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# Insulin and fatty acids regulate the expression of the fat droplet-associated protein adipophilin in primary human trophoblasts

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**Objective:** This study was undertaken to test the hypothesis that insulin and fatty acids regulate adipophilin expression in cultured human trophoblasts.
**Study design:** Cytotrophoblasts isolated from term human placentas were cultured in the absence or presence of insulin (10 nmol/L), and a mix of oleic and linoleic acid in serum-free medium. The expression of adipophilin as well as the fatty acid transport proteins (FATP) 2, 3, 4 and 6 was examined. Fat accumulation was quantified by BODIPY staining and fat uptake determined using [$^3$H]-oleic acid.
**Results:** A combination of insulin and fatty acids enhanced the expression of adipophilin (2.3-fold, $P < .05$). In contrast, the expression of FATPs was unchanged. Furthermore, insulin and fatty acids increased the accumulation of fat droplets in trophoblasts by 4- to 5-fold ($P < .05$), but had no effect on oleic acid uptake.
**Conclusion:** Insulin and fatty acids enhance the expression of adipophilin and the formation of fatty acid droplets in term human trophoblasts.


Supply of fatty acids to the fetus is particularly high during the third trimester of pregnancy, when the fetus more than doubles its size.[1] Because de novo lipid synthesis in the placenta is insufficient, transplacental transport of maternal fatty acids is paramount to the developing fetus. Not surprisingly, maternal serum lipid levels are elevated during pregnancy.[1,2] Although the mix of fatty acids delivered to the fetus is largely determined by maternal serum lipoproteins and fatty acid composition, the placenta is capable of regulated preferential transfer of long chain polyunsaturated fatty acids. In addition, the fetus is unable to synthesize n-3 and n-6 unsaturated fatty acids, and therefore depends on maternal transport for these essential fatty acids.[1,2]

Cell types that rely on the supply of fatty acids, such as adipocytes, cardiocytes and hepatocytes, express specific fatty acid transport proteins. Several fatty acid

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transporters have been identified in trophoblasts, including fatty acid transport proteins (FATPs), placental membrane fatty acid binding protein (p-FABPpm), and fatty acid translocase (FAT/CD36).[1,3] Adipophilin and its murine ortholog, adipocyte differentiation-related protein (ADRP), are associated with cellular lipid droplets and implicated in cellular fatty acid uptake and storage of neutral lipids in adipocytes.[4-6] We previously demonstrated that adipophilin is expressed in human villous trophoblasts in vivo and in cultured primary human trophoblasts in vitro.[7] Moreover, we showed that the expression of adipophilin is regulated by the insulin-sensitizer peroxisome proliferator-activated receptor-γ (PPARγ),[7] a protein that plays a pivotal role in development of the murine fetoplacental unit and differentiation of human trophoblasts.[8,9] Consistent with these findings, Tarrade et al[10] demonstrated enhanced accumulation of fatty acids during cytotrophoblast differentiation into syncytiotrophoblasts. Ablation of PPARγ is associated with a reduced number of fatty acid droplets in the murine placenta.[8] Taken together, these findings suggest that accumulation of fatty acids in trophoblasts is necessary for fetal growth and is regulated during placental development and trophoblast differentiation.

The placenta in women with poorly controlled gestational diabetes mellitus (DM) and type-2 DM is exposed to hyperinsulinemia[11] and increased supply of nutrients. In the current work we surmised that insulin, acting in a hyperlipidemic environment, regulates fatty acid accumulation in human trophoblasts. We hypothesized that insulin combined with fatty acids regulate the expression of adipophilin and fat accumulation in trophoblasts. To test this hypothesis we analyzed the influence of insulin, in the presence or absence of fatty acids, on adipophilin expression as well as fatty acid uptake and accumulation in cultured term human trophoblasts.

## Materials and methods

### Trophoblast isolation and culture

Our study was approved by the Institutional Review Board of Washington University School of Medicine. Placentas were obtained immediately after term singleton deliveries after uncomplicated pregnancies. Cytotrophoblasts were isolated by the trypsin-DNase, percoll (Sigma, St. Louis, MO) gradient centrifugation method described by Kliman et al,[12] with modifications.[13] Nonadherent cells and syncytial fragments were removed after 4 hours by washing 3 times with phosphate-buffered saline (PBS) solution, and cytotrophoblasts were cultured in Earle's medium 199 supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and antibiotics in a standard tissue culture atmosphere of 5% $CO_2$ and 20% $O_2$. After 24 hours in culture the cells were washed and the medium replaced with fresh serum-containing or serum-free medium, supplemented where indicated by insulin (10 nmol/L, Sigma) dissolved in 0.1% insulin-free and fatty acid-free albumin (Sigma), a mixture of 400 μmol/L oleic acid and 800 μmol/L linoleic acid in albumin (Sigma), or vehicle control. These concentrations were selected on the based of published information[14,15] and the company's recommendations, and were designed to assure that the saturating levels of fatty acids were well dissolved in the culture medium. All cells were harvested 48 hours after plating. The phosphatidylinositol 3-kinase (PI3K) inhibitor LY 294002 (10 μmol/L, Cell Signaling Technology, Beverly, MA) was added to some of the cell cultures.

### RNA and protein expression

At the end of the culture period the plates were rinsed with PBS. RNA was purified with the use of TriReagent (Molecular Research Center, Inc, Cincinnati, OH) according to the manufacturer's instructions. DNAse I (Ambion, Austin, TX) was added to the purified RNA for 1 hour at 37°C to remove contaminating DNA. Complementary DNA (cDNA) was prepared from 1 μg RNA with the use of the TaqMan Gold RT-PCR kit with the supplied random hexamer primers (Applied Biosystems, Foster City, CA). Polymerase chain reaction (PCR) was performed on reverse transcripts (RTs) by using primer pairs listed in the Table. Specificity of each primer pair was confirmed by BLAST analysis. Quantitative real-time PCR was performed on duplicate 3 μL samples of cDNA with the use of SYBR Green PCR Master Mix (Applied Biosystems) in a total reaction volume of 50 μL containing 300 nmol/L each of forward and reverse primers. Reactions were run and analyzed with the use of an Applied Biosystems Geneamp 5700 System. Dissociation curves were run on all reactions to ensure amplification of a single product with the appropriate melting temperature. Samples were normalized to parallel reactions with the use of primers specific for 18S RNA. The fold increase relative to control cultures was determined by the $2^{-\Delta\Delta C_T}$ method.[16] The fold change values were calculated by using geometric means of individual assays.

Western immunoblotting was performed as we previously described.[7] Samples consisted of 20 to 30 μg per lane, which were analyzed with the use of monoclonal anti-adipophilin antibody (1:100 RDI, Flanders, NJ) or polyclonal goat anti-β–actin (1:1000, Santa Cruz Biotech, Santa Cruz, Calif), followed by the corresponding horseradish peroxidase-linked secondary antibodies (1:2000, Santa Cruz). Chemiluminescence was analyzed with the use of Epichemi-3 darkroom (UVP BioImaging