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Insulin Stimulates Gene Expression of Ferritin Light Chain in Osteoblast Cells

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ABSTRACT

We explored to identify differentially expressed gene(s) by insulin in osteoblast-like UMR-106 cells by employing annealing control primer (ACP)-based GeneFishing PCR. UMR-106 cells were treated with insulin and total RNA was isolated. The GeneFishing differential display (DD) PCR was carried out and the profiles of expressed genes were compared between control and insulin treated group, and followed by cloning, sequencing and screened by a BLAST search. It has been found that expression of a PCR product was significantly increased by insulin: It was identified as ferritin light chain. It was further confirmed by reverse transcriptase-PCR (RT-PCR) analysis that insulin significantly stimulates the mRNA expression of ferritin light chain. In a Western blot analysis, insulin also increased the protein expression of ferritin light chain. When ERK inhibitor or Casein Kinase II inhibitor (DRB) was pretreated, the stimulation of ferritin light chain expression by insulin was significantly inhibited, suggesting that ERK I/II or Casein Kinase II may play a role in the insulin stimulated increase of ferritin light chain. These results suggest that insulin may play an important role in Fe metabolism in osteoblast cells. J. Cell. Biochem. 111: 1493–1500, 2010.

KEY WORDS: INSULIN; GENE EXPRESSION; L-FERRITIN LIGHT CHAIN; OSTEOBLAST

Signal transduction networks permit cells to receive external stimuli and respond to the signals in an appropriate manner. Many cytoplasmic enzymes including mitogen-activated protein (MAP) kinase, MAP kinase kinase (MEK), and casein kinase II (CKII) are activated after insulin receptor activation, but signaling progresses to the nucleus remains poorly understood [Cheatham and Kahn, 1995]. Insulin action on both cytoplasmic and nuclear processes is dependent on activation of p70S6 kinase (p70S6K) [Kim and Kahn, 1997a]. Insulin induces a rapid translocation of MEK from cytoplasm to the nucleus and activates resident nuclear MAP kinase (ERK I/II) and CKII [Kim and Kahn, 1997b]. The mitogen-activated protein kinase (MAPK) signaling pathways play an important role in signal transduction in eukaryotic cells.

The metal accumulation in plasma level including aluminum [Cournot-Witmer et al., 1981; Hahn, 1989], iron [de Vernejoul et al., 1982], cadmium [Kaji et al., 1990], and lead [Long et al., 1990] related with mineralizing matrix and primarily disturb in bone metabolism [Kaji et al., 1990; Long et al., 1990; Diamond et al., 1991; Ebina et al., 1991]. The intracellular accumulation of these metals leading to osteoblast toxicity may be a contributing factor and some evidence for a causal relationship between cellular iron overload and metabolic bone disease [de Vernejoul et al., 1982, 1984]. Ferritin is a nanobox protein designed to contain and maintain in solution up to a few thousand iron atoms, which otherwise would aggregate in toxic precipitates. The typical 24-mer ferritin is a hetero-polymer consisting of H- and L-subunits [Harrison and Arosio, 1996; Richardson and Ponka, 1997] and a high-molecular weight (450 kDa) almost spherical shape that delimits a large cavity about 8 nm across, which can accommodate up to 4,000 Fe atoms in a ferric hydroxide core, the presence of which does not affect protein surface and possible interactions with other molecules. The proteins are also characterized by catalytic sites for iron oxidation and hydrophilic pores for exchange with the solvent. Intracellular iron is the master regulator of ferritin biosynthesis and accumulation. The best characterized regulatory system of ferritin expression is the post-transcriptional, iron-dependent machinery based on the interaction by the iron regulatory proteins 1 and 2 (*trans*-acting RNA-binding protein IRP1 and IRP2—A cytoplasmic proteins previously known as the iron-responsive element-binding protein, IRE-BP, and iron-responsive factor, IRF). IRPs control the synthesis of ferritin and transferrin receptor (TfR) by binding to IREs that are located in the 5′-untranslated region (UTR) and the 3′-UTR of their respective mRNAs. When levels of iron in the labile iron pool (LIP) are low, IRPs bind to

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IREs and this association blocks the translation of ferritin mRNA and stabilizes TfR mRNA. On the other hand, expansion of the LIP inactivates the RNA-binding activity of IRP1 and leads to degradation of IRP2, resulting in efficient translation of ferritin mRNA and rapid degradation of TfR mRNA [Richardson and Ponka, 1997; Rouault and Klausner, 1997; Mikulits et al., 1999; Eisenstein, 2000]. Enamours investigations suggested that the effects of NO on ferritin synthesis and TfR mRNA or protein levels, but the results obtained are notably controversial. Whereas, some investigators observed that NO caused a marked decrease in ferritin synthesis [Weiss et al., 1993; Juckett et al., 1996] (as would be predicted based on NO-mediated activation of IRP-1 RNA binding activity), others reported that NO increased ferritin synthesis and accumulation [Oberle and Schroder 1997; Recalcati et al., 1998]. These latter findings are in agreement with numerous studies showing that inflammation and inflammatory cytokines stimulate ferritin synthesis [Konijn and Hershko 1977; Konijn 1994]. Moreover, the NO-mediated increase in IRP1-binding activity was coincident with the translational repression of ferritin synthesis in fibroblasts [Pantopoulos and Hentze 1995], macrophages [Weiss et al., 1993], and hepatoma cells [Phillips et al., 1996]. Insulin activate nuclear ERK I/II in the UMR-106 cells [Kim and Kim, 1997], it may phosphorylate osteoblast transcription factors, which, in turn, stimulate transcription of iNOS gene. These increased iNOS translation play a role for the stimulation of NO production. Even though evidence in insulin-stimulated MAPK-signaling pathway and the NO production that might lead to ferritin synthesis, but there is no direct evidence of insulin-stimulated ferrous metabolism and metabolic bone disorders in hyperinsulinema condition. However, it is unclear whether signaling pathways reportedly activated ferritin synthesis and alter the iron metabolism in osteoblast cells is relevant to the physiological actions of insulin in situ.

To understand as to how insulin could affect bone cell metabolism, we explored gene expression profiles in response to insulin in UMR-106 osteoblast-like cells using a specific differential display (DD) PCR technique with annealing control primer (ACP). We present evidence that insulin induces ferritin light chain synthesis and regulates the ferrous ion in osteoblast-like UMR-106 cells. The role of ERK on ferrous ion production by insulin is discussed.

MATERIALS AND METHODS

MATERIALS

Pork insulin was obtained from Elanco Products Co. (Indianapolis, IN). The reagents used for polyacrylamide gel electrophoresis were from Bio-Rad. Nitrocellulose filters (0.45 mm) were purchased from Schleicher & Schuell. Ferritin light chain antibody (Cat No: FERL14-A) was purchased from Alpha Diagnostic (San Antonio, TX) and ECL kit was purchased from Amersham. UMR-106 cells were purchased from ATCC. β-actin primer set was purchased from Clontech Laboratories, Inc. The reagents used for polyacrylamide gel electrophoresis were from Bio-Rad. Nitrocelluose filters (0.45 mm) were purchased from Schleicher & Schuell. HRP-conjugated mouse IgG antibody-H&L (ab6728) were purchased from Abcam (England). Accupower® RT PreMix Kit (Cat. No.: K-2041) and AccuPower® PCR PreMix (Cat. No.: K-2012) were purchased from Bioneer (Korea). Reagents used for reverse transcription such as 5́-reaction buffer, RNasin® RNase inhibitor (40 U/μl), and Moloney murine leukemia virus reverse transcriptase (200 U/μl) were purchased from Promega (Madison, WI). The GENCLEAN II Kit was purchased from Q-BIO gene (Carlsbad, CA). The TOPO TA cloning vector was purchased from Invitrogen (Carlsbad, CA). The ECL kit was purchased from Amersham. The 100 bp DNA Ladder marker (Cat. No.: EBM-1001) was purchased from ELPAS Biotech (Korea). The GeneFishing™ kit was purchased from Seegene (Korea). TRIzol® and all other chemicals were purchased from Sigma Chemical Co.

CELL CULTURE

UMR-106 cells were grown to confluence in 75-cm^2 flasks in DMEM media containing with 10% fetal calf serum and 100,000 U/ml penicillin G potassium at 37°C in a humidified 5% CO_2 environment. Cells were then seeded on 12-well cell culture plates and used the next day. Cells from passages 10–24 were used for the experiments. Before administration of insulin, cells were starved of fetal calf serum for 12–16 h. After treatment with insulin for 24 h, cells were cooled on ice and rinsed with ice-cold phosphate-buffered saline (PBS). Cells were then scraped into 200 μl of lysis buffer (10 mM Tris–HCl pH 7.4; 50 mM NaCl; 5 mM EDTA; 30 mM NaF; 0.1 mM sodium orthovanadate; 1% Triton X–100; 0.5%NP–40; 1 μg/ml leupeptin; 1 μg/ml aprotinin) and disrupted with a 1 ml syringe. The lysate was kept overnight at ice in refrigerator. Then centrifuge at 13,000 rpm for 15 min, the supernatant was allowed to protein assays.

ISOLATION OF TOTAL RNA

After cells were treated with insulin for 24 h, the cells were washed three times with 0.1% DEPC-treated PBS and 900 μl of TRIzol reagent was added, followed by homogenization. Then, 100 μl of chloroform was added to the homogenate and subjected to centrifugation at 12,000 rpm at 4°C. To the resulting supernatant, 100 μl of isopropanol was mixed and incubated for 45 min at −20°C, then followed by centrifugation to precipitate the pellet. The pellet was washed with 70% DEPC-ethanol and the final pellet was air-dried and resuspended with DEPC-water.

FIRST-STRAND CDNA SYNTHESIS

Total RNAs were extracted using TRIzol reagent and used for the synthesis of first-strand cDNAs by reverse transcriptase. Reverse transcription was performed for 1.5 h at 42°C in a final reaction volume of 20 μl containing 3 μg of the purified total RNA, 4 μl of 5× reaction buffer (Promega), 5 μl of dNTPs (2 mM each), 2 μl of 10 μM dT-ACP1 (5́-CTGTGAATGCTGCGACTACGATIIIIIT(18)-3́), 0.5 μl of RNasin® RNase Inhibitor (40 U/μl, Promega), and 1 μl of Moloney murine leukemia virus reverse transcriptase (200 U/μl, Promega). First-strand cDNAs were diluted by the addition of 80 μl of ultra-purified water for GeneFishing™ PCR and stored at −20°C until use.

ACP-BASED GENE FISHING™ PCR

Differentially expressed genes were screened by the ACP-based PCR method, using GeneFishing™ DEG kits (Seegene, Seoul, South

Korea). Briefly, second-strand cDNA synthesis was conducted at 50°C during one cycle of first-stage PCR in a final reaction volume of 20 μl containing 3–5 μl (about 50 ng) of diluted first-strand cDNA, 1 μl of dT-ACP2 (10 μM), 1 μl of 10 μM arbitrary ACP, and 10 μl of 2× Master Mix (Seegene). The PCR protocol for second-strand synthesis was one cycle at 94°C for 1 min, followed by 50°C for 3 min, and 72°C for 1 min. Once second-strand DNA synthesis was completed, the second-stage PCR amplification protocol was 40 cycles at 94°C for 40 s, followed by 65°C for 40 s, 72°C for 40 s, followed by a 5 min final extension at 72°C. The amplified PCR products were separated in 2% agarose gel stained with ethidium bromide.

CLONING AND SEQUENCING OF DIFFERENTIALLY EXPRESSED PCR PRODUCTS

The differentially expressed bands were extracted with a GENCLEAN II Kit (Q-BIO gene) and cloned into a TOPO TA cloning vector (Invitrogen) following the manufacturer's instructions. In order to verify the identity of insert DNA, isolated plasmids were sequenced automatically using M13 forward primer (5'-CGCCAGGGTTTTCC-CAGTCACGA-3') or M13 reverse primer (5'-AGCGGATAACAATTT-CACACAGGA-3') with an API PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). All sequences were used to perform a Basic Local Alignment Search Tool (BLAST) for gene identification at the National Center for Biotechnology Information (NCBI) GenBank.

REVERSE TRANSCRIPTASE PCR

Isolated total RNA (2 μg) was added to the Accupower RT PreMix and incubated for 5 min at 70°C. Then, DEPC-water was added to make the final volume of 20 μl. This was followed by incubation for 60 min at 42°C and for 5 min at 94°C to produce cDNA. For PCR, the reaction mixtures contained 1 μl cDNA, 1 μl forward primer 10 pM), 1 μl reverse primer (10 pM), 16 μl distilled water. They were subjected to PCR reaction: 94°C for 5 min and 25 cycles of reaction (94°C for 45 s, 55°C for 45 s, 72°C for 45 s) and 72°C for 10 min for extension. The final PCR mixture was applied to 1.2% agarose gel and subjected to electrophoresis and visualized under UV illumination.

The Primer sequence of ferritin light chain was as follows:

Forward: tgc ccg cac aga ccc tca ct

Reverse: ttc aga gtg agg cgc tca aag aga

MEASUREMENT OF IRON

The Iron content in the culture lysate was assessed using the Quantichrom iron assay (BioAssay Systems, Hayward, CA) with the manufacturer's protocol. Briefly, 50 μl of standards or samples containing 10^6 cells were mixed with 200 μl Quantichrom Working Reagent in a 96-well plate (in triplicate) and incubated at room temperature overnight. The iron concentration in experimental samples was determined by comparison of the optical density at 565 nm with the standard curve.

WESTERN BLOT ANALYSIS

The protein content of the soluble fraction was assessed by the method of Bradford [1976]. Protein (50 μg/lane) was electrophoretically separated in 10% polyacrylamide gels containing SDS. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) was carried out for 1 h at 100 V (constant) as described by Towbin et al. [1992]. The filter papers were preincubated for 1 h at 23°C with PBS containing 0.1% Tween 20, and 3% bovine serum albumin and washed with PBS containing 0.1% Tween 20 three times for 10 min each. Followed by the blots were probed with primary antibody directed against Ferritin light chain (1:500), GAPDH (1:1,000) for 2 h at room temperature or overnight at 4°C diluted in blocking buffer. The blots were then incubated with HRP-conjugated anti-rabbit IgG for 1 h at room temperature and washed with PBS containing Tween 20 three times for 10 min each. The detection of immobilized specific antigens was carried out by ECL (NEN). The images analyzed using Image J software.

STATISTICS

All values were expressed as the mean ± SE. Comparison between groups was performed by Student's *t*-test.

RESULTS

PCR PRODUCTS INDUCED BY INSULIN USING A SPECIFIC DIFFERENTIAL DISPLAY PCR

In the ACP system, seven arbitrary 37-mer primers having a 10 target nucleotide sequence (10-mer) at their 3́-end core positions were screened against one anchored oligo-dT primers. DD reactions yielded between 5 and 15 discreet cDNA bands ranging from 100 and 1.5 kb, which can be detected on agarose gels. One differentially expressed cDNA band with primer 8 was clearly detected and insulin (100 nM) for 24 h significantly stimulated its expression compared with the control group in UMR-106 cells (Fig. 1). Insulin stimulated the ferritin light chain mRNA expression by 2.44-fold as compared to control (Fig. 1). This PCR product was cloned into a TOPO TA cloning vector and followed by sequence analysis and computer searching against the Gen-Bank. It has been revealed that the PCR product shared more than 95% identity with ferritin light chain.

EFFECT OF INSULIN ON THE MRNA EXPRESSION OF FERRITIN LIGHT CHAIN

To further explore ferritin light chain expression in response to insulin for 24 h, ferritin light chain mRNA levels were analyzed by RT-PCR. Total RNA was isolated and subsequently reverse transcribed to cDNA. The cDNA was amplified with specific ferritin light chain primer sets. The PCR products were separated by agarose gel electrophoresis and identified by ethidium bromide staining. Insulin (100 nM) caused Ferritin light chain mRNA expresion in Osteoblast like UMR 106 cell lines (Fig. 2). The ratio of ferritin light chain to β-actin was increased by 2.88-fold in response to insulin as compared to control (Fig. 2).

EFFECT OF INSULIN ON THE PROTEIN EXPRESSION OF FERRITIN LIGHT CHAIN

To further confirmation of ferritin light chain mRNA expression in response to insulin, ferritin protein levels were analyzed by Western blot analysis with the anti-ferritin light chain antibody. There is little ferritin protein detected under basal conditions and 1 and 3 h,

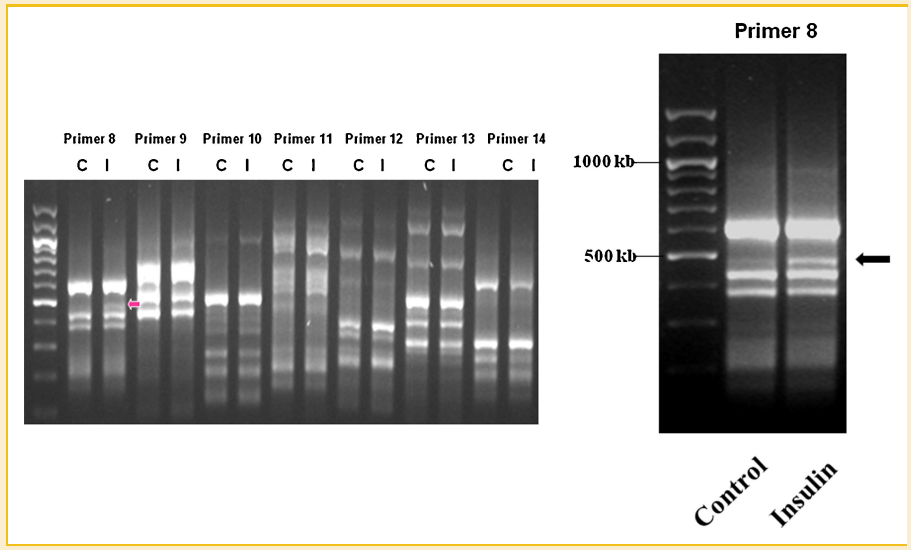

Fig. 1. PCR products induced by insulin using a specific DD PCR in UMR-106 cells. Following treatment with insulin (100 nM) for 24 h in UMR-106 cells, total RNA was isolated and followed by DD PCR with annealing control primers (ACP). PCR products were separated by 2% agarose gel electrophoresis with ethidium bromide and identified under UV illumination. GP1-10 indicate an arbitrary ACP. Arrow indicated differentially expressed gene.

whereas insulin treatment for 24 h caused a significant increase in the ferritin protein levels as evidenced by the increase of 14 kDa ferritin protein on the SDS-PAGE (Fig. 3). These results suggest that the normalized ferrous iron concentration in 24 h by insulin could be caused by ferritin protein induction. Dose-response relationship was also tested with increasing concentrations of insulin treatment for 24 h (Fig. 4). Insulin stimulated the expression of ferritin light chain in a dose-dependent manner by 1.7, 2.3, and 2.9-fold as compared to control at 1, 10, and 100 nM, respectively.

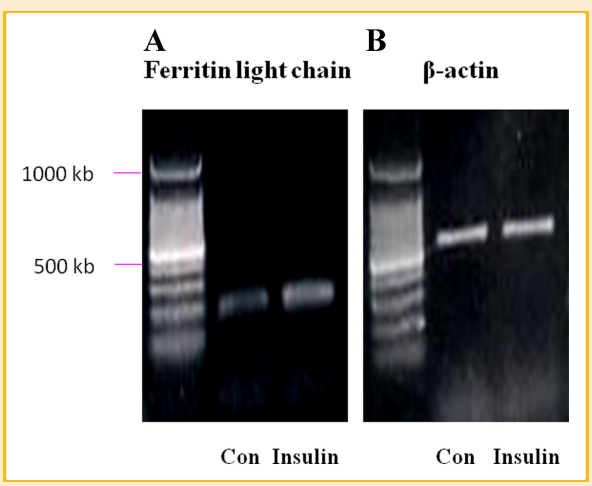

Fig. 2. Stimulation of ferritin light chain gene expression by insulin in UMR-106 cells. Following treatment with insulin (100 nM) for 24 h in UMR-106 cells, total RNA was isolated and followed by reverse transcriptase PCR with specific primers against ferritin light chain and β-actin as described in the Materials and Methods section. PCR products were separated by 1.2% agarose gel electrophoresis and identified under UV illumination.

EFFECT OF LY294002, PD98059, DRB ON THE INSULIN-INDUCED FERRITIN LIGHT CHAIN EXPRESSION

To delineate the mechanism of ferritin production induced by insulin, LY 294002 (PI_3-Kinase inhibitor), PD98059 (MEK inhibitor), and 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB, a cell permeable Casein Kinase II inhibitor) were employed. The cells were preincubated with these inhibitors for 1 h and then further incubated

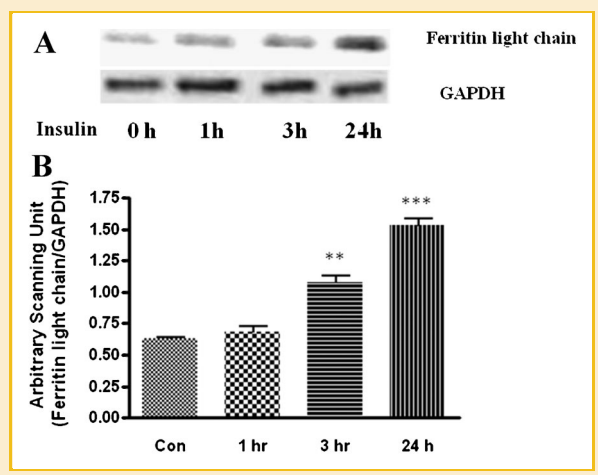

Fig. 3. Western blot analysis of ferritin light chain in response to insulin in UMR-106 cells. Following treatment with insulin (100 nM) for 24 h in UMR-106 cells, the cells were washed with PBS and scraped into an isolation buffer, then followed by homogenation. The resulting homogenate was subjected to SDS-polyacrylamide gel electrophoresis and Western blot analysis with a specific antibody against ferritin light chain and GAPDH as a internal control as described in the Materials and Methods section (A). The blots were subjected to scanning densitometry and expressed as a ratio of ferritin light chain to GAPDH (B). The data shown are the mean values ± SEM of three experiments $^{**}P < 0.01$; $^{***}P < 0.001$.

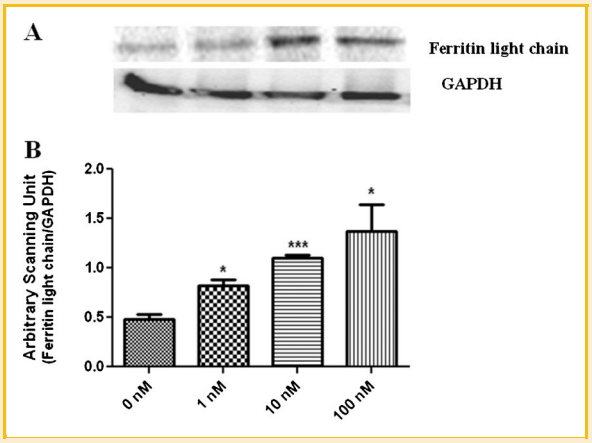

Fig. 4. Dose–response expression of ferritin light chain in response to insulin in UMR-106 cells. Following treatment with insulin (1, 10, and 100 nM) for 24 h in UMR-106 cells, the cells were washed with PBS and scraped into an isolation buffer, then followed by homogenation. The resulting homogenate was subjected to SDS–polyacrylamide gel electrophoresis and Western blot analysis with a specific antibody against ferritin light chain and GAPDH as an internal control as described in the Materials and Methods section (A). The blots were subjected to scanning densitometry and expressed as a ratio of ferritin light chain to GAPDH (B). The data shown are the mean values ± SEM of three experiments $^{*}P < 0.05$; $^{***}P < 0.001$.

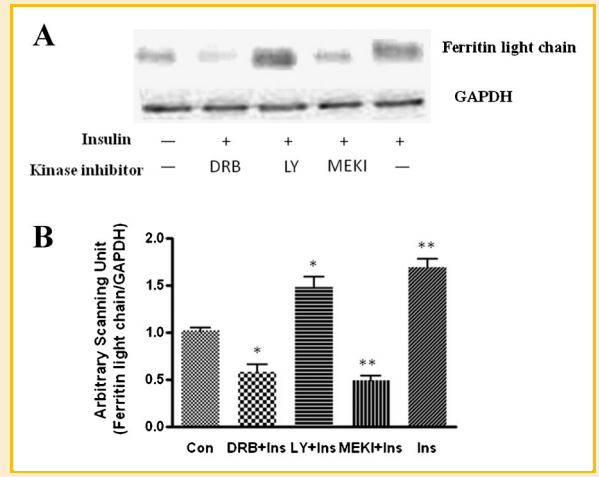

Fig. 5. Effect of protein kinase inhibitors on the expression of ferritin light chain in UMR-106 cells. Following pretreatment with DRB (1 mM), LY compound (5 μM), and MEKI (50 μM), cells were treated with insulin for 24 h in the presence of the inhibitors. Then the cells were lysed and subjected to SDS–PAGE and Western blot analysis with anti-ferritin light chain antibody as described in the Materials and Methods section (A). The blots were subjected to scanning densitometry and expressed as a ratio of ferritin light chain to GAPDH (B). The data shown are the mean values ± SEM of three experiments $^{*}P < 0.05$; $^{**}P < 0.01$.

with insulin for 24 h in the presence of the inhibitors. Preincubation of LY compound (5 μM) had little effect on the insulin induced ferritin production while MEK inhibitor (50 μM) and DRB (1 mM) almost completely inhibited the insulin-stimulated ferritin light chain production. The fact that MEKI and DRB significantly inhibited the insulin-stimulated ferritin light chain expression indicates that both ERK I/II and casein kinase II lies in the signal-transduction pathway for the insulin-stimulated ferritin production (Fig. 5).

EFFECT OF INSULIN ON THE IRON CONCENTRATION

To understand the mechanism of ferritin light chain expression in response to insulin, ferrous iron concentration levels were analyzed by Quantichrom iron assay kit. The UMR-106 cells were incubated with 100 nM insulin for 1, 3, and 24 h, the iron concentration of the cell lysate was measured. 1 and 3 h Insulin-treated cells significantly increased iron concentration and 24 h insulin-treated cells become a normal as compared to controls (Fig. 6).

DISCUSSION

IGF-1, PDGF, TGF-β, IL, and TNF like growth factors are synthesized in bone cell [Nicholas et al., 1990; Manolagas and Jilka, 1995; Kaji et al., 1997], suggesting that growth factor mediated signal transduction plays significant roles in bone cells. Insulin is an anabolic hormone that stimulates the cellular uptake of many nutrients, including hexoses, amino acids, cations, and anions. Interestingly, insulin receptors and glucose transporters were identified in osteoblasts [Thomas et al., 1996]. In addition, ERK activities were shown to be regulated by insulin in the osteoblasts. In the present study, we employed a specific DD PCR technique to search for insulin-regulated gene(s) and have shown that insulin significantly stimulates expression of ferritin light chain in UMR-106 osteoblast-like cells.

Recently, gene expression profiling techniques draw great attention to many researchers such as DNA microarray and conventional DD technique. However, one of the most critical drawbacks associated with these techniques is that many false-positive artifacts are appearing artificially. For the successful identification of genes of interest, it is necessary to discriminate artifacts from genuine genes. In the present study, we employed a

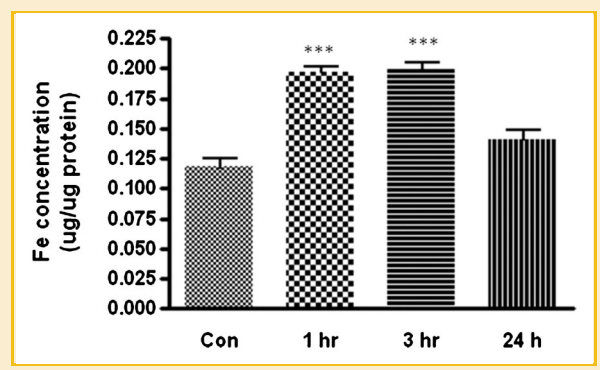

Fig. 6. Effect of insulin on the Fe levels in UMR-106 cells. Following treatment with insulin (100 nM) for the indicated times in UMR-106 cells, the cells were washed with PBS and scraped into an isolation buffer, then followed by homogenation. The resulting homogenate was subjected to the determination of Fe as described in the Materials and Methods section. The data shown are the mean ± SEM of three experiments $^{***}P < 0.001$.

specific DD PCR technique with ACP, in which artificial binding of PCR primers to RNA is known to be significantly diminished as evidenced by a number of publications [Hwang et al., 2004; Kim et al., 2004; Pohjanvirta et al., 2004]. Using the same technique, we have successfully identified a gene, ferritin light chain, and found that its mRNA expression is significantly stimulated by insulin.

The UMR-106 cells have been widely used for the study of hormonal regulation of the osteoblast. This cell line has conserved the differentiated properties of osteoblast such as high expression of alkaline phosphatase, activation of adenylate cyclase by PTH and expression of bone matrix protein collagen [Patridge et al., 1983; Forrest et al., 1985; Patridge et al., 1985]. In addition, the proteoglycans synthesized by UMR-106 cells are similar to those synthesized by cultured human osteoblast [Beresford et al. 1987].

Iron-binding protein ferritin is known to be expressed in osteoblast cells in vitro and in vivo [Spanner et al., 1995], suggesting that osteoblast cells contain a functional ferritin-dependent iron uptake and storage system and ferrous iron may play an essential role in bone metabolism. It has been suggested that iron first accumulates in the cytoplasm and promotes ferritin translation with subsequent incorporation of iron into the ferritin core [Arosio and Levi, 2002]. This dynamic process will then results in a reduction of metabolically available iron in the cytoplasm with subsequent activation of iron-regulatory protein (IRP) binding affinity leading to increased expression of ferritin: most likely via IRP-mediated stabilization of the mRNA [Hentze et al., 2004]. This is also reflected by our observation of increased ferrous ion-concentration and ferritin mRNA and ferritin protein expression in insulin-treated cells such as C6 glial cells [Yokomori et al., 1991; Schumann et al., 1999].

However, iron is not the only player that controls IRPs, because IRP activities/levels can also be affected by various forms of oxidative stress and NO [Richardson and Lok, 2008]. IRP1 is homologous to mitochondrial aconitase, an [Fe-S] protein whose activity is modulated by NO [Wardrop et al., 2000]. Hence, it is not surprising that IRP1-binding activity can also be affected by NO, which was shown to increase transferring receptor (TfR) mRNA levels [Hentze and Kühn, 1996; Ponka et al., 1998]. Furthermore, our previous study also demonstrates that the insulin (100 nM) had significant stimulatory effects on NO production in the UMR-106 osteoblast cells [Kim et al., 2000]. Recent research also supports this conclusion that cellular iron homeostasis appears to be regulated not only by iron levels but also by other factors that manifest during inflammation, a condition associated with increased NO production. Kim and Ponka [2000] demonstrated that nitrogen monoxide-mediated degradation of IRP2 leads to a dramatic up-regulation of ferritin synthesis in macrophages. Moreover, this is the first report, to our knowledge, showing that insulin stimulated ferritin synthesis in UMR 106 osteoblast cells, may play to store the intracellular iron. Increasing evidence has accumulated to indicate that there is close relationship between iron metabolism and diabetes and insulin resistance [Fernandez-Real et al., 2002; It has been suggested that total body iron stores are positively connected to the onset of impaired glucose tolerance and type II diabetes [Salonen et al., 1998]. In addition, serum ferritin concentrations are positively related with insulin resistance [Forouhi et al., 2007]. Iron exerts a potent oxidative stress, which could lead to tissue damages; this is an important factor contributing to the development of chronic microvascular complications of diabetes.

We also searched for a mechanism to link insulin action to ferritin expression by employing specific inhibitors PD98509 (50 μM), LY294002 (5 μM), and DRB (1 mM) for ERK I/II, PI3-Kinase and Casein Kinase II, respectively. The inhibitors were preincubated for 1 h, and followed by insulin stimulation for 24 h in the present of each inhibitors. To test if ERK, PI3 Kinase, and CKII are involved in the insulin-stimulated ferritin light chain expression, we incubated the cells with each inhibitors in the presence of insulin. Preincubation of ERK inhibitor caused a dramatic decrease in the insulin-stimulated ferritin expression; furthermore, preincubation of DRB also block the ferritin expression. These results suggest that insulin may stimulate ferritin light chain protein expression via ERK I/II and/or Casein Kinase II. This is consistent with our previous findings showing that treatment of osteoblast with insulin leads to iNOS production [Kim and Kim, 1997]. It is reasonable to propose that insulin-stimulated ERK I/II and/or Casein Kinase II may phosphorylate osteoblast transcription factors which, in turn, stimulate expression of ferritin light chain mRNA. These could lead to the increase of ferritin translation, which in turn, might play a role for the stimulation of ferritin expression in response to insulin. We have published a number of papers that insulin stimulates ERK in the UMR-106 cells (Kim and Kim, 1997; Kim et al., 2000). With regard to PI3-Kinase activity, it is well established that upon insulin receptor activation IRS-1 undergoes tyrosine phosphorylation and followed by its binding and activation of PI3-Kinase. We have previously found that IRS-1is activated by insulin in the UMR-106 cells [Seol and Kim, 2003], suggesting that PI3-Kinase could bind to tyrosine phosphorylated IRS-1 and activated. Thus, we speculate that there is functionally active PI3-Kinase in the insulin-signaling pathway in the UMR-106 cells. In terms of Casein Kinase II, insulin also stimulates Casein Kinase II in CHO (Hirc) overexpressing insulin receptor [Kim and Kahn, 1997b] and UMR-106 cells express Casein Kinase II [Sfeir and Veis, 1996], suggesting that insulin-sensitive Casein Kinase II lies in the insulin-signaling pathway in the UMR-106 cells.

Regarding the effect of inhibitors for ERK and casein kinase II on iron concentration, we suspect that their effect on iron concentration could be minimal. Since the insulin has significant effect on ferritin light chain protein expression in 24 h whereas iron concentration is transiently increased by insulin in 1–3 h. In addition, there is evidence that iron plays a role in the activation of both ERK and casein kinase II, suggesting both kinases lie downstream of iron-mediated signaling [Kaomongkolgit et al., 2008; Wang et al., 1995]. We propose that insulin increases iron uptake, thereby stimulating ferritin gene expression that require both ERK and casein kinae II.

The finding that the preincubation of LY compound failed to block the insulin-stimulated ferritin expression confirms that the PI3 Kinase may not play a role in expression of ferritin. Our previous study also indicates that the insulin-mediated NO production via the ERK I/II in the UMR-106 cells [Kim et al., 2000]. Considering the correlation of these studies, it is reasonable to speculate that after insulin binding to the cell leads to the activation of insulin receptor,

IRS proteins undergo tyrosine phosphorylation. The phosphorylated IRS proteins serve as docking proteins to bind and activate ERK I/II, recruiting serine kinases and phosphorylating osteoblast transcription factors. These sequential events could lead to the stimulation of NO production and to the expression of ferritin light chain in response to insulin. A study conducted by Kim and Ponka [2000] also supports our findings that the excess iNOS production may play the role in increasing ferrous iron concentration and inducing ferritin light chain expression in the UMR-106 osteoblast cells. Regarding Casein Kinase II involvement, it has been found that Casein Kinase phosphorylates NF-κB p65 subunit, stimulating iNOS gene transcription [Chantome et al., 2004]. This result raises a possiblity that insulin may activates CKII, which could lead to the phosphorylation of NF-κB p65 subunit; subsequent enhancement of iNOS gene transcription and NO production could cause the expression of ferritin light chain in the UMR-106 osteoblast cells.

Insulin's stimulatory effect on ferritin light chain expression was also observed at insulin concentration between 1 and 10 nM, suggesting that it could exert the same action in physiological conditions. The UMR-106 cells contain approximately 80,000 high affinity insulin-binding sites per cell. We cannot rule out the possibility that insulin binds to IGF-1 receptor, thereby stimulating ferritin light chain gene expression; however, it has been suggested that the affinity of IGF-1 to insulin receptor is weaker than insulin in the UMR-106 cells. For example, we have previously reported that IGF-1 is much weaker in the ability to produce NO as compare to insulin in the UMR-106 cells. Also, insulin receptor tyrosine kinase is known to be activated at the insulin concentrations between 1 and 100 nM. Thus, we believe that insulin's effect on ferritin light chain gene expression is mediated by its binding to insulin receptor.

In conclusion, we have presented evidence that insulin significantly stimulates gene and protein expression of ferritin light chain in UMR-106 osteoblast cells. In addition, we have found that ERK I/II and Casein Kinase II may play a role in the insulin-regulated ferritin light chain expression. These results strongly suggest that insulin takes part in the control of iron metabolism in UMR-106 cells via stimulating ferritin light chain expression. Iron homeostasis and ferritin expression may be functionally related to the transcriptional events that control the positive actions of insulin in multiple tissues, and understanding the regulation of iron metabolism may help us to understand the physiology of insulin actions in the cell.

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