



Leptin and high glucose stimulate cell proliferation in MCF-7 human breast cancer cells: reciprocal involvement of PKC- α and PPAR expression

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Abstract

Glucose concentration may be an important factor in breast cancer cell proliferation, and the prevalence of breast cancer is high in diabetic patients. Leptin may also be an important factor since plasma levels of leptin correlated with TNM staging for breast cancer patients. The effects of glucose and leptin on breast cancer cell proliferation were evaluated by examining cell doubling time, DNA synthesis, levels of cell cycle related proteins, protein kinase C (PKC) isozyme expression, and peroxisome proliferator-activated receptor (PPAR) subtypes were determined following glucose exposure at normal (5.5 mM) and high (25 mM) concentrations with/without leptin in MCF-7 human breast cancer cells. In MCF-7 cells, leptin and high glucose stimulated cell proliferation as demonstrated by the increases in DNA synthesis and expression of cdk2 and cyclin D1. PKC- α , PPAR γ , and PPAR α protein levels were up-regulated following leptin and high glucose treatment in drug-sensitive MCF-7 cells. However, there was no significant effect of leptin and high glucose on cell proliferation, DNA synthesis, levels of cell cycle proteins, PKC isozymes, or PPAR subtypes in multidrug-resistant human breast cancer NCI/ADR-RES cells. These results suggested that hyperglycemia and hyperleptinemia increase breast cancer cell proliferation through accelerated cell cycle progression with up-regulation of cdk2 and cyclin D1 levels. This suggests the involvement of PKC- α , PPAR α , and PPAR γ .

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1. Introduction

Plasma leptin concentrations may be an important factor in breast cancer cell proliferation. Because it has been reported that a strong and statistically significant inverse association of leptin levels with breast cancer prevalence in premenopausal women [1] and plasma leptin levels correlate with TNM staging [2]. Leptin is a 16 kDa protein encoded by the *ob* gene and is secreted from adipocytes [3].

Although the putative role of leptin appears to control satiety by providing information to the central nervous system, the function of leptin was demonstrated to be more complex [4–6] and its role has not been fully elucidated in various cell functions. Since epidemiological studies reveal that obesity has profound effects on breast cancer risk [7,8], leptin could be a plausible factor contributing to the pathogenesis of breast cancer. Furthermore, a recent report suggests the possible use of plasma leptin levels as a clinical marker of breast cancer [2]. Although leptin expression in breast tumors has been demonstrated [9], its role in breast cancer cell proliferation is not well understood.

Glucose concentrations may also be an important factor in breast cancer cell proliferation because the prevalence of breast cancer is high in diabetic patients [10–13]. We reported that MCF-7 human breast cancer cell proliferation was increased when glucose concentration in the culture medium was increased [14].

Abbreviations: cdk, cyclin-dependent kinases; TNF- α , tumor necrosis factor- α ; PKC, protein kinase C; cPKC, classical PKC; nPKC, novel PKC; aPKC, atypical PKC; PPARs, peroxisome proliferator-activated receptors; MDR, multidrug resistance; FBS, fetal bovine serum; DPBS, Dulbecco's phosphate buffered saline; PMSE, phenylmethylsulfonyl fluoride; TBS, Tris-buffered saline; TBS-T, TBS containing 0.1% Tween-20; ECL, enhanced chemiluminescence; SD, standard deviation

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Cyclin-dependent kinases (cdks) are enzymes responsible for the coordinated progression of the cell cycle [15]. Of nine cdks, cdk2 is involved in the control of cell cycle progression from G1 to S phase. Cdks are activated by association with D-type cyclins (cyclin D1, D2, and D3), and cyclin D1 has an important role in G1 phase of cell cycle progression in association with cdk2 [16]. Cyclin D1 is a rate-limiting activator of G1-to-S phase transition [17,18], since changes in cyclin D1 and cdk2 expression might reflect G1-to-S phase transition rate. Hence, we examined cyclin D1 and cdk2 expression following glucose exposure to demonstrate G1-to-S phase transition. In MCF-7 cells, tumor necrosis factor- α (TNF- α) has anti-mitogenic activity in early G1 phase as reflected by a decreased number of cells in S phase [19]. Therefore, we treated MCF-7 cells with TNF- α as a negative control of G1-to-S phase transition in the process of cell proliferation.

Protein kinase C (PKC) is a complex family of at least 11 isozymes classified into three groups: classical PKCs (cPKC) (PKC- α , - β I, - β II, and - γ), which require diacylglycerol (DAG), phospholipids and Ca^{2+} for full activity; novel PKCs (nPKC) (PKC- δ , - ϵ , - η , and - θ), which are phospholipid and DAG-dependent but Ca^{2+} -independent; and atypical PKCs (aPKC) (PKC- ζ , - (ι) λ , and - μ), which require only phospholipids [20–22]. There is significant but indirect evidence to suggest that one or more of the individual PKC isozymes may play a major role in cell growth and differentiation [23]. Alterations in the intracellular concentration of certain PKC isozymes induce cell differentiation [24]. In addition, we have demonstrated that high glucose-induced decreases of PKC- β II protein and mRNA levels might be important in response to high glucose levels [14]. Cellular PKC- β II levels may have to fall below a threshold for stimulation of cell proliferation. In some cells, glucose-induced decrease in PKC- β II level may not fall below this threshold for stimulation of cell proliferation. This can explain the differences in the proliferative response of different cells to high glucose medium. PKC isozymes may have various roles in the regulation of breast cancer cell proliferation, however, the relationship between PKC function and regulation of cell proliferation has not been fully elucidated. We studied the effects of high glucose and leptin on expression of PKC- α , - θ , and - ζ , representing cPKC, nPKC, and aPKC, families of isozymes, respectively.

Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors that can be activated by a variety of compounds such as thiazolidinediones and fatty acids [25–28]. Three PPAR subtypes, designated PPAR α , PPAR β and PPAR γ , have been described [25]. Although PPAR γ has been reported to have a role in the breast cancer cell differentiation [29], the precise functions of PPAR in regulation of cell cycle related to control of cell proliferation have not been studied. We examined the effect of high glucose and leptin on expression of PPAR α and γ .

On the basis of epidemiological data and our previous study [14], we now describe the effects of leptin and glucose

on MCF-7 human breast cancer cell proliferation, and the expression of PKC isozymes and PPARs. Furthermore, we examined the effects of high glucose and leptin on cell proliferation and expression of PKC isozymes and PPARs were evaluated in two unrelated cell lines, MCF-7 cells and NCI/ADR-RES [30,31], in order to detect cell line related difference to glucose and leptin.

2. Materials and methods

2.1. Cells and cell culture

Drug-sensitive MCF-7 human breast cancer cells and multidrug-resistant NCI/ADR-RES human breast cancer cells were obtained from Dr. P.B. Desai, University of Cincinnati, College of Pharmacy, OH. Multidrug-resistant NCI/ADR-RES cells were developed by Dr. K.H. Cowan of NCI, Bethesda, MD. Both cell lines were passaged in RPMI 1640 media (Gibco-BRL, Gaithersburg, MD) supplemented with 5% fetal bovine serum (FBS) (Gibco-BRL), 50 U/ml penicillin G and 50 $\mu\text{g}/\text{ml}$ streptomycin sulfate, and 5.5 mM glucose at 37 °C in a humidified, 5% CO_2 , 95% air atmosphere. Cells were subcultured weekly to maintain logarithmic growth in 6-well plates. NCI/ADR-RES was passaged in drug-free media for at least eight passages before testing. We compared the effects of leptin and glucose on proliferation of MCF-7 and NCI/ADR-RES cells to identify cell line-related differences. Although NCI/ADR-RES cells were previously referred to as MCF-7/ADR cells, it must be emphasized that they are not genetically related to, or derived from MCF-7 human breast cancer cells [30,31].

2.2. Cell proliferation

Cells in secondary cultures were maintained in RPMI 1640 containing 5% FBS with 5.5 mM glucose. Cells were trypsinized and seeded on 6-well plates (Costar, Cambridge, MA) at 1×10^4 cells/well in 3 ml of RPMI 1640 containing 5% FBS with 5.5 or 25 mM glucose. Leptin (Pepro Tech EC, London, UK) and TNF- α (Pepro Tech EC) were added to achieve a final concentration of 1 μM per well. Since TNF- α suppressed cell proliferation through down-regulation of cell cycle-related proteins in MCF-7 cells [19], we treated cells with TNF- α as a negative control. To count cell number, cells were washed with Dulbecco's phosphate buffered saline (DPBS) (Gibco-BRL), trypsinized for 3 min at 37 °C, and centrifuged at $350 \times g$ for 5 min to remove trypsin. The cell pellet was resuspended with DPBS containing 0.4% trypan blue (Gibco-BRL) for viable cell counts with a haemocytometer. Total viable cell counts were performed every day without changing media for up to 6 days.

To examine the effect of leptin concentration on the proliferation of cultured MCF-7 cells, cells were seeded at approximately 1×10^4 cells/well in 3 ml of RPMI 1640 containing 5% FBS with 5.5 mM glucose. MCF-7 cells were

maintained in media in the absence or presence of varying concentrations of leptin at 10^{-9} to 10^{-5} M per well. Total viable cell counts were determined every day without changing media for up to 6 days using a different culture each day from set of replicate cultures as described above.

2.3. DNA synthesis assay

DNA synthesis was determined by measuring the [3 H]-thymidine (dThd) incorporation into DNA of MCF-7 and NCI/ADR-RES cells cultured in media containing 5.5 and 25 mM glucose. The effect of leptin and TNF- α on DNA synthesis were also evaluated. Cultures were trypsinized and seeded at approximately 1×10^4 cells/well in 6-well plates with 3 ml of the same medium. When cultures reached 80% confluence, they were incubated with RPMI 1640 with 5.5 or 25 mM glucose containing 5% FBS for 72 h. Leptin and TNF- α were added so that the final concentration of 1 μ M was achieved in each well for 24 h. Cells were then incubated with 3 ml of the same media containing 1.0 μ Ci/ml [3 H]dThd (Du Pont-NEN, Boston, MA) for 1 h at 37 $^{\circ}$ C. After incubation, cells were washed twice with ice-cold phosphate-buffered saline (0.15 M NaCl in 0.1 M K_2PO_4 buffer, pH 7.4), acid-soluble radioactivity was removed by a 20-min treatment with 5% trichloroacetic acid (TCA), and washed twice with ethanol. Cells were solubilized with 1 N NaOH and neutralized with 1 N HCl. The [3 H] radioactivity in the acid-soluble pools were determined by multipurpose scintillation counter LS6500 (Beckman Instruments, CA, USA). DNA synthesis was estimated as cpm/ml per hour.

2.4. Western blot analysis

The changes in immunoreactive cdk2, cyclin D1, PKC isozymes, and PPAR subtypes following 5.5 and 25 mM

glucose exposure with/without leptin or TNF- α were measured in MCF-7 and NCI/ADR-RES cells by Western blot analysis. Cell cultures maintained in RPMI 1640 containing 5% FBS with 5.5 mM glucose were trypsinized and seeded on 6-well plates at a density of 1×10^6 cells/well in 3 ml of the same medium. When cells were grown to 80% confluency, cells were incubated with serum-free RPMI 1640 media containing of 5.5 mM glucose for 72 h. Then, media were switched to RPMI 1640 containing 5% FBS with 5.5 or 25 mM glucose and incubated with 1 μ M of leptin or TNF- α for 3 days. Subsequently, cells were washed with ice-cold DPBS and scraped from dishes with a rubber policeman. The cell suspension was centrifuged at $350 \times g$ for 5 min to remove DPBS, and the cell pellet was lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 145 mM NaCl, 10% (w/v) glycerol, 5 mM EDTA, 0.2 mM Na_2VO_4 , 0.1 mM phenylmethylsulfonylfluoride (PMSF) (Sigma), 10 μ g/ml leupeptin (Sigma), 10 μ g/ml aprotinin (Sigma), and 1% (w/v) nonyl phenoxy polyethoxy ethanol (NP-40) (Sigma). The suspension was sonicated for 5 s, centrifuged at $350 \times g$ for 5 min, and the resultant supernatant was used as the whole-cell lysate fraction. The protein concentration was determined by the method of Bradford [32] with bovine serum albumin (BSA) as a standard using protein assay kit (Bio-Rad, Hercules, CA). All procedures were performed at 4 $^{\circ}$ C. The same amount (25 μ g) of protein of each sample was dissolved in Laemmli's sample buffer containing 1% SDS [33] and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 4.5% (w/v) stacking gels and 10% (w/v) resolving gels in a RAPIDAS mini slab (ATTO, Japan) and electrophoretically transferred to nitrocellulose membranes Hybond-P $^{\circ}$ (Amersham LIFE SCIENCE, Buckinghamshire, England). Membranes were equilibrated in Tris-buffered saline (TBS; 20 mM Tris-HCl and 500 mM NaCl, pH 7.40), and non-specific binding sites were blocked by 5% dried milk in TBS containing 0.1%

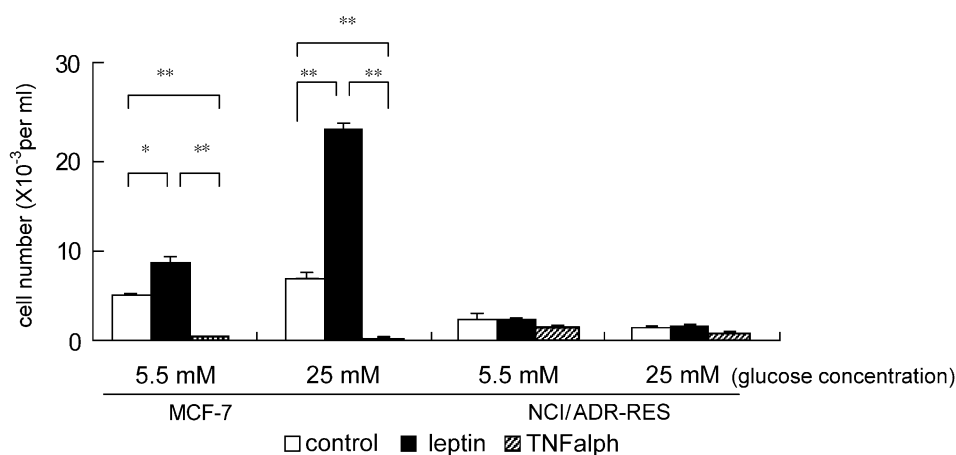


Fig. 1. The effects of leptin and glucose on cell proliferation were examined in MCF-7 and NCI/ADR-RES cells. Cells were seeded in 6-well plates at approximately 1×10^4 cells/well in 3 ml of RPMI 1640 containing 5% FBS with normal (5.5 mM) or high (25 mM) glucose concentrations with/without leptin or TNF- α . Total viable cell counts were determined daily using 0.4% trypan blue dye as described in Materials and methods. Data are the means \pm S.D. for triplicate determinations which were repeated in three separate experiments. * $P < 0.05$, ** $P < 0.01$ vs. control, $n = 3$.

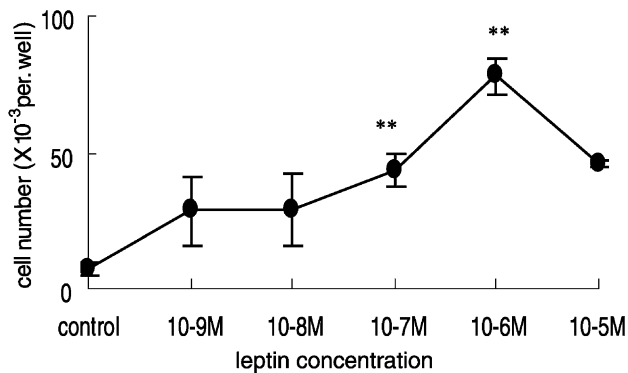


Fig. 2. Dose–response curve for leptin on MCF-7 cell proliferation. MCF-7 cells were maintained in the media for 5 days in various concentration of leptin. Data are the means \pm S.D. for triplicate determinations which were repeated in three separate experiments. ** $P < 0.01$ vs. control, $n = 3$.

Tween-20 (TBS-T) at room temperature for 1–2 h. After washing with TBS-T, membranes were incubated with in TBS containing the rabbit polyclonal antibodies to cdk2, cyclin D1, PKC- α , - θ , and - ζ , and PPAR α and γ (Santa Cruz, CA) (dilution ratio 1:1000) as primary antibody at room temperature for 3 h. After washing with TBS-T, the membrane was incubated in TBS-T containing a goat anti-rabbit γ -globulin coupled to horseradish peroxidase (Bio-Rad) (dilution ratio 1:1000) for 30 min. The blots were developed with the enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, IL), and visualized by exposure to Hyperfilm-ECL (Amersham). Specificity of protein bands was examined by blocking with antigens. The developed films were analyzed by a densitometric scanner linked to a Macintosh computer (Apple, Cupertino, CA).

2.5. Data analysis

Experiments were repeated separately at least three times to assure reproducible results. Results are expressed as the

means \pm standard deviation (S.D.) of the number of observations. The statistical significance was assessed by one-way analysis of variance (ANOVA). A P -value < 0.05 was considered statistically significant. Significance is determined after three or more separate experiments.

3. Results

3.1. Effect of leptin and glucose on MCF-7 cell proliferation

We examined MCF-7 and NCI/ADR-RES cell proliferation at two concentrations of glucose reflecting normal physiological glucose levels (5.5 mM) and hyperglycemic levels (25 mM) with/without leptin or TNF- α . Maximum MCF-7 and NCI/ADR-RES cell numbers were achieved following 5 days of culture in 6-well plates. In Fig. 1, MCF-7 and NCI/ADR-RES cell numbers on day 5 after seeding is shown. In physiological glucose, MCF-7 cell proliferation was increased compared to NCI/ADR-RES cells. In MCF-7 cells, proliferation was stimulated significantly by leptin exposure for 5 days with normal glucose, and increased more significantly as MCF-7 were grown in high glucose concentrations (Fig. 1). As reported previously [19], cells grown with TNF- α suppressed MCF-7 cell growth rate in normal and high glucose conditions. We had previously demonstrated that the osmotic control medium, 5.5 mM glucose with 19.5 mM mannitol, had no stimulatory effect on MCF-7 cell proliferation [14]. On the contrary, there were no significant differences in NCI/ADR-RES cell proliferation among cultures grown in normal and in high glucose concentration with leptin and TNF- α (Fig. 1).

We examined leptin-induced MCF-7 cell proliferation by varying concentrations of leptin with normal physiological glucose levels (5.5 mM) for 5 days. MCF-7 cell proliferation increased as the level of leptin concentration

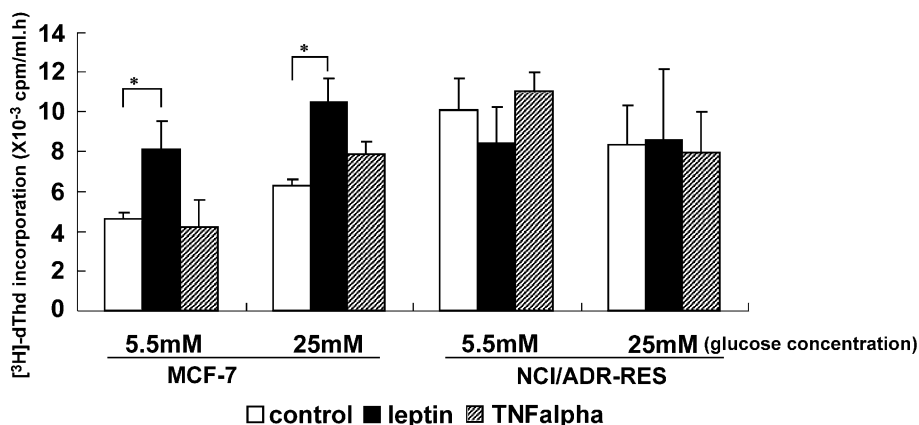


Fig. 3. DNA synthesis was determined by measuring [³H]-thymidine (dThd) incorporation into DNA in normal (5.5 mM) and high (25 mM) glucose with/without leptin or TNF- α in MCF-7 and NCI/ADR-RES. Cells were seeded in 24-well plates and subjected to serum-deprivation for 72 h. The cell cycle was initiated by switching to media containing serum, and [³H]-dThd incorporation was measured as described in Materials and methods. Data are the means \pm S.D. for triplicate determinations which were repeated in three separate experiments. * $P < 0.05$ vs. control (control), $n = 3$.

increased and maximal proliferation was observed at 10^{-6} M (Fig. 2).

3.2. Stimulatory effects of high concentration of glucose and leptin on [3 H]-thymidine (dThd) incorporation in MCF-7

To further examine the effects of glucose and leptin on cell proliferation, DNA synthesis was determined by dThd incorporation following serum addition to initiate the cell cycle in synchronized cells. In MCF-7 cells, the addition

of leptin caused an increase in dThd incorporation compared with the control. TNF- α did not change dThd incorporation regardless of glucose concentration (Fig. 3). However, leptin, glucose, and TNF- α had no significant effect on dThd incorporation in NCI/ADR-RES cells (Fig. 3).

To delineate whether leptin reduced cell death through the regulation of apoptosis, we examined morphologic changes by Hoechst 33258/propidium iodide (PI) staining, DNA fragmentation analysis, and cell death detection

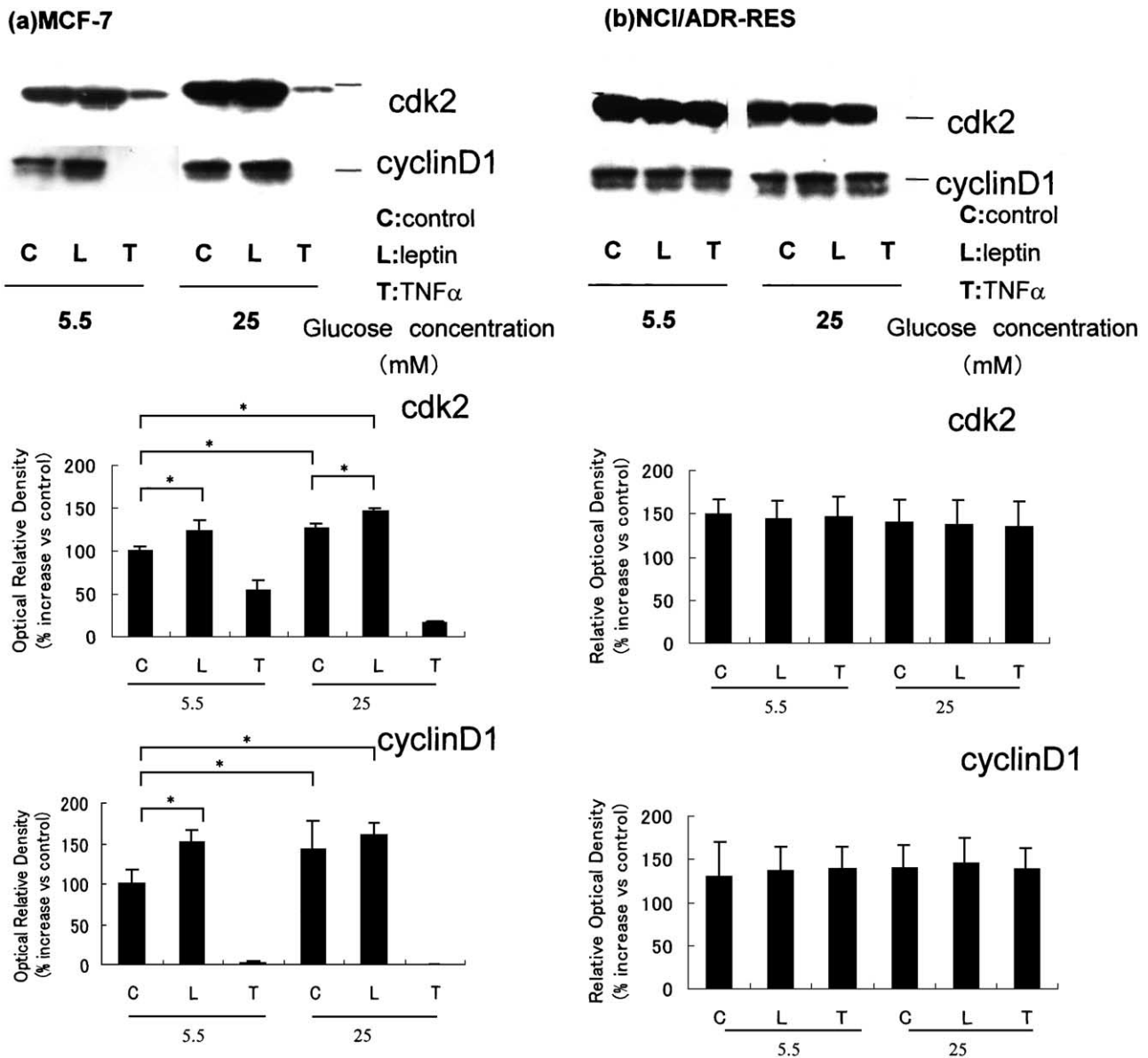


Fig. 4. Cyclin D1 and cdk2 immunoreactivity following leptin treatment of MCF-7 and NCI/ADR-RES cells was investigated. Cells were grown in 6-well plates, synchronized with serum-free media for 72 h, then the cell cycle was initiated by switching to media containing FBS with normal (5.5 mM) or high (25 mM) glucose concentrations and incubated with 1 μ M of leptin or TNF- α over a 3-day period. After incubation, cyclin D1 and cdk2 immunoreactivity in whole cell lysates was analyzed by Western blot using anti-cyclin D1- and cdk2-specific antibody (Santa Cruz) and analyzed by densitometric scanner as described in Materials and methods. Data are shown as relative optical density (control level in MCF-7; 100%). Data are the means \pm S.D. for triplicate determinations which were repeated in three separate experiments. * $P < 0.05$ vs. control (control), $n = 3$.

by ELISA assay in MCF-7 and NCI/ADR-RES cells. However, there were no significant effects of leptin on apoptosis or the expression of apoptotic markers such as Bax, Bcl-2, or Bcl-X_L (data not shown). Leptin induced MCF-7 cell proliferation without altering apoptosis. Further, leptin stimulated increase in dThd-incorporation into cells indicating that the mitotic cycle is accelerated in response to leptin.

3.3. Stimulatory effects of leptin and glucose on the acceleration of cell cycle demonstrated by the expression of cyclin D1 and cdk2 in MCF-7

Cdks and cyclin D1 are overexpressed as S phase checkpoints in the cell cycle [15,16], in MCF-7 cells [19]. Therefore, we examined if changes in cdk2 and cyclin D1 protein expression occurred with leptin, high glucose, and

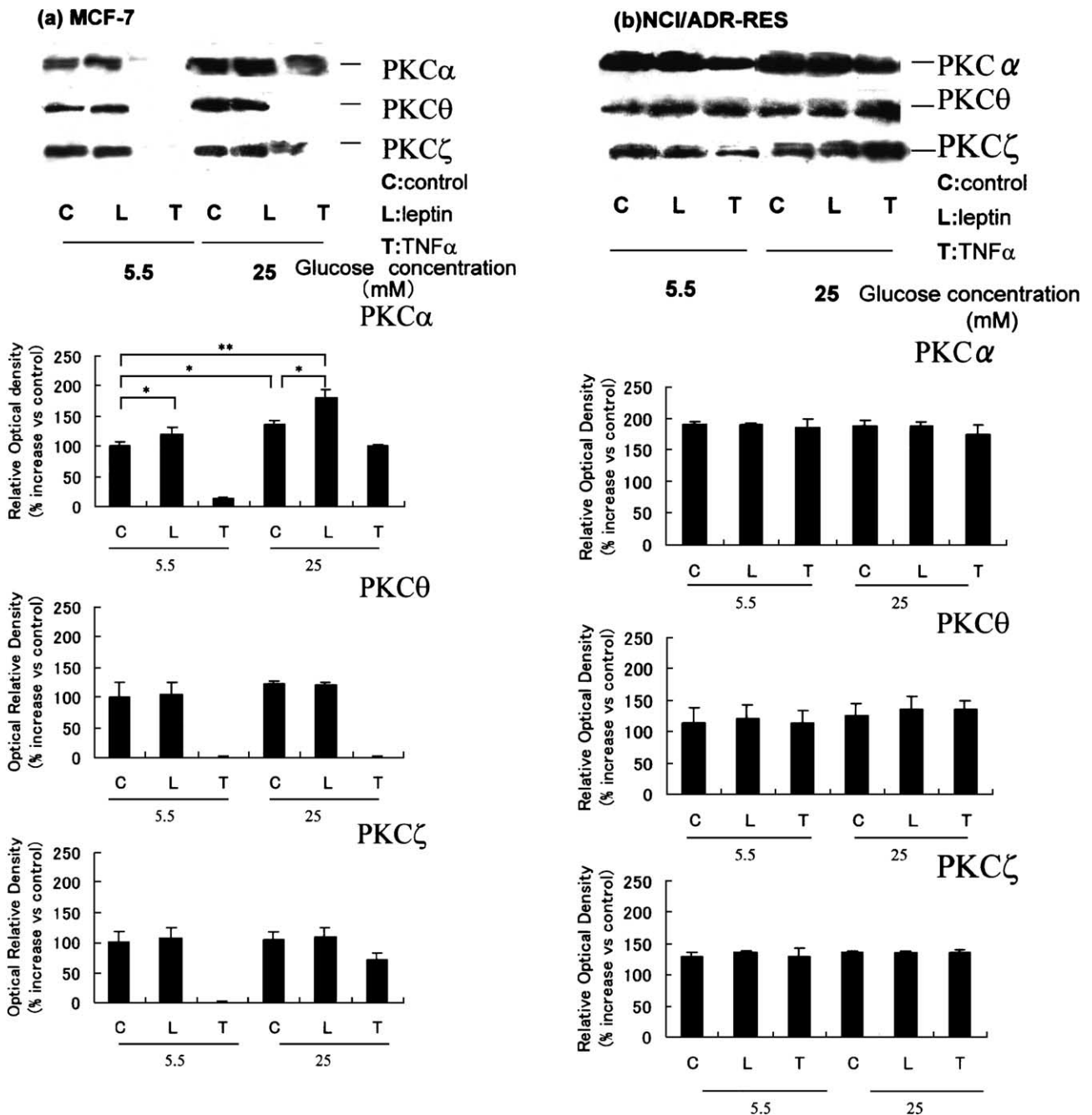


Fig. 5. PKC- α , - θ and - ζ immunoreactivity following leptin and high glucose treatment of MCF-7 and NCI/ADR-RES cells was investigated as described in Materials and methods. Data are shown as relative optical density (control level in MCF-7; 100%) analyzed by densitometric scanner. Similar results were obtained in three separate experiments. Data are the means \pm S.D. for triplicate determinations which were repeated in three separate experiments. * $P < 0.05$ vs. control (control), $n = 3$.

TNF- α treatment of MCF-7 and NCI/ADR-RES cells to relate possible changes in G1-to-S transition in cell cycle progression. Cyclin D1 and cdk2 protein levels increased with leptin and high glucose treatment and decreased with TNF- α treatment in MCF-7 cells, although these levels were not changed in NCI/ADR-RES cells (Fig. 4). Leptin and high glucose may have increased cell proliferation through acceleration of cell cycle with up-regulation of cell cycle-regulated proteins such as cyclin D1 and cdk2 in MCF-7 cells. However, in multidrug-resistant NCI/ADR-RES cells, the checkpoint of cell cycle regulation may be altered through modified functions of cell cycle-regulated proteins.

We used three methods to evaluate cell proliferation following increases in glucose levels and leptin treatment. In Fig. 1, we demonstrated variable cell numbers following glucose or leptin treatment for 5 days. In Fig. 3, DNA synthesis was measured by [3 H]-thymidine uptake for 1 h following glucose or leptin treatment. In Fig. 4, we evaluated cell cycle checkpoint protein expression to demonstrate cell cycle progression after 72 h treatment of glucose or leptin using Western blot analysis. When the methods are examined together, they present a cohesive description of glucose and leptin effects in MCF-7 cells, despite modest variations in their effect.

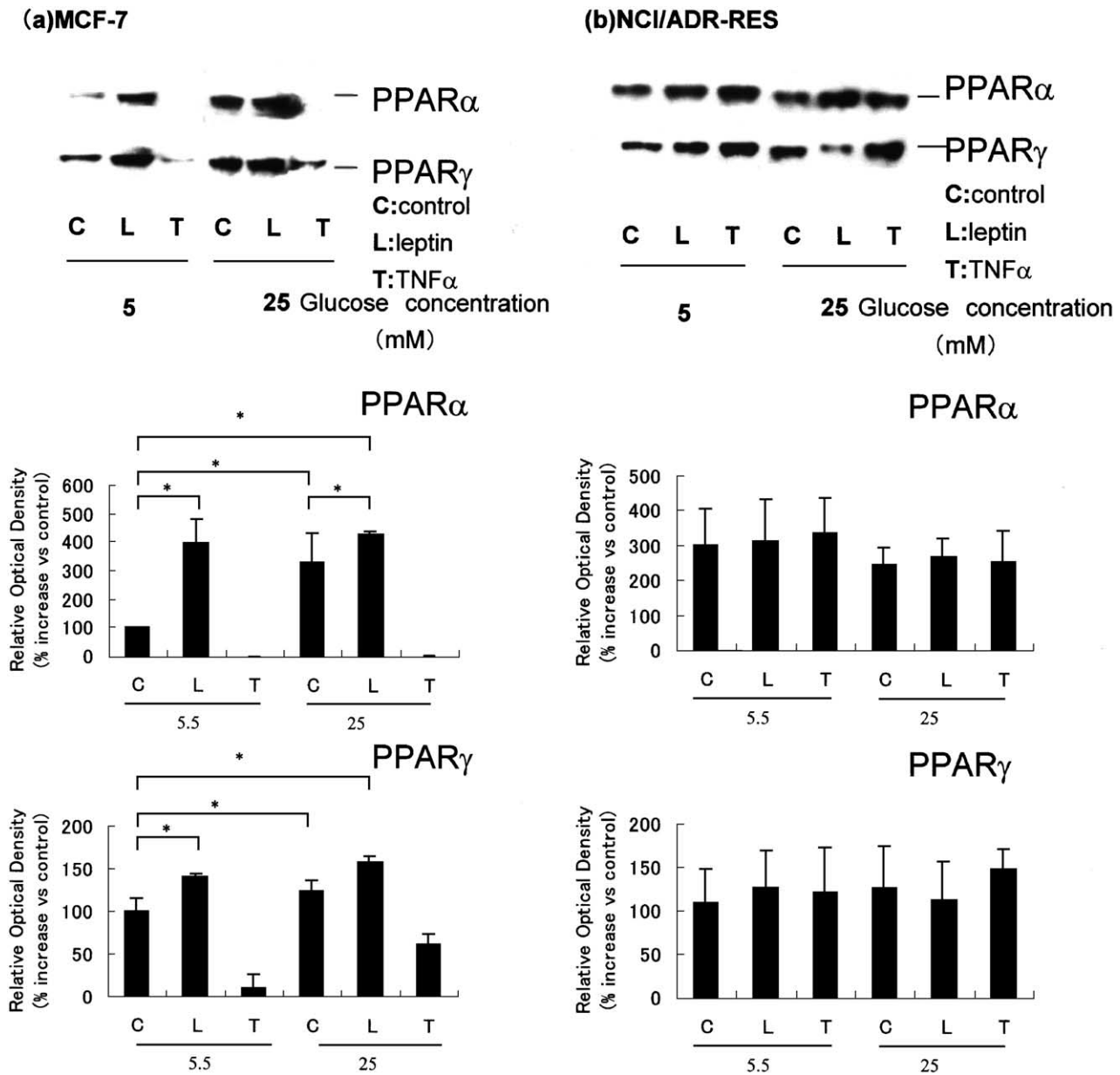


Fig. 6. PPAR γ and α immunoreactivity following leptin and high glucose treatment of MCF-7 and NCI/ADR-RES cells was investigated as described in Materials and methods. Data are shown as relative optical density (control level in MCF-7; 100%) analyzed by densitometric scanner. Similar results were obtained in three separate experiments. Data are the means \pm S.D. for triplicate determinations which were repeated in three separate experiments. * P <0.01 vs. control (control), $n=3$.

3.4. Effect of leptin and glucose on the expression of PKC- α , - θ and - ζ in MCF-7

Leptin may have accelerated the cell cycle. To examine if this was the case, we determined the expression of PKC- α , - θ , and - ζ using Western blot analysis in MCF-7 and NCI/ADR-RES treated with leptin in normal and high glucose concentrations. Leptin increased PKC- α expression in MCF-7 cells, and high glucose had an additive effect. TNF- α appeared to suppress PKC- α expression during the cell cycle, as shown in Fig. 5. No significant changes in PKC- θ and - ζ protein levels were detected with high glucose and leptin treatments, but PKC- θ and - ζ protein levels decreased when MCF-7 cells were exposed to TNF- α (Fig. 5). In NCI/ADR-RES, there were no significant differences of PKC- α , - θ and - ζ levels in cells treated with leptin, glucose, and TNF- α (Fig. 5). These results suggested that PKC- α may be involved in the regulation of leptin- and glucose-induced cell proliferation in MCF-7 cells.

3.5. Effects of leptin and glucose on the expression of nuclear hormone receptors (PPAR α , γ) in MCF-7

PPAR γ is expressed in human breast cancer cells and has an important role in cell growth [29]. We examined possible changes in PPAR γ and PPAR α levels in cells treated with leptin and glucose. As shown in Fig. 6, in MCF-7 cells, leptin and glucose increased PPAR γ and PPAR α levels, whereas TNF- α decreased their expression. However, there was no significant effect on PPAR γ or PPAR α levels in NCI/ADR-RES cells treated with leptin, glucose, or TNF- α .

4. Discussion

Based on epidemiological studies [1,2,7,8], obesity and plasma leptin levels are risk factors in breast cancer, since plasma leptin levels are higher in breast cancer patients than control subjects. Leptin is implicated in the regulation of food intake and energy balance. Leptin is also a regulatory signal for a variety of other physiological processes in addition to body weight maintenance [3]. However, the function of leptin appears to be more complex because several non-adipose sources of leptin have been reported [4–6]. Recently, leptin mRNA and protein have been reported in breast cancer cell lines [9]. In this study, we indicated that leptin increased cell proliferation through cell cycle progression in MCF-7 breast cancer cells, but not in multidrug-resistant NCI/ADR-RES cells as shown by measurement of cell proliferation, DNA synthesis, and cdk2/cyclin D1 expression. Cdk2 and cyclin D1 may have also important roles in neoplastic transformation and cell cycle progression through the G1–S border. Ectopic expression of cyclin D1 contributes to the oncogenic transformation of cells [15,16], and cyclin D1 overexpression in the mammary gland of

transgenic mice induces mammary carcinoma [34]. Our finding that leptin and high glucose induced up-regulation of cdk2 and cyclin D1 indicates that MCF-7 cell proliferation was activated through the alteration of cell checkpoints that accelerate cell cycle progression. Since TNF- α has an anti-mitogenic activity in MCF-7 cells through suppression of cells in S phase [19], we examined TNF- α effects on cell proliferation, cdk2, cyclin D1, and PPAR γ expression. TNF- α significantly suppressed cell proliferation and expression for cdk2, cyclin D1, and PPAR γ as demonstrated previously [19].

PKC levels were related to cell cycle progression in earlier studies. We previously demonstrated that PKC- β II is overexpressed in MCF-7/ADR cells and PKC- β II has an important role in glucose-induced MCF-7 cell proliferation [14]. PKC isozyme expression and subcellular distribution are tissue-specific [23]. Our findings indicated that PKC- α may have roles in leptin-induced cell proliferation in MCF-7 cells, in particular, at the cell cycle progression checkpoint similar to cdk2 and cyclin D1. On the other hand, in NCI/ADR-RES cells, glucose- and leptin-induced changes of PKC isozymes were not observed. Increasing PKC- α levels may have an important role for the regulation of cell proliferation. PKC- α overexpression induces a more aggressive [35] or more transformed [36] neoplastic phenotype in MCF-7. We also have examined that overexpression of PKC- α stimulated cell proliferation compared to wild-type of MCF-7 cells (data not shown). These facts indicated that PKC- α has a stimulatory role in the regulation of cell proliferation in MCF-7 cells.

PPARs are lipid-activated transcription factors which control the promoters of multiple genes encoding enzymes in the lipid metabolic pathways [25–28]. In this study, leptin and high glucose treatment increased PPAR γ and PPAR α in MCF-7 cells. However, in NCI/ADR-RES cells, leptin and high glucose had no significant effects on expression of these transcription factors. Up-regulation of PPAR γ and PPAR α may be a step for the acceleration of cell proliferation in MCF-7 cells. Motomura et al. [37] reported that PPAR γ was highly expressed in the nuclei of pancreatic adenocarcinoma cells, whereas normal pancreatic duct epithelial cells expressed no PPAR γ . Overexpression of PPAR γ has an important association with carcinogenicity, since increased expression of PPAR γ is reported in colon adenocarcinoma [38], lung cancer [39], breast cancer [40], and prostate cancer [41]. These facts indicate that up-regulation of PPAR γ may have a molecular link with increased risk of cancer. However, the relationship between up-regulation of PPARs and function of PKC isozymes should also be elucidated.

This is the first demonstration that leptin and high glucose exposure increases MCF-7 cell proliferation. Our findings that leptin and high glucose stimulate cell cycle progression with up-regulation of PKC- α , PPAR γ and PPAR α may be relevant in terms of diabetes and obesity as risk factors for breast cancer. Leptin- or glucose-induced

changes in PKC- α levels may be characteristic of MCF-7 breast cancer cells.

Leptin and high glucose may contribute to increased rates of cell cycle progression by increasing PPAR γ and PPAR α levels. Our findings that leptin and high glucose act through PKC- α might provide further insight into the regulation of cell proliferation in MCF-7 human breast cancer cells.

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