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Amyloid Precursor Protein Expression Is induced by Tumor Necrosis Factor α in 3T3-L1 Adipocytes

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ABSTRACT

Amyloid precursor protein (APP) has been characterized as an adipocyte-secreted protein that might contribute to obesity-related insulin resistance, inflammation, and dementia. In the current study, regulation of APP by the proinflammatory and insulin resistance-inducing cytokine tumor necrosis factor (TNF) α was determined in 3T3-L1 adipocytes. Interestingly, APP protein synthesis and mRNA expression were significantly increased by TNFα in a time-dependent manner with maximal induction observed after 24 h of treatment. Furthermore, TNFα induced APP mRNA expression dose-dependently with maximal 6.4-fold upregulation seen at 100 ng/ml effector. Moreover, inhibitor experiments suggested that TNFα-induced APP expression was mediated by nuclear factor κ B. Taken together, we show for the first time a potent upregulation of APP by TNFα suggesting a potential role of this adipocyte-secreted protein in TNFα-induced insulin resistance and inflammatory disease. J. Cell. Biochem. 108: 1418–1422, 2009.

KEY WORDS: 3T3-L1 ADIPOCYTE; ADIPOKINE; AMYLOID PRECURSOR PROTEIN; TNFα

Adipocyte-derived factors, so-called adipokines, have been proposed to link insulin resistance, obesity, and chronic inflammation. Among those, adiponectin is an insulin-sensitizing and anti-atherogenic fat-secreted factor [Yamauchi et al., 2001; Ouchi et al., 2003]. Furthermore, tumor necrosis factor (TNF) α, interleukin (IL)-1β, and IL-6 have been suggested to induce insulin resistance [Hotamisligil et al., 1993; Fasshauer and Paschke, 2003; Lagathu et al., 2006].

Most recently, amyloid precursor protein (APP) has been introduced as a novel proinflammatory adipokine which might link obesity, insulin resistance, inflammation, and dementia [Lee et al., 2008]. It is interesting to note in this context that adiposity is associated not only with insulin resistance, hypertension, and inflammation but also with the risk for dementia and Alzheimer's disease (AD) [Gorospe and Dave, 2007]. Thus, a Swedish representative 70-year-old female cohort showed an increased risk for dementia by 36% with every increase of the body mass index (BMI) by 1 kg/m^2 [Gustafson et al., 2003]. Furthermore, a long-time study of Whitmer et al. [2005] demonstrated that obesity is a risk factor for AD in both men and women. Moreover, AD patients show larger waist circumferences, higher triglyceride, and glucose concentrations, as well as lower HDL-cholesterol levels, compared to control individuals [Razay et al., 2007]. Adipose tissue processing of APP is a potential link between increased body weight and AD. Thus, APP and its fragments amyloid $\beta_{1\text{-}40}$ and amyloid $\beta_{1\text{-}42}$ are expressed in fat tissue [Lee et al., 2008]. Furthermore, Lee et al. [2008] were the first to demonstrate that APP is overexpressed in subcutaneous abdominal adipocytes from obese patients. Moreover, APP synthesis was independently correlated to indices of insulin resistance in vivo and with expression of proinflammatory genes including monocyte chemoattractant protein-1, macrophage inflammatory protein-1α, and IL-6. It is interesting to note in this context that APP cross-linking induced the release of the proinflammatory cytokines IL-1β, IL-6, and IL-8 in monocytic lineage cells [Sondag and Combs, 2006]. In addition, it has long been known that insulin, amyloid $\beta_{1\text{-}40,}$ and amyloid $\beta_{1\text{-}42}$ directly compete for insulin-degrading enzyme [Qiu et al., 1998] and binding to the insulin receptor (IR) [Xie et al., 2002].

TNFα has been established as an adipokine which impairs insulin sensitivity and induces proinflammatory gene expression in various

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tissues [Hotamisligil et al., 1993]. However, the effect of this proinflammatory cytokine on expression of APP in adipocytes has not been elucidated so far. Therefore, we determined regulation of APP by TNFα in fat cells in the current study.

MATERIALS AND METHODS

MATERIALS

Cell culture reagents were obtained from PAA (Pasching, Austria), oligonucleotides from MWG-Biotech (Ebersberg, Germany). Dexamethasone, insulin, isobutylmethylxanthine, and TNFα were purchased from Sigma Aldrich (Steinheim, Germany). LY294002, MG132, parthenolide, PD98059, and SB203580 were from Calbiochem (Bad Soden, Germany). The IC_{50} value of parthenolide for NFκB-driven transcription in THP-1 and HEK293 cells is $9.76 \pm 0.83\,\mu M$ [Dell'Agli et al., 2009]. Parthenolide inhibits a common step in NFκB activation by preventing the TNFα-mediated induction of I kappa B kinase (IKK) and IKK β [Hehner et al., 1999]. In contrast, MG132 is a cell-permeable proteasome inhibitor that blocks the processing of the p105 precursor of the p50 subunit of NFκB [Palombella et al., 1994].

CULTURE AND DIFFERENTIATION OF 3T3-L1 CELLS

3T3-L1 cells (American Type Culture Collection, Rockville, MD) were differentiated into adipocytes as described [Fasshauer et al., 2001b]. In brief, preadipocytes were grown to confluence in DMEM containing 25 mM glucose (DMEM-H), 10% fetal bovine serum, and antibiotics (culture medium). After this period, cells were induced for 3 days in culture medium further supplemented with 1 μM insulin, 0.5 mM isobutylmethylxanthine, and 0.1 μM dexamethasone. Subsequently, they were grown for 3 days in culture medium with 1 μM insulin and for additional three to six more days in culture medium. Various effectors were added to cells starved in DMEM-H only for the indicated periods of time. At the time of the stimulation experiments, at least 95% of the cells had accumulated fat droplets.

ANALYSIS OF APP PROTEIN EXPRESSION BY WESTERN BLOTTING

Detection of APP protein synthesis was performed essentially as described previously [Fasshauer et al., 2001a]. Briefly, after indicated stimulation periods, cells were harvested in lysis buffer (50 mM HEPES, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM $Na_4P_2O_7$, 10 mM NaF, 2 mM EDTA, 10% glycerol, 1% igepal CA-630, 2 mM vanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 2 mM phenylmethylsulfonyl fluoride, pH 7.4). Lysates were clarified and 100 μg total protein was solubilized directly in Laemmli sample buffer. Proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, blocked for 20 min, and immunoblotted with a rabbit APP antibody directed against the C-terminus of the protein (Sigma–Aldrich, Taufkirchen, Germany, Cat. number A8717) overnight. Specifically bound primary antibodies were detected with peroxidase-coupled secondary antibody and enhanced chemiluminescence. One APP band at approximately 100 kDa was observed in accordance with the data provided by the manufacturer. β-Actin was used as a loading control and detected with an antibody from Sigma–Aldrich (Cat. number A2066).

ANALYSIS OF APP mRNA EXPRESSION

APP mRNA was quantified by quantitative real-time polymerase chain reaction (qPCR) in a fluorescent temperature cycler (ABI Prism 7000, Applied Biosystems, Darmstadt, Germany) as described previously [Fasshauer et al., 2001b]. Briefly, total RNA was isolated from 3T3-L1 adipocytes with TRIzol reagent (Invitrogen, Life Technologies, Inc., Carlsbad, CA) and 1 μg RNA was reverse transcribed using standard reagents (Invitrogen, Life Technologies, Inc.). Two microliter of each reverse transcription reaction was amplified in a PCR with a total volume of 26 μl. After initial denaturation at 95°C for 10 min, 40 PCR cycles were performed using the following conditions: 95°C for 15 s, 60°C for 1 min, and 72°C for 1 min. The following primer pairs were used: APP (accession no. NM_007471.2), 5′-CAAAGGCGCCATCATCGGACTC-AT-3′ (sense) and 5′-CTGTGGCGCGGGTGGGGCTTAG-3′ (antisense); 36B4 (accession no. NM_007475) 5′-AAGCGCGTCCTGGCATTG-TCT-3′ (sense) and 5′-CCGCAGGGGCAGCAGTGGT-3′ (antisense). SYBR Green I fluorescence emissions were monitored after each cycle and synthesis of APP and 36B4 mRNA was quantified using the second derivative maximum method of the ABI Prism 7000 software (Applied Biosystems). In brief, crossing points of individual samples were determined by an algorithm identifying the first turning point of the fluorescence curve. 36B4 was used as internal control due to its resistance to hormonal regulation [Laborda, 1991], and APP expression was calculated relative to 36B4. Specific transcripts were confirmed by melting curve profiles (cooling the sample to 68°C and heating slowly to 95°C with measurement of fluorescence) at the end of each PCR and the specificity of the PCR was further verified by subjecting the amplification products to agarose gel electrophoresis.

STATISTICAL ANALYSIS

Results are shown as mean ± SE. Differences between various treatments were analyzed by Mann–Whitney *U*-tests with *P* values <0.05 considered significant.

RESULTS

TNFα INDUCES APP PROTEIN SYNTHESIS

APP protein expression was quantified in differentiated 3T3-L1 cells in the presence and absence of 20 ng/ml TNFα. Interestingly, TNFα induced APP protein synthesis significantly after 8 and 24 h of treatment as compared to control conditions (Fig. 1).

APP mRNA EXPRESSION IS INDUCED TIME- AND DOSE-DEPENDENTLY BY TNFα IN 3T3-L1 ADIPOCYTES

Treatment with 20 ng/ml TNFα increased APP mRNA expression in a time-dependent manner (Fig. 2). Thus, significant 1.5-fold up-regulation of APP mRNA was seen after 4 h of TNFα stimulation ($P < 0.05$) and maximal 4.1-fold stimulation after 24 h ($P < 0.05$) (Fig. 2).

Furthermore, TNFα induced APP synthesis in a dose-dependent manner after 16 h of treatment (Fig. 3). Here, significant 1.8-fold stimulation was observed at TNFα concentrations as low as 0.33 ng/

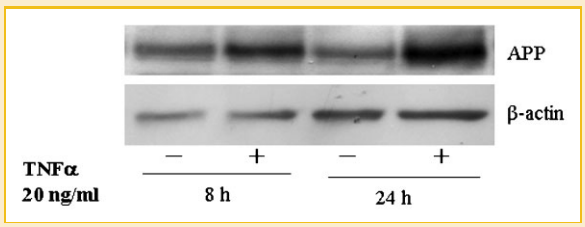

Fig. 1. TNFα stimulates APP protein synthesis. Fully differentiated 3T3-L1 adipocytes were serum-deprived overnight before TNFα (20 ng/ml) was added for the indicated periods of time. Total protein was isolated and immunoblotted as described in Materials and Methods Section. β-Actin expression was used as a loading control. A representative blot of four independent experiments is shown.

ml ($P < 0.05$), and maximal 6.4-fold ($P < 0.05$) upregulation was seen at 100 ng/ml effector (Fig. 3).

NFκB MEDIATES THE EFFECT OF TNFα ON APP EXPRESSION

We elucidated which signaling molecules implicated in TNFα signaling might mediate the positive effect of TNFα on APP expression. To this end, 3T3-L1 cells were treated with specific pharmacological inhibitors for nuclear factor κ B (NFκB) (parthenolide, 50 μM), p44/42 mitogen-activated protein (MAP) kinase (PD98059, 50 μM), p38 MAP kinase (SB203580, 20 μM), and phosphatidylinositol (PI) 3-kinase (LY294002, 10 μM) before TNFα (20 ng/ml) was added. Treatment of 3T3-L1 adipocytes with each of the four inhibitors for 17 h had no significant effect on basal APP expression (Fig. 4A). Again, APP expression was increased nearly threefold after 16 h of TNFα treatment ($P < 0.05$) (Fig. 4A). This induction was completely reversed to 25% of control levels in cells pretreated with the pharmacological inhibitor of NFκB ($P < 0.05$)

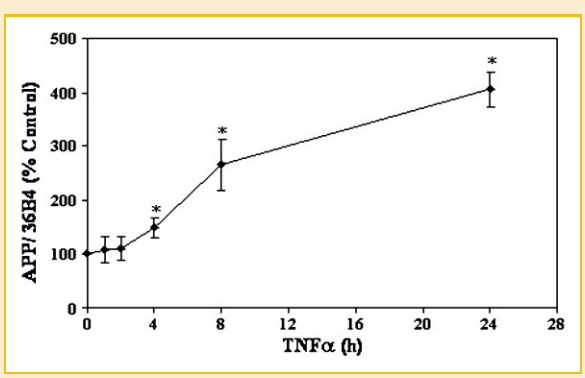

Fig. 2. TNFα stimulates APP mRNA expression time-dependently. Fully differentiated 3T3-L1 adipocytes were serum-deprived overnight before TNFα (20 ng/ml) was added for the indicated periods of time. Total RNA was extracted and subjected to qPCR determining APP mRNA levels normalized to 36B4 expression relative to untreated control cells (=100%) as described in Materials and Methods Section. Results are the means ± SE of at least four independent experiments. *$P < 0.05$ comparing TNFα-treated with non-treated cells.

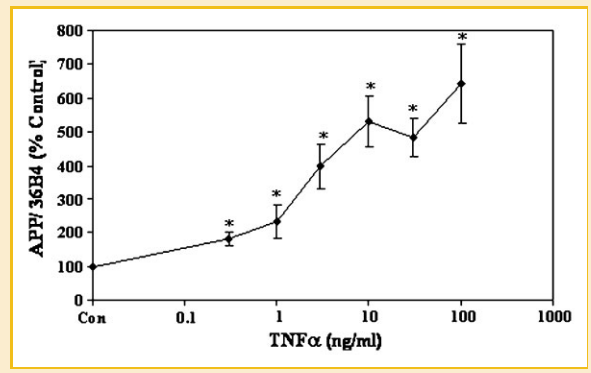

Fig. 3. TNFα stimulates APP mRNA expression dose-dependently. 3T3-L1 fat cells were serum-starved for 6 h before various concentrations of TNFα were added for 16 h. Total RNA was extracted and subjected to qPCR to determine APP mRNA levels normalized to 36B4 expression as described in Materials and Methods Section. Data are expressed relative to untreated control (Con) cells (=100%). Results are the means ± SE of at least three independent experiments. Asterisk (*) denotes $P < 0.05$ comparing TNFα-treated with non-treated cells.

(Fig. 4A). In contrast, PD98059, SB203580, and LY294002 treatment did not significantly influence TNFα-induced APP expression (Fig. 4A). Additional experiments were performed using MG132 as a second pharmacological NFκB inhibitor. Treatment of 3T3-L1 adipocytes with MG132 (50 μM) had no significant effect on basal APP expression (Fig. 4B). Again, APP mRNA synthesis was increased 2.9-fold after 16 h of TNFα treatment ($P < 0.01$) (Fig. 4B). This induction was completely reversed to 61% of control levels in 3T3-L1 adipocytes pretreated with MG132 ($P < 0.01$) (Fig. 4B).

DISCUSSION

Identification and characterization of downstream signaling molecules of the adipokine TNFα has become a focus of current research since novel pharmacological targets for the treatment of obesity and associated diseases including insulin resistance, type 2 diabetes mellitus, chronic inflammation, dementia, and AD might be derived from these studies. In the current study, we show for the first time that TNFα significantly stimulates APP mRNA and protein expression in 3T3-L1 fat cells. These findings suggest that adipocyte-derived APP might contribute to TNFα-induced insulin resistance and inflammation. Furthermore, TNFα-stimulated APP synthesis might contribute to the increased risk for dementia and AD when body weight is gained [Gustafson et al., 2003; Whitmer et al., 2005; Gorospe and Dave, 2007] since TNFα expression in fat and circulating levels of the adipokine are increased in obese subjects [Fasshauer and Paschke, 2003]. However, other mechanisms including TNFα-mediated upregulation of insulin resistance-inducing IL-6 [Fasshauer et al., 2003] and downregulation of insulin-sensitizing adiponectin [Fasshauer et al., 2002] probably also contribute. Furthermore, TNFα-induced serine phosphorylation of IR substrate (IRS)-1 has been suggested as an important mechanism for the insulin resistance-inducing effects of the

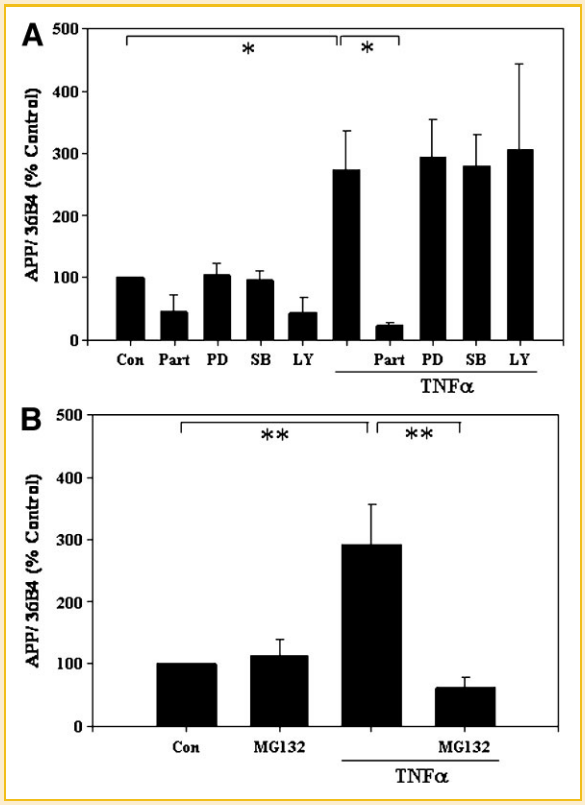

Fig. 4. APP mRNA induction by TNFα is mediated via NFκB. After serum-starvation for 6 h, 3T3-L1 adipocytes were cultured in the presence or absence of (A) parthenolide (Part, 50 μM), PD98059 (PD, 50 μM), SB203580 (SB, 20 μM), or LY294002 (LY, 10 μM) or (B) MG132 (50 μM) for 1 h before TNFα (20 ng/ml) was added for 16 h. Total RNA was extracted and subjected to qPCR to determine APP normalized to 36B4 expression as described in Materials and Methods Section. Data are expressed relative to non-treated control (Con) cells (=100%). Results are the means ± SE of (A) four and (B) five independent experiments. Double asterisk (**) denotes $P < 0.01$, asterisk (*) denotes $P < 0.05$ comparing untreated with inhibitor-pretreated or TNFα-treated cells, as well as comparing TNFα-treated with inhibitor-pretreated adipocytes.

adipokine. Thus, serine phosphorylated IRS-1 inhibits autokinase activity of the IR, resulting in decreased activity of insulin signaling molecules including PI 3-kinase and glucose transporter 4 in murine adipocytes [Hotamisligil et al., 1996; Stephens et al., 1997].

It is interesting to note in the context of our study that neuronal cell expression of APP and regulation by inflammatory cytokines and NFκB has been elucidated in more detail recently. Thus, stimulation of primary neuronal cultures with the inflammatory cytokine IL-1β potently upregulates the expression of NFκB subunits that specifically bind to the NFκB-binding sites located on the APP gene [Grilli et al., 1996]. The influence of TNFα among other cytokines and growth factors on APP promoter activity in PC12 neuronal cells has been determined and an increase of reporter gene activity after stimulation with TNFα is reported [Ge and Lahiri, 2002]. Another study demonstrates upregulated secretion of the APP metabolite amyloid-β after treatment with TNFα in neuronal cells [Yamamoto et al., 2007]. This increase is due to upregulation of β-site APP cleaving enzyme (BACE1) expression that in turn promotes APP-processing [Yamamoto et al., 2007].

In the current study, we elucidate by which signaling molecules TNFα induces APP mRNA production in fat cells. Binding of TNFα to its transmembrane receptors TNFR1 and TNFR2 leads to stimulation of various signaling cascades [Vilcek and Lee, 1991]. While NFκB activation is obligatory for TNFα action [Bluher et al., 2000; Ruan et al., 2002], p44/42 and p38 MAP kinases [Engelman et al., 2000; Valladares et al., 2001], as well as PI 3-kinase [Fuortes et al., 1999] are also implicated as important molecules in TNFα signaling. Pharmacological inhibition of NFκB by parthenolide reverses TNFα-mediated upregulation of APP mRNA expression. However, it has to be pointed out that parthenolide significantly decreases cell viability assessed by trypan blue staining as compared to untreated 3T3-L1 adipocytes (data not shown). Since we could not exclude the possibility that downregulation of APP after parthenolide treatment might at least partly be due to a toxic effect of the inhibitor, MG132 has been used as a second NFκB inhibitor in the present study. MG132 at a concentration of 50 μM does not decrease cell viability (data not shown). Again, TNFα-mediated induction of APP mRNA expression is reversed to control levels in cells pretreated with MG132. These findings support the notion that NFκB is an important mediator of TNFα-induced APP. Parthenolide significantly inhibits TNFα-induced APP transcription below basal levels. Interestingly, a similar trend is seen in the experiments using MG132. The reasons for these findings remain unclear at present. One explanation is that TNFα also has an inhibitory effect on APP mRNA expression which is mediated by signaling molecules independent from NFκB and becomes unmasked after pharmacological NFκB inhibition. This hypothesis should be tested in future experiments. P44/42 MAP kinase, p38 MAP kinase, and PI 3-kinase are probably not involved in regulation of APP by TNFα.

Taken together, the current study reveals for the first time that the proinflammatory and insulin resistance-inducing cytokine TNFα potently stimulates APP expression in adipocytes. This upregulation might be an important factor contributing to the association between obesity on one hand and insulin resistance, inflammation, dementia and AD on the other hand.

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