Chlamydophila pneumoniae Inhibits Differentiation of Progenitor Adipose Cells and Impairs Insulin Signaling

Yu Shi,1 Youan Liu,1 Andrew Murdin,2 Ausra Raudonikiene-Mancevski,2 Bilal B. Ayach,1,4 Zhiwen Yu,3 I. George Fantus,1,3,4 and Peter P. Liu1,4

1Toronto General Hospital Research Institute, 2Sanofi Pasteur Canada, 3Banting and Best Diabetes Centre, and 4University of Toronto, Toronto, Ontario, Canada

Background. Recent clinical studies have shown Chlamydophila pneumoniae seropositivity to be related to overweight status and inversely related to insulin sensitivity. The present study was performed to investigate the potential effects of C. pneumoniae infection of adipocytes.

Methods. 3T3-L1 cells and primary epididymal preadipocytes were infected with C. pneumoniae either before or after induction of differentiation, and the effects on adipogenesis and insulin signaling were determined. Tumor necrosis factor (TNF)–α signaling was examined by assessing the effects of C. pneumoniae infection in preadipocytes isolated from epididymal adipose tissue of both wild-type and TNF-α−/− mice.

Results. C. pneumoniae successfully infected both undifferentiated and differentiated 3T3-L1 cells in vitro. The bacteria were also detected in adipose tissue of infected low-density lipoprotein receptor–deficient mice. TNF-α protein levels were significantly increased in cells infected with either live or heat-killed C. pneumoniae or treated with lipopolysaccharide or heat-shock protein 65; this increase was associated with inhibition of adipocyte differentiation and down-regulation of insulin-stimulated tyrosine-phosphorylated insulin receptor and its substrate. In contrast, C. pneumoniae infection in TNF-α−/− adipocytes produced no apparent changes, but addition of recombinant TNF-α reversed this effect.

Conclusions. We demonstrate for the first time that C. pneumoniae can infect murine pre- and postdifferentiated adipocytes and, through a TNF-α–mediated inflammatory mechanism, can impair differentiation and insulin signaling.

There are compelling findings demonstrating the existence of a subclinical inflammatory reaction associated with obesity and a predictor of the onset of type 2 diabetes [1]. However, the cause of the inflammatory state is uncertain, and whether infection with agents such as Chlamydophila pneumoniae is involved has not been studied.

Adipose, muscle, and liver tissue are insulin-sensitive tissues [2]. Adipose tissue, in addition to storing excess energy, is also an active endocrine organ with the ability to secrete adipokines, such as tumor necrosis factor (TNF)–α, adiponectin, and leptin [3]. The cytokine TNF-α has been reported to play a role in insulin resistance in vivo and in vitro by inhibiting insulin receptor (IR) phosphorylation and IR substrate 1 (IRS-1) tyrosine phosphorylation without affecting the number of receptors or their insulin binding capacity [4]. Recent studies have shown that overexpression of TNF-α in adipose tissue is responsible, to a significant extent, for insulin resistance in obese mice and humans [5–7].

To date there have been few studies on the relationship between the bacteria and host metabolic disorder. One study [8] found that 42% of men <50 years old and 45% of men >50 years old with persistently elevated
levels of IgG and IgA antibodies suggestive of chronic *C. pneumoniae* infection were overweight, compared with 20% and 26% of men without markers of chronic infection, respectively. In subjects who were seropositive for *C. pneumoniae*, the relationship between the quantitative seropositivity index and insulin sensitivity was strengthened [9]. Interestingly, a recent study has shown that human adenoviruses have adipogenic potential [10].

Adipose tissue, however, has not previously been reported as a target for *C. pneumoniae* infection. Because directly isolated primary adipocytes always float on the surface of the medium, it is impossible to infect the cells with *C. pneumoniae* by centrifugal force. In these experiments, we used a mouse-derived preadipocyte 3T3-L1 cell line model to evaluate the possibility of *C. pneumoniae* infection and inflammatory signaling. Previous studies have demonstrated the reliability of this cell line for adipocyte differentiation studies [11, 12], insulin signaling [13], adiponectin [14], and leptin-expression profiling [15]. Moreover, 3T3-L1 cells are capable of adhering and maintaining fibroblast shape and therefore can be centrifugally infected by *C. pneumoniae*. Furthermore, as preadipocytes, 3T3-L1 cells display some features of macrophages [16], which have been shown both in vivo and in vitro to be hosts for *C. pneumoniae* growth [17].

In the present study, we infected 3T3-L1 cells and primary epididymal preadipocytes with *C. pneumoniae* and evaluated how this infection affects inflammatory and metabolic activity, in particular insulin signaling and differentiation of adipocytes.

**MATERIALS AND METHODS**

**Cell culture and adipocyte differentiation.** Culturing of 3T3-L1 cells (American Type Culture Collection [ATCC]) and their differentiation to adipocytes was performed in accordance with methods described elsewhere [18]. In brief, cells were propagated in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS; Invitrogen) and penicillin/streptomycin (100 U/mL) and allowed to reach confluence. After 2 days (day 0), the medium was changed to differentiation medium 1 (10% FCS, 160 nmol/L insulin, 250 μmol/L dexamethasone, and 0.5 mmol/L 3-isobutyl-1-methylxanthine). Two days later (day 2), the medium was switched to differentiation medium 2 (10% FCS and 160 nmol/L insulin). After another 2 days (day 4), the cells were switched back to DMEM supplemented with 10% FCS.

**Adipogenesis assay.** Adipocyte differentiation was evaluated by oil red O staining (Sigma), as described elsewhere [19]. Cultures in 24-well plates were fixed for 1 h with 10% formalin and then washed with water, stained for 2 h by complete immersion in a working solution of 0.3% oil red O, and exhaustively rinsed with water. After evaporation of excess water, 0.25 mL of isopropyl alcohol was added to the stained culture wells to extract the dye by gentle pipetting, and absorbance was monitored by spectrophotometry at 490 nm.

**Animals.** Specific pathogen–free C57BL/6 (wild-type [WT]), TNF-α knockout (KO) (TNF-α−/−), and low-density lipoprotein receptor–deficient (LDLR−/−) male mice (Jackson Laboratory) were housed under standard conditions at the animal facility at the University Health Network and were treated in accordance with our institutional animal care and ethics regulations. During the duration of this study, male LDLR−/− mice were fed an atherogenic high-cholesterol diet (D12108; Research Diets) and tap water ad libitum.

**C. pneumoniae infection and staining.** *C. pneumoniae* (strain AR-39) was originally purchased from ATCC and provided by Sanofi Pasteur. Mycoplasma-free bacteria were cultured on HL cells, and challenge inocula for cell culture and animal studies were prepared and stored as described elsewhere [20]. LDLR−/− mice were infected biweekly with 10⁶ inclusion-forming units (ifu) of *C. pneumoniae* in 28 μL of sucrose-phosphate-glutamate (SPG) buffer administered intranasally for total of 10 times (drops were applied to the nares under light anesthesia with ketamine hydrochloride [Ketalar] and xylazine [Rompun]).

Cells were infected with either live or heat-killed *C. pneumoniae* at an MOI of 1 ifu/cell or were mock infected with SPG buffer. *C. pneumoniae* were inoculated onto confluent monolayers. To prepare heat-killed *C. pneumoniae*, bacteria were heated at 90°C for 30 min. After centrifugation at 800 g and incubation at 37°C for 1 h, the inoculum was removed, and cells were washed twice with PBS and then cultured in DMEM containing 10% heat-inactivated FCS and 1 μg/mL cycloheximide (Sigma). Cells were grown on glass coverslips in 6-well plates, fixed with ice-cold acetone for 20 min, and then rinsed with PBS. The cells were incubated with fluorescein isothiocyanate (FITC)–conjugated mouse monoclonal anti–*C. pneumoniae* antibody (TT-401, IgG; Department of Pathology, University of Washington), and nuclei were counterstained with 0.05 mg/mL propidium iodide in Antifade reagent (Bio-Rad) to identify infected cells. After being washed in distilled water, slides were mounted with coverslips, and cells were imaged and quantified with an Olympus BX60 fluorescence microscope.

**Experimental protocols.** For evaluation of cell viability and inclusion-forming units, cells were infected either before or after differentiation and analyzed at various time points (days 0, 1, 4, 7, 10, 14, and 18). To examine the effect of treatment on adipocyte differentiation, cells were pretreated (day 1) and maintained with either 10 μg/mL bacterial lipopolysaccharide (LPS) [21], 5 μg/mL hsp65 [22], or 0.2 ng/mL recombinant TNF-α (Sigma) and then analyzed on days 0, 1, 4, 7, 10, 14, and 18. The concentration of recombinant TNF-α used in the experiment was based on the result of measuring medium TNF-α concentration when 3T3-L1 cells were being infected with *C. pneumoniae*.
nul antibody against chlamydial major outer membrane protein (clone RR402; mouse IgG3; Dako) after treatment with 0.3% H₂O₂ for 20 min to quench the endogenous peroxidase. The primary antibody binding was probed with a goat anti–mouse IgG conjugated with horseradish peroxidase (Vectastain ABC Kit; Vector Laboratories). Chlamydia 16S rRNA was detected using a DNA extraction and purification kit (Qiagen). DNA from adipose tissue was applied to polymerase chain reaction (PCR), with a forward primer of 5’-GGATTTATGGGCTAAAGG-3’ and a reverse primer of 5’-TCCACATCAAGTATGCAGT-3’, as described elsewhere [23].

**Inclusion-forming unit assay.** The *C. pneumoniae* titer was determined by infection of HeLa cells (ATCC) plated in 24-well plates, as described elsewhere [20]. Cell lysates were sonicated, centrifuged to remove coarse debris, and inoculated on monolayer of HeLa cells in 24-well plates. The burst size of the inclusions grown in preadipocytes and adipocytes was determined by dividing the total number of inclusion-forming units obtained from the titration of the lysates on HeLa cells by the number of inclusion-forming units used to initially infect preadipocytes and adipocytes [24].

**Apoptosis analysis.** Cell viability was analyzed with the Annexin V–FITC Apoptosis Detection Kit (APOAF-50TST; Sigma).

**Western blot analysis.** Protein was extracted by homogenization from the freshly prepared cells with Celllytic-M cell lysis buffer (Sigma). After gel electrophoresis, the protein was transferred onto polyvinylidene fluoride membranes (Millipore). Membranes were first blocked with 5% nonfat dried milk for 1 h and then incubated with anti–mouse peroxisome proliferator–activated receptor–γ (PPAR-γ) monoclonal antibody (1:200; Santa Cruz Biotechnology) after treatment with 0.3% H₂O₂ for 20 min to quench the endogenous peroxidase. The primary antibody binding was probed with a goat anti–mouse peroxisome proliferator–activated receptor–γ (clone RR402; mouse IgG3; Dako) after treatment with 0.3% H₂O₂ for 20 min to quench the endogenous peroxidase. 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lated cytokine production [26]. Using an apoptosis kit to examine cell death in infected cells on day 4 after infection, we found significantly more apoptosis in adipocytes than in preadipocytes (P < .01), and >90% of infected adipocytes were dead on day 14 (figure 2A). Figure 2B shows representative photographs of apoptotic cells on days 4 and 14.

**Inhibition of adipocyte differentiation by C. pneumoniae infection through increased expression of TNF-α.** As a first step to investigate the potential functional consequences of *C. pneumoniae* infection, its effect on preadipocyte differentiation was determined. TNF-α has been shown to be one of the most important inflammatory cytokines after injury or infection, and it has been shown to inhibit adipocyte differentiation [27]. TNF-α mRNA levels in 3T3-L1 cells were significantly increased in *C. pneumoniae*-infected cells compared with uninfected cells 4 days after infection (arbitrary densitometric units, 62 ± 5.5 vs. 9.9 ± 1.6; P < .001) (figure 3A). Furthermore, the secretion of TNF-α in culture medium, as determined by ELISA, was significantly increased in both undifferentiated and differentiated 3T3-L1 cells, with a peak secretion on day 1 (figure 3B). *C. pneumoniae* infection in 3T3-L1 cells induced an increase in TNF-α secretion, possibly mediated via cell stimulation by LPS. LPS is a cell-surface membrane of gram-negative bacteria that interacts with host-cell receptors [21]. The LPS receptor on 3T3-L1 cells has been characterized as Toll-like receptor 4, which is responsible for NF-κB-mediated production of the proinflammatory cytokine TNF-α and activates an innate immune response [28]. Stimulation by LPS has also been reported to induce interleukin (IL)-6 production in primary adipocytes and to cause insulin resistance [21]. Similarly, 3T3-L1 cells have been found to se-

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**Figure 1.** *Chlamydia pneumoniae*-infected fat cells. A, Presence of inclusion bodies of *C. pneumoniae* in cytoplasm of 3T3-L1 cells 72 h after infection. Propidium iodine–stained nuclei appear red, and fluorescein isothiocyanate (FITC)–labeled *C. pneumoniae* antibody appear green. The white arrow indicates lipid droplets in adipocyte. B, Image showing a preadipocyte that is positive for anti-*C. pneumoniae* antibody (brown color, black arrow). The adipose tissue in this picture is from a low-density lipoprotein receptor–deficient (LDLR−/−) mouse after 10 intranasal inoculations with *C. pneumoniae* infection. C, Polymerase chain reaction (PCR) for chlamydial DNA. D, Inclusion-forming unit assay. Photomicrographs show representative HeLa cells inoculated with cell lysates harvested from infected or differentiated 3T3-L1 cells on day 4 after infection. *C. pneumoniae* particles appear green (FITC-labeled *C. pneumoniae* antibody). Quantitative data are expressed as mean ± SE values (n = 3). Cpn, *C. pneumoniae*. 

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![Figure 1](image-url)
crete IL-6 in the presence of TNF-α, and this mechanism was impaired in the absence of TNF-α [29]. Thus, TNF-α may exert an important autocrine function regulating other inflammatory cytokines, such as IL-6 [30].

To evaluate the effects of infection on adipogenesis, cells in 24-well plates were inoculated with live or heat-killed C. pneumoniae at an MOI of 1 or treated with bacterial LPS or hsp65 before induction of differentiation. As figure 3C shows, C. pneumoniae infection completely inhibited cellular differentiation, whereas LPS, heat-killed C. pneumoniae, and hsp65 showed partial but significant inhibitory effects from day 10 onward (P < .05 vs. SPG), suggesting that live bacteria exert more effect than do their structural components. Similarly, this effect was correlated with the secretion of TNF-α in culture medium (figure 3D). As previously reported [31], the observed decrease in TNF-α level on day 1 before an increase on day 4 may have resulted from the use of dexamethasone to induce differentiation.

To investigate whether the higher TNF-α levels were responsible for the inhibition of differentiation by C. pneumoniae infection, preadipocytes were isolated from WT and TNF-α KO mice and then infected with C. pneumoniae before differentiation was induced. Compared with complete inhibition in WT preadipocytes, KO cells significantly differentiated, with a peak on day 7 after infection; however, this effect was reversed by the addition of recombinant TNF-α (figure 3E). Although it has been reported that TNF-α plays an early role in eradicating pathogens [32], in our study the numbers of bacterial inclusion-forming units did not differ between WT, KO, and KO + TNF-α cells (figure 3F). Because all preadipocytes infected with bacteria resulted in a large number of apoptotic cells (figure 3G), this may explain the drastic decrease in oil red O–stained lipid in KO preadipocytes (figure 3E) and may

Figure 2. Chlamydia pneumoniae–induced cell apoptosis. The Annexin V–FITC Apoptosis Detection Kit (Sigma) allows fluorescent detection of annexin V bound to apoptotic cells and quantitative determination by flow cytometry. It uses annexin V conjugated with fluorescein isothiocyanate (FITC) to label phosphatidylserine sites on the membrane surface and propidium iodide (PI) to label the cellular DNA in necrotic cells where the cell membrane has been totally compromised. This combination allows the differentiation among early apoptotic cells (annexin V positive, PI negative), necrotic cells (annexin V positive, PI positive), and viable cells (annexin V negative, PI negative). A, Quantitative apoptosis determination by flow cytometry. Results are expressed as mean ± SE values (n = 3). B, Representative photomicrographs of cell apoptosis.
imply that a TNF-α–independent mechanism induces apoptosis after C. pneumoniae infection.

**Impairment of insulin signaling in differentiated adipocytes by C. pneumoniae infection.** Because insulin signaling plays a key role in adipocyte differentiation, we evaluated whether C. pneumoniae infection impaired insulin signaling and thus differentiation. Immunoblotting revealed that, although total IR and IRS-1 protein content was not altered, insulin-induced tyrosine phosphorylation of both IR and IRS-1 was significantly impaired in infected cells compared with controls (P < .001) (figure 4A). PPAR-γ is a transcription factor expressed predominantly in adipose tissue and functions promi-
Figure 4. Requirement of tumor necrosis factor (TNF–α) for Chlamydia pneumoniae infection–associated inhibition of preadipocyte differentiation and decreased insulin signaling. Differentiated adipocytes, either infected with C. pneumoniae for 4 days or uninfected, were stimulated with insulin for 5 min, homogenized, insulin receptor (IR)/IR substrate 1 (IRS-1) immunoprecipitated, and separated by SDS-PAGE, as described in Materials and Methods. C. pneumoniae infection for 4 days decreased proliferator-activated receptor–γ (PPAR-γ) and phosphorylated tyrosine (pTyr) of IR β subunits and IRS-1 in 3T3-L1 adipocytes (A). Whole-cell lysates (20 µg of protein) were separated by SDS-PAGE and immunoblotted for PPAR-γ, IR, and IRS-1 (see text for details). C. pneumoniae infection for 4 days decreased insulin signaling in wild-type (WT) adipocytes but not in TNF-α–/– adipocytes (B). Quantitative data are expressed as mean ± SE values (n = 3). *P < .05, for the comparison with uninfected cells. Cpn, C. pneumoniae; KO, knockout.
nently in differentiation, promoting lipid accumulation in adipocytes [33]. Importantly, C. pneumoniae infection completely inhibited the expression of PPAR-γ (figure 4A), which is likely to contribute to the inhibition of differentiation (figure 3C).

**Requirement of TNF-α for C. pneumoniae infection and impairment of insulin signaling by TNF-α.** First, to confirm our observation in differentiated 3T3-L1 cells as described above, the effects of C. pneumoniae infection on insulin action was assessed using primary differentiated WT mouse cells. Even though there was no change in total unphosphorylated IR or IRS-1 levels, IR pTyr and IRS-1 pTyr were significantly decreased after infection (figure 4B). Preadipocytes derived from TNF-α⁻/⁻ mice had no alterations in IR pTyr and IRS-1 pTyr levels, but this effect was reversed with the addition of recombinant TNF-α (figure 4B). These findings indicate that TNF-α plays a key role in the impairment of insulin signaling.

**DISCUSSION**

In the present study, we demonstrate for the first time that C. pneumoniae can infect pre- and postdifferentiated murine adipocytes and, through a TNF-α–mediated mechanism, can inhibit differentiation by down-regulating PPAR-γ and can impair insulin signaling through down-regulation of IR and IRS-1 tyrosine phosphorylation.

Although C. pneumoniae could infect both murine preadipocytes and adipocytes in vitro, the latter showed less susceptibility to infection. The reason for this differential sensitivity is unclear. In vivo immunostaining also showed the presence of the bacteria in preadipocytes. C. pneumoniae, although classically classified as a respiratory pathogen, has been shown to infect not only lung epithelial cells but also human-derived epithelial cell lines, vascular cells, endothelial and vascular smooth muscle cells, and macrophages, which are important in atherogenesis [34]. Recent studies using PCR and electron microscopy have demonstrated the presence of chlamydial organisms in the central nervous system of patients with multiple sclerosis [35]. C. pneumoniae was also detected in the joint of a patient with common variable immunodeficiency [36], and this organism appears to infect different types of host cells, suggesting that it is more ubiquitous than previously thought.

Although less susceptible to C. pneumoniae infection, adipocytes were more prone to C. pneumoniae–induced apoptosis. Because infection-induced TNF-α levels were similar between undifferentiated and differentiated 3T3-L1 cells (figure 3B) and because the apoptotic effect was not abolished by use of TNF-α KO cells, some TNF-α–independent mechanisms may be involved. Interestingly, chlamydial infection has been reported to inhibit apoptosis through induction of IL-10, an anti-inflammatory cytokine [37]. A recent article indicated that a Chlamydia protein (CADD) interacts with the death domains of TNF-α receptor 1, Fas, DR4, and DR5 [38], which might contribute to live C. pneumoniae–induced apoptosis in TNF-α KO cells. This is also supported by the observation that heat-killed C. pneumoniae did not increase apoptosis (figure 3G).

It has been shown that C. pneumoniae infection can stimulate chronic inflammation by its various antigen components, including chlamydial hsp60, which is particularly immunogenic [39, 40]. The heat-shock protein family comprises hsp60 in mammals, mycobacterial homologue mhs65, chlamydial hsp60, and the E. coli homologue GroEL [41]. A study [39] has shown that chlamydial hsp60 can induce TNF-α synthesis. Using mycobacterial hsp65, we also detected a significant increase in TNF-α production in cell culture medium on day 4 after treatment (P < .05) (figure 3D). Treatment with bacterial LPS and heat-killed C. pneumoniae also induced significant production of TNF-α. As expected, live C. pneumoniae, with all the antigen components, induced significantly higher TNF-α levels, but these dropped after day 4 because of apoptotic cell loss.

Although TNF-α was not critical for C. pneumoniae–induced apoptosis, it was responsible for inhibiting differentiation. TNF-α levels correlated with the extent of adipocyte differentiation, as evaluated by oil red staining, and were significantly reduced in all treated groups (live or heat-killed C. pneumoniae, LPS, and hsp65) compared with SPG-treated control cells (figure 3C). In a previous report [42], heat-killed C. pneumoniae did not induce TNF-α production in macrophages. Aside from the different cell types we used, the reason for this discrepancy is not clear.

The role played by TNF-α in inhibiting adipocyte differentiation was demonstrated using TNF-α⁻/⁻–derived preadipocytes, which showed an increase in differentiation that was reversed by the addition of recombinant TNF-α (figure 3E). One mechanism by which TNF-α inhibited differentiation may be explained by recent findings of Cho et al. [43] demonstrating that C. pneumoniae infection reduces the steroid responsiveness of human peripheral blood mononuclear cells via a TNF-α–dependent pathway.

As another major mechanism, TNF-α impairs insulin-dependent differentiation by decreasing IR and IRS-1 tyrosine phosphorylation. Using adipocytes derived from TNF-α⁻/⁻ KO mice, we demonstrated that TNF-α is required for the inhibition of differentiation after C. pneumoniae infection and that the addition of recombinant TNF-α to KO adipocytes reversed this effect (figure 4B). Our findings are consistent with previous reports that TNF-α contributes to insulin resistance by blocking tyrosine phosphorylation of IR and IRS-1 [13]. Another study [44] supports our finding by showing that TNF-α does have profound effects on adipocytes, including suppression of differentiation and inhibition of insulin-stimulated glucose, free fatty acid uptake, and lipogenesis, resulting in lipolysis and altered regulation of gene expression. It has also been demonstrated that TNF-α mediates inhibition through a TNF-α receptor 1 mechanism and that reversal of adipocyte differentiation is accompa-
nied by suppression of PPAR-γ, a marker and positive regulator of adipocyte differentiation [45, 46].

*C. pneumoniae* infection and its role in human insulin resistance or metabolic syndromes have not been well studied. Our data suggest that further study in mouse models and human subjects is warranted to determine whether *C. pneumoniae* infection contributes to adipose tissue dysfunction, insulin resistance, and its complications.

References

36. Ardeniz O, Gülbahar O, Mete N, et al. *Chlamydia pneumoniae* infection and its role in human insulin resistance or metabolic syndromes have not been well studied. Our data suggest that further study in mouse models and human subjects is warranted to determine whether *C. pneumoniae* infection contributes to adipose tissue dysfunction, insulin resistance, and its complications. *Chlamyphoria* in Adipocytes • JID 2008:197 (1 February) • 447