Abstract

Aim. – This study aimed to investigate whether NF-κB contributes to insulin resistance in type 2 diabetes (T2DM).

Methods. – Subcutaneous abdominal adipose tissue was obtained from T2DM patients and non-diabetic control subjects. Pre-adipocytes were cultured and differentiated into adipocytes in vitro. Upon insulin stimulation, IRS-1 tyrosine and AKT (Ser473) phosphorylation were examined by immunoprecipitation and immunoblotting, while levels of inflammatory mediators IL-6 and MCP-1, and the DNA-binding activity of NF-κB, were examined by ELISA and electrophoretic mobility shift assay (EMSA), respectively. NF-κB decoy molecules were introduced into T2DM adipocytes, and their effects on all these molecular events evaluated.

Results. – Compared with cells from non-diabetic subjects, adipocytes from T2DM patients showed signs of insulin resistance, with significantly reduced IRS-1 tyrosine and AKT (Ser473) phosphorylation levels in response to insulin stimulation. At the same time, T2DM cells displayed elevated levels of IL-6 and MCP-1, and NF-κB activity. Introduction of NF-κB decoy molecules significantly inhibited both IL-6 secretion and NF-κB activity, while enhancing insulin-stimulated IRS-1 tyrosine and AKT (Ser473) phosphorylation in T2DM adipocytes.

Conclusion. – Abdominal subcutaneous fat cells from T2DM patients display signs of insulin resistance and microinflammatory status. NF-κB decoy molecules inhibited NF-κB overactivation and also partly reversed insulin resistance. These results provide evidence of a link between inflammation and insulin resistance in T2DM cells, suggesting a potential contribution of inflammation to the mechanism of insulin resistance.

Keywords: NF-κB decoy; NF-κB activation; Microinflammation; Insulin resistance; Type 2 diabetes mellitus; Adipocytes; In vitro study

Résumé

Effet de leurre de NF-κB sur la résistance à l’insuline des adipocytes de patients atteints de diabète de type 2.

Objectif. – Cette étude visait à déterminer si NF-κB contribuait à l’insulinorésistance observée dans le diabète de type 2 (DT2).

Méthodes. – Du tissu adipeux sous-cutané abdominal a été prélevé chez des patients atteints de DT2 et des témoins non diabétiques. Les préadipocytes ont été cultivés et se sont différenciés en adipocytes in vitro. Dans des conditions de stimulation par l’insuline, la phosphorylation de la tyrosine d’IRS-1 et d’Akt-Ser 473 a été mesurée par immunoprécipitation et immunoblotting, les concentrations d’IL-6, de MCP-1 et l’activité de liaison à l’ADN de NF-κB ont été mesurées respectivement par Elisa et par Emsa. Les molécules-leurre pour NF-κB ont été introduites dans les adipocytes provenant des patients atteints de DT2 et leur effet sur l’ensemble des événements moléculaires a été évalué.

Résultats. – En comparaison avec les adipocytes des patients non diabétiques, les adipocytes des DT2 présentaient des signes d’insulinorésistance, avec une diminution significative de la phosphorylation de la tyrosine d’IRS-1 et d’Akt-Ser473 en réponse à la stimulation par l’insuline. Parallèlement, les adipocytes provenant des DT2 présentaient une augmentation d’IL-6 et de l’activité NF-κB. L’introduction de molécules-leurre de NF-κB a inhibé significativement à la fois la sécrétion d’IL-6 et l’activité NF-κB, et a amélioré la phosphorylation de la tyrosine d’IRS-1 et d’Akt-Ser473 en réponse à la stimulation par l’insuline dans les adipocytes provenant de patients atteints de DT2.

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Mots clés : Leurre de NF-κB ; Activation ; NF-κB ; Micro-inflammation ; Insulinorésistance ; Diabète de type 2 ; Adipocytes ; Études in vitro
1. Introduction

Insulin resistance and defects in β-cell secretion are two important mechanisms in the pathogenesis of type 2 diabetes (T2DM). However, the mechanism of insulin resistance has not been fully elucidated. Recently, the aetiology of insulin resistance has been linked to inflammation [1,2] and, as a result, the study of the relationship between inflammation and insulin resistance in adipose tissues has become a research focus. Studies have shown that cytokines and other proinflammatory factors secreted from adipocytes, and especially white adipocytes, may participate in insulin resistance by inhibiting phosphorylation of the insulin receptor tyrosine, thereby affecting insulin signal transduction [3–5].

Nuclear factor-kappa-B (NF-κB) is a multifunctional transcription factor participating in the transcriptional regulation of a variety of cytokines, inflammatory mediators, enzymes and adhesion molecules [6,7]. It has also been shown to be involved in insulin resistance [8], so inhibition of NF-κB activity may offer a new treatment strategy for insulin resistance. In recent years, studies have shown that decoy oligodeoxynucleotides (ODNs) can inhibit the activity of NF-κB [9], but whether or not NF-κB contributes to insulin resistance in T2DM, and what is the effect of NF-κB decoy molecules on T2DM, remain unknown.

In the present study, the coexistence of altered insulin signaling and elevated inflammatory status was identified in adipocytes from T2DM patients, thereby further confirming the relationship between NF-κB and insulin resistance via the effects of NF-κB blockage on insulin resistance. These findings may help to advance our understanding of insulin resistance and offer a mechanistic basis for the treatment of diabetes.

2. Materials and methods

2.1. Participants

A total of 10 T2DM patients and 10 non-diabetic subjects, all of whom had undergone cholecystectomy at our hospital, were enrolled in the study. The study protocol was approved by the hospital ethics committee, and all patients gave their informed consent to participate. The diabetic patients included six women and four men, aged 45 ± 6.8 years; the non-diabetic subjects comprised five women and five men, aged 46 ± 3.8 years. All participants had no active liver disease, heart damage, kidney disease, infectious diseases, cancer, coronary heart disease or history of cerebral vascular accident.

Prior to surgery, clinical examinations were performed on all study participants, and the relevant clinical parameters for the diabetic group are shown in Table 1. About 5 g of abdominal subcutaneous adipose tissue was obtained from each subject during the operation.

2.2. Reagents

Dulbecco’s modified Eagle medium/nutrient mixture F-12 (DMEM/F12; 1:1) and fetal calf serum were used (HyClone, Thermo Scientific). Bovine serum albumin (BSA) was purchased from Roche Applied Science, and collagenase II, hydrocortisone, insulin, transferrin, biotin, pantothenate, triiodothyronine and 3-isobutyl-1-methylxanthine were all from Sigma-Aldrich. Antibodies against IRS-1, phosphotyrosine and phosphorylated AKT (Ser473) were all from Cell Signaling Technology (Danvers, MA, USA). The enhanced chemiluminescence (ECL) kit was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the enzyme-linked immunosorbent assay (ELISA) kit was purchased from AMDL (ADL, Tustin, CA, USA). Lipofectamine 2000 was obtained from Invitrogen Corporation (Carlsbad, CA, USA), and the NF-κB DNA probe was synthesized and biotin-labeled by Invitrogen also.

2.2.1. Synthesis of NF-κB decoy and decoy control oligodeoxynucleotides

Sequences of the two strands for the NF-κB decoy were 5′-CCT TGA AGG GAT TTC CCT CC-3′ and 5′-GGA ACT TTC CTA AAG GGA GG-5′. Sequences of a control molecule of a random ODN were 5′-TTG CCG TAC CTG ACT TAG CC-3′ and 5′-AAC GGC ATG GAC TGA ATC GG-5′. All sequences were phosphorothiate-modified, and some were labeled with fluorescent fluorescein isothiocyanate (FITC) at the 5′ end. All syntheses and modifications were carried out by Invitrogen.

2.3. Methods

2.3.1. Isolation and culture of human abdominal subcutaneous preadipocytes

Under sterile conditions, 5 g of abdominal subcutaneous adipose tissue was removed from each participant and cut into small pieces. After digestion with collagenase II for 1.5 h in the presence of BSA, single cells were filtered and centrifuged...
at 1000 rev/min for 10 min. The cell pellet was resuspended in DMEM/F12 to 2 x 10^4/cm² and inoculated into a 50-mL culture flask, then passaged at 80% confluence and used within three or four passages.

2.3.2. Real-time quantitative polymerase chain reaction (PCR)

Total RNA was isolated from abdominal subcutaneous preadipocytes by the guanidinium method. Total RNA (500 ng) was used as a template for reverse transcription (RT) using an RNA RT kit from ABI (Applied Biosystems, Warrington, UK). The RT reaction was set up in a 20-μL mixture containing 50 mmol/L of KCl, 10 mmol/L of Tris–HCl, 5 mmol/L of MgCl², 1 mmol/L of deoxynucleoside triphosphate (dTTP), 2.5 μmol/L of random hexamers, 20 U of RNAsin and 50 U of Moloney murine leukemia virus (MoMuLV) reverse transcriptase. Incubations were performed in a DNA Thermal Cycler 9700 (Applied Biosystems, Foster City, CA, USA) for 10 min at room temperature, followed by 30 min at 42 °C and 5 min at 99 °C.

Real-time quantitative PCR was performed with a TaqMan ABI 7000 Sequence Detection System using TaqMan Green PCR Master Mix (Applied Biosystems, UK). The Thermal Cycler conditions included holds for 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 20 s at 95 °C, 20 s at 55 °C and 30 s at 72 °C. The relative amount of mRNA was calculated using a comparative Ct method, and β-actin served as the reference housekeeping gene. Amplification efficiencies of the target and reference were shown to be approximately equal, with a slope of log input amount at Ct < 0.1.

The following oligonucleotide primers were used: For human peroxisome proliferator-activated receptor (PPAR)-γ, upper 5′-TCAGGCCTGCACTTGCC-3′, lower 5′-GCTTTTGCCATACCTGTGATCTC-3′; for human sterol regulatory element-binding protein (SREBP)-1, upper 5′-CGCAAGGGCCATCGACTACAT-3′, lower 5′-GACCTAGGTTCCTCCTGTTGAGTTC-3′; for human phospho-AKT (Ser473) and 3′-GCCCATCCACCAGGAGTA-3′. The primers were designed by Primer Express Software version 2.0 (Applied Biosystems, USA).

2.3.3. Differentiation and identification of human abdominal subcutaneous preadipocytes

Preadipocytes in good growth condition were inoculated into six-well plates. They were switched to a serum-free medium and cultured for 16 days, after which the culture medium was removed, and the cells rinsed and fixed with 10% formaldehyde. After rinsing with 60% isopropyl alcohol, Oil Red O working solution was added. Cells were incubated for 30 min, and then the Oil Red O stain was rinsed off. The differentiated cells were then observed under a light microscope and photographed.

2.3.4. Transfection of the NF-κB decoy

Transfection was carried out with Lipofectamine 2000 according to product instructions from the manufacturer. First, 4.0 μg of DNA and 10 μL of liposomes were each diluted with 250 μL of Opti-MEM media (Invitrogen) in separate tubes, then mixed to allow the formation of a liposome/DNA complex. Cells in the six-well plate were changed to 2 mL of fresh serum-free medium after washing with the same medium, after which 500 μL of Lipofectamine/DNA complexes were added into the wells, drop by drop, and gently mixed. After incubation at 37 °C for 4 h, cells were switched to a complete culture medium, and culture continued for another 24 h. Cells transfected with fluorescent-labeled ODN served as a control for visualization of transfected cells under a fluorescence microscope. Cells transfected with non-fluorescent NF-κB decoy DNA were used in the subsequent experiments.

2.3.5. Protein extraction

Differentiated fat cells, with or without the above-mentioned transfection, were treated with 100 nM of insulin for 15 min. After washing, radioimmunoprecipitation assay (RIPA) buffer and phenylmethylsulphonyl fluoride (PMSF) were added; cells were then scraped off and transferred to a vortex tube, and incubated on ice for 30 min. The tube was centrifuged at 4 °C, and the supernatant transferred to another 1.5-ml tube. Protein concentration was measured using a Coomassie Brilliant Blue protein quantitative kit, and samples were stored in −80 °C.

2.3.6. Detection of AKT (Ser473) phosphorylation by Western blotting

Protein samples (40 μg) were applied to sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a membrane. After the blot was blocked with 5% skimmed milk for 2 h, mouse anti-human phospho-AKT (Ser473) antibody was added to the blocking solution, and the incubation continued overnight. After washing with Tris-buffered saline with Tween (TBST), the blot was incubated with HRP-goat anti-mouse secondary antibody for 2 h, washed three times, reacted with ECL reagent and exposed to an X-ray film. Optical densities of the protein bands were qualified by Quantity One software. Band intensities for phospho-AKT (Ser473) were normalized with that of β-actin. The experiment was repeated twice.

2.3.7. Detection of IRS-1 tyrosine phosphorylation by immunoprecipitation

Phosphate buffered saline (PBS)-washed protein A sepharose beads (50 μL) were mixed with 10 μL of IRS-1 antibody, centrifuged at 4 °C, washed with PBS again and resuspended in 50 μL of PBS to obtain a protein A sepharose–IRS-1 antibody complex, which was then added to 100-μg protein samples obtained by the above steps. After incubation at 4 °C under rotation for 2 h, centrifugation and PBS washing, the protein-bound beads were resuspended in 50 μL of 1X SDS gel loading buffer, and the bound proteins released by denaturation at 100 °C. The supernatant was transferred to a new tube and stored at −20 °C. The samples were then subjected to immunoblotting as described above.

2.3.8. Detection of IL-6 and MCP-1 levels by ELISA

Culture fluid was removed from cells with or without transfection of the decoy molecules, and centrifuged at 3000 rev/min...
for 5 min at 4°C to remove cell debris. Supernatant was collected and subjected to ELISA to detect interleukin-6 (IL-6) and monocyte chemotactic protein (MCP)-1 levels, according to the manuals provided with the ELISA kit and microplate reader. IL-6 and MCP-1 concentrations were expressed as ng/L, and each sample was measured in duplicate.

2.3.9. Detection of NF-κB DNA-binding activity by EMSA

This procedure involved two steps. The first was the extraction of nuclear proteins from fat cells. Cells were collected and resuspended in 400 μL of pre-chilled nuclear protein extract solution A, lysed by ultrasonic force and incubated on ice. After the addition of 33 μL of 10% NP-40, cells were shaken vigorously and centrifuged. The pellet was then resuspended in 50 μL of pre-cooled nuclear protein extract solution B, shaken vigorously at 4°C and centrifuged. The supernatant containing nuclear proteins was transferred to aliquots to new tubes and stored at −80°C.

In the second step to obtain DNA-binding reactions, each 10-μL reaction contained 2 μL of nuclear protein extract, 1 μL of biotin-labeled NF-κB oligonucleotide probe (5′-AGT TGA GGG GAC TTT CCC AGG C-3′), 2 μL of 5X binding buffer and 5 μL of deionized water. The result was five groups, each containing either the labeled NF-κB DNA probe with or without the cold probe as competitor, or the labeled probe alone with no protein samples (control). All reactions were carried out at room temperature for 30 min, then 1 μL of 10X loading buffer was added, and the sample subjected to 6.5% non-denaturing PAGE and transferred to an X-ray film for autoradiography. This experiment was repeated twice.

2.4. Statistical analysis

All data are presented as means ± SD. Student’s t and Newman–Keuls (q) tests were used to compare the differences between two and among three groups, respectively. P values < 0.05 were considered statistically significant. All statistical analyses were performed with SAS statistical analysis software.

3. Results

3.1. In-vitro differentiation and transfection of human preadipocytes

Abdominal subcutaneous preadipocytes were isolated from both T2DM patients and non-diabetic subjects, and primary cultures established (Fig. S1, A, B; see supplementary material associated with this article online). The preadipocytes spontaneously differentiated into mature adipocytes after being cultured in serum-free medium for 21 days, and the lipid droplets within the mature adipocytes were identified as red particles after Oil Red O staining (Fig. S1, C; see supplementary material associated with this article online). Mature fat cells are known to be difficult to transfet. However, using Lipofectamine 2000 and an optimized protocol, we were able to transfect the cells with NF-κB ODN decoy molecules. The FITC-labeled ODNs were localized within the cell nuclei (Fig. S1, D; see supplementary material associated with this article online). The expression of SREBP-1 and PPAR-γ mRNA was more greatly increased in mature adipocytes than in preadipocytes (Fig. S1, E; see supplementary material associated with this article online).

3.2. Levels of IRS-1 tyrosine and AKT (Ser473) phosphorylation

As shown in Fig. S2 (see supplementary material associated with this article online), IRS-1 tyrosine and AKT (Ser473) phosphorylation occurred in adipocytes from both diabetic and non-diabetic groups in response to insulin stimulation, but the levels of both types of phosphorylation were significantly lower in cells from T2DM patients than in those from non-diabetic controls (P < 0.05), indicating a reduced insulin response or insulin resistance.

Furthermore, in T2DM cells after being transfected with NF-κB decoy molecules, insulin-stimulated IRS-1 tyrosine and AKT (Ser473) phosphorylation levels were both significantly higher than in all of the control groups (untreated cells, cells that were mock-transfected with liposomes alone and cells transfected with the decoy control ODN, Fig. 1; P < 0.05). Levels of both types of phosphorylation showed no significant differences among the three control groups (P > 0.05).

3.3. IL-6 and MCP-1 levels

By ELISA, the IL-6 and MCP-1 levels in the culture supernatant from adipocytes of T2DM patients were significantly higher than in those from the non-diabetic control groups (P < 0.05; (Fig. S3, A, C; see supplementary material associated with this article online). In T2DM cells after transfection with NF-κB decoy molecules, IL-6 and MCP-1 levels in supernatants were significantly decreased compared with those in all three control groups (untreated cells, cells mock-transfected with Lipofectamine alone and cells transfected with decoy control molecules). All differences were statistically significant between decoy-transfected cells and any of the control groups (P < 0.05), but there was no significant difference among the control groups themselves (Fig. S3, B, D; see supplementary material associated with this article online).

3.4. DNA-binding activity of NF-κB

As a well-accepted means of in vitro detection of the transcriptional activity of NF-κB, electrophoretic mobility shift assay (EMSA) using a labeled NF-κB DNA-binding element showed that the binding of NF-κB to the probe was significantly stronger in cells from T2DM patients than in those from the non-diabetic controls (lanes 1 and 3, Fig. 2; P < 0.01). Binding specificity was verified by the competition of the excess amount of unlabeled DNA seen with the same NF-κB target sequence (lanes 2 and 4, Fig. 2; P < 0.01). After transfection with NF-κB decoy molecules, DNA-binding activity in the T2DM cells was significantly decreased compared with the mock-transfected cells or those treated with Lipofectamine alone (Fig. 3, P < 0.05).
Fig. 1. Levels of IRS-1 tyrosine and AKT (Ser473) phosphorylation in T2DM adipocytes after transfection as shown by immunoprecipitation and immunoblot analyses, respectively: (A) protein samples from T2DM adipocytes before transfection (lanes 1 and 2), or mock-transfected with Lipofectamine alone (lanes 3 and 4), or transfected with decoy control molecules (lanes 5 and 6) or NF-κB decoy molecules (lanes 7 and 8); (B, C) quantitative comparisons based on band intensities in the gel. Data are expressed as means ± SD from 10 replicates.

Fig. 2. NF-κB DNA-binding activity in adipocytes from T2DM patients and control groups as shown by EMSA reactions using labeled and competitive probes: (A) Lane 1, sample from T2DM cells with labeled probe; lane 2, sample from T2DM cells with the competitor probe; lane 3, sample from non-diabetic cells with labeled probe; lane 4, sample from non-diabetic cells with competitor probe; and lane 5, control reaction with only labeled probe and no protein samples; (B) quantitative comparison of band intensities of lane 3 (non-DM cells) and lane 1 (T2DM cells) in the gel.

4. Discussion

Insulin resistance is one of the hallmarks of T2DM. The accumulated evidence indicates that insulin regulation of blood sugar levels in the body is mainly through the IR/IRS-1/Pi3 K/PDK1/AKT/GLUT-4 signaling pathway [10]. Dysfunction of one or more components of this pathway can lead to insulin resistance and the onset of diabetes [11,12]. IRS-1 is the main substrate of the insulin receptor tyrosine kinase. After binding to the β subunit of the receptor and the subsequent tyrosine phosphorylation by the receptor tyrosine kinase, IRS-1 changes its conformation to an active form with a number of Src homology region 2 (SH2) binding sites [13]. In this pathway, phosphatidylinositol 3-kinase (Pi3 K) is the major insulin signaling molecule downstream of IRS-1 [12,14], and AKT is an important serine/threonine kinase downstream of Pi3 K. By activating phosphatidyl fructokinase-2, AKT enhances glyco-
sis while inhibiting glycogen synthesis through inactivation of glycogen synthase kinase-3 (GSK-3). Activation of AKT can also regulate the synthesis, secretion and transport of glucose transporter-4 (GLUT-4) [7]. Thus, decreased IRS-1 and PI3 K activities can directly inhibit insulin signal transduction, and are important molecular events in the onset and progression of diabetes.

In the present study, the expression of IRS-1 tyrosine and AKT (Ser473) phosphorylation was upregulated in response to insulin stimulation in T2DM patients. The present results further confirm this mechanism. It is well known that there are many proposed mechanisms for insulin resistance, including some that are related to infection and inflammation. Acute infections can cause significant hyperglycaemia. In non-diabetic people, an acute response to a variety of infections can temporarily increase levels of blood glucose and serum insulin, creating a short-term insulin-resistant state; however, in diabetic patients, infection can decrease the control of blood glucose and worsen the already existing insulin resistance. Also, inflammatory cytokines can activate serine kinases in the insulin–PI3 K pathway that, in turn, phosphorylate serine/threonine residues in the IRS-1 and PI3 K subunits, thereby impairing phosphorylation of the tyrosine residues of these molecules, resulting in the inhibition of insulin signaling and, ultimately, insulin resistance [15,16].

The present research was mainly focused on the regulation of the NF-κB/IL-6 signaling pathway in an insulin-resistant state. The results show that T2DM cells displayed elevated IL-6 levels and NF-κB activity. As a key inflammatory mediator, NF-κB can stimulate high levels of expression of multiple cytokines, adhesion molecules and acute-phase response proteins [17], which can further activate NF-κB via a positive-feedback regulatory system and further augment inflammation [18,19]. In theory, blocking the activity of NF-κB is superior to blocking each of the different inflammatory mediators, and is also safer than the use of glucocorticoids. Thus, blocking the transcription activities of NF-κB or its binding to cis elements in its target genes may present an effective strategy to reverse insulin resistance. Gene-trapping using decoy ODNs interferes with
gene expression at the terminal end of signal transduction, and has a higher efficiency and specificity than other gene-therapy strategies. In addition, ODNs are easily synthesized, and exert their antagonistic effects without being expressed inside the cell.

The present study has identified at least two abnormalities in the insulin-signaling pathway in adipose tissue from T2DM patients, as well as coexisting inflammation. The NF-κB decoy competitively inhibited the binding of NF-κB to DNA cis elements, and interfered with the activity of transcription factors and expression of inflammatory cytokines, thus significantly reducing the insulin resistance of T2DM adipocytes. Our study also confirms that NF-κB can affect the insulin-signaling pathway and contribute to insulin resistance. Such reduction or blockage of NF-κB activity offers benefits in the treatment of insulin resistance, as evidenced by the clinical effects of anti-inflammatory drugs such as glucocorticoids. However, the use of ODNs against NF-κB is a novel treatment strategy, and further studies are needed to clarify the different mechanisms of actions between glucocorticoids and ODNs, and to evaluate any non-specific or potentially toxic effects of NF-κB decoys in vivo. Nevertheless, our study provides evidence of a link between inflammation and insulin resistance in T2DM cells, suggesting a contribution of inflammation to the mechanism of insulin resistance and offering potential targets for future treatments.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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Appendix A. Supplementary material

Supplementary material (Figs. S1–S3) associated with this article can be found at http://www.sciencedirect.com, at doi:10.1016/j.diabet.2011.04.004

References


