Tumor Necrosis Factor Receptor-1 Can Function through a Goq/11-β-Arrestin-1 Signaling Complex*

Received for publication, July 17, 2007 Published, JBC Papers in Press, July 30, 2007, DOI 10.1074/jbc.M705869200

Yuji Kawamata1, Takeshi Imamura1, Jennie L. Babendure, Juu-Chin Lu, Takeshi Yoshizaki, and Jerrold M. Olefsky2

From the Department of Medicine, Division of Endocrinology and Metabolism, University of California, San Diego, La Jolla, California 92093-0673

Tumor necrosis factor-α (TNFα) is a proinflammatory cytokine secreted from macrophages and adipocytes. It is well known that chronic TNFα exposure can lead to insulin resistance both in vitro and in vivo and that elevated blood levels of TNFα are observed in obese and/or diabetic individuals. TNFα has many acute biologic effects, mediated by a complex intracellular signaling pathway. In these studies we have identified new G-protein signaling components to this pathway in 3T3-L1 adipocytes. We found that β-arrestin-1 is associated with TRAF2 (TNF receptor-associated factor 2), an adaptor protein of TNF receptors, and that TNFα acutely stimulates tyrosine phosphorylation of Goq/11 with an increase in Goq/11 activity. Small interfering RNA-mediated knockdown of β-arrestin-1 inhibits TNFα-induced tyrosine phosphorylation of Goq/11 by interruption of Src kinase activation. TNFα stimulates lipolysis in 3T3-L1 adipocytes, and β-arrestin-1 knockdown blocks the effects of TNFα to stimulate ERK activation and glycerol release. TNFα also led to activation of JNK with increased expression of the proinflammatory gene, monocyte chemoattractant protein-1 and matrix metalloproteinase 3, and β-arrestin-1 knockdown inhibited both of these effects. Taken together these results reveal novel elements of TNFα action; 1) the trimeric G-protein component Goq/11 and the adapter protein β-arrestin-1 can function as signaling molecules in the TNFα action cascade; 2) β-arrestin-1 can couple TNFα stimulation to ERK activation and lipolysis; 3) β-arrestin-1 and Goq/11 can mediate TNFα-induced phosphatidylinositol 3-kinase activation and inflammatory gene expression.

Chronic low grade inflammation is a critical feature of a number of common, non-immune diseases such as insulin resistance/obesity, type 2 diabetes mellitus, certain forms of cancer, and neurodegenerative disorders. The cytokine, TNFα3, is a key signaling molecule that activates proinflammatory effects in target cells. TNFα binds to the TNF receptor-1 (TNF-R1) and receptor-2 (TNF-R2) and stimulates the NFκB pathway through complexes that includes the TRAF, RIP (receptor-interacting protein), and TRADD (TNF-R-associated death domain) families (1, 2). Numerous studies have reported TNFα signaling to NFκB activation; however, the mechanisms underlying TNFα stimulates ERK, cdc42, or PI 3-kinase actually remain incompletely understood. It is well known that chronic TNFα treatment can lead to insulin resistance both in vitro (3) and in vivo (4), and elevated levels of TNFα have been reported in obese and/or diabetic individuals. Thus, further insights into the mechanisms of TNFα signaling are of interest. Recent findings reported that TNF-R1 is observed in plasma membrane microdomains, termed lipid rafts and/or caveolae (5, 6), in which various G-proteins, 7-transmembrane (TM) receptors (7-TMRs), and many other signaling components are collected. For example, the endothelin-1 (ET-1) receptor ETAR, a Gq-coupled 7-TM receptor, is detected in caveolae, and insulin receptors are also found in this membrane structure (7). Interestingly, it has been shown that the insulin receptor can couple into the heterotrimeric G-protein Gaq/11 (8) and that chronic ET-1 treatment can induce insulin resistance by heterologous desensitization of Gaq/11 signaling (9, 10).

Based on these facts, we sought to determine whether TNFα signaling could couple with G-protein components to mediate biologic effects. In this report we show that in 3T3-L1 adipocytes, TNFα signals through the TNF-R1-TRAF2 receptor complex and that this can couple into a trimeric G-protein Gaq/11 and an adaptor protein β-arrestin-1 to mediate TNFα activation of ERK, lipolysis, and stimulation of the proinflammatory pathway.

EXPERIMENTAL PROCEDURES

Materials—Mouse monoclonal anti-phosphotyrosine (PY-20) antibody was purchased from BD Biosciences. Horseradish peroxidase-linked anti-rabbit, -mouse, -goat antibodies, anti-c-Jun N-terminal kinase (JNK), -cdc42, TRAF2, -p110 subunit of PI 3-kinase, and -pan-Src polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-specific anti-ERK and -JNK antibodies were from Cell Signaling, Inc. TNF-R1 antibody and mouse- and human-TNFα were from

*This work was supported by National Institutes of Health Research Grant DK 33651, University of California Discovery Program Project bio03-10383 (BioStar), and United States and Israel Binational Scientific Foundation Grant 2003238. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: University of California, San Diego, Dept. of Medicine (0673), 9500 Gilman Dr., La Jolla, CA 92093. Tel.: 858-534-6651; Fax: 858-534-6653; E-mail: jolefsky@ucsd.edu.

2 Both authors contributed equally to this work.

3 The abbreviations used are: TNF, tumor necrosis factor; TNF-R, TNF receptor; 7-TMR, 7-transmembrane (TM) receptor; ET-1, endothelin-1; TRAF2, TNF receptor-associated factor 2; JNK, c-Jun NH2-terminal kinase; ERK, extracellular signal-regulated kinase; MMP3, matrix metalloproteinase 3; MCP-1, monocyte chemoattractant protein-1; PI 3-kinase, phosphatidylinositol 3-kinase; IP, immunoprecipitation; βar, β-arrestin; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; PLC, phospholipase C; RT, reverse transcription; siRNA, small interfering RNA.
TNF-R1 Can Function as a β-Arrestin-1-Gq-coupled Receptor

R&D Systems (Minneapolis, MN), Mouse TNFα was used in the experiments unless otherwise specified. Anti- Gqα11, antibody, Src-kinase inhibitor (PP2), PD98059, LY294002, and tocin-B were from Calbiochem. [γ-32P]GTPγ-sialodiimide was from Affinity Labeling Technologies, Inc. (Lexington, KY). RT-PCR primers were from Invitrogen, and the oligonucleotide sequences used were: matrix metalloproteinase 3 (MMP3) forward, 5′-TGACCCACTCATTCTCCTC-3′; MMP3 reverse, 5′-GCCTTGGCTGAGGTGATAG-3′; monocyte chemotactic protein-1 (MCP-1) forward, 5′-AGACCCAGGCAAC- TCTCAC-3′; MCP-1 reverse, 5′-TCTGAGACCACCTTCTC-7′; TNF-R1 forward, 5′-GACCGGGAAGAGGGTAGAT-3′; TNF-R1 reverse, 5′-CAGCGACTGGAAGTGTCTT-3′; TNF-R2 forward, 5′-AAATGCAACAGATGCAG-3′; TNF-R2 reverse, 5′-TCTGGATTTCTCCTCAG-3′. Cell culture materials and other radioisotopes were from ICN (Costa Mesa, CA). All other reagents were purchased from Sigma.

siRNA Transfection—3T3-L1 adipocytes (day 8 after differentiation) were transiently transfected by electroporation (GENE PULSER, Bio-Rad) with 2 nmol of siRNA duplex, as previously described (11). 24–120 h after electroporation, the efficiency of each siRNA was analyzed by RT-PCR or Western blotting every 24 h, and the time point for the maximum efficiency of each siRNA was determined. All experiments with siRNA were performed 48–96 h after electroporation or micro-injection. SiGENOME SMARTpool siRNA against TRAF2 and all other custom siRNAs were purchased from Dharmacon Research Inc. (Lafayette, CO). The target sequences of the siRNAs were: β-arrestin 1-A, 5′-GGCGCTGGTTGTGCTGAT- TAT-3′; β-arrestin 1-B, 5′-AGCTTCTTCTGCTGAGAC-3′; Gqα11-A, 5′-GCTGGGTGTATCAGAACATC-3′; Gqα11-B, 5′-TCCATATGATGCAAATA-3′; Gqα11-A, 5′-ACTCACACTTGGTGACATTA-3′; Gαi2-B, 5′-GGTGGGTGTCACAGGACATC-3′; cdc42, 5′-GTTATCCACAGACAGTGT-3′; TNF-R1, 5′-GATGCTCCTACAAAGAC-3′; TNF-R2, 5′-ACTCCA- AGATCCTTAC-3′. Each one of the paired siRNAs (A and B) was used separately, and this confirmed the absence of off-target effects. All sequences were confirmed to have no homology to any other genes by BLAST search (NCBI, National Institutes of Health).

Photoaffinity GTP-loading Assay—Photoaffinity GTP-labeling of membrane-associated G-proteins was performed as we described previously (12) with some modifications. Membrane fractions were semi-purified from scraped 3T3-L1 adipocytes (1 × 10⁸ cells) by Dounce homogenization and centrifugation (18,000 × g, 10 min) in a buffer containing 50 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM Na₂VO₄, 15 mM NaF, 1 mM phenylmethylsulfonyl fluoride and then immediately incubated with 10 μM [γ-32P]GTP-sialodiimide (12.2 mCi/μmol) for 3 min followed by UV irradiation (254 nm) for 1 min on ice. Samples were lysed with the addition of Nonidet P-40 (final concentrations, 1%), then immunoprecipitated with anti-Gqα, Gqα11, or Goi11, antibody. Immunoprecipitates were resolved by SDS-PAGE, and signals were quantitated by PhosphorImager (GE Healthcare).

Insoluble Phosphorus Production Assay—3T3-L1 adipocytes were incubated with [3H]inositol (ICN, Costa Mesa, CA) (2 μCi/ml) in inositol-free Dulbecco’s modified Eagle’s medium for 16 h before assay. After washing cells twice with serum-free Dulbecco’s modified Eagle’s medium, cells were stimulated with TNFα (20 ng/ml) for 15 min. The accumulation of total 3H-labeled (IP1 and IP2 plus IP3) was measured as previously described (13).

cdc42 Assay—cdc42 activity was measured according to the manufacturer’s instructions (Upstate Biotechnology, Inc., Lake Placid, NY). 3T3-L1 adipocytes were starved for 16 h and stimulated with 17 nm insulin or 20 ng/ml TNFα for the indicated time periods, washed once with ice-cold phosphate-buffered saline, and lysed with lysis buffer containing 25 mM HEPES (pH 7.5), 150 mM NaCl, 1% IGEPAL CA-630, 10 mM MgCl₂, 1 mM EDTA, 10% glycerol, 1 mM Na₂VO₄, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 25 mM NaF for 15 min at 4°C. Insoluble materials were removed by centrifugation. For a negative control cell lysate was incubated with 1 mM GDP for 15 min at 30°C. 5 μg of p21-activated kinase 1-agarose beads, which specifically bound to active cdc42, were added to the cell lysates and incubated for 1 h at 4°C. Agarose beads were washed with lysis buffer three times and boiled in Laemmli sample buffer. Samples were resolved by SDS-PAGE and immunoblotted with anti-cdc42 antibody.

Lipolysis Assay—Triglyceride hydrolysis (lipolysis) was measured using free glycerol reagent (Sigma) according to the manufacturer’s specifications. Briefly, 3T3-L1 adipocytes were washed twice and incubated with L-buffer (Dulbecco’s modified Eagle’s medium without phenol red plus 0.1% bovine serum albumin) then stimulated with or without TNFα (20 ng/ml) for 16 h. To measure lipolysis, 150 μl of free glycerol assay reagent was incubated with 10 μl of culture supernatants and glycerol standards for 15 min, and the absorbance was read at 540 nm. A standard curve constructed from the glycerol standards was used to calculate the concentration of glycerol in the culture supernatants.

Cell Culture and Other Methods—3T3-L1 adipocyte cell culture (8), immunoprecipitation with dithiobis(succinimidyl propionate) cross-linking (12), Src kinase assay (14), RT-PCR and PI 3-kinase assay (15), arachidonic acid release assay (16), and cyclic AMP assay (17) were performed as we previously described.
results in Src-kinase activation (18). To see if this was also the case for TNFα, we measured Src-kinase activity, and we detect that, like ET-1, TNFα acutely stimulated Src-kinase activity (2.5-fold above basal), as shown in Fig. 1B. Using RNA interference, electroporation with siRNA against β-arrestin-1 (βar1-A or βar1-B) decreased protein expression in 3T3-L1 adipocytes (Fig. 1C), and we found that acute stimulation of Src kinase activity with TNFα was inhibited by siRNA knockdown of β-arrestin-1 (Fig. 1D). These results suggest that TNFα-induced Src-kinase activation is dependent on β-arrestin-1, similar to 7-TMR signaling.

Umemori et al. (19) reported that the activation of Goq11 is coincident with phosphorylation of tyrosine residue 356 in the C terminus of Goq11. In response to ET-1 stimulation, we have previously reported that Src kinase plays a key role, leading to tyrosine phosphorylation of Goq11 (14). Accordingly, we examined the effects of TNFα on Goq11 and found that TNFα acutely induced tyrosine phosphorylation of Goq11 at 3 min after stimulation and that this effect was inhibited by siRNA-mediated β-arrestin-1 knockdown (Fig. 1E). Because two different siRNA against β-arrestin-1 (βar1-A or βar1-B in the upper panel) showed quite comparable results, this confirms that off-target effects do not contribute to our results. Furthermore, the Src-kinase inhibitor, PP2, also inhibited TNFα-induced Goq11 tyrosine phosphorylation (Fig. 1F), similar to its effects to block ET-1-stimulated phosphorylation (14). Interestingly, PP2 did not inhibit insulin-induced Goq11 phosphorylation, suggesting that the insulin receptor kinase itself can phosphorylate Goq11, independent of Src kinase activity. These results indicate that β-arrestin-1 mediates the effect of TNFα to phosphorylate Goq11 via the activation of Src-kinase.

TNFα Stimulates Goq11 Activity Without Leading to Phospholipase Cβ (PLC-β) Activation—To confirm the activation of Goq11, we performed a GTP-loading assay using photoaffinity GTP labeling method, as described under “Experimental Procedures.” As shown in Fig. 2A, TNFα stimulation increased the amount of GTP bound to Goq11 (3-fold) but had little effect on GTP loading of Goq or Gaq. Consistent with this, TNFα stimulation did not lead to changes in the cellular cyclic AMP level (Fig. 2B). It is generally thought that activated Goq11 stimulates PLC-β, leading to the induction of diacylglycerol, inositol phosphate-3, and subsequent arachidonic acid release. We measured the total amount of with siRNA against β-arrestin-1 (βar1-A or βar1-B) or scrambled sequence (Scr) as a control, then cells were serum-starved for 4 h and stimulated with or without TNFα (20 ng/ml) for 3 min. Cell lysates were immunoprecipitated with control IgG or anti-phosphotyrosine (PY20) antibody, and immunoprecipitates were analyzed by Western blotting with anti-Goq11 antibody. The result shown in the lower panel is a representative image from three independent experiments. The scanned bar graphs are shown as % maximum of basal condition (mean ± S.E. of three independent experiments). * represents statistical significance (p < 0.05; **, p < 0.01) in a two-tailed Student t test compared with basal conditions. 

FIGURE 1. TNFα stimulates Src-kinase activity to phosphorylate Goq11, via β-arrestin-1 in 3T3-L1 adipocytes. A, serum-starved 3T3-L1 adipocytes expressing either FLAG-tagged β-arrestin-1 (FL) or enhanced green fluorescent protein control (Ct) were stimulated with TNFα (20 ng/ml) for the indicated time periods. Cells were then washed twice with ice-cold phosphate-buffered saline and incubated with dithiobis(succinimidyl propionate) cross-linker for 1 h on ice, as described under “Experimental Procedures.” The resulting cell lysates were immunoprecipitated with control IgG or anti-TRAF2 antibody (Ab), and immunoprecipitation (IP) samples were analyzed by Western blotting with biotin-conjugated anti-FLAG antibody. The IP efficiency of TRAF2 antibody was the same in each lane. The result shown in the lower panel is a representative image from three independent experiments, and the scanned bar graph (upper panel) shows % maximum of the basal condition (mean ± S.E. of three independent experiments). WCL, whole cell lysates from cells expressing FLAG-tagged β-arrestin-1. Asterisk, statistical significance (*, p < 0.05; **, p < 0.01) in a two-tailed Student t test compared with basal conditions. B, serum-starved 3T3-L1 adipocytes were stimulated with insulin (100 ng/ml), ET-1 (10 nM), or TNFα (20, or 100 ng/ml) for 3 min. Cell lysates were immunoprecipitated with anti-pan Src antibody, and Src-kinase activity in the IP samples was assayed for the ability to phosphorylate the Src-kinase substrate peptide, as described under “Experimental Procedures.” Data are presented as the mean ± S.E. of three independent experiments. C, differentiated 3T3-L1 adipocytes were electroporated with specific siRNA against β-arrestin-1 (βar1-A or βar1-B) or a scrambled sequence (Scr), 24–120 h after electroporation, protein expression of β-arrestin-1 was analyzed by Western blotting every 24 h, and the maximum efficiency of RNA was observed during 24–96 h after the transfection. The images shown are representative results from three independent experiments. The scanned bar graphs are shown as % maximum of control (Scr) siRNA-transduced cells (mean ± S.E. of three independent experiments). D, 3T3-L1 adipocytes transduced with siRNA against β-arrestin-1 (βar1) were serum-starved for 4 h then stimulated with TNFα for 3 min. Src kinase activity was measured as described above. Data are represented as % maximum of scrambled (Scr) siRNA-transfected cells (mean ± S.E. of three independent experiments). E, 3T3-L1 adipocytes were incubated for 96 h after transfection...
TNF-R1 Can Function as a β-Arrestin-1-Gq-coupled Receptor

FIGURE 2. TNFα stimulates Gαq/11 activity without PLC-β activation in 3T3-L1 adipocytes. A, after photomixing labeling with [γ-32P]GTP-azidoanilide, solubilized membranes were immunoprecipitated with anti-Gαq/11 or Gαi antibody. Immunoprecipitates (IP) were analyzed by SDS-PAGE and Phosphorimage, as described under “Experimental Procedures.” Data were quantitated by Phosphorimage and represent the mean ± S.E. of three independent experiments (upper panel). A representative image is shown in lower panel. B, serum-starved 3T3-L1 adipocytes were stimulated with either TNFα (20 ng/ml), isoprotenerol (ISO, 10 μM), ET-1 (10 nM), or insulin (100 ng/ml) for 15 min. Intracellular cyclic AMP levels were normalized with cellular protein concentrations. Data are presented as -fold over basal (mean ± S.E. of three independent experiments). C, 3T3-L1 adipocytes were incubated with [3H]inositol (left panel) or [3H]arachidonic acid (right panel) for 16 h, then washed and stimulated with either TNFα (20 ng/ml), ET-1 (10 nM), or insulin (100 ng/ml) for 15 min. [3H]levels in the samples were quantitated and normalized for cellular protein concentration, as described in under “Experimental Procedures.” Data were quantitated by Phosphorimage and represent the mean ± S.E. of three independent experiments. D, differentiated 3T3-L1 adipocytes were serum-starved for 4 h and stimulated with TNFα (20 ng/ml) or ET-1 (10 nM) for the indicated time periods. Cell lysates were immunoprecipitated with control (Ctrl) IgG, anti-ETAR, or TNF-R1 antibody, and IP samples were analyzed by Western blotting with anti-PLC-β antibody. The IP efficiency of anti-TNF-R1 or ETAR antibody was the same in each lane (data not shown). The representative image is from two independent experiments. WCL, whole cell lysates.

To confirm these signaling events, we tested the effect of kinase inhibitors on TNFα signaling. Consistent with the above results, TNFα-stimulated JNK activation was inhibited by pretreatment of the cells with the Src inhibitor (PP2), the PI 3-kinase inhibitor (LY294002, LY), or the cdc42 inhibitor (toxin-B, TB) but not by MEK inhibitor (PD98059, PD) (Fig. 4A). Furthermore, we confirmed that TNFα stimulates cdc42 activity (Fig. 4B) and cdc42-associated PI 3-kinase activity (Fig. 4C), and these effects were inhibited by PP2 treatment (*, p < 0.01) (Fig. 4, B and C). Taken together, these data indicated that TNFα signaling goes through the TNF-R1-TRAF2 → β-arrestin-1-Src-kinase → Gαq/11 → cdc42/PI 3-kinase pathway to activate JNK, a proinflammatory pathway in 3T3-L1 adipocytes.

It has been reported that TNFα-induced ERK activation was regulated by Src kinase (27), although the full mechanisms of ERK activation remain unclear. In 3T3-L1 adipocytes TNFα-induced ERK phosphorylation was maximal at 15 min after

first focused on JNK, a proinflammatory pathway mediator, since the mechanism of JNK activation is still unclear. Using RNA interference-induced knockdown of TNF-R1, TNF-R2, or TRAF2 (Fig. 3, A and B, left panel), we found that TNFα (20 ng/ml, 8 min) stimulated JNK phosphorylation and that this effect was strongly inhibited by TNF-R1 or TRAF2 knockdown but not by TNF-R2 knockdown (Fig. 3C), consistent with the view that a TNF-R1-TRAF2 complex mediates TNFα signaling to JNK activation. It has been reported previously that activation of JNK is mediated by cdc42 (22) and PI 3-kinase (23) and that TNFα can stimulate cdc42 (24) and PI 3-kinase activities (25), although the mechanisms for activation of these kinases are unclear. Because activated Gαq/11 can lead to stimulation of cdc42 and cdc42-associated PI 3-kinase (26), we assessed this pathway with respect to TNFα stimulation. Using RNA interference showing in Figs. 1C and 3B, we found that TNFα-stimulated JNK phosphorylation was inhibited by β-arrestin-1, Gαq/11, or cdc42 knockdown in 3T3-L1 adipocytes (Fig. 3D). Two different siRNAs against β-arrestin-1 (β1-A in Fig. 3D, left panels, and β1-B in right-side panels) or Gαq/11 (Gαq/11, Gαq/11, Gαq/11, Gαq/11-A in Fig. 3D, left panels, and Gαq/11 + Gαq/11-B in right side panels) showed quite similar results, indicating that there were no off-target effects in these siRNAs.

Biological Roles of β-Arrestin-1-Gαq/11 as Signaling Mediators for TNFα Action—To investigate which biological effects of TNFα are mediated by β-arrestin-1/Src kinase and Gαq/11, we

inositol phosphates (Fig. 2C, left panel) and arachidonic acid release (Fig. 2C, right panel) in 3T3-L1 adipocytes and found that TNFα stimulation interestingly did not increase either of these metabolites, suggesting that TNFα does not activate PLC-β. This is also observed in insulin receptor signaling (Fig. 2C), in which Gαq/11 is activated (8), and importantly, both receptors do not have a 7-TM type structure. Several studies have revealed that the second and/or third intracellular loops of 7-TM receptors are required for PLC-β activation (20, 21), and these motifs are not present in the insulin and TNF receptors. Therefore, we hypothesized that non-7-TM type receptor cannot associate with PLC-β. We tested the co-immunoprecipitation with receptor antibody and found no co-immunoprecipitation of PLC-β3 (the main isofrom of the PLC-β family in 3T3-L1 adipocytes) with anti-TNF-R1 antibody, although PLC-β3 was modestly co-immunoprecipitated with anti-ETAR-Ab after ET-1 treatment (Fig. 2D).

Biological Roles of β-Arrestin-1-Gαq/11 as Signaling Mediators for TNFα Action—To investigate which biological effects of TNFα are mediated by β-arrestin-1/Src kinase and Gαq/11, we
TNF-R1 Can Function as a β-Arrestin-1-Gq-coupled Receptor

In other systems we (29) and others (30) have shown that β-arrestin-1 is an upstream regulator of ERK activity, and recent reports by Kang et al. (31) revealed that β-arrestin-1 can be translocated to the nucleus after ligand stimulation, where it participates in regulation of gene expression. Accordingly, we measured mRNA expression levels of two TNFα target genes, MMP3 and MCP-1, and found that expression of these genes was strongly stimulated by TNFα treatment (Fig. 5C). Because TNFα stimulation of both ERK and JNK1 appears to be β-arrestin-1-dependent and because both ERK and JNK1 can regulate gene expression, we investigated the effects of β-arrestin-1 knockdown and found that TNFα-mediated MCP-1 and MMP3 expression was inhibited by depletion of β-arrestin-1 (*, p < 0.03).

Another biologic effect of TNFα in adipocytes is stimulation of lipolysis. We found a role for β-arrestin-1 signaling in this process by showing that TNFα-induced lipolysis was partially inhibited by β-arrestin-1 knockdown (*, p < 0.05) but not by Gαq/11 knockdown, as seen in Fig. 5D, left panel. Because β-arrestin-1 mediates TNFα-induced ERK activation, we treated the cells with the MEK inhibitor PD98059 and found that this also partially inhibited TNFα-induced lipolysis (*, p < 0.05) (Fig. 5D, right panel), consistent with a previous report (32).

DISCUSSION

TNFα stimulation induces a wide range of biologic effects related to innate immunity, inflammation, insulin resistance/diabetes, and other conditions. Chronic inflammation is increasingly recognized as a major cause of decreased insulin sensitivity. By activating pro-inflammatory pathways (33), TNFα can cause insulin resistance in vitro (3) and in animals treated with TNFα (4). The molecular mechanisms of TNFα action have been extensively studied, and many elements of this pathway have been defined (2). In this study we have identified new elements of a novel TNFα signaling pathway in adipocytes, which flows through a TNF-R1 signaling pathway that includes the use of the classical 7-TMR signaling components, β-arrestin-1, and Gαq/11. We found that β-arrestin-1 can mediate TNFα effects on Src kinase and ERK activation and that Gαq/11 can couple TNFα action to cdc42, PI 3-kinase, and JNK activation, leading to...
TNF-R1 Can Function as a β-Arrestin-1-Gαq-coupled Receptor

It is well known that β-arrestin-1 plays several roles in 7-TMR function, including receptor desensitization and internalization (34). Interestingly, it has also been reported that β-arrestin-1 interacts with non-7-TM type receptors, such as the insulin-like growth factor-I, epidermal growth factor, and insulin receptors (35), and here we show that the TNF-R1-TRAF2 complex can signal through β-arrestin-1 to mediate TNFα biological effects in 3T3-L1 adipocytes. As such, these studies further demonstrate the diverse functions of β-arrestin-1 as an adaptor protein mediating the signaling properties of receptor systems distinct from its classical role in 7-TMR action. Our studies also show that the G-protein component, Goq/11, can also function in the TNFα signaling pathway. To accomplish this task, TNFα signaling uses β-arrestin-1 effects to activate Src kinase, causing Goq/11 tyrosine phosphorylation and activation. Together these effects can mediate downstream signaling events such as activation of ERK, PI 3-kinase, and lipidosis.

We investigated the downstream TNFα effects transduced by β-arrestin-1 and/or Goq/11, and one of them is JNK activation. It is well known that JNK can propagate the effects of TNFα to induce proinflammatory gene expression, and this can result in insulin resistance. Previous studies have shown that TNFα-induced JNK activation is mediated by TRAF2 (36),cdc42 (22), Src (37), and PI 3-kinase (38). There may be multiple ways in which TNFα can cause JNK activation, and consistent with these reports, our findings show that TNFα can signal through β-arrestin-1-Src-kinase to activate JNK. Similarly, previous studies revealed multiple mediators for TNFα-induced ERK activation, including TRAF2, MADD, and Src kinase (27, 39). Ligand stimulation can induce diverse signaling pathways leading to ERK activation; e.g. insulin-like growth factor-I receptor can signal through β-arrestin-1-Gαq, as well as SHC to activate ERK (30, 40). Consistent with this formulation, siRNA-induced β-arrestin-1 knockdown did not completely inhibit TNFα-induced ERK phosphorylation (Fig. 5B), indicating the presence of another pathway(s) from the TNF receptor to this biologic end point. Because NFκB pathway may also be involved in lipolytic effects (41), this may also explain why β-arrestin-1 knockdown only partially inhibits TNFα-stimulated lipolysis (Fig. 5D).

Previous studies have shown that β-arrestin-1 is an upstream mediator of ERK activation in various receptor signaling systems (42) and that β-arrestin-1 can translocate into the nucleus to exert transcriptional effects (31). In addition, activated JNK the induction of lipolysis and proinflammatory gene expression in 3T3-L1 adipocytes.

FIGURE 4. TNF-R1 signaling activates JNK through cdc42/PI 3-kinase pathway. A, serum-starved 3T3-L1 adipocytes were pretreated with LY294002 (LY, 10 μM, 30 min), toxin-B (TB, 50 ng/ml, 2 h), PP2 (400 nM, 30 min), PD98059 (PD, 25 μM, 30 min), or Me2SO (DMSO) vehicle for 30 min, then stimulated with or without TNFα (20 ng/ml, 8 min). Cell lysates were analyzed, and the results were shown as described in Fig. 3A. The scanned bar graphs represent the mean ± S.E. of three independent experiments. Asterisk, statistical significance (p < 0.01) in a two-tailed Student t test comparing with control (Me2SO) treatment. B, cdc42 activity was measured by the association with GST-p21-activated kinase 1 beads, which specifically recognize activated cdc42. Serum-starved 3T3-L1 adipocytes were pretreated with or without PP2 (400 nM, 30 min) and stimulated with TNFα (20 ng/ml) for 3 min. Cell lysates were incubated with GST-p21-activated kinase 1 beads, and the precipitates were analyzed by Western blotting with anti-cdc42 antibody (400 nM, 30 min) for 3 min. PI 3-kinase activity in the immunoprecipitates measured as described under “Experimental Procedures.” Data were quantitated by PhosphorImager and represent the mean ± S.E. of three independent experiments. Asterisk, statistical significance (p < 0.01) versus control in a two-tailed Student t test.
TNF-R1 Can Function as a β-Arrestin-1-Gq-coupled Receptor

FIGURE 5. Effects of TNFα on ERK, lipolysis, and gene expression are β-arrestin-1-dependent. A, 48 h after transduction with specific siRNA against TNF-R1 (R1), TNF-R2 (R2), TRAF2, or scrambled (Scr) sequence, serum-starved 3T3-L1 adipocytes (day 10) were stimulated with mouse TNFα (20 ng/ml), human TNFα (20 ng/ml), or ET-1 (10 μM) for 15 min, and cell lysates were analyzed by Western blotting with anti-ERK (middle panel) or phospho (P)-specific ERK antibody (top panel). The scanned bar graphs (bottom panel) are shown as % maximum of scrambled (Scr) siRNA-transduced cells (mean ± S.E. of three independent experiments). B, 3T3-L1 cells transduced with specific siRNAs against β-arrestin-1 (bar1-A), Gq_11-A, or scrambled (Scr) sequence were pretreated with pertussis toxin (PTX, 10 ng/ml, 4 h) (right panel) or phospho (P)-specific ERK antibody (left panel). The scanned bar graphs (bottom panel) are shown as % maximum of scrambled (Scr) siRNA-transduced cells (mean ± S.E. of three independent experiments). C, total RNA samples were purified from serum-starved 3T3-L1 adipocytes stimulated with or without mouse TNFα (20 ng/ml) for 2 or 4 h after transduction with siRNA against β-arrestin-1 (bar1-A) or scrambled (Scr) sequence. The expression levels of MMP3, MCP-1, and β-actin were analyzed by RT-PCR. Data were adjusted to the expression level of β-actin as an internal control and are shown as % maximum control (mean ± S.E. of four independent experiments). D, 3T3-L1 adipocytes transduced with siRNA as shown above (B) or pretreated with PD98059 (PD, 25 μM, 30 min) or Me2SO (DMSO) vehicle were stimulated with mouse TNFα (20 ng/ml) for 15 h. Glycerol concentration was measured in the resulting culture supernatant, normalized to cellular protein concentration. Data represented the mean ± S.E. of three or five independent experiments. Asterisk, statistical significance versus control in a two-tailed Student t test.

stimulates c-Jun, which translocates into the nucleus as a transcription factor activating pro-inflammatory gene expression (43). These findings suggested that β-arrestin-1 might play a role in TNFα-induced gene expression. Consistent with this, our results show that siRNA-knockdown of β-arrestin-1 exerts inhibitory effects on TNFα-stimulated MCP-1 and MMP3 gene expression.

TNFα stimulation induces a wide range of biologic effects related to innate immunity, inflammation, insulin resistance/diabetes, and other conditions. Chronic inflammation is increasingly recognized as a major cause of decreased insulin sensitivity, and by activating proinflammatory pathways (33), TNFα can cause insulin resistance in vitro (3) and in animals treated with TNFα (4). Last, it is well known that elevated circulating free fatty acid levels can be a cause of insulin resistance (44). In this study we have identified new elements in TNFα signaling pathways in adipocytes, which can lead to inflammatory pathway activation and lipolysis (Fig. 6). This pathway flows through a TNF-R1-TRAF2 receptor complex and includes the use of classical 7-TMR signaling components, β-arrestin-1 and Gq11, showing a novel signaling paradigm for these molecules distinct from their traditional functions mediating signals from an array of 7-TMRs.

Acknowledgment—We thank Elizabeth J. Hansen for editorial assistance.

REFERENCES

1. Barnes, P. J., and Karin, M. (1997) N. Engl. J. Med. 336, 1066–1071
2. Chen, G., and Goeddel, D. V. (2002) Science 296, 1634–1635
3. Hotamisligil, G. S., Murray, D. L., Choy, L. N., and Spiegelman, B. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4854–4858
4. Miles, P. D., Romeo, O. M., Higo, K., Cohen, A., Rafaat, K., and Olefsky, J. M. (1997) Diabetes 46, 1678–1683
TNF-R1 Can Function as a β-Arrestin-1-G_q-coupled Receptor

5. Feng, X., Gaeta, M. L., Madge, L. A., Yang, J. H., Bradley, J. R., and Poiber, J. S. (2001) J. Biol. Chem. 276, 8341–8349
6. Doan, J. E., Windmiller, D. A., and Riches, D. W. (2004) J. Immunol. 172, 7654–7660
7. Gustavsson, J., Parpali, S., Karlsson, M., Ramsing, C., Thorn, B., Borg, M., Lindroth, M., Petersson, K. H., Magnusson, K. E., and Stålfors, P. (1999) FASEB J. 13, 1961–1971
8. Imamura, T., Vollenweider, P., Egawa, K., Clodi, M., Ishibashi, K., Nakashima, N., Ugi, S., Adams, J. W., Brown, J. H., and Olefsky, J. M. (1999) Mol. Cell. Biol. 19, 6765–6774
9. Ishibashi, K., Imamura, T., Sharma, P. M., Huang, J., Ugi, S., and Olefsky, J. M. (2001) J. Clin. Investig. 107, 1193–1202
10. Usui, I., Imamura, T., Babendure, J. L., Satoh, H., Lu, J. C., Hupfeld, C. J., and Olefsky, J. M. (2005) Mol. Endocrinol. 19, 2760–2768
11. Liao, W., Nguyen, M. T., Imamura, T., Singer, O., Verma, I. M., and Olefsky, J. M. (2006) Endocrinology 147, 2245–2252
12. Imamura, T., Huang, J., Usui, I., Satoh, H., Beverly, J., and Olefsky, J. M. (2003) Mol. Cell. Biol. 23, 4892–4900
13. Shimizu, N., Guo, J., and Gardella, T. J. (2001) J. Biol. Chem. 276, 49003–49012
14. Imamura, T., Huang, J., Dalle, S., Ugi, S., Usui, I., Luttrell, L. M., Miller, W. E., Lefkowitz, R. J., and Olefsky, J. M. (2001) J. Biol. Chem. 276, 43663–43667
15. Usui, I., Imamura, T., Satoh, H., Huang, J., Babendure, I. L., Hupfeld, C. J., and Olefsky, J. M. (2004) EMBO J. 23, 2821–2829
16. Hinuma, S., Habata, Y., Fujii, R., Kawanaga, Y., Hosoya, M., Fukusumi, S., Kitada, C., Masuo, Y., Asano, T., Matsumoto, H., Sekiguchi, M., Kurokawa, T., Nishimura, O., Onda, H., and Fujino, M. (1998) Nature 393, 272–276
17. Hupfeld, C. J., Dalle, S., and Olefsky, J. M. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 161–166
18. Miller, W. E., Maudsley, S., Ahn, S., Khan, K. D., Luttrell, L. M., and Lefkowitz, R. J. (2000) J. Biol. Chem. 275, 11312–11319
19. Umemori, H., Inoue, T., Kume, S., Sekiyama, N., Nagao, M., Itoh, H., Nakaniishi, S., Mikoshita, K., and Yamamoto, T. (1997) Science 276, 1878–1881
20. Wang, H. L. (1997) J. Neurochem. 68, 1728–1735
21. Thompson, J. B., Wade, S. M., Harrison, J. K., Salafranca, M. N., and Neubig, R. R. (1998) J. Pharmacol. Exp. Ther. 285, 216–222
22. Coso, O. A., Chiarriello, M., Yu, J. C., Teramoto, H., Crespo, P., Xu, N., Miki, T., and Gutkind, J. S. (1995) Cell 81, 1137–1146
23. Lopez-Ilasaca, M., Li, W., Uren, A., Yu, J. C., Kazlauskas, A., Gutkind, J. S., and Heidaran, M. A. (1997) Biochem. Biophys. Res. Commun. 232, 273–277
24. Puls, A., Eliopoulos, A. G., Nobes, C. D., Bridges, T., Young, L. S., and Hall, A. (1999) J. Cell Sci. 112, 2983–2992
25. Reddy, S. A., Huang, J. H., and Liao, W. S. (2000) J. Immunol. 164, 1355–1363
26. Usui, I., Imamura, T., Huang, J., Satoh, H., and Olefsky, J. M. (2003) J. Biol. Chem. 278, 13765–13774
27. van Vliet, C., Bukczynska, P. E., Puryer, M. A., Sadek, C. M., Shields, B. J., Tremblay, M. L., and Tiganis, T. (2005) Nat. Immunol. 6, 253–260
28. Lewis, M., Tartaglia, L. A., Lee, A., Bennett, G. L., Rice, G. C., Wong, G. H., Chen, E. Y., and Goeddel, D. V. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 2830–2834
29. Dalle, S., Imamura, T., Rose, D. W., Worrall, D. S., Ugi, S., Hupfeld, C. J., and Olefsky, J. M. (2002) Mol. Cell. Biol. 22, 6272–6285
30. Luttrell, L. M., Roudabush, F. L., Choy, E. W., Miller, W. E., Field, M. E., Pierce, K. L., and Lefkowitz, R. J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 2449–2454
31. Kang, J., Shi, Y., Xiang, B., Qu, B., Su, W., Zhu, M., Bao, G., Wang, F., Zhang, X., Yang, R., Fan, F., Chen, X., Pei, G., and Ma, L. (2005) Cell 123, 833–847
32. Greenberg, A. S., Shen, W. J., Muliro, K., Patel, S., Souza, S. C., Roth, R. A., and Kraemer, F. B. (2001) J. Biol. Chem. 276, 45456–45461
33. Xu, H., Barnes, G. T., Yang, Q., Tan, G., Yang, D., Chou, C. J., Soley, I., Nichols, A., Ross, J. S., Tartaglia, L. A., and Chen, H. (2003) J. Clin. Investig. 112, 1821–1830
34. Lefkowitz, R. J., Rajagopal, K., and Whalen, E. J. (2006) Mol. Cell 24, 643–652
35. Dalle, S., Ricketts, W., Imamura, T., Vollenweider, P., and Olefsky, J. M. (2001) J. Biol. Chem. 276, 15688–15695
36. Natoli, G., Costanzo, A., Ianni, A., Templeton, D. J., Woodgett, J. R., Balsano, C., and Leverero, M. (1997) Science 275, 200–203
37. Tateno, M., Nishida, Y., and Adachi-Yamada, T. (2000) Science 287, 324–327
38. Timokhina, I., Kissel, H., Stella, G., and Besmer, P. (1998) EMBO J. 17, 6250–6262
39. Schievella, A. R., Chen, J. H., Graham, J. R., and Lin, L. L. (1997) J. Biol. Chem. 272, 12069–12075
40. Roudabush, F. L., Pierce, K. L., Maudsley, S., Khan, K. D., and Luttrell, L. M. (2000) J. Biol. Chem. 275, 22583–22589
41. Laurencikiene, J., van Harmelen, V., Arvidsson Nordstrom, E., Dicker, A., Blomqvist, L., Naslund, E., Langin, D., Arner, P., and Ryden, M. (2007) J. Lipid Res. 48, 1069–1077
42. Lefkowitz, R. J., and Shenoy, S. K. (2005) Science 308, 512–517
43. Sluss, H. K., Barrett, T., Derijard, B., and Davis, R. J. (1994) Mol. Cell. Biol. 14, 8376–8384
44. Groop, L. C., Bonadonna, R. C., DelPrato, S., Ratheiser, K., Zyczk, K., Ferrannini, E., and DeFronzo, R. A. (1989) J. Clin. Investig. 84, 205–213