Insulin Up-regulates Tumor Necrosis Factor-α Production in Macrophages through an Extracellular-regulated Kinase-dependent Pathway*

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Hyperinsulinemia has recently been reported as a risk factor for atherosclerotic diseases such as coronary heart disease; however, the effect of insulin on the development of atherosclerosis is not well understood. Here we have investigated the direct effect of insulin on macrophages, which are known to be important in the atherosclerotic process. We treated THP-1 macrophages with insulin (10^{-7}mol/L) and examined the gene expression using nucleic acid array systems. The results of array analysis showed that insulin stimulated gene expression of tumor necrosis factor-α (TNF-α) the most among all genes in the analysis. In addition, insulin administration to macrophages enhanced both mRNA expression and protein secretion of TNF-α in a dose-dependent manner. To determine the signaling pathway involved in this TNF-α response to insulin, we pretreated the cells with three distinct protein kinase inhibitors: wortmannin, PD98059, and SB203580. Only PD98059, which inhibits extracellular signal-regulated kinases, suppressed insulin-induced production of TNF-α mRNA and protein in THP-1 macrophages. These observations indicate that insulin stimulates TNF-α production in macrophages by regulating the expression of TNF-α mRNA and that the extracellular signal-regulated kinase signaling pathway may have a critical role in stimulating the production of TNF-α in response to insulin in macrophages.

Macrophages are important for host defense and inflammation, in which their functions include the secretion of various cytokines in physiological response, and phagocytosis of bacteria and denatured lipids (1–3). In addition, macrophages play a significant role in the process of atherosclerosis: they take up and degrade modified low density lipoprotein via scavenger receptors (4, 5), developing into macrophage foam cells; and infiltrate atherosclerotic lesions, and microscopy shows that early lesions of atherosclerosis are characterized by the infiltration of monocyte macrophages, the proliferation of medial smooth muscle cells, and the presence of macrophage foam cells (6).

Diabetes mellitus is a strong risk factor in all manifestations of atherosclerotic vascular disease; however, the pathophysiology of atherosclerosis in diabetic patients has not yet been elucidated. Several prospective studies in nondiabetic and diabetic patients have shown that there is an association between hyperinsulinemia resulting from insulin resistance and developed atherosclerotic disorders such as cardiovascular diseases (7, 8). In addition, evidence has accumulated that insulin has actions that may be related to the development of atherosclerosis (9–11). The insulin-resistant state that occurs in hyperinsulinemia often clusters with other risk factors, including hypertension, glucose tolerance, and hypertriglyceridemia, that increase the risk of cardiovascular disease, presumably due to promotion of atherosclerosis (9–11). Several studies have demonstrated that exogenous insulin accelerates the development of atherosclerosis (12, 13). Therefore, insulin action has been widely investigated in cells and tissues that construct arterial walls, such as arterial endothelial cells and vascular smooth muscle cells. For example, it has been suggested that insulin acts as a growth factor for vascular smooth muscle cells (14, 15). However, the action of insulin on macrophages involved in the initiation and development of atherosclerosis has been poorly studied.

Here, to examine the direct effect of insulin on macrophages, we have investigated the expression of genes induced by insulin using a membrane array technique. The results show that insulin is able to up-regulate the expression of genes, including the TNF-α gene, in macrophages derived from the cloned cell line THP-1. Our study demonstrates that insulin can also increase the release of TNF-α protein from THP-1-derived and human monocyte-derived macrophages after an increase in TNF-α mRNA and that this effect involves the insulin-mediated activation of extracellular signal-regulated kinases (ERKs), members of the mitogen-activated protein kinase (MAPK) family.

**EXPERIMENTAL PROCEDURES**

**Materials**—RPMI 1640 medium, human insulin, phorbol 12-myristate-13-acetate, lipopolysaccharide (LPS), and wortmannin were purchased from Sigma. Insulin was dissolved in sterile water acidified (pH 2.0) with a small volume of HCl to make a 10^{-8} mol/L stock solution. The monoclonal antibody against TNF-α was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PD98059 and SB203580 were purchased from Calbiochem.

**Cell Culture**—The human THP-1 monocytic leukemia cell line was purchased from Dainippon Pharmaceuticals (Tokyo, Japan). THP-1...
cells were grown in RPMI 1640 medium containing 10% fetal bovine serum supplemented with 100 units/ml penicillin and 100 mg/ml streptomycin. We differentiated the THP-1 cells by resuspending them for 2 days in medium containing phorbol 12-myristate-13-acetate (40 ng/ml), which had been prepared as a 10^4 times concentrated stock solution in MeSO. Such treatment with phorbol 12-myristate-13-acetate induced the conversion of THP-1 into mature cells that function as macrophages (16, 17). We used these mature cells, designated “THP-1-derived macrophages,” in all experiments.

Human peripheral blood mononuclear cells were obtained from three healthy volunteers by density centrifugation using Lymphoprep™ (Nycomed, Oslo, Norway). The collected mononuclear cells were cultured in RPMI 1640 medium supplemented with 10% autologous serum for 7 days for differentiation into macrophages.

These cells were always pretreated with media lacking fetal bovine serum and phorbol 12-myristate-13-acetate for at least 8 h before insulin stimulation to remove the effects of these reagents.

**Nucleic Acid Arrays**—The nylon membrane on which a large number of different human cDNA probes were spotted was purchased from CLONTECH (Palo Alto, CA). THP-1 macrophages were washed with phosphate-buffered saline twice and then incubated in RPMI 1640 medium or in RPMI 1640 medium containing 10⁻⁷ M insulin. After 6 h, total RNA was prepared from cells by using the acid guanidinium-phenol-chloroform method and treated with DNase I (100 units/mg). The RNA was electrophoresed on a 1% agarose gel containing formaldehyde and transferred to a nylon membrane for Northern blot hybridization. In some experiments, we prepared poly(A)⁺ RNA as described above and used 1 µg for electrophoresis. To construct the probes for human TNF-α for hybridization, we carried out reverse transcription-polymerase chain reaction on RNA that had been extracted from THP-1 macrophages using the primers 5'-CTCTCCTTCCCTGATCGTGG-3' and 5'-CTCTCCTCTCTCTCGCTCGG-3', which were designed on the basis of the TNF-α sequence reported in GenBank™ (accession number X01394). The 266-base pair amplified fragments were analyzed by the dideoxy chain termination method and labeled to generate the 3²P-labeled probes used for Northern blot hybridization. After Northern blot hybridization, the membrane was washed in 2× SSC + 1% SDS at 58 °C for 30 min. The autoradiographs were quantified by densitometry with the BAS2000 Bioimaging Analyzer (Fuji Film). Each densitometric result of mRNA was normalized to the densitometric result of 28 S rRNA or elongation factor-1α mRNA in the same sample.

**Western Blot Analysis**—The differentiated THP-1 macrophages were stimulated with insulin for the indicated times, and the cells were lysed by phosphate-buffered saline containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and some protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 10 µg/ml aprotinin). The solubilized proteins were centrifuged, and the supernatants were used for protein analysis. Protein concentration was determined with a modified procedure of the Lowry assay (18), and equal volumes of proteins were separated by 13.5% SDS-polyacrylamide gel electrophoresis and then electrotransferred to a polyvinylidene difluoride membrane. The membrane was treated with primary antibody specific to human TNF-α. After incubation with horseradish peroxidase-conju-
Effect of Insulin on TNF-α Production in Macrophages

**Fig. 1.** Induction of TNF-α mRNA expression by insulin in THP-1-derived macrophages. (a) THP-1-derived macrophages were pretreated with media without serum for 8 h and then stimulated with insulin for 24 h. In some experiments, cells were pretreated with other chemicals as indicated for 1 h. TNF-α release in medium was measured by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (R&D Systems, Minneapolis, MN), following the manufacturer’s protocols. In each well, residual cells that had been deprived of medium were lysed with 0.1 N NaOH, and cellular protein was measured with a modified procedure of the Lowry assay. The TNF-α content in the medium was normalized to cell protein values.

**ERK Enzyme Assay—**THP-1 macrophages were lysed with cold lysis buffer (10 mM Tris, 150 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Cell lysates were centrifuged for 20 min, and the supernatant was collected for measurement. ERK enzyme activity was determined using a commercial kit (Amersham Pharmacia Biotech) as follows. Briefly, the supernatants were mixed with the synthetic substrate peptide, and then a solution of magnesium and [32P]ATP was added. After incubation at 30 °C for 30 min, the reaction was terminated with buffer containing orthophosphoric acid, and samples of each of the reaction solutions were spotted onto paper disks. The paper disks were washed to remove any nonspecifically bound [32P]ATP, and ERK activity was determined by measuring the radioactivity of phosphorylated peptide bound to the paper disks using a scintillation counter.

**Statistical Analysis—**All experiments were repeated at least twice, and similar results were observed in each experiment. The results are expressed as the mean ± S.D. Group data are compared using Student’s t test. p < 0.05 is considered to be statistically significant.

**RESULTS**

We investigated the effect of insulin on gene expression in THP-1-derived macrophages. In Table I, we show a sample selection of 25 genes (the names or the GenBank™ accession number of representative genes including TNF-α are shown in Table I) that had values of radioactivity in the highest range and 6 housekeeping genes. To calculate the fold induction, we divided the radioactivity detected from stimulated cells by that from unstimulated cells. The results showed that 6 h of insulin stimulation in macrophages up-regulated mRNA levels of TNF-α. The average fold induction of the housekeeping genes was 1.036 ± 0.203, whereas that of TNF-α was much higher (2.452). The increase in TNF-α expression was also higher as compared with the average fold induction of the other sample genes, which was 1.186 ± 0.366.

We then carried out Northern blotting to confirm the effect of insulin on TNF-α mRNA expression that we had observed in THP-1 macrophages by using the membrane array technique. Treatment with insulin resulted in significant increases in TNF-α mRNA in THP-1-derived macrophages (Fig. 1a). After incubating the macrophages with 10−7 M insulin for 6 h, the level of TNF-α mRNA increased about 1.5-fold when analyzing poly(A)1 RNA (Fig. 1a) and 1.6-fold when analyzing poly(A)2 RNA (Fig. 1a and c) as compared with cells incubated in the absence of insulin. Similar results were observed after 24 h of insulin incubation. This stimulatory effect of insulin was dose-dependent in a range between 10−9 and 10−7 M (Fig. 1a, b, and c).

Next, we examined the effect of insulin on the biosynthesis of TNF-α protein in macrophages by Western blotting analysis of a whole-cell lysate. After 24 h of insulin treatment, the amount poly(A) RNA was isolated and analyzed for TNF-α and elongation factor-1α mRNA. In c, results at 6 h are expressed as relative activity in insulin-treated cells compared with unstimulated cells (n = 3). *, p < 0.05 versus unstimulated control; **, p < 0.01 versus unstimulated control.
of TNF-α was increased in whole cells (Fig. 2). These results confirmed that insulin stimulated the transcription of TNF-α in THP-1 macrophages and that the increase in TNF-α protein observed in cells was comparable with the increase observed in mRNA.

Inflammatory chemicals such as interferon-γ and LPS are shown to increase the secretion of TNF-α (19, 20). We therefore measured TNF-α protein mass in the cell medium by ELISA to determine the effect of insulin on TNF-α secretion. Treatment of THP-1-derived macrophages with insulin for 24 h resulted in an increase of TNF-α secretion (Fig. 3). To determine whether the concentration of insulin used for incubation affects the amount of TNF-α secreted, we incubated THP-1 macrophages in increasing concentrations of insulin for 24 h. Insulin up-regulated the secretion of TNF-α in a dose-dependent manner (Fig. 3a). Up-regulation of TNF-α secretion was also observed after incubating the macrophages for 48 h with a concentration of 10^{-7} M insulin (Fig. 3b). Similar effects of insulin were observed in human monocyte-derived macrophages (Fig. 4) and also in the presence of LPS (data not shown).

When cells are stimulated with insulin, two distinct signaling pathways are activated via the insulin receptor: the phosphatidylinositol 3-kinase pathway and the Raf/ERK pathway. We investigated the involvement of these pathways in insulin-stimulated TNF-α mRNA expression and protein production using a specific inhibitor of each pathway. The increase in TNF-α mRNA expression induced by insulin in THP-1-derived macrophages was markedly inhibited by pretreatment of the cells with PD98059, which is a specific inhibitor of ERK-1 and ERK-2, at a concentration of 25 μM (Fig. 5). This concentration has been reported to be sufficient to inhibit more than 90% of ERK activity (21). In contrast, pretreatment of the macrophages with 100 nM wortmannin, which is a specific inhibitor of phosphatidylinositol 3-kinase activity, or with 25 μM SB203580, which is a specific inhibitor of p38 MAPK, had no significant effect on the insulin stimulation of TNF-α gene expression (Fig. 5).

Next, we examined the effect of these inhibitors on the insulin-induced increase in TNF-α protein release. As with the gene expression results, the measurement of TNF-α protein mass in the cell medium showed that the ERK inhibitor markedly suppressed TNF-α secretion from insulin-stimulated THP-1
macrophages. Interestingly, the inhibitory effect of PD98059 was so potent that the production of TNF-α in cells treated with PD98059 was not only less than that in cells treated with insulin but was also less than that in the untreated control cells. This inhibitory effect of PD98059 was not due to a general toxic effect because the total protein production was not reduced by treatment with this compound (data not shown). The p38 MAPK inhibitor also suppressed TNF-α secretion, but the effect was much weaker than that of the ERK inhibitor, and the phosphatidylinositol 3-kinase inhibitor had no effect on the effect was much weaker than that of the ERK inhibitor, and expression of TNF-α mRNA and elongation factor-1α mRNA was analyzed by Northern hybridization. TNF-α RNA levels, normalized by comparison with levels of elongation factor-1α RNA, are shown (n = 4). Results are expressed as relative activity in insulin-treated cells compared with unstimulated cells (n = 3). **, p < 0.01 versus unstimulated control.

Our findings indicated that the Raf/ERK pathway and its activation are required for insulin-induced production of TNF-α in THP-1 macrophages; therefore, we next tried to determine whether insulin treatment leads to the direct activation of ERKs in THP-1 macrophages and whether PD98059 can inhibit this activation. We therefore determined ERK activity by measuring the radioactivity of substrates phosphorylated with 32P. Treatment of macrophages with 10⁻⁷ M insulin induced the activation of ERKs within the first 5 min of incubation; this activation increased until 15 min and decreased gradually thereafter until 30 min. However, this insulin-induced activation was completely blocked by PD98059. ERK activity decreased sharply within the first 5 min of co-incubation with insulin and PD98059 and continued to decline throughout the duration of the experiment. At 30 min, PD98059 treatment had reduced the ERK activity to 57% of the pretreatment levels, despite the presence of insulin (Fig. 7).

**FIG. 5.** Inhibitory effect of PD98059 on insulin-induced mRNA expression in THP-1-derived macrophages. THP-1-derived macrophages were incubated with media without serum for 8 h. After pretreatment with or without the inhibitors PD98059 (PD), SB203580 (SB), and wortmannin (WT) for 1 h, cells were stimulated with 10⁻⁷ M insulin for 6 h. Total RNA was isolated after insulin stimulation, and expression of TNF-α mRNA was analyzed by Northern hybridization. TNF-α RNA levels, normalized by comparison with levels of elongation factor-1α RNA, are shown (n = 4). Results are expressed as relative activity in insulin-treated cells compared with unstimulated cells (n = 3). **, p < 0.01 versus unstimulated control.

**FIG. 6.** Inhibitory effect of PD98059 on insulin-induced release of TNF-α in THP-1-derived macrophages. a, THP-1-derived macrophages were incubated in media without serum for 8 h. After pretreatment with or without the inhibitors PD98059 (PD), SB203580 (SB), and wortmannin (WT) for 1 h, cells were stimulated with 10⁻⁷ M insulin for 24 h. b, cells were pretreated with the inhibitors PD98059 (PD) and SB203580 (SB) for 1 h and then stimulated with or without 10⁻⁷ M insulin for 24 h. Supernatant was collected after 24 h of insulin stimulation, and TNF-α content was analyzed by ELISA. The amount of TNF-α was normalized to the value of total cell protein (n = 5). *, p < 0.05 versus control; **, p < 0.01 versus control.

**DISCUSSION**

Current studies demonstrate novel effects of insulin action on the gene expression and protein production of TNF-α in macrophages differentiated from human monocyte or monocytic THP-1 cells, which have been shown to be differentiated into mature cells with macrophage functions by treatment with reagents such as phorbol ester (17, 22). There have not been many reports on the influence of insulin on gene expression in cultured macrophages, but using a membrane array system, we observed the induction of some genes as shown in Table I, including TNF-α, in THP-1 macrophages after incubation with insulin. We also confirmed that the expression of TNF-α mRNA was up-regulated by insulin treatment using Northern blot analysis. TNF-α is a potent inflammatory mediator secreted from macrophages, and it is important in many macrophage-associated reactions such as immune and inflammation processes (1–3). Some inflammatory mediators such as bacterial LPS or interferon-γ have also been reported to mediate TNF-α release from primary macrophages and macrophage cell lines.
(19, 20). In particular, LPS, which is a powerful inducer of TNF-α release in macrophages, has been reported to increase TNF-α production by regulating mRNA expression directly in macrophages (23). We therefore expected that the insulin-stimulated increase in TNF-α mRNA in macrophages would lead to a corresponding increase in production and release of TNF-α protein, as we have shown in this study. Furthermore, we have investigated the effect of insulin on TNF-α production in other types of cells (3T3-L1 adipocytes and vascular smooth muscle cells in which TNF-α is synthesized); however, we could not find any significant increase in TNF-α production in these cells.

The signaling pathway that leads to the induction of TNF-α gene and protein in insulin-stimulated macrophages has not been reported. However, it has been reported that some protein kinases are required for stimulation of the biosynthesis of cytokines in monocytes and macrophages (24–26). In addition, recent data indicate that MAPKs play a key role in the induction of cytokine production and mRNA expression in macrophages, and, in particular, ERK-1 and ERK-2 (also called p44 and p42 MAPK, respectively) have been reported to be necessary for the stable or inducible expression of TNF-α in macrophages by some reagents, such as LPS and macrophage colony-stimulating factor (27, 28). Our results showed that ERK plays an important role in the basal biosynthesis of TNF-α in THP-1 macrophages, whereas p38 MAPK may have only a minor role.

Previously, several hormones such as growth hormone, progesterone, α-melanocyte-stimulating hormone, and insulin-like growth factor I (IGF-1) have been found to regulate TNF-α production in macrophages (29–32). Most of these factors have been reported to inhibit TNF-α synthesis, but insulin-like growth factor I induces TNF-α synthesis in murine macrophages via its own receptor (29). On the other hand, the effect of insulin on TNF-α production in macrophages has not been fully investigated. Our results, which show that insulin enhanced TNF-α production at a concentration of 10−8 to 10−6 M, indicate that insulin may directly regulate TNF-α production in macrophages and that insulin may not function effectively through the IGF-1 receptor because the affinity of insulin for the IGF-1 receptor is 1000 times less than that of IGF-1 (33). However, the signal of insulin at high concentrations may partially be mediated through the insulin-like growth factor I receptor pathway.

People with insulin resistance acquire a cluster of coronary risk factors including obesity, hypertension, dyslipidemia, and glucose intolerance. In the past decade, many reports have suggested that hyperinsulinemia associated with insulin resistance might be a risk factor in atherosclerotic diseases such as coronary heart disease (9–11), which is one of the major causes of death in such patients (34). Macrophages play an important role in the initial stages of atherosclerosis by taking up modified low density lipoprotein, which is accompanied by a morphological change into foam cells and secretion of various cytokines (4, 35, 36). In addition, many cholesterol-enriched foam cells have been observed to be a characteristic aspect of the developed atherosclerotic lesions (6). Here we have attempted to determine the effect of insulin action on macrophage function to gain insights into the pathogenesis of atherosclerosis in lesion development, and we have shown that high concentrations of insulin within physiological concentrations increase TNF-α biosynthesis in macrophages. TNF-α induces various chemotactic proteins and growth factors of vascular endothelial and smooth muscle cells, such as matrix metalloproteinases, vascular cell adhesion molecule 1, and heparin-binding epidermal growth-factor-like growth factor (37–40), which are involved in the migration and adhesion of cells that promote the development of atherosclerosis (41). Moreover, in atheromatous plaques, TNF-α mRNA has been confirmed by hybridization techniques to be synthesized by macrophages (42). These findings indicate that TNF-α secreted from macrophages may contribute to the pathophysiology of the atherosclerotic plaque.

In conclusion, we have demonstrated that the expression and biosynthesis of TNF-α are induced by insulin through the ERK-MAPK pathway in THP-1 macrophages.
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