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Original Research

Macrophages Inhibit Insulin Signalling in Adipocytes: Role of Inducible Nitric Oxide Synthase and Nitric Oxide

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ABSTRACT

Objectives: The interaction of immune cells with adipocytes within the adipose tissues in obese persons with diabetes mellitus may play a role in insulin resistance. We examined in vitro whether nitric oxide (NO) and inducible nitric oxide synthase (iNOS) play a role in impaired insulin signalling in adipocytes exposed to activated macrophages.
Methods: We used a co-culture system in which Raw264.7 macrophages were plated over differentiated, low passage 3T3-L1 cells (dif3T3) at a cell density ratio of 1:2. Inflammation was induced by a challenge with bacterial lipopolysaccharide.
Results: Significantly ($p<0.001$) enhanced iNOS expression and NO synthesis was observed in activated co-cultures. In the co-cultures as compared with Raw264.7 cells alone, iNOS protein was induced up to 11-fold above background, and NO release was significantly ($p<0.001$) increased up to 2.8-fold. Co-culturing dif3T3 and Raw264.7 cells as compared to dif3T3 alone reduced insulin-induced Akt phosphorylation by 50% and AS160 phosphorylation by 42%. This was correlated with reduced glucose consumption when dif3T3 was exposed to 1,3-morpholinosydnonimine. Adiponectin, GLUT4 and AS160 mRNA were reduced by 4-fold, 5-fold and 2-fold, respectively, in co-cultures as compared to dif3T3 alone. On the contrary, GLUT1 mRNA levels were increased by 2-fold in co-cultures as compared to dif3T3. NG-monomethyl-L-arginine abolished NO production with modest reversal of Akt/AS160 phosphorylation.
Conclusions: This study demonstrated a potential association between iNOS/NO-mediated inflammation and insulin resistance.

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Mots clés :
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RÉSUMÉ

Objectifs : L'interaction entre les cellules immunitaires et les adipocytes dans les tissus adipeux chez les personnes obèses souffrant de diabète sucré peut jouer un rôle sur l'insulinorésistance. Nous avons examiné *in vitro* si l'oxyde nitrique (NO) et l'oxyde nitrique synthase inductible (iNOS) joue un rôle dans l'altération de la signalisation de l'insuline des adipocytes exposés à l'activation des macrophages.
Méthodes : Nous avons utilisé un système de coculture dans lequel les macrophages Raw264.7 ont été ensemencés dans des plaques sur des cellules 3T3-L1 différenciées à faible passage (dif3T3) selon un ratio de densité cellulaire de 1:2. L'inflammation a été induite par une stimulation avec le lipopolysaccharide bactérien.
Résultats : L'amélioration significative ($p < 0{,}001$) de l'expression de l'iNOS et de la synthèse du NO a été observée dans les cocultures activées. Dans les cocultures comparativement aux cellules Raw264.7 seules, la protéine iNOS a été induite jusqu'à 11 fois au-dessus de la normale, et la libération du NO a été significativement ($p < 0{,}001$) augmentée jusqu'à 2,8 fois. La coculture de la dif3T3 seule a réduit de 50 % la phospholyration de l'Akt induite par l'insuline et de 42 % la phosphorylation de l'AS160. Cela a été corrélé à la réduction de la consommation de glucose lorsque la dif3T3 était exposée au 3-morpholinosydnonimine (ou SIN-1). L'adiponectine, l'ARNm de GLUT4 et d'AS160 ont été respectivement réduits de 4 fois, 5 fois et 2 fois dans les cocultures comparativement à la dif3T3 seule. À l'inverse, les concentrations de l'ARNm de GLUT1 ont été augmentées de 2 fois dans les cocultures

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comparativement à la dif3T3. La G-monométhyl-L-arginine a aboli la production de NO par le renversement modeste de la phosphorylation de l'Akt et de l'AS160.
Conclusions : Cette étude a démontré un lien potentiel entre l'inflammation médiée par l'iNOS et le NO, et l'insulinorésistance.

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Introduction

Obesity is widely considered a state of chronic low-grade inflammation that contributes to insulin resistance and type 2 diabetes (1), but there is no clear understanding of the precise cause of insulin resistance. Insulin resistance in obese persons is associated with adipose tissue macrophages infiltration (2,3), which is speculated to be through nitric oxide (NO) generation, lipolysis and free fatty acids activation of TLR4 complex (4,5). Secretion of proinflammatory adipokines and chemokines through lipolysis may further potentiate inflammation (6). Macrophages express inducible nitric oxide synthase (iNOS), which catalyzes the synthesis of NO; the latter may impair insulin-stimulated PI3K/Akt activation (7–9) and play a role in obesity-related insulin resistance. Mice with deletion of iNOS are protected from high fat diet-induced insulin resistance and decreased Akt activation (10). Conversely, NO has been reported to stimulate glucose transport through GLUT4 translocation (11,12). Clinical studies show differential circulating and skeletal muscle NO levels depending on the adiposity of type 2 diabetes patients (13).

We have recently observed that co-culturing adipocytes with myocytes impairs insulin signalling through increased interleukin-6 expression and secretion (14). Here, we examine the effects of co-culturing adipocytes with macrophages, iNOS expression and NO release on insulin signalling and glucose uptake. The 3T3-L1 cells are well-established alternative model systems, and when adequately differentiated (dif3T3) can provide a physiological model mimicking adipocytes (15), and these lipid-laden adipocytes parallel the in vivo upregulation of transcription factors and the formation of characteristic fat depots (16). Raw264.7 cells are also known to be similar to bone marrow macrophages in expression of CD14 and F4/80 and bacterial lipopolysaccharide (LPS)–induced NO production and represent a common point in the monocyte-macrophage differentiation pathway (17). Direct coculture of dif3T3 with Raw264.7 may represent adipose tissue infiltration by macrophages. In the present study, we used the Raw264.7 and dif3T3 coculture model and the pharmacological manipulation of NO production with 3-morpholinosydnonimine (SIN-1) for NO generation or NG-monomethyl-L-arginine (LNMMA) for inhibition of iNOS together with proinflammatory activation by LPS.

Methods

Cell culture design

Raw264.7 cell line and murine macrophage and 3T3-L1 preadipocyte (pre3T3) cell lines, originally derived from murine fibroblasts, were purchased from the American Type Culture Collection (ATCC). Raw264.7 cells were grown and maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 1% penicillin/streptomycin (growth medium) and 10% fetal bovine serum (FBS). For use in experiments, cells were seeded in multiple-well plates and grown to confluence as recommended by ATCC. The pre3T3 cells were differentiated into dif3T3 according to a previously described method (18). Briefly, 2 days postconfluence, pre3T3 cells were induced in DMEM supplemented with 10% FBS, 250 nmol/L dexamethasone, 25 nmol/L insulin and 0.5 mmol/L isobutyl methylxanthine for 3 to 5 days. Seven days after induction of differentiation, at which time more than 95% of cells were observed to show multiples of fat droplets, the dif3T3 cells were used in co-culture with Raw264.7 cells. For co-culture experiments, the Raw264.7 cells were trypsinized and 5×10^5 cells were plated in each well of 6-well plates containing approximately 1×10^6 dif3T3 cells 7 days after induction of differentiation (i.e. cellular ratio of 1:2, respectively). All cell culture medium and supplements were from Invitrogen (Carlsbad, CA, USA).

Protein extraction and immunoblotting

The antibody phospho-Akt (Ser 474) was purchased from Abcam; iNOS, pAS160 and alpha-tubulin were all from Cell Signaling (Beverly, MA). Chemical reagents LPS, SIN-1, LNMMA and wortmannin were from Sigma. The isozyme selective Akt1/2 kinase inhibitor (iAkt) was from Sigma and used in cells at a dose of 5 μM. Cells treated with LPS, SIN-1, LNMMA and iAkt where appropriate and control cells were harvested and washed with phosphate-buffered saline; total cell proteins were extracted by radio-immunoprecipitation assay buffer containing a protease inhibitor cocktail, antiphosphatases I and II (Sigma-Aldrich, St Louis, MO). Protein concentration was measured with BCA assay reagents (Thermo Scientific Pierce, Rockford, IL). Protein lysates were mixed with sample buffer (20% glycerol, 4% sodium dodecyl sulfate [SDS], 10% 2-mercaptoethanol, 0.05% bromophenol blue and 1.25 M Tris-HCl, pH 6.8). Cell lysate proteins from treated and control cells were resolved using SDS-PAGE in 10% to 12% polyacrylamide. For a given Western analysis, equivalent protein loading controls and pre-stained molecular weight markers (Thermo Fisher, Rockford, IL) were included. After electrophoresis, the proteins were transferred onto PVDF membrane (Amersham, Pittsburgh, PA) in a buffer containing 25 mMTris-HCl, 192 mM glycine, 20% methanol and 0.01% SDS (pH 8.5) using Bio-Rad transblot semidry apparatus at 14V for 1.5 hours. Residual protein-binding sites on the membranes were blocked by incubation for 1 hour in Tris-buffered saline and Tween 20 buffer (20 mMTris-HCl, pH 7.6, 250 mM NaCl, 0.05% Tween-20) containing 5% nonfat dry milk (Bio-Rad, Hercules, CA). The membranes were incubated with primary antibody overnight at 4°C. After washing with Tris-buffered saline and Tween 20 buffer, a secondary antibody (anti-immunoglobulin G conjugated with horseradish peroxidase) was added for 1 hour. Finally, the protein bands were visualized by autoradiography using an enhanced chemiluminescence Thermo Fisher detection system. Blot spot densities were adjusted for total protein loading indicated by alpha-tubulin.

RNA extraction and complementary DNA synthesis

Total RNA extraction and purification was carried out using a Promega kit following manufacturer's instructions (Promega, Madison, WI). Briefly, the total RNA extraction was processed by adsorbing total nucleic acids to the column matrix; adsorbed DNA was hydrolyzed with DNase enzymatic digestion buffer and removed by further washing. The purified RNA was eluted into

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