Insulin Inhibits Apoptosis of Macrophage Cell Line, THP-1 Cells, via Phosphatidylinositol-3-Kinase–Dependent Pathway

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Abstract—Hyperinsulinemia has recently been reported as a risk factor for atherosclerotic diseases such as coronary heart disease; however, its precise mechanism is not well understood. To elucidate the role of insulin in the development of atherosclerosis, we have investigated the effect of insulin on cell survival in macrophages, which are known to be important in the atherosclerotic process. Apoptosis was induced in macrophage cell lines derived from human monocytes or murine macrophages by serum starvation. Insulin administration retarded macrophage apoptosis by means of DNA laddering, dimethylthiazol diphenyltetrazolium bromide assay, and annexin V binding assay. Insulin also enhanced mRNA expression and protein production of the antiapoptotic Bcl-XL gene in a dose-dependent manner within the range of physiological concentrations. In the exploration of the signaling pathway involved in these antiapoptotic effects of insulin, pretreatment of cells with a specific inhibitor of phosphatidylinositol-3-kinase significantly suppressed insulin-mediated cell survival and insulin-induced Bcl-XL expression in macrophages. These data indicate that the survival effect of insulin on the apoptosis of macrophages is associated with the upregulation of Bcl-XL expression, and it may be mediated through the phosphatidylinositol-3-kinase signaling pathway. These mechanisms could be involved in the possible role of insulin in the development of atherosclerosis. (Arterioscler Thromb Vasc Biol. 2002;22:380–386.)

Key Words: insulin ■ apoptosis ■ macrophages ■ Bcl-X ■ phosphatidylinositol-3-kinase

Macrophages play important roles in host defense and inflammation; their functions include the secretion of various cytokines in physiological responses. In addition, macrophages also play a significant role in the phagocytosis of bacteria and denatured substances, and in this phagocytotic process, macrophages aggravate the development of atherosclerosis by taking up and degrading modified LDL via their scavenger receptors. In fact, many macrophage foam cells/macrophages can be observed in atherosclerotic lesions, and microscopy has shown that early lesions of atherosclerosis are characterized by the infiltration of monocytes/macrophages, the proliferation of medial smooth muscle cells, and the presence of macrophage foam cells.

Diabetes mellitus is a strong risk factor in all manifestations of atherosclerotic vascular diseases. However, the pathophysiological mechanism of atherosclerotic development in diabetic patients has not yet been elucidated fully. Several prospective studies in nondiabetic and diabetic patients have shown that there is an association between hyperinsulinemia resulting from insulin resistance and atherosclerotic disorders such as cardiovascular disease, which is one of the major causes of death in such patients. In the past decade, much evidence has indicated that high-insulin conditions cause atherosclerosis in patients with high insulin resistance. In particular, the insulin-resistant state associated with hyperinsulinemia is often clustered with other risk factors, including hypertension, glucose tolerance, and hypertriglyceridemia; thus, the risk of cardiovascular disease increases, presumably because of the promotion of atherosclerosis. How atherosclerosis develops in patients with hyperinsulinemia remains unclear, but some reports have demonstrated that insulin has a direct effect on cells (e.g., arterial endothelial cells and vascular smooth muscle cells), suggesting that insulin accelerates atherosclerotic lesion development. In contrast, the action of insulin on macrophages (in particular, the effects of insulin on the apoptosis of macrophages) has been poorly investigated, despite the fact that these cells are involved in the initiation and development of atherosclerosis.

Insulin is a metabolic hormone that acts on various cells via its receptor to reduce blood glucose. It has also been shown that insulin can rescue many types of cells from apoptotic cell death and that these antiapoptotic effects of insulin and some hormones are mediated by activation of the phosphatidylinositol-3 (PI3)-kinase signaling pathway. In addition, the expression of antiapoptotic proteins, such as

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380
B-cell lymphoma-2 (Bcl-2) protein families, has been suggested to be involved in the antiapoptotic effects of these factors. In particular, Bcl-X, which is a member of the Bcl-2-related protein family, plays a significant role in the regulation of apoptosis in many types of hematopoietic cells, including macrophages.

Recently, numerous studies have demonstrated that cells can die in atherosclerotic plaques through apoptosis, and they have suggested that apoptosis of macrophages could be involved in the atherosclerotic process. In the present study, to gain insight into the direct effect of insulin on macrophages, we have examined whether insulin can affect apoptosis in macrophages that have been serum-starved to induce apoptosis. We show that insulin inhibits apoptosis in these cells and that insulin increases mRNA and protein expression of the apoptosis-regulatory protein Bcl-X subtype, Bcl-XL. We also show that the signaling pathway of these effects of insulin in macrophages involves PI3-kinase.

**Methods**

**Materials**

RPMI 1640, phorbol 12-myristate 13-acetate (PMA), dimethylthiazol diphenyltetrazolium bromide (MTT), and wortmannin were purchased from Sigma Chemical Co. PD98059 was purchased from Calbiochem. Human insulin was obtained from Roche. Rabbit polyclonal antibody against Bcl-X and goat polyclonal antibody against lamin B were purchased from Santa Cruz Biotechnology. Anti-phosphoryrosine antibody (4G10) was from Upstate Biotechnology. Anti-insulin receptor antibody (29B4) and anti-insulin-like growth factor-1 (IGF-1) receptor antibody (aIR3) were purchased from Oncogene Science.

**Cell Culture**

The human THP-1 monocytic leukemia cell line was purchased from Dainippon Pharmaceuticals and grown in RPMI 1640 culture medium containing 10% FBS. The differentiation of THP-1 cells was achieved by resuspending the cells in medium with the addition of PMA (40 ng/mL) for 2 days. Such a treatment with PMA has been shown to induce the conversion of THP-1 into mature cells that function as macrophages.

The murine macrophage cell line RAW 264.7 was a gift from Dr Nakauchi (Tsukuba University, Ibaraki, Japan) and was grown in DMEM culture medium containing 10% FBS.

**DNA Fragmentation Assay**

Cells (2x10⁶) lysed in 200 μL lysis buffer (10 mMol/L Tris, pH 7.4, 10 mMol/L EDTA, and 0.5% Triton X-100) were treated with RNase A and then proteinase K. The nuclear DNA in the supernatant was isolated by isopropanol precipitation. DNA samples resuspended in 10 mMol/L Tris-HCl (pH 7.4) were electrophoresed on agarose gel (0.8%) and stained with SYBR Green I (Wako). The autoradiographs of phosphorylated phosphatidylinositol were detected with the BAS2000 Bioimaging Analyzer (Fuji Film).

**MTT Assay**

THP-1 cells were differentiated in 24-well plates (2x10⁶ cells per well). After differentiation, cells were deprived of serum for 8 hours and treated with the indicated chemicals for 24 hours. MTT (5 mg/mL) was added for the last 3 hours of incubation, and then the medium was removed. The converted MTT dye in the cells was solubilized with 0.04N HCl in absolute isopropanol. Absorbance of dye was measured at a wavelength of 570 nm with background subtraction at 690 nm.

**Annexin V Binding Assay**

To assess the cellular apoptosis, 2x10⁶ cells were stained with annexin V by using the Annexin-V-FLUOS staining kit (Roche Diagnostics) according to the manufacturer’s instructions. To exclude necrotic cells from the analysis, cells were also incubated with propidium iodide. Cells were analyzed (10 000 cells per sample) on a FACSscan (Beckton Dickinson) by using CELLQUEST flow cytometric analysis software. Propidium iodide–positive cells are necrotic and were excluded from analysis.

**RNA Analysis**

Total RNA prepared from the cells was transferred to a nylon membrane after electrophoresis on a formaldehyde-containing 1% agarose gel. To confirm the subtype of Bcl-X expressed in THP-1 macrophages and to construct the probes for hybridization, polymerase chain reaction (PCR) with reverse transcription (RT-PCR) was performed. Total RNA extracted from THP-1 macrophages was reverse-transcribed and amplified by using primer pairs as follows: 5′-GGAGGCGGGAGCTGAATG 3′ and 5′-GGTGGAGGGTAGAGTGTTG 3′ (GenBank, accession Nos. Z23115 and Z23116). Human cDNA of the antiapoptotic gene Bcl-2 was a gift from Dr Tsujimoto (Osaka University, Osaka, Japan), and a human cDNA fragment of Bax was generated by RT-PCR with the use of primer pairs as follows: 5′-TCAAGGCAAGCAAGTTTGAG 3′ and 5′-GGTGGAGGGTAGAGTGTTG 3′ (GenBank, accession No. L22473). Each cDNA fragment was labeled with 32P and hybridized to the membrane. The autoradiographs were quantified by densitometry with the BAS2000. Each densitometric result of mRNA was normalized to the densitometric result of 28S RNA or elongation factor 1α mRNA in the same sample.

**Western Blot Analysis**

THP-1 macrophages were lysed by PBS containing 1% NP-40, 0.5% sodium deoxychylate, 0.1% SDS, and 1 mMol/L phenylmethylsulfonyl fluoride. Each sample containing equal protein mass was separated on 13.5% SDS-PAGE and electrotransferred to a polyvinyldene difluoride membrane. The membrane was incubated with an antibody specific for Bcl-X for 1 hour at room temperature. Immunoreactive signal was detected by using the ECL chemiluminescence detection system (Amersham), and each result was normalized to the result of lamin B in the same sample.

**Statistical Analysis**

The results are expressed as mean±SD, and the group means are compared with the Student t test. The accepted level of significance was set at P<0.05.

**Results**

**Insulin Treatment Inhibits THP-1 Macrophages From Apoptosis**

We examined whether insulin administration could rescue THP-1 macrophages from apoptosis by a DNA fragmentation assay. Cells were incubated for 8 hours in a serum-free medium and then placed in fresh serum-free medium in the absence or presence of 10⁻⁷ mol/L insulin for the indicated times (Figure 1a). Apoptosis is accompanied by the rapid

Iida et al
cleavage of extranuclear DNA into multiples of 180 bp, and DNA from apoptotic cells forms a 180-bp ladder on electrophoresis gels. We found that DNA from cells treated with or without insulin was uncleaved after 24 hours of incubation. After 36 hours, DNA from cells that were untreated showed ladder formation, whereas DNA from insulin-treated cells was still uncleaved. After 48 hours, DNA from insulin-treated cells was partially cleaved, but the DNA from untreated control cells was fragmented completely. A similar result was also observed in RAW 264.7 cells after 24 hours of incubation in the absence or presence of insulin (Figure 1b). These findings suggest that insulin can retard the apoptosis of macrophages.

PI3-Kinase Is Involved in Antiapoptotic Effect of Insulin in THP-1 Macrophages

We examined the involvement of PI3-kinase in the antiapoptotic effect of insulin in macrophages. First, we determined whether insulin treatment leads to the direct activation of PI3-kinase in THP-1 macrophages. Induction of PI3-kinase activity was determined by measuring the radioactivity of phosphatidylinositol that had been phosphorylated with $^{32}$P-labeled ATP. The results showed that the treatment of cells with insulin at concentrations of $10^{-7}$ to $10^{-8}$ mol/L for 15 minutes potently stimulated PI3-kinase activity in THP-1 macrophages (Figure 2a). This activation was markedly inhibited by wortmannin, which is a specific inhibitor of PI3-kinase.

Next, to examine the involvement of PI3-kinase, we determined the effect of wortmannin on the antiapoptotic effect of insulin in THP-1 macrophages. As shown in Figure 2b, 100 nmol/L wortmannin abolished the ability of insulin to prevent DNA fragmentation. An inhibition of the antiapoptotic effect of insulin by wortmannin was also observed in RAW 264.7 cells (data not shown). These results suggest that the PI3-kinase pathway is involved in the antiapoptotic effect of insulin in macrophages.

To confirm the effect of insulin and the central role of PI3-kinase on insulin action in cell survival quantitatively, we also performed MTT assays, which measure mitochondrial activity and, hence, indicate cellular survival rate. The results showed that insulin affected cell survival in THP-1 macrophages in a dose-dependent manner after 24 hours of incubation (Figure 3a) and also after 36 hours of incubation. The absorbance of MTT dye in the supernatant, which is a direct indication of cell survival, was significantly decreased by serum starvation. Compared with no treatment, treatment with insulin increased the absorbance in cells, and this insulin-induced increase in the survival rate was significantly inhibited by 100 nmol/L wortmannin. The effect of insulin on cellular apoptosis and the involvement of PI3-kinase on insulin action were also assessed by annexin V staining. Similar results showing that insulin rescued THP-1 macrophages from apoptosis in a dose-dependent manner were observed, and wortmannin inhibited this antiapoptotic effect of insulin in THP-1 macrophages (Figure 3b).

Insulin Stimulates Expression of Apoptotic-Associated Bcl-XL Gene and Protein in THP-1 Macrophages in a PI3-Kinase–Dependent Manner

We examined the gene expression of Bcl-X, which is a member of the Bcl-2–related apoptosis-associated protein family, in THP-1 macrophages treated with insulin. Because 2 species of Bcl-X, Bcl-XL and Bcl-XS, are generated through alternative splicing, we initially determined the
expression of both subtypes in untreated macrophages by RT-PCR. PCR primers were selected for simultaneous detection of these 2 isoforms of Bcl-X. RT-PCR that was performed on RNA derived from THP-1 macrophages showed that the 462-bp Bcl-XL fragment was strongly amplified, whereas the 273-bp Bcl-XS fragment was barely detected (Figure 4a). This result showed that in THP-1 macrophages the predominant subtype of Bcl-X is Bcl-XL, irrespective of stimulation with insulin.

Next, we analyzed the effect of insulin on the expression of Bcl-XL in THP-1 macrophages (Figure 4b). Within 6 hours, expression of Bcl-XL was induced ∼1.6-fold by stimulation with 10⁻⁷ mol/L insulin compared with no insulin stimulation (control). This increase was sustained for 24 hours after stimulation, and the mRNA levels remained ∼1.5-fold higher than control levels. In contrast, the expression of 2 other genes, the antiapoptotic gene Bcl-2 and the apoptosis-accelerating gene Bax, had not changed after stimulation with insulin.

Because we had shown that the PI3-kinase pathway is involved in the mechanism of insulin inhibition of apoptosis, we next determined whether wortmannin would affect the insulin-stimulated induction of Bcl-XL mRNA. In cells treated with insulin and wortmannin, compared with cells treated only with insulin, Bcl-XL mRNA expression was inhibited by a 25% decrease (Figure 5a). In contrast, PD98059, which is the specific inhibitor of extracellular signal-regulated kinase (ERK), had no significant effect on insulin-induced Bcl-XL expression.

We also performed Western blotting to determine whether there was an increase of Bcl-X protein production corresponding to the increase in mRNA. Insulin stimulation for 24 hours in THP-1 macrophages significantly increased the production of 29-kDa Bcl-XL protein in a dose-dependent manner, as was predicted from the results shown in RNA analysis. In addition, the treatment of THP-1 macrophages with 100 nmol/L wortmannin significantly inhibited the insulin-stimulated induction of Bcl-XL, which remained at basal levels of expression (Figure 5b).

**Insulin Action on Apoptosis and Bcl-XL Expression and Its Specific Receptor in THP-1 Macrophages**

It is possible that insulin at a high concentration acts not only through its own receptor but also through an IGF-1 receptor. To investigate which receptor is mainly involved in the effects of insulin observed in THP-1 macrophages, we used a neutralizing antibody against each receptor: an antibody against the insulin receptor (29B4) and an antibody against the IGF-1 receptor (αIR3). As shown in Figure 6a, the antiapoptotic effect of insulin assessed by annexin V staining was significantly attenuated by the addition of 2 μg/mL 29B4. By contrast, αIR3 at the same concentration did not
Insulin affects the inhibitory effect of insulin on apoptosis. Similar results were observed in Bcl-XL protein production. Although αIR3 had no significant effect on Bcl-XL production, the addition of 29B4 significantly inhibited the insulin-induced increase of Bcl-XL protein production in THP-1 macrophages (Figure 6b). A normal mouse IgG as a control had no significant effects on the result of annexin V staining. These results suggest that the antiapoptotic effect of insulin associated with an increase of Bcl-XL production is mainly mediated through the insulin receptor in THP-1 macrophages.

Discussion

Insulin is well known as an anabolic hormone that stimulates the translocation of several transporters to the plasma membrane, resulting in a reduction in blood glucose. It is also a potent growth factor and antiapoptotic factor that regulates gene expression in various cells.11–13 In the present study, to investigate the effect of insulin on macrophages, we examined cell survival in macrophages differentiated from monocytic THP-1 cells. These cells have been shown to be differentiated into mature cells with macrophage functions after treatment with agents such as phorbol ester.24 Our results show that in THP-1 macrophages and also in the murine macrophage cell line (RAW 264.7 cells), insulin inhibited cell death induced by serum starvation in a dose-dependent manner. The results from DNA fragmentation assays indicate that insulin may inhibit apoptosis in these cells.

The molecular mechanisms that govern apoptosis have not been elucidated completely; however, it has been suggested that the members of the Bcl-2-related protein family play key roles in the regulation of apoptosis in a number of cell types.26–28 Bcl-XL, a member of the Bcl-2 family, is a potent repressor of apoptosis28 and is important in protecting various hematopoietic progenitor cells and hemocytic mature cells, including macrophages from apoptosis.18–20 It is also shown that Bcl-XL is implicated in the antiapoptotic effect of some growth factors, such as IGF-1.29 We found that THP-1 macrophages express Bcl-XL mRNA and that treatment of macrophages with insulin at concentrations that inhibited cell death induced by serum starvation upregulated Bcl-XL mRNA and protein expression. The antiapoptotic regulator Bcl-2 has also been reported to be upregulated by IGF-1 and interleukin-4 to promote cell survival in other cells; however, we found that although Bcl-2 was expressed in THP-1 macrophages, it was not regulated by insulin. These findings suggest that insulin inhibits macrophage cell death by inducing an increase in Bcl-XL production, thereby preventing apoptosis.

Recent data indicate that protein tyrosine kinases are required for the promotion of cell survival by some growth factors, including insulin. In particular, PI3-kinase has been reported to play a key role in the signaling pathway of the antiapoptotic effects of insulin and other growth factors in some cell types.14,15 It has also been reported that PI3-kinase is required for the regulation of the gene expression of...
members of the Bcl-2 protein family. We hypothesized that the insulin receptor PI3-kinase–dependent signaling pathway is involved in the insulin effect on the inhibition of apoptosis and the induction of Bcl-XL synthesis in macrophages, and our data in the present study are consistent with this idea. The antiapoptotic effect of insulin on macrophages is significantly blocked by an antagonistic antibody against the insulin receptor or by wortmannin, a specific inhibitor of PI3-kinase. Also, the increase in Bcl-XL production induced by insulin was inhibited similarly. These findings suggest that the involvement of the PI3-kinase signaling pathway in the antiapoptotic mechanism of insulin is associated with the regulation of Bcl-XL expression. Insulin has 2 distinct signaling pathways: the PI3-kinase pathway described above and the ERK (a kind of mitogen-activated protein kinase) pathway. Therefore, we examined the potential involvement of ERK in the insulin-stimulated biosynthesis of Bcl-XL by using PD98059, the specific inhibitor of ERK; however, the results showed that this reagent had no significant effect on Bcl-XL expression in insulin-stimulated THP-1 macrophages.

In the present study, our data link the antiapoptotic effect of insulin in THP-1 macrophages with PI3-kinase, whereas THP-1 cells have been reported to lack the insulin receptor substrate (IRS) subtypes, IRS-1 and IRS-2, which are important for signal transduction between the insulin receptor and PI3-kinase. However, by measuring the radioactivity of 32P-phosphorylated phosphatidylinositol, we demonstrated that insulin activated PI3-kinase in THP-1 macrophages despite lacking these IRSs. We examined whether the expression of IRS proteins can be induced on the differentiation of THP-1 cells; however, neither IRS-1 nor IRS-2 was detected in insulin-stimulated THP-1 macrophages by Western blot analysis in immunoprecipitation (data not shown). These results indicate the possibility that there is another protein that assists the insulin-induced PI3-kinase activation in THP-1 macrophages. Recently, several novel substrate proteins that mediate interactions between the insulin receptor and PI3-kinase have been reported. These proteins might be involved in the insulin–PI3-kinase signaling pathway in THP-1 cells.

During the past decade, accumulating evidence has shown that hyperinsulinemia has a causal role in the development of atherosclerosis. Therefore, we examined the effect of insulin on macrophages, which are important in the development of atherosclerosis, to understand the pathogenesis of atherosclerosis. We found that insulin inhibits the apoptosis of macrophages at high, but still physiological, concentrations. These findings may contribute to the development of atherosclerosis in patients with hyperinsulinemia.

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