin-A [α_2 Heremans–Schmid glycoprotein (*AHSG*)] is synthesized primarily in human liver and secreted into serum. In human beings, it is associated with insulin resistance and Type 2 diabetes [1–3]. Fetuin-A is an endogenous inhibitor of insulin receptor tyrosine kinase phosphorylation and abrogates insulin-stimulated downstream signals [4–6]. This inhibitory activity of fetuin-A has been evaluated in a number of *in vitro* and *in vivo* studies across different species levels, including human beings and was found to be strictly conserved [7,8]. Fetuin-A-knockout mice exhibit improved insulin sensitivity [6,9]. The human fetuin-A gene is located on chromosome 3q27 and has been identified as the Type 2 diabetes susceptibility locus [10]. Although fetuin-A is poised to be a potential factor in affecting insulin resistance and Type 2 diabetes, little is known about the regulation of its synthesis in liver. However, a few reports suggest an involvement of lipid on fetuin-A synthesis. In fatty livers which favour the development of Type 2 diabetes [11], a significant increase in fetuin-A levels [12] and its mRNA expression [13] could be detected, whereas a decrease in liver fat in humans is associated with the decline of plasma fetuin-A [12]. Consistently higher levels of circulatory fetuin-A have been observed in high-fat-fed animals and obese diabetic patients [12,13], whereas fetuin-A-null mice are shown to be protected against obesity and insulin resistance [6]. All of these reports indicate a relationship between lipid and fetuin-A to promote insulin resistance, but direct evidence of its up-regulation by lipid is still lacking.

A few recent reports indicate that, despite fetuin-A's classical inhibitory effect on insulin resistance through the down-regulation of insulin receptor activity, its association with adipogenesis may also be an additional factor that amplifies fetuin-A's attenuating effect on insulin activity, thus aggravating insulin resistance. Human visceral adiposity associated with incident diabetes in older persons is a consequence of higher fetuin-A levels [3]. A polymorphism in the gene encoding human fetuin-A is found to adversely affect insulin action in adipocytes [14]. Fetuin-A has also been shown to repress adiponectin production in animals and humans [15], which will obviously adversely affect adipogenesis. This information indicates fetuin-A's inhibitory effect on adipocyte function, but specific information on this aspect of fetuin-A's effects is still scant.

It should be evident from the above description that fetuin-A is closely associated with insulin resistance and, since this is central to the pathophysiology of Type 2 diabetes, understanding of the regulation of fetuin-A synthesis is extremely important. In the present paper, we report that FA (fatty acid) significantly enhanced fetuin-A expression in the human liver cell line HepG2 by increasing NF-κB (nuclear factor κB) binding to its promoter. In lipid-induced fetuin-A expression, C/EBPβ

Abbreviations used: ADM, adipocyte differentiation medium; aP2, adipocyte protein 2; CD36, cluster of differentiation 36; C/EBPβ, CCAAT/enhancer-binding protein β; ChIP, chromatin immunoprecipitation; DMEM, Dulbecco's modified Eagle's medium; 2-DOG, 2-deoxyglucose; EMSA, electrophoretic mobility-shift assay; FA, fatty acid; FAT, fatty acid translocase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT4, glucose transporter 4; HBSS, Hanks buffered saline solution; HFD, high-fat diet; IL-6, interleukin 6; KRP, Krebs–Ringer phosphate; MEM, minimum essential medium; NEFA, non-esterified ('free') fatty acid; NF-κB, nuclear factor κB; NP-40, Nonidet P40; PDTC, pyrrolidine dithiocarbamate; PPARγ, peroxisome-proliferator-activated receptor γ; RT, reverse transcription; siRNA, short interfering RNA; TNFα, tumour necrosis factor α.

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(CCAAT/enhancer-binding protein β) has no role; however, it was shown to be involved in glucocorticoid-induced fetuin-A expression [16]. In NF-κB-knockout HepG2 cells, FA fails to augment fetuin-A synthesis, whereas its expression is greatly increased in the cells transfected with pCMV-NF-κBp65 vector. Up-regulated fetuin-A expression due to lipid may worsen insulin resistance by impairing adipocyte function.

MATERIALS AND METHODS

Reagents and antibodies

All tissue culture materials were obtained from Gibco-BRL/Life Technologies. FAs were purchased from Sigma. [^{3}H]Leucine (specific activity 1000 Ci/mmol), [^{3}H]2-DOG (2-deoxyglucose) (specific activity 12.0 Ci/mmol), [γ-^{32}P]ATP (specific activity 6000 Ci/mmol) and [^{14}C]palmitate (specific activity 60.0 mCi/mmol) were obtained from GE Healthcare. Antibodies utilized included anti-(rabbit pNF-κBp65), anti-NF-κBp65, anti-fetuin-A, anti-adiponectin, anti-FAT (fatty acid translocase)/CD36 (cluster of differentiation 36), anti-aP2 (adipocyte protein 2), anti-PPARγ (peroxisome-proliferator-activated receptor γ) and anti-C/EBPβ antibodies were purchased from Santa Cruz Biotechnology. Alkaline-phosphatase-conjugated goat anti-rabbit secondary antibody was purchased from Sigma. Glucose estimation kit was procured from Autospan. Recombinant fetuin-A protein and a fetuin-A ELISA kit were procured from R&D Systems. Serum NEFA [non-esterified ('free') fatty acid] levels was measured by acyl-CoA synthase and acyl-CoA oxidase methods (Roche Diagnostics). All other chemicals were purchased from Sigma.

Cell lines and cell culture

The human hepatoma HepG2 and mouse pre-adipocyte 3T3-L1 cell lines were gifts from Dr Partha Banerjee, Georgetown University Medical Center, Washington, DC, U.S.A. The HepG2 cells were cultured in MEM (minimal essential medium) containing Earle's salts and non-essential amino acids supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10 % (v/v) FBS (fetal bovine serum), penicillin (100 units/ml) and streptomycin (100 μg/ml) in a humidified 5 % CO_2 atmosphere at 37 °C. 3T3-L1 pre-adipocytes were maintained in DMEM (Dulbecco's modified Eagle's medium) containing 10 % (v/v) FBS and antibiotics. When the cells reached confluence, they were cultured in ADM (adipocyte differentiation medium) containing DMEM supplemented with 10 % (v/v) FBS, 5 μg/ml insulin, 0.5 mmol/l 3-isobutyl-1-methylxanthine and 1 μmol/l dexamethasone.

Animal experiments

Adult male Sprague–Dawley rats weighing approx. 175–200 g were conditioned at 25 $\pm$ 2 °C with a 12 h light/12 h dark cycle and fed on a standard diet *ad libitum*. An insulin-resistant rat model was reared on an HFD (high-fat diet) for 12 weeks. In terms of total energy, the HFD consisted of 32.5 % lard, 32.5 % corn oil, 20 % sucrose and 15 % protein, whereas the standard diet contained 57.3 % carbohydrate, 18.1 % protein and 4.5 % fat. The energy content of the standard diet was 15 kJ/g and the HFD was 26 kJ/g. Control (C57BLKS/6J) and *db*/*db* [BKS.Cg-m$^{+/+}$ Lepr(db)/J, stock # 000642] mice obtained from the Jackson Laboratory aged 12–18 weeks were housed under a 12 h light/12 h dark cycle at 23 $\pm$ 2 °C (humidity 55 $\pm$ 5 %) with access *ad libitum* to food and water. Blood was collected from HFD-fed rats, *db*/*db* mice and their controls for the estimation of serum glucose, NEFA and fetuin-A levels. All animal experiments were performed following the guidelines prescribed by the Animal Ethics Committee.

Primary culture

Adult male Sprague–Dawley rats (weighing 175–200 g) were anaesthetized followed by opening of the abdomen. The liver was perfused via the portal vein by using modified Hanks medium. Perfused liver was isolated, minced and digested with type IV collagenase in original Hanks medium for 1 h at 37 °C. Digested cell suspension was filtered through two layers of nylon mesh and supernatant was removed. Cells were then resuspended in MEM supplemented with antibiotics (penicillin/streptomycin) and 0.2 % BSA. Viability of the cells was determined using the Trypan Blue exclusion method. Cells were plated in collagen-coated plates and cultured in a humidified 5 % CO_2 atmosphere at 37 °C.

Human subjects

Visceral adipose tissue was obtained from 12 patients (subjects without diabetes) who were admitted to the S.S.K.M. Hospital and underwent abdominal surgery. We obtained consent from all subjects included in the study and the approval of the ethical committee of the I.P.G.M. E&R, S.S.K.M. Hospital, Kolkata. Adipose tissue was rinsed with sterile 0.9 % NaCl solution. The tissue was then cleaned in HBSS (Hanks buffered saline solution) supplemented with 5.5 mM glucose. Adipose tissue was digested in HBSS containing 5.5 mM glucose, 5 % (w/v) FA-free BSA and 3.3 mg/ml type II collagenase for 30 min in a 37 °C water bath. The digestion mixture was passed through a tissue sieve. The pre-adipocyte-containing fraction was collected and washed several times by centrifugation at 585 ***g*** for 5 min. The supernatant was discarded, and the pellet containing pre-adipocytes was resuspended in standard medium consisting of DMEM supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml) and 10 % (v/v) FBS. Cells were plated in culture plates and kept in a humidified 5 % CO_2 atmosphere at 37 °C. For adipogenesis, the medium was further replaced by ADM.

Cell culture treatments

Confluent HepG2 cells were subcultured by trypsinization and subsequently seeded in six-well culture plates containing MEM with essential supplements. Cells were serum-starved for 24 h before starting the experiments. Lipid-containing media were prepared by conjugating lipid to BSA following our previously described method [17]. Confluent HepG2 cells were incubated for different periods (0, 2, 4, 6 and 8 h) or at different doses (0.25, 0.5, 0.75, 1.0 and 1.5 mM) in the absence or presence of palmitate. Cells were also incubated with different types of FAs (0.75 mM). When inhibitors were used, cells were pre-incubated with inhibitors such as SN-50 (50 μg/ml), actinomycin D (1 μM), cycloheximide (10 μM) or PDTC (pyrrolidine dithiocarbamate) (50 μM) for 1 h before treatment with palmitate. Rosiglitazone (10 μM) was co-incubated along with fetuin-A protein in pre-adipocytes. At the end of the incubation, medium was collected or cells were lysed and centrifuged at 10 000 ***g*** for 10 min and the supernatant was collected. The protein concentration of the supernatant and medium was determined by following the method described previously [18]. NF-κBp65 siRNA (short interfering RNA), C/EBPβ siRNA and pCMV-NF-κBp65 vector were transfected using Lipofectamine™ 2000 (Invitrogen) following

Table 1 Primers used in the present study

Primer	Direction	Sequence
NF-κBp65	Forward	5′-CCATCAGGGCAGATCTCAAACC-3′
	Reverse	5′-GCTGCTGAAACTCTGAGTTGTC-3′
Fetuin-A	Forward	5′-CCAGTGTCATTCCACCAGA-3′
	Reverse	5′-CGCAGCTATCACAAACTCCA-3′
C/EBPβ	Forward	5′-GGACGCAGCGGAGCCCGC-3′
	Reverse	5′-CTCGGCGGGCCACTGCTAG-3′
PPARγ	Forward	5′-ATCATCTACACGATGCTGGCC-3′
	Reverse	5′-CTCCCTGGTCATGAATCCTTG-3′
aP2	Forward	5′-TGATGCCTTTGTGGGAACCT-3′
	Reverse	5′-GCAAAGCCCACTCCCACTT-3′
CD36	Forward	5′-GAGCCATCTTTGAGCCTTCA-3′
	Reverse	5′-TCAGATCCGAACACAGCGTA-3′
IL-6	Forward	5′-ATTTCCTCTGGTCTTCTGGA-3′
	Reverse	5′-TCCTTAGCCACTCCTTCTGT-3′
TNFα	Forward	5′-TCTCAGCCTCTTCTCATTCC-3′
	Reverse	5′-ACTTGGTGGTTTGCTACGAC-3′
GAPDH	Forward	5′-GCCATCAACGACCCCTTC-3′
	Reverse	5′-AGCCCCAGCCTTCTCCA-3′

the manufacturer's protocol. 3T3-L1 and human pre-adipocytes were incubated with medium containing or not 1.5 mM Ca^{2+} in the presence of 100 μg/ml fetuin-A for 12 h followed by culture of cells in ADM. FITC-labelled fetuin-A was incorporated into adipocytes in the presence or absence of Ca^{2+}. Fetuin-A protein was FITC-labelled with the help of an FITC labelling kit (Calbiochem) following the manufacturer's protocol. Differentiated 3T3-L1 and primary adipocytes were stained with Oil Red O stain following the manufacturer's protocol (Cayman Chemical).

Western blots

Protein (50 μg) from cell lysates and media was resolved by SDS/PAGE (10 % gels) and transferred on to PVDF membranes (Millipore) using Semi-Dry Trans-Blot® Apparatus (Bio-Rad Laboratories). The membranes were first incubated with different primary antibodies at 1:1000 dilutions followed by goat anti-rabbit secondary antibody conjugated to alkaline phosphatase at the same dilutions using SNAP i.d.™ apparatus (Millipore). The protein bands were detected by using BCIP (5-bromo-4-chloroindol-3-yl phosphate)/NBT (Nitro Blue Tetrazolium).

RT (reverse transcription)–PCR and real-time PCR

Total RNA was extracted from different incubations using TRI Reagent (Sigma) according to the manufacturer's instructions. RT–PCR was performed using the Revert Aid™ first-strand cDNA synthesis kit (Fermentas Life Sciences). Alteration in gene expression was confirmed further by real-time PCR (Applied Biosystems). PCR was performed using gene-specific primers with the following reaction conditions: initial activation step at 95 °C for 15 min, then 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and final extension at 72 °C for 30 s. *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase) was simultaneously amplified in separate reactions. The C_t value was corrected using corresponding *Gapdh* controls. Primer sequences used were as shown in Table 1.

EMSA (electrophoretic mobility-shift assay)

EMSAs were performed using nuclear extracts prepared from different incubations with oligonucleotide probes specific for the NF-κB-binding site [wild–type, 5′-GCACCTGGGTTG-GTCCCGAAGC-3′; mutant, 5′-GCACCTGG**C**TTGGTCCC-GAAGC-3′ (mutated residue is in bold and underlined)] within the fetuin-A promoter. The probes were end-labelled with [γ-^{32}P]ATP with T4 polynucleotide kinase and then they were incubated with 10 μg of nuclear extracts in a 20 μl reaction volume for 45 min on ice. For the supershift assay, 2 μg of anti-NF-κBp65 antibody was added to the nuclear extract and the reaction mixture was resolved on a 5 % (w/v) polyacrylamide gel and visualized using a PhosphorImager (GE Healthcare).

ChIP (chromatin immunoprecipitation) assay

ChIP was performed using a ChIP assay kit (Upstate Biotechnology) following the manufacturer's protocol using anti-NF-κBp65 and anti-C/EBPβ antibodies. Primers used for amplification of human fetuin-A promoter sequence were 5′-CA-GAGCACCTGGTTGGT-3′ (forward) and 5′-GCCCCAGAG-CTGAGCAA-3′ (reverse), PCR products were resolved on an ethidium-bromide-stained 1.5 % agarose gel, and the image was captured by the Bio-Rad gel documentation system using Quantity One software.

[^{3}H]Leucine-incorporation study

HepG2 cells were serum-starved in KRP (Krebs–Ringer phosphate) buffer supplemented with 0.2 % BSA. To determine the rate of protein synthesis, serum-starved cells were incubated with 10 μCi/ml [^{3}H]leucine. Cells were incubated without or with palmitate or with palmitate plus actinomycin D. The medium was collected, and fetuin-A was pulled down using anti-fetuin-A antibody. Radioactive count was measured in a liquid scintillation counter (PerkinElmer Tri-Carb 2800TR).

[^{14}C]Palmitate uptake

Adipocytes transducted without or with fetuin-A were incubated with 1 μCi/ml [^{14}C]palmitate for 3 h. Cells were washed three times with ice-cold KRP buffer. Cells were solubilized with 1 % (v/v) NP-40 (Nonidet P40), and [^{14}C]palmitate was measured in a liquid scintillation counter.

[^{3}H]2-DOG uptake

Skeletal muscle tissue was dissected out from control and HFD-fed rats and *db*/*db* mice. The muscle tissue was washed thoroughly and subjected to digestion in DMEM in the presence of 0.02 % trypsin and 0.5 % collagenase for 30 min at 37 °C under 5 % CO_2. Isolated skeletal muscle cells and 3T3-L1 adipocytes were incubated in KRP buffer supplemented with 0.2 % BSA followed by 30 min of incubation in the presence of insulin (100 nM). [^{3}H]2-DOG (0.4 nmol/ml) was added to each incubation 5 min before the termination of incubation. Cells were washed three times with ice-cold KRP buffer in the presence of 0.3 mM phloretin. Cells were solubilized with 1 % NP-40 and [^{3}H]2-DOG was measured in a liquid scintillation counter.

Reporter assay

A pFetA-luc construct containing the 985 bp promoter region (chr3:187812685–187813673) of the human fetuin-A gene (GenBank®/EMBL accession number NM_001622) was purchased from SwitchGear Genomics. pFetA-luc served as a template for the generation of mutant plasmids with the help of the QuikChange® site-directed mutagenesis system (Stratagene). For mutated fetuin-A plasmid construction, sense oligonucleotides

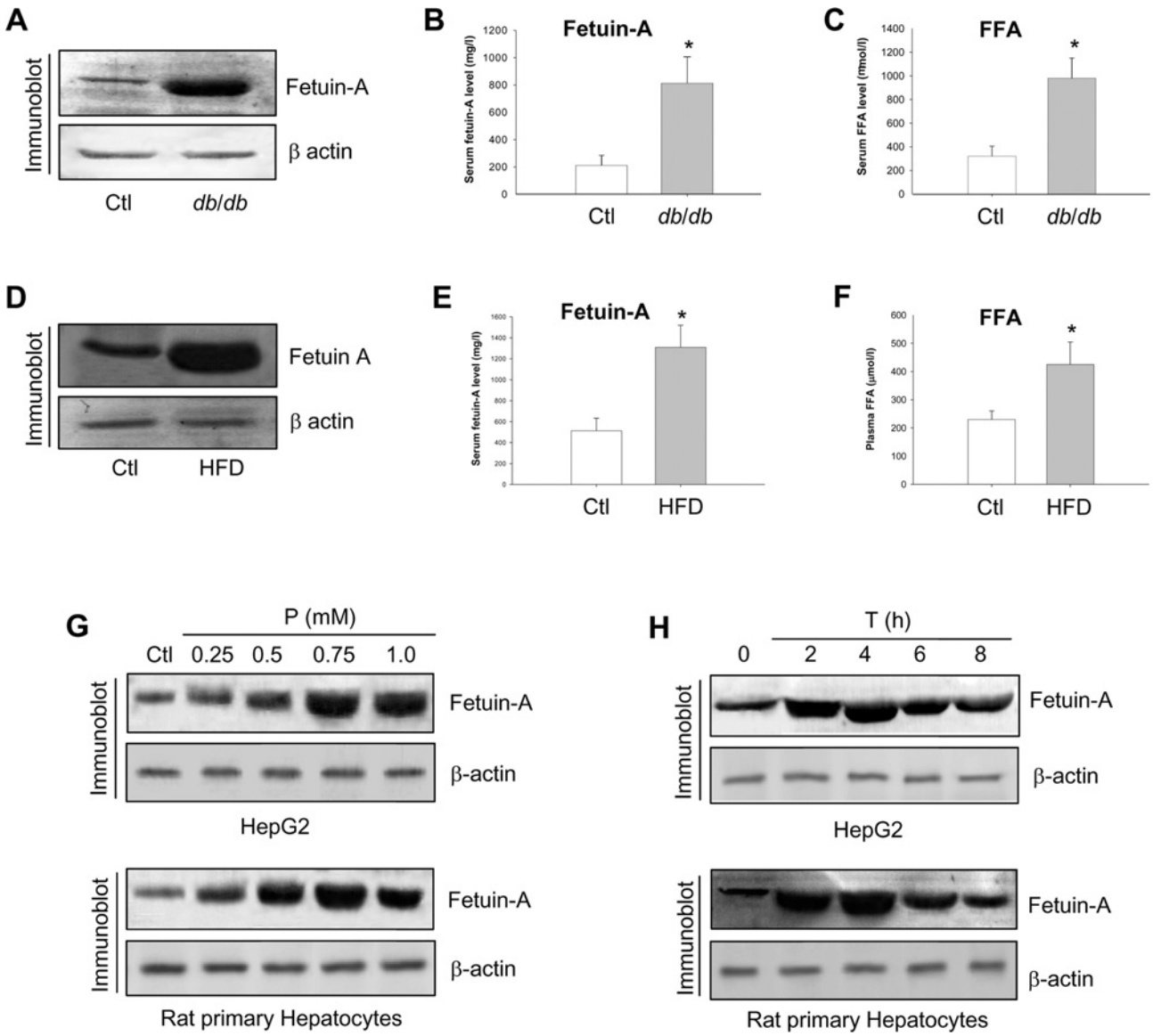

Figure 1 Interrelationship between FAs and fetuin-A

(**A**) Serum collected from control (Ctl) and *db*/*db* mice was subjected to immunoblot for fetuin-A. β-Actin was used as a loading control. (**B**) Serum fetuin-A levels were determined by ELISA. $^*P < 0.001$ compared with Ctl. (**C**) Serum NEFA ('FFA') levels were estimated from the above mice. Means $\pm$ S.E.M. were calculated from three experiments, with each experiment containing five control (Ctl) and five experimental animals. $^*P < 0.001$ compared with Ctl. (**D**) Serum fetuin-A levels in HFD-fed rats were measured by immunoblotting with anti-fetuin-A antibody. (**E**) Serum fetuin-A levels were estimated by ELISA. $^*P < 0.001$ compared with Ctl. (**F**) Estimation of serum NEFA ('FFA') levels in HFD-fed and control rats. $^*P < 0.001$ compared with Ctl. (**G**) HepG2 cells and rat primary hepatocytes were incubated with different concentrations of palmitate (P) for 4 h. On termination of incubations, medium was subjected to immunoblotting for fetuin-A. (**H**) The effect of palmitate (0.75 mM) on hepatocytes at different periods (T) was observed by Western blot analysis using an anti-fetuin-A antibody.

included oligohfetΔN1 (5′-AGGTCTGGAGAAGGAAGAGAACCCCACACGTT-3′), oligohfetΔN2 (5′-CCCACACGTTTTGCTCACCGTGGTCCTGCC-3′), oligohfetΔN3 (5′-CAAGAATCTTCCCCCAAAATCTTATACACATCTGTACCTTTGCT-3′), oligohfetΔN4 (5′-GATCACAGTAGAAGACCTGCCAAACCCATGGC-3′), oligohfetΔN5 (5′-GGTGTTTTTTTTTTTCTTTTGAACCACATCCTGTATCCTTATGCAATTCTTC-3′) and oligohfetΔN6 (5′-CTCTGGGGCAGCCTCGTCCTGCTCCT-3′) covering the deletion of fetuin-A $-251/-242$, $-277/-268$, $-395/-386$, $-559/-568$, $-794/-785$ and $-957/-948$ sequences respectively. HepG2 cells were transfected with wild-type or mutated pFetA-luc plasmid using Lipofectamine™ 2000. The luciferase activity was measured by the Steady-Glo luciferase assay system (Promega) following the manufacturer's instructions.

Statistical analysis

Data were analysed by one-way ANOVA where the F value indicated significance, means were compared by a post hoc multiple range test. All values are means $\pm$ S.E.M.

RESULTS

FA stimulates fetuin-A release by enhancing its gene expression

To have additional information on the association of lipid with fetuin-A, we selected *db*/*db* mice as it is a well known model for Type 2 diabetes with the hyperlipidaemic conditions and compared the serum profile with its control littermate. There was a significant ($P < 0.001$) increase in fetuin-A levels

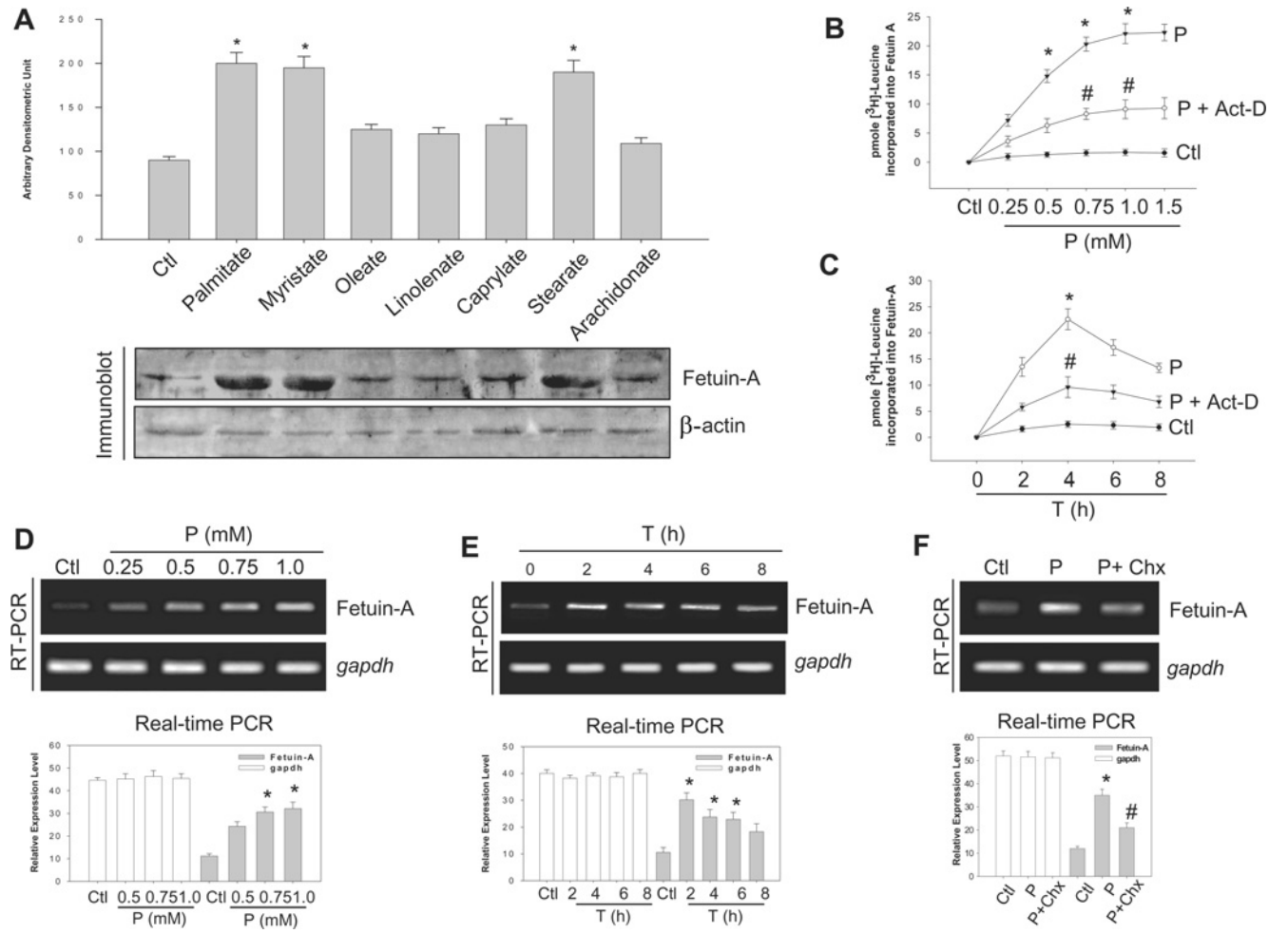

Figure 2 Effect of FAs on fetuin-A gene and protein expression

(**A**) HepG2 cells were incubated without (Ctl) or with different FAs (0.75 mM) for 4 h. On termination of incubations, medium was collected and subjected to immunoblot to determine fetuin-A. β-Actin served as an internal control. Means $\pm$ S.E.M. were calculated from four independent experiments. $^*P < 0.001$, compared with Ctl. (**B**) HepG2 cells were incubated in different concentrations of palmitate (P) in the presence of 10 μCi of [^{3}H]leucine without or with 1 μM actinomycin D (P+Act-D). Fetuin-A from the medium was pulled down by anti-fetuin-A antibody and processed for radioactive counting. (**C**) The same experiment was performed with hepatocytes incubated with 0.75 mM palmitate for different periods (T). Means $\pm$ S.E.M. were calculated from four independent experiments. $^*P < 0.001$, $^\#P < 0.001$, compared with Ctl. (**D**) HepG2 cells were incubated with different concentrations of palmitate (P). RNA extracted from the cells was subjected to RT–PCR and real-time PCR analysis using fetuin-A-specific primers taking *Gapdh* as an internal control. $^*P < 0.001$ compared with Ctl. (**E**) RNA extracted from HepG2 cells incubated with palmitate for different periods (T) was analysed by RT–PCR and real-time PCR. Means $\pm$ S.E.M. were calculated from three independent experiments. $^*P < 0.001$, compared with Ctl. (**F**) Effect of cycloheximide (Chx) on palmitate-incubated cells (P) was determined by RT–PCR and real-time PCR. Means $\pm$ S.E.M. were calculated from three independent experiments. $^*P < 0.001$, compared with Ctl; $^\#P < 0.01$, compared with P.

(Figures 1A and 1B) in *db*/*db* mice along with an increase in serum NEFAs (Figure 1C). We also investigated this in the HFD rat model where the significant increase in body weight (see Supplementary Figure S1A at http://www.BiochemJ.org/bj/429/bj4290451add.htm), blood glucose level (see Supplementary Figure S1B) and glucose uptake by skeletal muscle (see Supplementary Figure S1C) suggested development of insulin resistance. HFD-fed rats showed a similar trend of fetuin-A (Figures 1D and 1E) and NEFA (Figure 1F) levels to that observed in *db*/*db* mice. These findings suggest an association between FAs and fetuin-A. To have direct evidence, we incubated HepG2 cells and rat primary hepatocytes with palmitate. Consistent with *in vivo* observations, it was found that palmitate significantly increased fetuin-A secretion into the medium which was dose- (Figure 1G) and time- (Figure 1H) dependent. To observe whether all FAs are similarly involved in enhancing fetuin-A secretion, we incubated HepG2 cells with different FAs and results indicated that only long-chain saturated FAs were effective (Figure 2A). Since fetuin-A is a hepatic secretory protein, increased release could be related to its enhanced synthesis. To examine this, we incubated HepG2 cells with palmitate in the presence of [^{3}H]leucine and observed a significant elevation of fetuin-A protein synthesis ($P < 0.001$) which was inhibited by actinomycin D (Figures 2B and 2C). Palmitate strikingly up-regulated fetuin-A mRNA expression which was concentration- and time-dependent (Figures 2D and 2E). However, the palmitate augmentary effect was not direct as it was suppressed by cycloheximide (Figure 2F), indicating an involvement of a protein(s) mediator.

FA-induced fetuin-A synthesis is mediated through NF-κB

To search for this protein mediator, we considered NF-κB as a probable candidate because of its dependence on lipid for

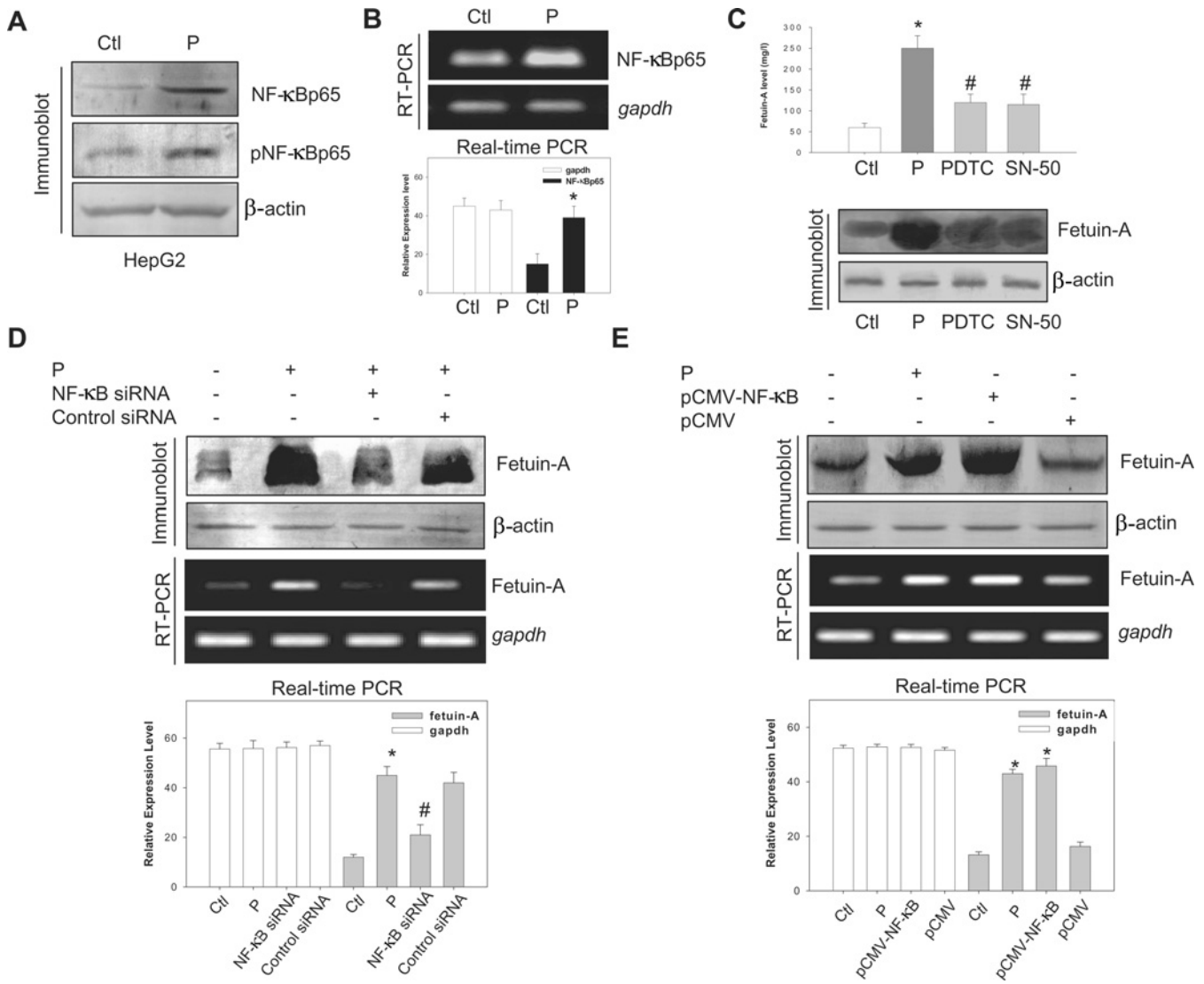

Figure 3 Palmitate stimulation of fetuin-A expression is mediated through NF-κB

(**A**) HepG2 cells were incubated without (Ctl) or with (P) 0.75 mM palmitate for 4 h. Cells were lysed and immunoblotted with anti-NF-κBp65 or anti-pNF-κBp65 antibodies. β-Actin served as an internal control. (**B**) RNA extracted from the above incubations was subjected to RT–PCR and real-time PCR using NF-κB-specific primers where *Gapdh* served as an internal control. Means $\pm$ S.E.M. were calculated from three independent experiments. $^*P < 0.001$, compared with Ctl. (**C**) HepG2 cells were incubated without (Ctl) or with (P) palmitate in the presence of PDTC or SN-50; fetuin-A released into the medium was determined by ELISA (upper panel) and immunoblot (lower panel). Means $\pm$ S.E.M. were calculated from four independent experiments. $^*P < 0.001$, compared with Ctl; $\#P < 0.001$, compared with P. (**D**) Control (Ctl) or NF-κB siRNA-transfected HepG2 cells were incubated with palmitate (P), and fetuin-A released into the medium was estimated by immunoblot analysis using an anti-fetuin-A antibody. RNA was extracted from the above mentioned incubated cells and subjected to RT–PCR and real-time PCR. (**E**) HepG2 cells were incubated without (Ctl) or with (P) palmitate or transfected with pCMV-NF-κBp65 or pCMV empty vector, and fetuin-A levels in the medium were determined by immunoblot analysis. RT–PCR and real-time PCR was performed with the RNA extracted from the above mentioned incubated cells. (**D** and **E**) Means $\pm$ S.E.M. were calculated from four independent experiments. $^*P < 0.001$, compared with Ctl; $\#P < 0.001$, compared with P.

its activation and nuclear translocation in liver cells [19,20]. In addition, we have recently reported an increase in NF-κB expression by palmitate that adversely affects insulin sensitivity [17]. Interestingly, palmitate also enhanced both NF-κB activity (Figure 3A) and expression (Figures 3A and 3B) in HepG2 cells and primary hepatocytes (see Supplementary Figures S2A and S2B at http://www.BiochemJ.org/bj/429/bj4290451add.htm). Palmitate stimulation of fetuin-A secretion from HepG2 cells could be attenuated by inhibitors of NF-κB, such as PDTC and SN-50 (Figure 3C), indicating that its effect on fetuin-A may be through NF-κB. We then checked whether alterations of NF-κB levels in HepG2 cells could be commensurate with fetuin-A synthesis. Transfection of NF-κB siRNA to HepG2 cells abrogated palmitate-induced fetuin-A protein and gene expression (Figure 3D). In contrast, forced expression of NF-κB in HepG2 cells enhanced fetuin-A protein and gene expression (Figure 3E) in the absence of palmitate. Considering that NF-κB may be up-regulating fetuin-A gene expression through the activation of the fetuin-A promoter, we performed a ChIP assay and found that NF-κB binding to the fetuin-A promoter was greatly enhanced due to palmitate, which was suppressed by SN-50 and NF-κB siRNA (Figure 4A, upper panel). Since salicylate has been shown to modulate the NF-κB pathway, we determined its effects on palmitate stimulation of NF-κB binding to the fetuin-A promoter

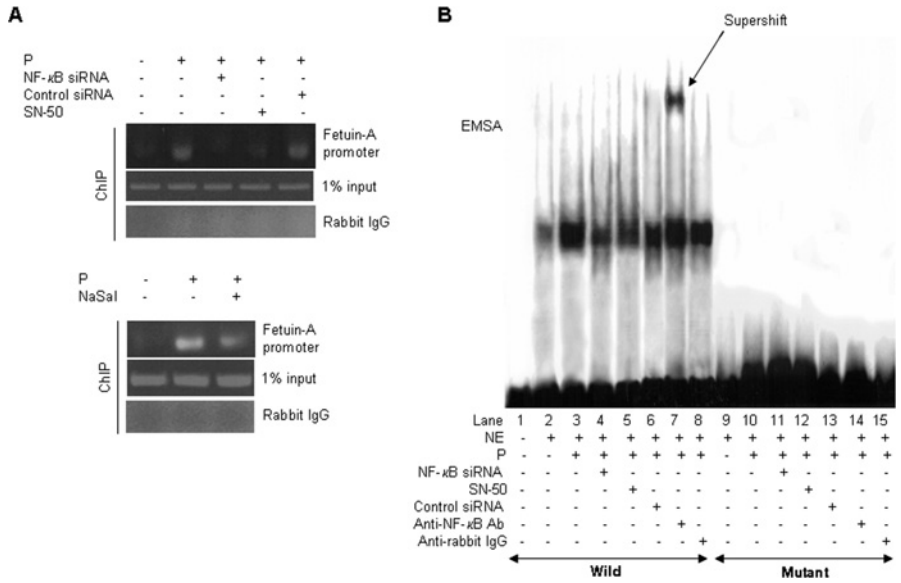

Figure 4 Palmitate-enhanced NF-κB binding to the fetuin-A promoter

(**A**) HepG2 cells transfected without or with NF-κB siRNA were incubated with palmitate (P) or with palmitate plus SN-50 (upper panel). HepG2 cells were incubated with palmitate or with palmitate plus sodium salicylate (NaSal) (lower panel). NF-κB binding to the fetuin-A promoter was determined by ChIP assay. (**B**) Nuclear extracts (NE) from the above mentioned incubated cells were subjected to EMSA. For the supershift assay, an anti-NF-κBp65 antibody was used. Mutant probe and non-specific anti-IgG antibody was used to determine specific binding of NF-κB on the fetuin-A promoter.

and found that salicylate inhibited the palmitate augmentary effect (Figure 4A, lower panel). We further confirmed it by EMSA, which showed that NF-κB binding activity increased in nuclear extract from palmitate-incubated cells, and the addition of anti-NF-κBp65 antibody supershifted the binding complex, indicating that this band constitutes the p65 subunit. To examine the specificity of the NF-κBp65–DNA interaction, we used a mutant oligonucleotide probe where binding activity was not observed (Figure 4B). These results demonstrate that palmitate stimulation of fetuin-A expression requires NF-κB.

Palmitate stimulation of fetuin-A promoter activity is NF-κB-dependent, but independent of C/EBPβ

To have better insight into the palmitate stimulation of fetuin-A through NF-κB, there is a need to demonstrate regulation of the fetuin-A promoter activity by NF-κB and to detect specific NF-κB-binding sites on the fetuin-A promoter, and then whether the occupation of those sites by NF-κB could activate the promoter. To achieve these objectives, we transfected pFetA-Luc vector to HepG2 cells, co-transfected HepG2 cells with NF-κB siRNA followed by palmitate incubation or transfected HepG2 cells with pCMV-NF-κBp65 vector and then incubated them in the absence of palmitate. Palmitate enhanced fetuin-A reporter activity which was significantly attenuated in NF-κB-knockout cells, whereas reporter activity markedly increased in cells transfected with pCMV-NF-κBp65 in the absence of palmitate. Interestingly, salicylate which is an inhibitor of NF-κB activity, also reduced luciferase activity indicating this to be a reflection of reduced NF-κB binding to the fetuin-A promoter (Figure 5A). Hence, palmitate regulation of fetuin-A expression is NF-κB-dependent; once the critical concentration of NF-κB within the cell is achieved, fetuin-A up-regulation may occur in the absence of palmitate. We then searched for NF-κB-binding sites on the fetuin-A promoter. There are six putative binding sites (termed N1, N2, N3, N4, N5 and N6) as detected by using the Match computer program with a sequence homology with classical NF-κB-response elements (see Supplementary Table S1 at http://www.BiochemJ.org/bj/429/bj4290451add.htm). To investigate whether these elements were involved in fetuin-A promoter activation due to NF-κB, we transfected wild-type and mutated pFetA-Luc vector construct into HepG2 cells and incubated them with palmitate. Constructs lacking N1, N4 or N5 but containing N2, N3 or N6 increased luciferase activity due to palmitate, whereas deletion of N2, N3 or N6 abolished reporter gene activity (Figure 5B). Furthermore, luciferase activity was attenuated in NF-κB-silenced HepG2 cells transfected with pN2-N3-N6-FetA-Luc construct; this was reversed in NF-κB-overexpressed cells (Figure 5C). Therefore N2, N3 and N6 elements are essential for palmitate-induced NF-κB-mediated fetuin-A promoter activation. On finding evidence in favour of palmitate stimulation of fetuin-A promoter activation due to NF-κB binding, we considered whether C/EBPβ has any association with the palmitate stimulatory effect on the fetuin-A promoter as glucocorticoid up-regulates fetuin-A through C/EBPβ. The fetuin-A promoter has several binding sites for C/EBPβ, and occupation of them augmented fetuin-A expression [16]. However, palmitate did not alter C/EBPβ binding to the fetuin-A promoter (Figure 5D); it also had no effect on C/EBPβ expression (Figure 5E) or fetuin-A protein and gene expression in C/EBPβ-knockout cells (Figure 5F). Moreover, NF-κB- and C/EBPβ-binding sites on the fetuin-A promoter are different. These findings suggest that, in lipid-induced up-regulation of fetuin-A, NF-κB plays the role of a mediator, whereas C/EBPβ remains uninvolved.

Impairment of adipocyte function by fetuin-A

On studying the mechanism of lipid-induced regulation of fetuin-A synthesis in liver cells, we wondered whether such robust expression of fetuin-A could only be attributed to its known adverse effects on the phosphorylation of insulin receptor tyrosine

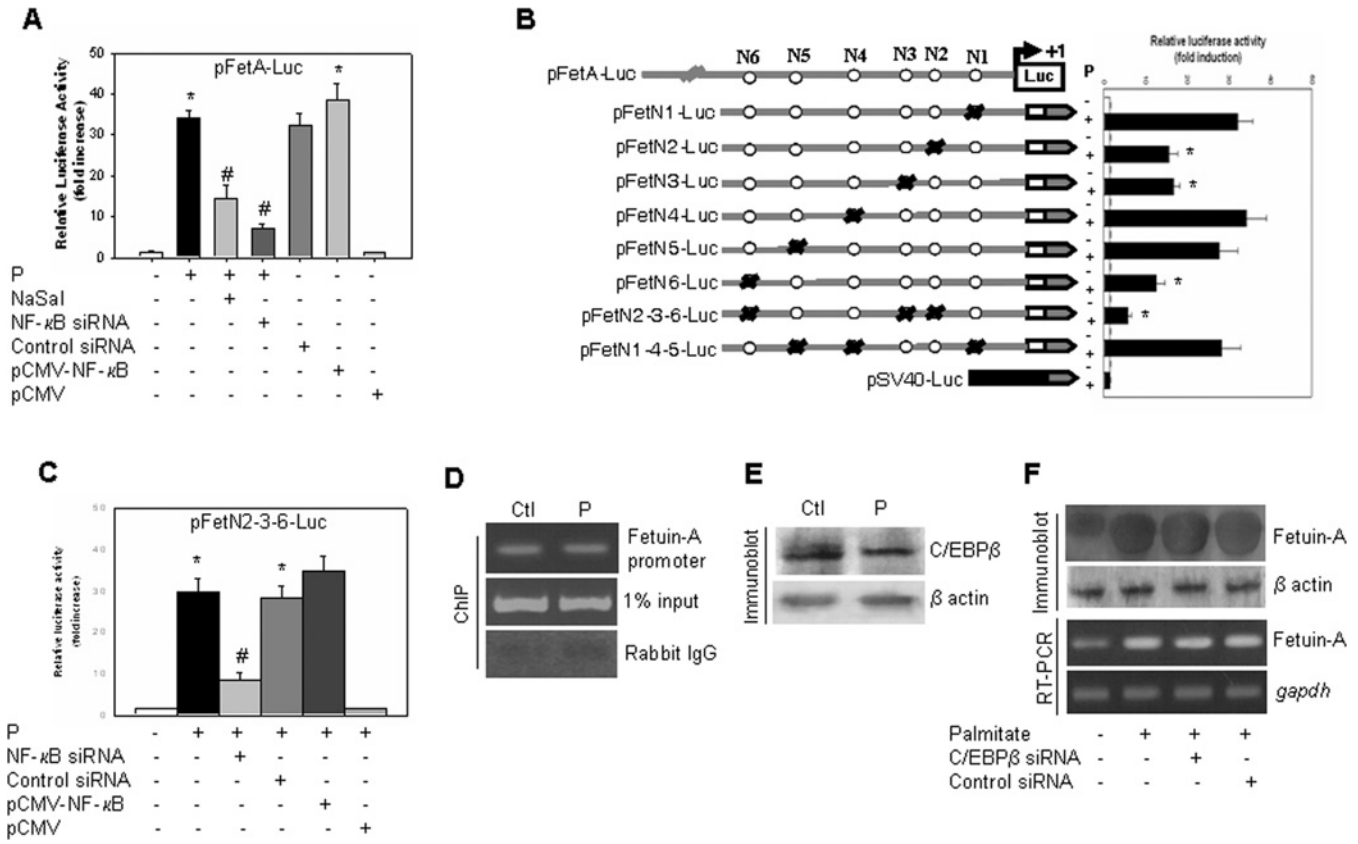

Figure 5 Increase in fetuin-A promoter activity by NF-κB

(**A**) pFetA-luc-transfected HepG2 cells or HepG2 cells co-transfected with NF-κB siRNA or pCMV-NF-kBp65 vector were incubated with palmitate (P) or palmitate plus sodium salicylate (P+NaSal). On termination of incubations, reporter activity was determined by using luciferase assay. Means $\pm$ S.E.M. were calculated from three independent experiments. *$P < 0.001$, compared with Ctl; #$P < 0.001$, compared with P. (**B**) A cartoon shows pFetA-luc vector with the six putative NF-κB-binding elements (N1, N2, N3, N4, N5 and N6). On the right-hand side, luciferase activity of palmitate-incubated HepG2 cells transfected with mutated pFetA-luc vector demonstrates the requirement of N2, N3 and N6 for its activity. (**C**) Luciferase activity was also observed in cells transfected with pN2-N3-N6-FetA-luc construct or co-transfected with NF-κB siRNA or pCMV-NF-κBp65. Means $\pm$ S.E.M. were calculated from five independent experiments. *$P < 0.001$, compared with Ctl; #$P < 0.001$, compared with P. (**D**) HepG2 cells were incubated without (Ctl) or with (P) palmitate and subjected to ChIP assay using an anti-C/EBPβ antibody. (**E**) HepG2 cells were incubated without (Ctl) or with palmitate (P) and immunoblotted for C/EBPβ. β-Actin was used as an internal control. (**F**) HepG2 cells transfected with C/EBPβ siRNA was incubated without or with palmitate (P) and immunoblotted with an anti-fetuin-A antibody or subjected to RT–PCR using fetuin-A-specific primers.

kinase or could it also target other impairments associated with insulin resistance and Type 2 diabetes. A few reports directed our attention towards adipocytes. It is reported that when fetuin-A-null mice were fed on an HFD, there is a significant decrease in body fat, and they are resistant to weight gain [6]. These results imply that the presence of fetuin-A would promote fat accumulation. In haemodialysis patients, fetuin-A is associated with truncal obesity and dyslipidaemia [21]. Moreover, an anti-diabetic thiazolidinedione class of drug, known for its plasma NEFA-reducing effect, also decreased fetuin-A [22]. We therefore considered adipocytes to be another target and examined the role of fetuin-A on adipocyte function. Fetuin-A uptake in cells has been shown to be Ca^{2+}-dependent [23,24]. We utilized this information to determine whether fetuin-A acts from the outside or whether its entry into the cell is obligatory. Our experiments therefore included adipocyte incubations with or without 1.5 mM Ca^{2+} (less than the normal circulatory level [25]). Interestingly, fetuin-A was readily translocated into the cells in the presence of Ca^{2+} (Figure 6A). Since Ca^{2+} remains bound to albumin in circulation, determination of the level of free Ca^{2+} that permitted maximum uptake of fetuin-A is important. We have found that 1.5 mM $CaCl_2$ produced 0.25 μM free ionized Ca^{2+} in adipocytes which induced maximum fetuin-A entry into the cells, whereas 2 mM $CaCl_2$ resulting in 0.28 μM free Ca^{2+} in cells had no additional effect on fetuin-A entry (see Supplementary Figures S3A and S3B at http://www.BiochemJ.org/bj/429/bj4290451add.htm). These results suggest 0.25 μM free Ca^{2+} as the threshold level for permeating fetuin-A entry. We transducted fetuin-A to mouse 3T3-L1 and human pre-adipocytes and traced its activity in differentiated adipocytes by culturing them in ADM followed by the determination of lipid content with the help of Oil Red O staining. There was a significant reduction in lipid droplet size and numbers (Figure 6B) and diminished lipid content (Figure 6C) due to fetuin-A in both cases. In contrast, rosiglitazone, a PPARγ agonist, increased lipid accumulation, which was substantially reduced by fetuin-A (Figures 6B and 6C). That uptake of lipid by adipocytes is impaired due to fetuin-A was observed with [^{14}C]palmitate, and it was found that addition of fetuin-A to 3T3-L1 incubation significantly inhibited [^{14}C]palmitate uptake (Figure 6D).

We examined fetuin-A's effect on PPARγ, a well-known adipogenic factor [26,27] and observed a significant reduction in its protein (Figure 7A) and gene (Figure 7B) expression in fetuin-A-treated cells. We then checked the protein and gene expressions

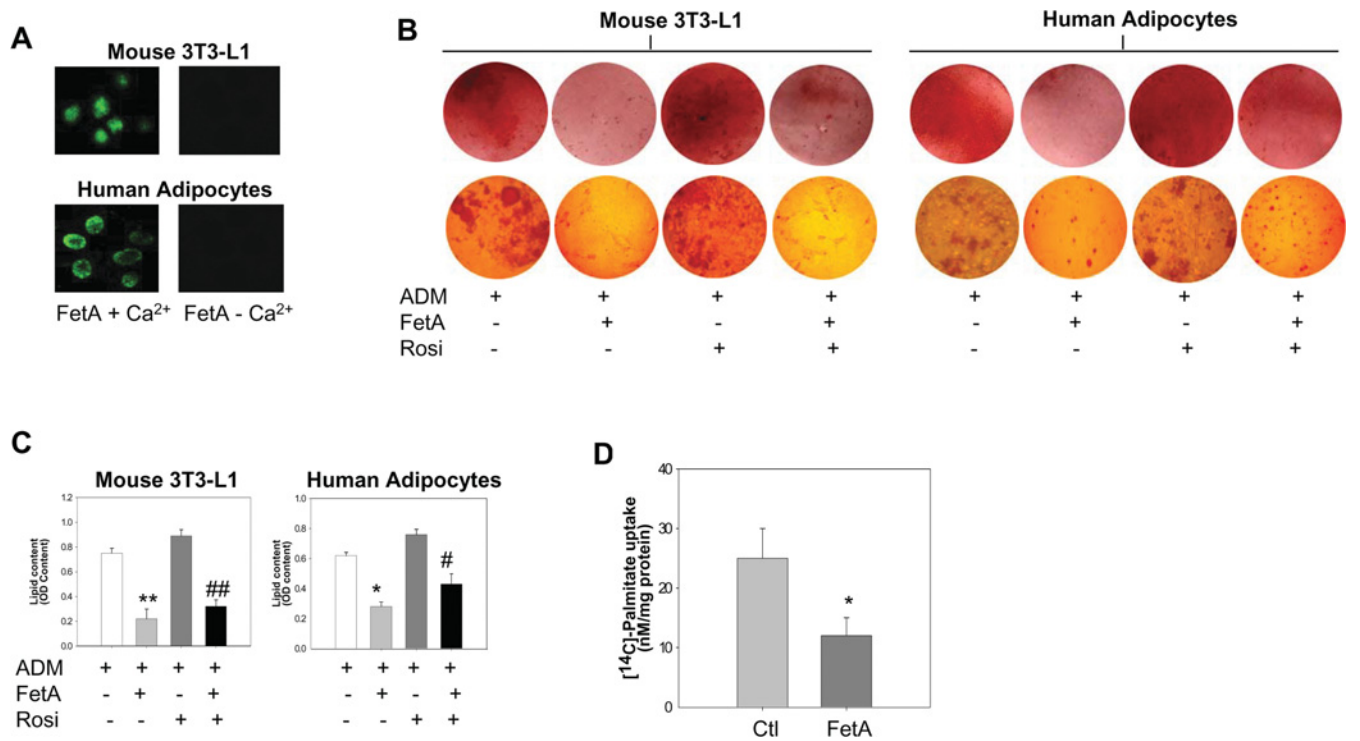

Figure 6 Fetuin-A impairs adipocyte function

(**A**) FITC-conjugated fetuin-A (FetA) was incubated with mouse 3T3-L1 and human adipocytes in the absence or presence of Ca^{2+}. (**B**) Mouse 3T3-L1 and human adipocytes were incubated with or without fetuin-A (FetA) or co-incubated with rosiglitazone (Rosi) in ADM. Adipogenic differentiation was measured by Oil Red O staining (upper panel) and were observed under microscopy (lower panel). (**C**) Lipid content was determined by the dye-extraction method. Means ± S.E.M. were calculated from four independent experiments. $^{**}P < 0.001$, $^{*}P < 0.01$ compared with ADM; $\#\#P < 0.001$, $\#P < 0.01$ compared with ADM+Rosi. (**D**) 3T3-L1 adipocytes were incubated with [^{14}C]palmitate (Ctl) or [^{14}C]palmitate plus fetuin-A (FetA) followed by the determination of the radioactive count from cell lysates. Means ± S.E.M. were calculated from five independent experiments. $^{*}P < 0.01$, compared with Ctl.

of downstream molecules of PPARγ such as adiponectin, aP2 and FAT/CD36 (Figures 7A and 7B) and found all of them to be down-regulated by fetuin-A. Fetuin-A imposed defects on adipocytes were also indicated by the up-regulation of TNFα (tumour necrosis factor α) and IL-6 (interleukin 6) (Figures 7A and 7B). Presumably, all of these would inflict a severe damage to adipocyte function and thereby cause insulin resistance, which is evident from the inhibition of insulin-stimulated [^{3}H]2-DOG uptake and GLUT4 (glucose transporter 4) translocation (Figure 7C).

DISCUSSION

On the basis of the reports that have accumulated for the last several years, it is reasonable to consider fetuin-A as an important factor associated with insulin resistance and Type 2 diabetes. The fetuin-A gene is expressed in the liver and the protein, once synthesized, is immediately secreted into the circulation; its high level is linked to impairment of insulin sensitivity in animals and humans [1–3]. One site of its action is known: it adversely affects the insulin receptor tyrosine kinase and that inhibits insulin-stimulated downstream signalling [4–6]. However, certain important aspects still remain unclear. (i) It is not known which factor(s) regulates up-regulation of fetuin-A. Glucocorticoid is known to induce fetuin-A gene expression through the activation of its promoter by C/EBPβ. FA's role on insulin resistance is well known [28–31], therefore whether its effect is mediated through the same pathway requires investigation. (ii) On the other hand, involvement of lipid has been implicated in the increase in fetuin-A by many [11–13], which is logical as lipid is known to cause insulin resistance. However, how lipid influences fetuin-A up-regulation is unclear, as there is no direct evidence in favour of this. (iii) During insulin resistance and Type 2 diabetes, consistently high levels of fetuin-A indicate an alternative target(s) of fetuin-A besides its classical effect on insulin receptor tyrosine kinase, and a couple of recent reports suggested defects in adipogenesis [3,21]. This is pertinent as it would enhance FA levels and that in turn will adversely affect insulin sensitivity. However, here also clearer evidence is required to show that fetuin-A is anti-adipogenic. In the present study, we wanted to add new information, which may fill some gaps in our understanding of fetuin-A's regulation during insulin resistance.

We first set our examination on *in vivo* insulin-resistant and Type 2 diabetes models, as results from these experiments would indicate the nature of the relationship between lipid and fetuin-A. The ideal model is *db*/*db* mice, and in them it is clearly observed that a significant increase in NEFA circulatory levels coexisted with high levels of fetuin-A. A similar observation was made with HFD-fed rats. Taking this cue as meaningful, we performed experiments with the human liver cell line HepG2 and rat primary hepatocytes. In both cell culture systems, the type of results obtained are fairly uniform in nature, both depicted enhancement of fetuin-A expression to more than 4-fold in comparison with control. However, the palmitate effect is not direct; it is mediated through NF-κB. Our earlier observations on lipid-induced overexpression of NF-κB in insulin target cells [20] led us to presume that, since NF-κB is known to be involved

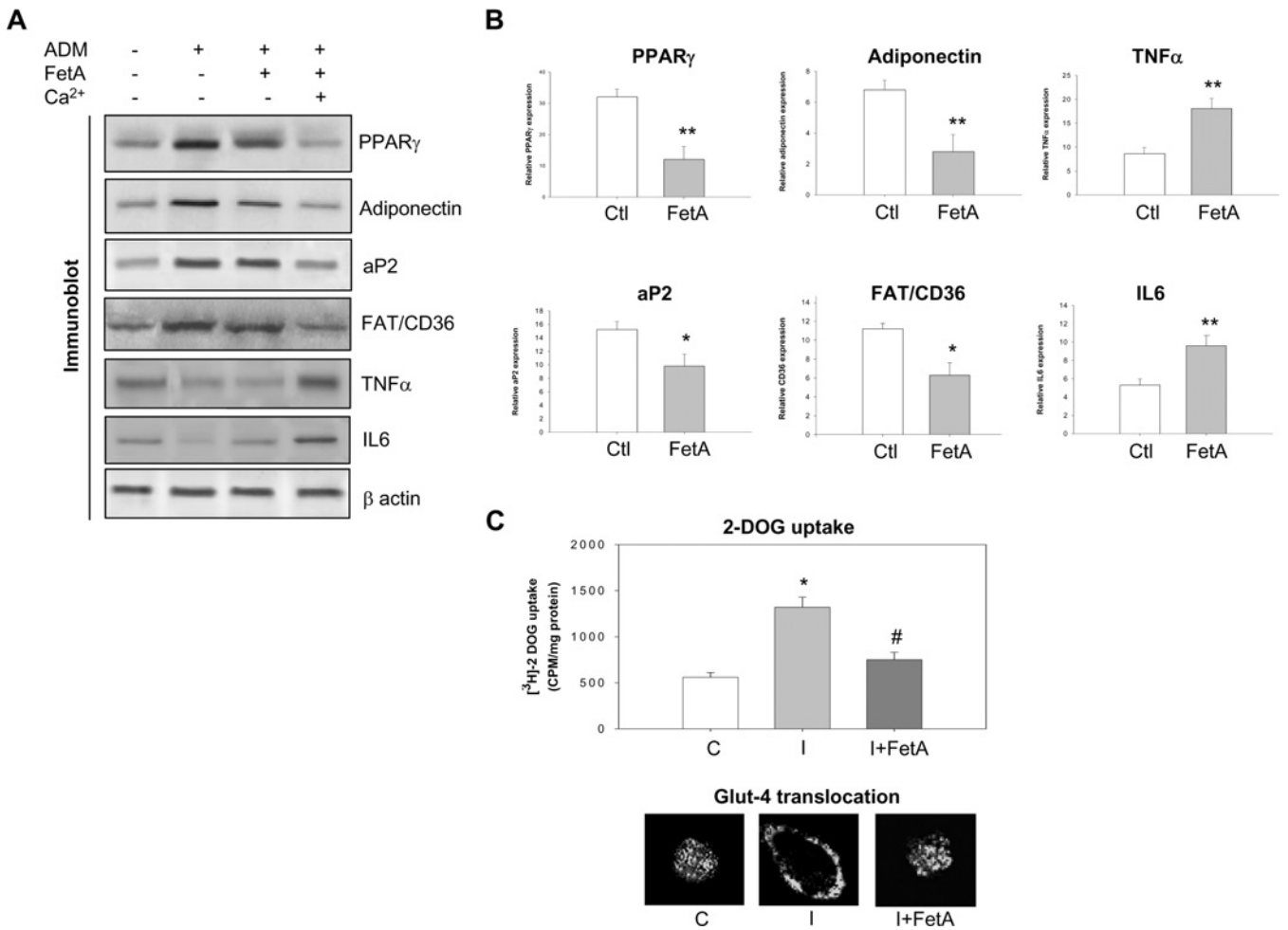

Figure 7 Fetuin-A down-regulates adipogenic factors and stimulates pro-inflammatory cytokines

(**A**) 3T3-L1 adipocytes were incubated with fetuin-A in the absence or presence of Ca^{2+} in ADM. Adiponectin, TNFα or IL-6 released in the medium was estimated by immunoblot analysis using anti-adiponectin, anti-TNFα and anti-IL-6 antibodies respectively. Cell lysates from different incubations were subjected to Western blot analysis using anti-PPARγ, anti-aP2 and anti-FAT/CD36 antibodies. β-Actin served as an internal control. (**B**) 3T3-L1 adipocytes were incubated without (Ctl) or with fetuin-A (FetA) in the presence of Ca^{2+} (1.5 mM). On termination of incubation, RNA was extracted and subjected to real-time PCR by using gene-specific primers of PPARγ, adiponectin, TNFα, aP2, FAT/CD36 and IL-6. Means $\pm$ S.E.M. were calculated from three independent experiments. $^*P < 0.01$, $^{**}P < 0.001$, compared with Ctl. (**C**) Fetuin-A-transducted 3T3-L1 adipocytes were incubated with insulin followed by [^{3}H]2-DOG. 2-DOG uptake by the cells was determined by radioactive count (upper panel). $^*P < 0.01$, compared with Ctl; $\#\ P < 0.01$ compared with I+FetA. GFP (green fluorescent protein)–GLUT4 plasmid-transfected 3T3-L1 adipocytes were incubated with insulin in the absence or presence of fetuin-A, and GLUT4 translocation was observed by confocal microscopy (lower panel).

in insulin resistance, its greater turnover and activity may be a causative factor in up-regulating fetuin-A. FA has been shown to activate NF-κB and its nuclear translocation compromised insulin sensitivity in insulin target cells [18,19]. Here also, palmitate elevated fetuin-A through a similar pathway, it increases fetuin-A promoter activity through the enhancement of NF-κB binding that significantly augmented fetuin-A gene and protein expression. Interestingly, it is not palmitate alone, but a few other long-chain FAs that are associated in fetuin-A expression as shown in the present study and these FAs also mediate their effect through NF-κB (results not shown). We used several specific inhibitors to block NF-κB, and all of them reduced palmitate-induced increased expression of fetuin-A in HepG2 cells. Since several authors have indicated salicylate to be an inhibitor of NF-κB activity [32,33], we also found that salicylate inhibited palmitate stimulation of NF-κB binding to the fetuin-A promoter which reduces promoter activity. Our findings support the validity of earlier reports on fetuin-A in diabetic patients and animals where it has been retained consistently at high levels in the serum [2,3,13].

Another interesting observation is the identification of NF-κB-response elements on the fetuin-A promoter. Since it is known that glucocorticoids induce up-regulation of fetuin-A expression through C/EBPβ [16], we examined the effect of palmitate on C/EBPβ and did not find any alteration of C/EBPβ expression or activity. This suggests that C/EBPβ is not involved in palmitate-induced fetuin-A up-regulation. To have a better insight, we searched for NF-κB-binding sites on the fetuin-A promoter and found that, out of six binding sites, three are responsible for fetuin-A promoter activation and these are different from what was reported in the case of C/EBPβ. Hence, it appears that FA takes a separate pathway in up-regulating fetuin-A expression which corroborates the condition that prevails during insulin resistance.

On studying the mechanism of lipid-induced regulation of fetuin-A synthesis in liver cells, we thought that this high expression of fetuin-A may not only be for its inhibition of insulin

receptor tyrosine kinase phosphorylation, but also possibly target other impairments associated with insulin resistance and Type 2 diabetes. At this juncture, two reports attracted our attention, one demonstrated that fetuin-A-null mice are protected against HFD-induced obesity [6] and another showed a decrease in adiponectin levels in adipocytes due to fetuin-A [15]. These reports suggest adipocytes to be another target of fetuin-A. We therefore expected that fetuin-A will inflict defects on adipocyte function. Our observation with both human and 3T3-L1 adipocytes demonstrated that fetuin-A effects several impairments, which abrogates uptake of lipid into the cells. Pro-adipogenic function of PPARγ, adiponectin, FAT/CD36 and aP2 are known, and all of them are down-regulated by fetuin-A. All these direct to a reasonable notion that such impairments may cause obesity and insulin resistance. Association of lipid and NF-κB with insulin resistance and Type 2 diabetes is well known, it is also fairly well known that fetuin-A is linked to insulin resistance. However, a connection between lipid, NF-κB and fetuin-A leading to the impairment of adipocyte function is not known. Our present work has contributed in understanding these shaded areas and pointed out that this pathway may serve as a novel therapeutic target for insulin resistance and Type 2 diabetes.

AUTHOR CONTRIBUTION

Suman Dasgupta and Sushmita Bhattacharya designed and conducted experiments, and co-wrote the paper. Anindita Biswas conducted the experiments on HFD-fed rats, Subeer Majumdar conducted experiments on *db*/*db* mice and co-wrote the paper. Satinath Mukhopadhyay evaluated results and co-wrote the paper. Sukanta Ray performed human adipocyte experiments. Samir Bhattacharya designed experiments, supervised the study, evaluated the results, and co-wrote the paper.

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REFERENCES

1 Stefan, N., Fritsche, A., Weikert, C., Boeing, H., Joost, H. G., Haring, H. U. and Schulze, M. B. (2008) Plasma fetuin-A levels and the risk of type 2 diabetes. Diabetes **57**, 2762–2767
2 Mori, K., Emoto, M., Yokoyama, H., Araki, T., Teramura, M., Koyama, H., Shoji, T., Inaba, M. and Nishizawa, Y. (2006) Association of serum fetuin-A with insulin resistance in type 2 diabetic and nondiabetic subjects. Diabetes Care **29**, 468
3 Ix, J. H., Wassel, C. L., Kanaya, A. M., Vittinghoff, E., Johnson, K. C., Koster, A., Cauley, J. A., Harris, T. B., Cummings, S. R. and Shlipak, M. G. (2008) Fetuin-A and incident diabetes mellitus in older persons. JAMA, J. Am. Med. Assoc. **300**, 182–188
4 Auberger, P., Falquerho, L., Contreres, J. O., Pages, G., Le Cam, G., Rossi, B. and Le Cam, A. (1989) Characterization of a natural inhibitor of the insulin receptor tyrosine kinase: cDNA cloning, purification, and anti-mitogenic activity. Cell **58**, 631–640
5 Rauth, G., Pöschke, O., Fink, E., Eulitz, M., Tippmer, S., Kellerer, M., Häring, H. U., Nawratil, P., Haasemann, M., Jahnen-Dechent, W. and Müller-Esterl, W. (1992) The nucleotide and partial amino acid sequences of rat fetuin: identity with the natural tyrosine kinase inhibitor of the rat insulin receptor. Eur. J. Biochem. **204**, 523–529
6 Mathews, S. T., Singh, G. P., Ranalletta, M., Cintrón, V. J., Qiang, X., Goustin, A. S., Jen, K. L., Charron, M. J., Jahnen-Dechent, W. and Grunberger, G. (2002) Improved insulin sensitivity and resistance to weight gain in mice null for the *Ahsg* gene. Diabetes **51**, 2450–2458
7 Srinivas, P. R., Wagner, A. S., Reddy, L. V., Deutsch, D. D., Leon, M. A., Goustin, A. S. and Grunberger, G. (1993) Serum α_2-HS-glycoprotein is an inhibitor of the human insulin receptor at the tyrosine kinase level. Mol. Endocrinol. **7**, 1445–1455
8 Grunberger, G. M. S., Mathews, S. T. and Deutsch, D. D. (2002) Tyrosine kinase inhibitors. In Insulin Signaling: from Cultured Cells to Animal Models (Grunberger, G. M. S. and Zick, Y., eds), pp. 281–298, Taylor and Francis, New York
9 Mathews, S. T., Rakhade, S., Zhou, X., Parker, G. C., Coscina, D. V. and Grunberger, G. (2006) Fetuin null mice are protected against obesity and insulin resistance associated with aging. Biochem. Biophys. Res. Commun. **350**, 437–443
10 Vionnet, N., Hani, E. H., Dupont, S., Gallina, S., Francke, S., Dotte, S., De Matos, F., Durand, E., Leprêtre, F., Lecoeur, C. et al. (2000) Genomewide search for type 2 diabetes-susceptibility genes in French whites: evidence for a novel susceptibility locus for early-onset diabetes on chromosome 3q27-qter and independent replication of a type 2-diabetes locus on chromosome 1q21-q24. Am. J. Hum. Genet. **67**, 1470–1480
11 Kelley, D. E., McKolanis, T. M., Hegazi, R. A., Kuller, L. H. and Kalhan, S. C. (2003) Fatty liver in type 2 diabetes mellitus: relation to regional adiposity, fatty acids, and insulin resistance. Am. J. Physiol. Endocrinol. Metab. **285**, 906–916
12 Stefan, N., Hennige, A. M., Staiger, H., Machann, J., Schick, F., Krober, S. M., Machicao, F., Fritsche, A. and Haring, H. U. (2006) α_2-Heremans–Schmid glycoprotein/fetuin-A is associated with insulin resistance and fat accumulation in the liver in humans. Diabetes Care **29**, 853–857
13 Lin, X., Braymer, H. D., Bray, G. A. and York, D. A. (1998) Differential expression of insulin receptor tyrosine kinase inhibitor (fetuin) gene in a model of diet-induced obesity. Life Sci. **63**, 145–153
14 Dahlman, I., Eriksson, P., Kaaman, M., Jiao, H., Lindgren, C. M., Kere, J. and Arner, P. (2004) α_2-Heremans–Schmid glycoprotein gene polymorphisms are associated with adipocyte insulin action. Diabetologia **47**, 1974–1979
15 Hennige, A. M., Staiger, H., Wicke, C., Machicao, F., Fritsche, A., Häring, H. U. and Stefan, N. (2008) Fetuin-A induces cytokine expression and suppresses adiponectin production. PLoS ONE **3**, e1765
16 Wöltje, M., Tschöke, B., Bülow, V., Westenfeld, R., Denecke, B., Gräber, S. and Jahnen-Dechent, W. (2006) CCAAT enhancer binding protein β and hepatocyte nuclear factor 3β are necessary and sufficient to mediate dexamethasone-induced up-regulation of α_2 HS glycoprotein/fetuin-A gene expression. J. Mol. Endocrinol. **36**, 261–277
17 Barma, P., Bhattacharya, S., Bhattacharya, A., Kundu, R., Dasgupta, S., Biswas, A., Bhattacharya, S., Roy, S. S. and Bhattacharya, S. (2009) Lipid induced overexpression of NF-κB in skeletal muscle cells is linked to insulin resistance. Biochim. Biophys. Acta **1792**, 190–200
18 Lowry, O. H., Rosebrough, N. J., Farr, A. E. and Randall, R. J. (1951) Protein measurement with Folin phenol reagent. J. Biol. Chem. **193**, 265–275
19 Boden, G., She, P., Mozzoli, M., Cheung, P., Gumireddy, K., Reddy, P., Xiang, X., Luo, Z. and Ruderman, N. (2005) Free fatty acids produce insulin resistance and activate the proinflammatory nuclear factor-κB pathway in rat liver. Diabetes **54**, 3458–3465
20 Cai, D., Yuan, M., Frantz, D. F., Melendez, P. A., Hansen, L., Lee, J. and Shoelson, S. E. (2005) Local and systemic insulin resistance resulting from hepatic activation of IKK-β and NF-κB. Nat. Med. **11**, 183–190
21 Chen, H. Y., Chiu, Y. L., Hsu, S. P., Pai, M. F., Lai, C. F., Peng, Y. S, Kao, T. W., Hung, K. Y., Tsai, T. J. and Wu, K. D. (2009) Association of serum fetuin-A with truncal obesity and dyslipidemia in non-diabetic hemodialysis patients. Eur. J. Endocrinol. **160**, 777–783
22 Mori, K., Emoto, M., Araki, T., Yokoyama, H., Lee, E., Teramura, M., Koyama, H., Shoji, T., Inaba, M. and Nishizawa, Y. (2008) Effects of pioglitazone on serum fetuin-A levels in patients with type 2 diabetes mellitus. Metab. Clin. Exp. **57**, 1248–1252
23 Chen, N. X., O'Neill, K. D., Chen, X., Duan, D., Wang, E., Sturek, M. S., Edwards, J. M. and Moe, S. M. (2007) Fetuin-A uptake in bovine vascular smooth muscle cells is calcium dependent and mediated by annexins. Am. J. Physiol. Renal Physiol. **292**, F599–F606
24 Mellgren, R. L. and Huang, X. (2007) Fetuin-A stabilizes m-calpain and facilitates plasma membrane repair. J. Biol. Chem. **282**, 35868–35877
25 Fuszek, P., Lakatos, P., Tabak, A., Papp, J., Nagy, Z., Takacs, I., Horvath, H. C., Lakatos, P. L. and Speer, G. (2004) Relationship between serum calcium and CA 19–9 levels in colorectal cancer. World J. Gastroenterol. **10**, 1890–1892
26 Tontonoz, P., Hu, E. and Spiegelman, B. M. (1994) Stimulation of adipogenesis in fibroblasts by PPARγ2, a lipid-activated transcription factor. Cell **79**, 1147–1156
27 Farmer, S. R. (2005) Regulation of PPARγ activity during adipogenesis. Int. J. Obes. **29**, S13–S16

28 Boden, G. (1997) Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. Diabetes **46**, 3–10
29 Santomauro, A. T., Boden, G., Silva, M. E., Rocha, D. M., Santos, R. F., Ursich, M. J., Strassmann, P. G. and Wajchenburg, B. L. (1999) Overnight lowering of free fatty acids with Acipimox improves insulin resistance and glucose tolerance in obese diabetic and nondiabetic subjects. Diabetes **48**, 1836–1841
30 Boden, G., Lebed, B., Schatz, M., Homko, C. and Lemieux, S. (2001) Effect of acute changes of plasma free fatty acids on intramyocellular fat content and insulin resistance in healthy subjects. Diabetes **50**, 1612–1617
31 Dey, D., Mukherjee, M., Basu, D., Datta, M., Roy, S. S., Bandyopadhyay, A. and Bhattacharya, S. (2005) Inhibition of insulin receptor gene expression and insulin signaling by fatty acid: interplay of PKC isoforms therein. Cell. Physiol. Biochem. **16**, 217–228
32 Kopp, E. and Ghosh, S. (1994) Inhibition of NF-κB by sodium salicylate and aspirin. Science **265**, 956–959
33 Yuan, M., Konstantopoulos, N., Lee, J., Hansen, L., Li, Z. W., Karin, M. and Shoelson, S. E. (2001) Reversal of obesity and diet-induced insulin resistance with salicylates or targeted disruption of IKKβ. Science **293**, 1673–1677

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online data

SUPPLEMENTARY ONLINE DATA

NF-κB mediates lipid-induced fetuin-A expression in hepatocytes that impairs adipocyte function effecting insulin resistance

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MATERIALS AND METHODS

Free Ca^{2+} ($[Ca^{2+}]_i$) measurements

$[Ca^{2+}]_i$ was estimated by following the method described by Kenny et al. [1] except for the variation of $CaCl_2$ concentrations in the incubation buffer. The Fura-2 fluorescence of cells was recorded using a Shimadzu dual-excitation-wavelength spectrofluorimeter with excitation at 340 and 380 nm and emission at 500 nm. Fura-2 fluorescence increased with the increasing $[Ca^{2+}]_i$, changes in fluorescence intensity reflected the changes in $[Ca^{2+}]_i$ concentrations.

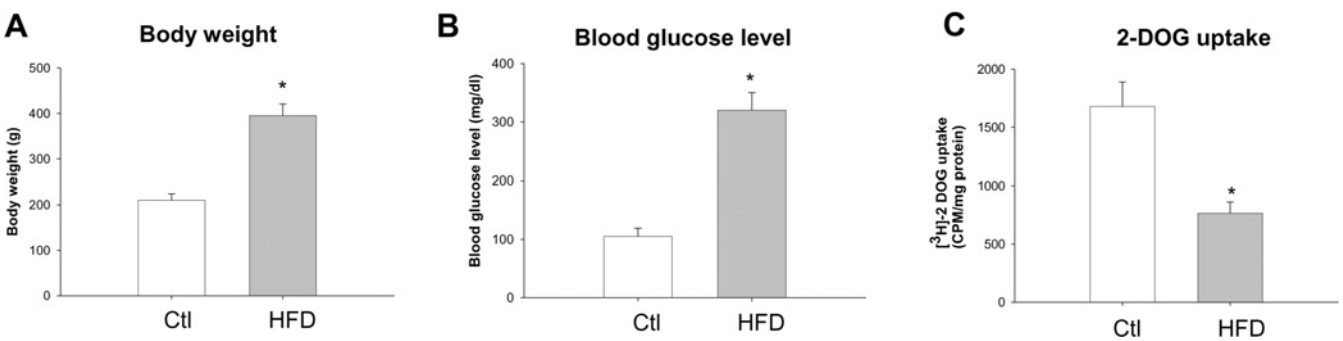

Figure S1 Insulin resistance in HFD-fed rats

(**A**) Adult rats were fed on a normal diet (Ctl) or on an HFD for 12 weeks. Comparison of body weight of normal and HFD rats. (**B**) Blood glucose levels of normal and HFD rats was measured using the glucose oxidase method. (**C**) Skeletal muscle cells isolated from control and HFD-fed rats were incubated *in vitro* with [^{3}H]2-DOG followed by the determination of its uptake. Means ± S.E.M. were calculated from six independent experiments. *$P < 0.001$, compared with Ctl.

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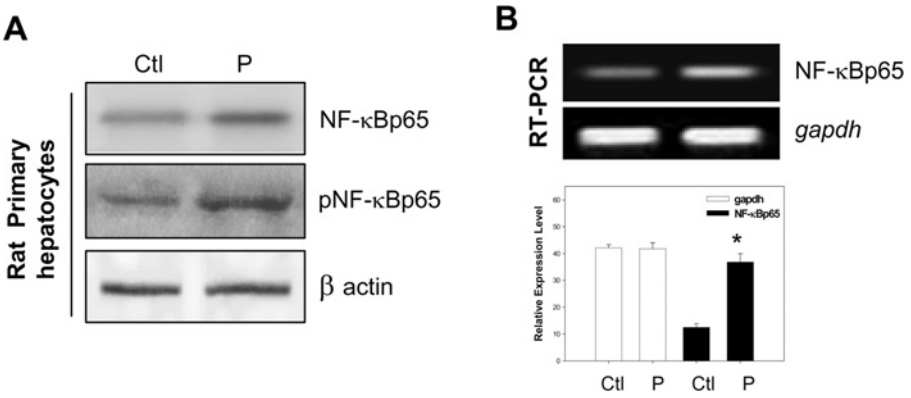

Figure S2 Palmitate stimulates NF-κB in rat primary hepatocytes

(**A**) Rat primary hepatocytes were incubated without (Ctl) or with (P) palmitate for 4 h. Cells were lysed and immunoblotted with anti-NF-κBp65 or anti-pNF-κBp65 antibodies. β-Actin served as an internal control. (**B**) RNA extracted from these incubations was subjected to RT–PCR and real-time PCR using NF-κB-specific primers where *Gapdh* served as an internal control. Means $\pm$ S.E.M. were calculated from three independent experiments. $^*P<0.001$, compared with Ctl.

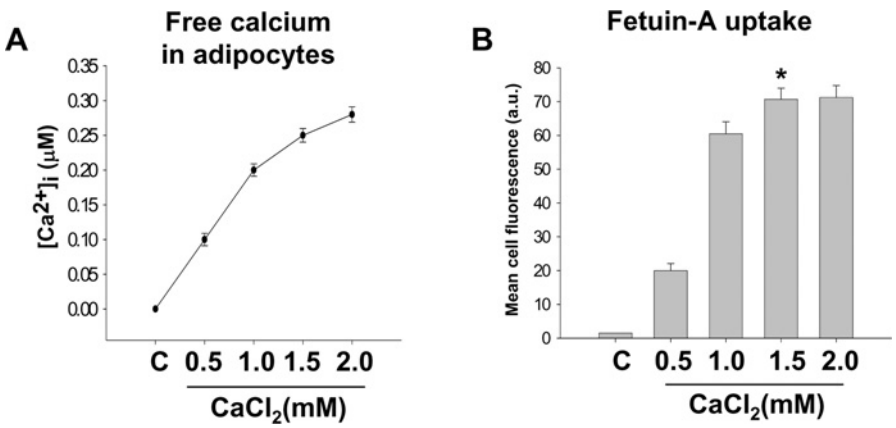

Figure S3 Effects of Ca^{2+} on fetuin-A uptake by 3T3-L1 adipocytes

(**A**) 3T3-L1 adipocytes were incubated with Fura-2 in Tris-balanced salt solution containing various concentrations of $CaCl_2$ (0.5, 1.0, 1.5 and 2.0 mM), and free Ca^{2+} was measured using a spectrofluorimeter. (**B**) 3T3-L1 adipocytes were incubated with FITC-conjugated fetuin-A in the absence or presence of various concentrations of $CaCl_2$ (0.5, 1.0, 1.5 and 2.0 mM). Cells were then washed thoroughly and lysed, and the supernatant was used to quantify the amount of internalized FITC-labelled fetuin-A using the spectrofluorimeter. Means $\pm$ S.E.M. were calculated from three independent experiments. $^*P<0.01$ for 1.5 mM $CaCl_2$ compared with C (control). a.u., arbitrary units.

Table S1 The putative NF-κB-binding sites on the pFetA-luc vector

Putative NF-κB-response elements on the fetuin-A promoter region of pFetA-luc vector and representative of the same in classical NF-κB. The position of six putative elements is given with respect to the transcription start site.

Putative elements	Position	Sequence
N1	−251/−242	5′-GGGGCAGGGA-3′
N2	−277/−268	5′-TGGTCATTTC-3′
N3	−395/−386	5′-TTGGCATCTC-3′
N4	−559/−568	5′-ATTTCCTCTG-3′
N5	−794/−785	5′-AAGCAGAAAT-3′
N6	−957/−948	5′-ATGAAGTCCC-3′
Classical NF-κB response element		5′-GGGRNNYYCC-3′

REFERENCE

1 Kenny, J. S., Kisaalita, W. S., Rowland, G., Thai, C. and Foutz, T. (1997) Quantitative study of calcium uptake by tumorigenic bone (TE-85) and neuroblastoma X glioma (NG108-15) cells exposed to extremely-low-frequency (ELF) electric fields. FEBS Lett. **414**, 343–348

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