Fetuin-A, a hepatic secretory protein, has been recently 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 our previous report, we show 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.
(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. [3H]Leucine (specific activity 1000 Ci/mmol), [3H]2-DG (2-deoxyglucose) (specific activity 12.0 Ci/mmol), [γ-32P]ATP (specific activity 6000 Ci/mmol) and [14C]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 were 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% CO2 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 mM/1 3-isobutyl-1-methylxanthine and 1 μmol/l dexamethasone.

Animal experiments

Adult male Sprague–Dawley rats (weighing 175–200 g) were anaesthetized following 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% CO2 atmosphere at 37°C.

Primary culture

Adult male Sprague–Dawley rats (weighing 175–200 g) were anaesthetized following 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% CO2 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% CO2 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 μM), 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 10000 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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Direction</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>NF-xBp65</td>
<td>Forward</td>
<td>5′-CCATCAGGGCCATCTCAACCC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-GTCGTCGAAACTGCTGGTTC-3′</td>
</tr>
<tr>
<td>Fetuin-A</td>
<td>Forward</td>
<td>5′-CCACTGTTATCTCCACCA-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-GCAGCTTCTACACAACTTCA-3′</td>
</tr>
<tr>
<td>C/EBPβ</td>
<td>Forward</td>
<td>5′-GGACCGACGAGGACGCGC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-CTGCCGCCGCCAATGCTGAC-3′</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Forward</td>
<td>5′-ATCATCCAGTAGCTGTCGCC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-CTTCTGCTGTTCACTTGG-3′</td>
</tr>
<tr>
<td>alp2</td>
<td>Forward</td>
<td>5′-TGAATCTTTGTGGAACCTG-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-GGCGGCGCTGACCTCTCCA-3′</td>
</tr>
<tr>
<td>CD36</td>
<td>Forward</td>
<td>5′-GAGGCATTCTTGGACCTTGA-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-TCAGATCCGAACACAGCGTA-3′</td>
</tr>
<tr>
<td>IL-6</td>
<td>Forward</td>
<td>5′-ATTCCTCCTGCTGCTCTGA-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-TTCTTGAGCACCCTTCTG-3′</td>
</tr>
<tr>
<td>TNFα</td>
<td>Forward</td>
<td>5′-TCCTAGGCATCTCTCATCC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-ACCTCGTGGTGTGGCTGAC-3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>5′-GCCATCAAGCTCCACCTCT-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-AGGGCAGGCCATTCTCCA-3′</td>
</tr>
</tbody>
</table>

The NF-κB-binding site [wild-type, 5′-GCACCTGGTTTG-GTCGGCAGGC-3′; mutant, 5′-GCACCTGGCCTTGCTCCCC-3′ (mutated residue is in bold and underlined)] within the fetuin-A promoter. The probes were end-labelled with [γ-32P]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-GGACACCTGTTTGGT-3′ (forward) and 5′-GCCCCAGAC-CTGACAA-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.

[3H]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 [3H]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).

[14C]Palmitate uptake

Adipocytes transduced without or with fetuin-A were incubated with 1 μCi/ml [14C]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 [14C]palmitate was measured in a liquid scintillation counter.

[3H]2-DG 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% CO2. 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). [3H]2-DG (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 [3H]2-DG 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 ± 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′-AGGTCTGGGAGGAGAGAAACCCACACGT-3′), oligohfetΔN2 (5′-CCCCACAGTTTGCTCACGGTGCTGCCC-3′), oligohfetΔN3 (5′-CAAGAATCTTCCTCCCCAAAATCTTTATACACTCTTCTTGCT-3′), oligohfetΔN4 (5′-GATCACAGTAGAAGACCTGCCAAAACCCATGGC-3′), oligohfetΔN5 (5′-GGTGTTTTTTTTTCTTTGAACACCACATCTCTTATCTTTGCAATCTTC-3′) and oligohfetΔN6 (5′-CTCTGGGGCCAGCCCTGCTGCTCCTCTC-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 ± 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...
Lipid-induced NF-κB up-regulates fetuin-A

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 ± S.E.M. were calculated from four independent experiments. *< 0.001, compared with Ctl. (B) HepG2 cells were incubated in different concentrations of palmitate (P) in the presence of 10 μCi of [3H]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 ± S.E.M. were calculated from four independent experiments. *< 0.001, #< 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. *< 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 ± S.E.M. were calculated from three independent experiments. *< 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 ± S.E.M. were calculated from three independent experiments. *< 0.001 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 [3H]leucine and observed a significant elevation of fetuin-A mRNA expression (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 ± 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 ± S.E.M. were calculated from four independent experiments. *P < 0.001, compared with Ctl; #P < 0.001, compared with P.

Figure 4  Palmitate stimulation of fetuin-A synthesis is mediated through NF-κB

(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 ± 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 by 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.
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-κ-B-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-κ-B 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 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-κ-B- 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...
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. Futuin-A uptake in cells has been shown to be Ca\textsuperscript{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\textsuperscript{2+} (less than the normal circulatory level [25]). Interestingly, futuin-A was readily translocated into the cells in the presence of Ca\textsuperscript{2+} (Figure 6A). Since Ca\textsuperscript{2+} remains bound to albumin in circulation, determination of the level of free Ca\textsuperscript{2+} that permitted maximum uptake of futuin-A is important. We have found that 1.5 mM CaCl\textsubscript{2} produced 0.25 μM free ionized Ca\textsuperscript{2+} in adipocytes which induced maximum fetuin-A entry into the cells, whereas 2 mM CaCl\textsubscript{2} resulting in 0.28 μM free Ca\textsuperscript{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\textsuperscript{2+} as the threshold level for permeating fetuin-A entry. We transduced 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 PPAR\textgreekgamma 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 [14C]palmitate, and it was found that addition of fetuin-A to 3T3-L1 incubation significantly inhibited [14C]palmitate uptake (Figure 6D).

We examined fetuin-A’s effect on PPAR\textgreekgamma, 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

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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-κBp65 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 ± 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 ± 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 palmitate (P) 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.
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 [3H]2-DG 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...
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

* Figures 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 Ca²⁺ 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 Ca²⁺ (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±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 [3H]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).
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.

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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 Ca2+ ([Ca2+]i) measurements
[Ca2+]i was estimated by following the method described by Kenny et al. [1] except for the variation of CaCl2 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 [Ca2+]i, changes in fluorescence intensity reflected the changes in [Ca2+]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 [3H]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 ± S.E.M. were calculated from three independent experiments. *P < 0.001, compared with Ctl.

Figure S3  Effects of Ca²⁺ 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 CaCl₂ (0.5, 1.0, 1.5 and 2.0 mM), and free Ca²⁺ 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 CaCl₂ (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 ± S.E.M. were calculated from three independent experiments. *P < 0.01 for 1.5 mM CaCl₂ 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.

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<th>Putative elements</th>
<th>Position</th>
<th>Sequence</th>
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<tbody>
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<td>5′-GGGGCAGGGGA-3′</td>
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<tr>
<td>N2</td>
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<td>Classical NF-κB response element</td>
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