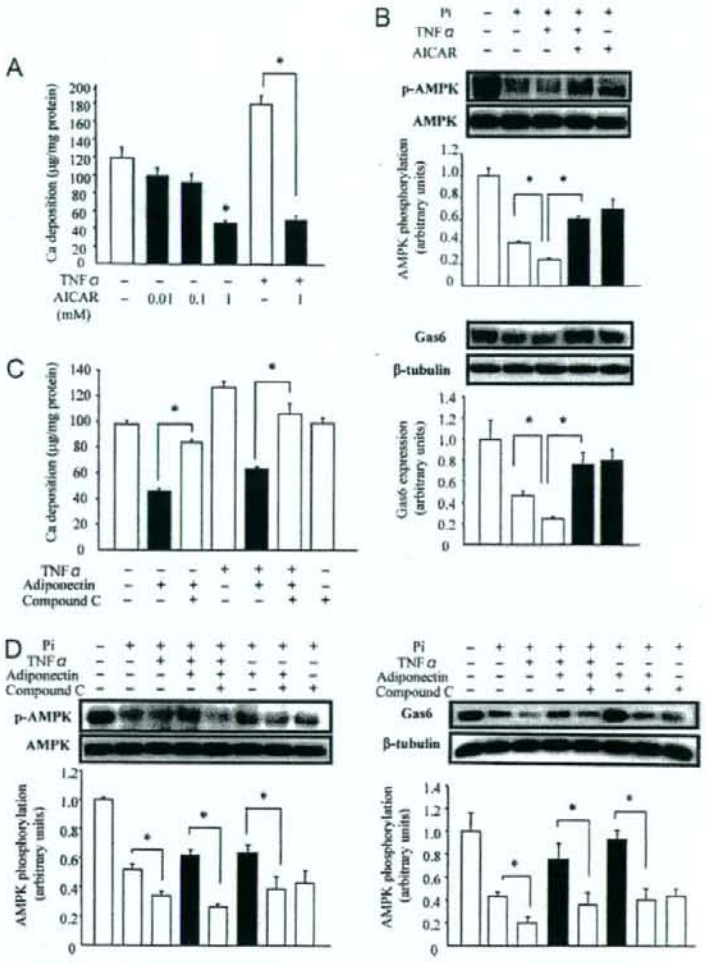

FIG. 5. AMPK plays an important role in Pi-induced calcification. HASMC were treated with or without AICAR (1 mM), a pharmacological activator of AMPK and TNFα (20 ng/ml) in calcification medium for 6 d. A and B, Ca deposition (n = 6) (A) was measured, and immunoblotting with antibodies to p-AMPK, AMPK, Gas6, and β-tubulin (B) was performed (n = 3). HASMC were cultured with or without compound C (1 μM), a chemical inhibitor of AMPK, adiponectin (300 ng/ml), and TNFα (20 ng/ml) in calcification medium for 6 d. C and D, Ca deposition (C) was evaluated (n = 6), and immunoblotting with antibodies to p-AMPK, AMPK, Gas6, and β-tubulin (D) was performed (n = 3). All values are presented as mean ± SE. *, $P < 0.05$ by Bonferroni test. Each experiment was performed in triplicate for each condition.

phorylation that was inhibited by Pi and TNFα in a calcification-promoting condition.

To clarify the causal relationship between AMPK and calcification, we tried to activate AMPK by treatment with 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) (25). In HASMC, AICAR significantly inhibited Ca deposition in a concentration-dependent manner (Fig. 5A). In addition, TNFα-stimulated Ca deposition was also blunted by AICAR. Interestingly, AICAR restored Gas6 expression down-regulated by Pi and TNFα (Fig. 5B). Next, to investigate whether the effect of adiponectin is dependent on AMPK, we tried to block AMPK using compound C, a chemical inhibitor of AMPK. As shown in Fig. 5C, compound C clearly abrogated the inhibitory effect of adiponectin both on Pi- and TNFα-induced calcification. The increase in Gas6 expression as well as AMPK phosphorylation by adiponectin was also abolished by compound C (Fig. 5D). These results suggest that AMPK regulates Gas6 expression, followed by regulation of Ca deposition in HASMC.

Transcription activity of Gas6 is regulated by adiponectin and TNFα via AMPK

To investigate whether Gas6 expression is transcriptionally regulated by adiponectin, TNFα, and AMPK, a promoter study was undertaken. Reporter assay using the −1.9-kb Gas6-luciferase DNA construct revealed that adiponectin completely reversed the down-regulation of Gas6 transcription activity by TNFα. Furthermore, compound C abrogated the effect of adiponectin on Gas6 transcription activity, indicating that adiponectin and TNFα regulate Gas6 expression at the transcription level via AMPK activity (Fig. 6).

Discussion

The present study showed that adiponectin has a protective effect against Pi-induced calcification and, furthermore,

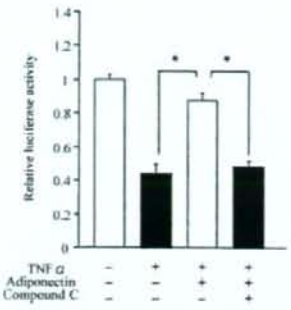

FIG. 6. Effect of adiponectin and TNFα on Gas6 promoter activity. HASMC were transfected with the Gas6 promoter-luciferase construct using lipofectamine 2000. Twenty-four hours after transfection, adiponectin (300 ng/ml), compound C (1 μM), and TNFα (20 ng/ml) were added. Cells were incubated for an additional 44 h. Luciferase activity was normalized to that of vehicle-treated cells. All values are presented as mean ± SE (n = 4). *, $P < 0.05$ by Bonferroni test. Each experiment was performed in triplicate for each condition.

has an antagonistic effect on TNFα-augmented calcification. Based on our previous finding that Pi-induced calcification is dependent on apoptotic cell death in HASMC, we examined the role of adiponectin and TNFα in Pi-induced apoptosis. As expected, we found that adiponectin had an inhibitory effect and TNFα had a stimulatory effect on Pi-induced apoptosis. This study also demonstrated the

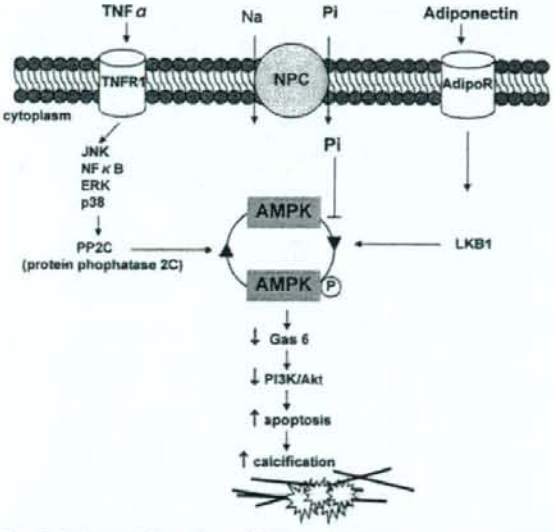

FIG. 7. Scheme of the effect of TNFα and adiponectin on Pi-induced calcification. In HASMC, exogenous Pi is internalized by sodium-dependent phosphate cotransporter (NPC, such as Pit-1) and inhibits AMPK phosphorylation, followed by down-regulation of the Gas6-mediated survival pathway. This pathway stimulates apoptosis, leading to subsequent development of calcification. TNFα directly suppresses AMPK activation by promoting PP2C activation via TNF receptor-1 (TNFR1). On the other hand, adiponectin activates LKB1-AMPK pathway via adiponectin receptors (AdipoR). AMPK activation modulated by TNFα and adiponectin contributes to the regulation of Pi-induced calcification.

regulation of Gas6 expression by TNFα and adiponectin, a suppressive effect and a promoting effect, respectively, at the transcriptional level. Akt, a critical downstream effecter of Gas6, was activated by adiponectin, whereas TNFα had an opposite action on its phosphorylation. Given that adiponectin and TNFα did not affect Gas6 expression and Akt phosphorylation in the absence of Pi (data not shown), the effects of adiponectin and TNFα on these molecules may dependent on Pi-induced responses. These results suggest that Gas6 is the target of adiponectin and TNFα in regulating Pi-induced apoptosis, accompanied by modulation of the Akt-dependent survival pathway.

As reported previously (6), Pi-induced VSMC calcification is associated with both phenotypic transition to osteoblastic cells via sodium-dependent phosphate cotransporter and apoptotic cell death. In our preliminary experiments, the expression of osteopontin, an osteoblastic marker, was not affected by TNFα and adiponectin (data not shown). Although this result suggests little influence of TNFα and adiponectin on osteoblastic differentiation of VSMC, extensive and systematic investigation including other markers of osteoblastic differentiation is needed to conclude this issue.

Multiple lines of clinical evidence show that adiponectin has protective actions on the cardiovascular system (26, 27). Circulating levels of adiponectin in humans are as high as 500–30,000 μg/ml (28). Therefore, the concentration of adiponectin (300 ng/ml) used in this study are within physiological levels. Especially, consistent with our findings, adiponectin has been implicated in apoptosis of cardiovascular cells (19, 23, 29). Adiponectin inhibits apoptosis in cardiac myocytes and fibroblasts that are exposed to hypoxia-reoxygenation stress (19). In endothelial cells, adiponectin has been reported to inhibit serum starvation-induced apoptosis (23). *In vivo* experiments have also shown that adiponectin-deficient mice develop larger myocardial infarcts due to increased myocardial cell apoptosis and TNFα expression (17). Taking these observations together with our results, the antiapoptotic actions of adiponectin contribute to the inhibition of VSMC calcification.

Most effects of adiponectin have been attributed to the activation of AMPK, which affects many aspects of cellular metabolism including glucose uptake (30, 31), glucose utilization (32), and fatty acid oxidation (33, 34). Recently, AMPK activation in VSMC has been suggested as a target to prevent or treat vascular disease (35, 36). AICAR-induced AMPK activation inhibited angiotensin II-stimulated VSMC proliferation, and administration of AICAR prevented neointimal formation in a rat balloon injury model (35). AMPK activation in VSMC elicited cell cycle arrest at the G1 phase and inhibited cell proliferation via p53 up-regulation (36). Furthermore, in the heart, the inhibitory effects of adiponectin on ischemic injury-induced apoptosis have been shown to be dependent on AMPK activation (19). The results of *in vitro* studies also revealed that AMPK signaling is essential for the antiapoptotic activities of adiponectin on endothelial cells (23). These observations are consistent with the finding of the present study that AMPK activated by adiponectin stimulated Gas6 expression to restore the survival pathway, leading to the suppression of calcification.

In the present study, we further demonstrated that adiponectin significantly augmented the transcriptional activity of Gas6

that was decreased by TNFα. Indeed, suppression of AMPK by compound C clearly abrogated this beneficial effect of adiponectin. This result suggests that AMPK participates in the transcriptional regulation of Gas6 by adiponectin and TNFα. Several studies support that AMPK regulates the expression of particular genes at the transcriptional level (37–39). For example, AMPK activation by AICAR enhanced activator protein 1-mediated proopiomelanocortin promoter activities, which were completely abolished by compound C (37). AMPK has been shown to mediate the transcription signal that leads to the repression of phosphoenolpyruvate carboxykinase expression, a key enzyme of gluconeogenesis, through phosphorylation of a transcription factor, AICAR-responsive element binding protein (38). It has also been observed that AICAR treatment is able to reduce nuclear factor-κB-regulated transcription, which is activated by TNFα (39).

Consistent with our findings, it has been recently reported that TNFα directly suppresses AMPK activation by promoting protein phosphatase 2C (PP2C) activity via TNF receptor-1 (40). PP2C has been proposed as one of modulators of the covalent regulation of AMPK (41). Increased PP2C levels account for the reduced AMPK activity and phosphorylation after TNFα treatment (40). On the other hand, LKB1 [also known as serine/threonine kinase II (STK II)] is the well-known, principal upstream kinase of AMPK (42, 43) that is regulated by adiponectin (44). AMPK activation by adiponectin is considered to be mediated by the cell surface receptors adiponectin receptors 1 and 2 (45). Another adiponectin receptor, T-cadherin, has recently been identified (46). In preliminary experiments, we found that all of the three adiponectin receptors were endogenously expressed in HASMC, and Pi did not affect their expression (data not shown). Taking these observations together, we hypothesized the mechanism of regulation by adiponectin and TNFα on Pi-induced vascular calcification (Fig. 7). However, further intensive investigations are required to elucidate the role of each player in VSMC calcification.

In summary, adiponectin inhibited VSMC calcification and antagonized the stimulatory effect of TNFα. This action was caused by preventing apoptosis via AMPK activation, followed by restoration of the Gas6-mediated survival pathway. AMPK regulated Gas6 expression at the transcriptional level. AMPK activation regulated by adiponectin and TNFα in vascular calcification might be a key to the management of cardiovascular disease.

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Address all correspondence and requests for reprints to: Masahiro Akishita, M.D., Ph.D., Department of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. E-mail: akishita-tky@umin.ac.jp.

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Raloxifene analogue LY117018 suppresses oxidative stress-induced endothelial cell apoptosis through activation of ERK1/2 signaling pathway

Jing Yu [a,b], Masato Eto [a], Koichi Kozaki [a], Masahiro Akishita [a], Tetsuro Okabe [b], Yasuyoshi Ouchi [a,*]

[a] Department of Geriatric Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan
[b] Department of Integrated Traditional Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

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ABSTRACT

A selective estrogen receptor modulator, raloxifene, has been shown to reduce cardiovascular events in relatively high-risk postmenopausal women with osteoporosis. However, the mechanisms by which raloxifene exerts a pharmacological effect on cardiovascular organs have not been fully elucidated. The present study was designed to examine whether the raloxifene analogue, 6-hydroxy-2-(*p*-hydroxyphenyl)-benzo(b) thien-3-yl-*p*-(2-(pyrrolidinyl)ethoxy phenyl ketone (LY117018), could inhibit apoptosis and to clarify the signaling pathway in vascular endothelial cells. LY117018 significantly inhibited hydrogen peroxide-induced apoptosis in bovine carotid artery endothelial cells. The anti-apoptotic effect of LY117018 was abolished by an estrogen receptor antagonist, 7α,7β-(9[(4,4,5,5,5-Pentafluoropentyl)sulfinyl]nonyl) estra-1,3,5(10)-triene-3,17-diol (ICI 182,780). Mitogen-activated protein kinases (MAPK), including p38, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated protein kinase1/2 (ERK1/2), and Akt, have been shown to act as apoptotic or anti-apoptotic signals. Phosphorylation of p38, JNK, ERK1/2 and Akt was examined. LY117018 increased ERK1/2 phosphorylation but did not enhance the phosphorylation of p38, JNK, or Akt. The anti-apoptotic effect of LY117018 was prevented by treatment with 2-[2′-amino-3′-methoxyphenyl]-oxanaphthalen-4-one (PD98059), an upstream inhibitor of ERK1/2. LY117018 stimulated an increase in ERK1/2 phosphorylation, which was diminished by ICI 182,780. The activation of ERK/1/2 by LY117018 was not inhibited by the transcription inhibitor, actinomycin D. These results suggest that estrogen receptors and the ERK1/2 signaling pathway are involved in the anti-apoptotic action of LY117018 in vascular endothelial cells.

1. Introduction

The incidence of clinical coronary heart disease in premenopausal women is very low. However, following the menopause, atherogenic risk factors increase and the rate of clinical coronary events accelerates to the level observed in men (Kannel et al., 1976). This difference has been considered to be attributable to the protective effects of estrogen before the menopause (Clarkson, 2007). Recent randomized placebo-controlled trials of hormone replacement therapy, however, have not shown any benefit in either the secondary or the primary prevention of cardiovascular events (Hulley et al., 1998; Grady et al., 2002; Rossouw et al., 2002).

Much current interest is focused on the therapeutic potential of selective estrogen receptor modulators. Interestingly, drugs of this class show estrogen-antagonist effects in the mammary gland and uterus, while they have estrogen-agonist effects in bone and other tissues (Delmas et al., 1997; Grady et al., 2004; Johnell et al., 2004; Cox et al., 2004; Sporn et al., 2004). Thus, they are expected to overcome the adverse effects found with conventional hormone replacement therapy.

Recently, the MORE (Multiple Outcomes of Raloxifene Evaluation) study showed that a representative selective estrogen receptor modulator, raloxifene, significantly reduced cardiovascular events in relatively high-risk postmenopausal women with osteoporosis (Barrett-Connor et al., 2002). The death of endothelial and vascular smooth muscle cells is implicated in several pathological vascular conditions, such as atherosclerosis and aneurysm formation. Endothelial damage/dysfunction plays a central role in the clinical manifestation of coronary atherosclerosis (Ross, 1990; Ross, 1999). It has been reported that selective estrogen receptor modulators show a variety of direct actions on vascular cells via estrogen receptors (Simoncini et al., 1999; Simoncini et al., 2002). However, the effect of selective estrogen receptor modulators on endothelial apoptosis has not been clarified.

The aim of this study was to examine the effect of a raloxifene analogue, 6-hydroxy-2-(*p*-hydroxyphenyl)-benzo(b) thien-3-yl-*p*-(2-(pyrrolidinyl)ethoxy phenyl ketone (LY117018), on endothelial apoptosis and to clarify the mechanisms of action.

* Corresponding author. Department of Geriatric Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Tel.: +81 3 5800 8830; fax: +81 3 5800 6529.
E-mail address: youchi-tky@umin.ac.jp (Y. Ouchi).

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2. Materials and methods

2.1. Chemicals and reagents

The raloxifene analogue LY117018 was provided by Eli-Lilly (Indianapolis, IN, USA). 1,3,5(10)-estariene-3,17β-diol (17β-estradiol), wortmannin and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma (St. Louis, MO, USA). Phenol red-free Medium199 (M199) was from Gibco (NY, USA). 7α,7β-(9[(4,4,5,5,5-Pentafluoropentyl) sulfinyl]nonyl) estra-1,3,5(10)-triene-3,17-diol (ICI 182,780) was purchased from AstraZeneca (Macclesfield, Cheshire, UK). Hydrogen peroxide (H_2O_2 30% solution) and actinomycin D were obtained from Wako (Osaka, Japan). The mitogen-activated protein/extracellular signal-regulated protein kinase (MEK)1 inhibitor, 2-[2'-amino-3'-methoxyphenyl]-oxanaphthalen-4-one (PD98059), and antibodies against Akt, phospho-Akt (Ser-473), c-Jun N-terminal kinase (JNK), phospho-JNK (Thr183/Tyr185), extracellular signal-regulated protein kinase1/2 (ERK1/2) and phospho-ERK1/2 (Thr202/Tyr204) were purchased from Cell Signaling (Beverly, MA, USA). Antibodies against p38 (A-12) and phospho-p38 (D-8) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The JNK inhibitor anthrax [1, 9-cd] pyrazol-6(2H)-one (SP600125) and the p-38 inhibitor 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) 1H-imidazole (SB203580) were from Calbiochem (Darmstadt, Gemany). Fetal bovine serum (FBS) was from CCT (Sanko Junyaku Co., Ltd., Tokyo, Japan). Charcoal-stripped fetal bovine serum was from MultiSer (ThermoTrace Ltd., Melbourne, Australia). Nitrocellulose membranes were from Amersham (Buckinghamshire, UK). LumiGLO Reserve Chemiluminescent Substrate Kit was from KPL (Gaithersburg, MD, USA). Cell Death Detection ELISA ^{plus} was purchased from Roche (Mannheim, Germany).

2.2. Cell culture

Bovine carotid endothelial cells (BCEC) were provided by Dr. Sudoh and prepared as described previously (Sudoh et al., 2001; Akishita et al., 1998). Cells were cultured in a 37 °C humidified atmosphere of 95% air/5% CO_2 in DMEM containing 10% FBS and 100 units/ml penicillin/100 μg/ml streptomycin. For all experiments, BCEC were used at passages 5 to 7, and plated at a concentration of 10^4 cells/ml. Raloxifene experiments were performed with phenol red-free M199. DMSO was used as a solvent for LY117018, 17β-estradiol, ICI 182,780 and PD98059. DMSO was present at equal concentrations (0.05%) in all groups, including the vehicle group.

2.3. Apoptosis induction

Apoptosis was induced by addition of hydrogen peroxide (H_2O_2). At 70–80% confluence, cells were washed with phosphate-buffered saline (PBS), and then replenished with phenol red-free M199 without serum, and proliferation was stopped. Cells were exposed to 100 μM H_2O_2 for 1 h after 6 h starvation, washed twice again with PBS (−), then replenished with phenol red-free M199 containing 5% DCC-FBS. In the same experiments, LY117018 or 17β-estradiol was added for 30 min before H_2O_2 stimulation in the apoptosis assay. In experiments on inhibitors, the inhibitors were added for 60 min before LY117018 addition. After 24 h of stimulation by H_2O_2, cell apoptosis was evaluated.

2.4. Assay of endothelial cell apoptosis (DNA fragmentation assay)

Cell apoptosis was quantified by means of DNA fragmentation, using a photometric enzyme-linked immunosorbent assay (Cell Death Detection ELISA ^{plus}) kit. Cells with each treatment were lysed in 300 μl lysis buffer, and a fraction of the supernatant was subjected to reaction for 2 h with the immunocomplex of anti-DNA conjugated with peroxidase, which binds to nucleosomal DNA, and antihistone–biotin, which interacts with streptavidin-coated wells in a microtiter plate. At the end of the incubation, substrate was added, and development was quantified at 405 nm wavelength.

2.5. Western blot analysis

After treatment with reagents, confluent monolayers of cells were washed two times in ice-cold phosphate-buffered saline and lysed with buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM PMSF. For Western blot analysis, total cell lysate was subjected to SDS-polyacrylamide gel electrophoresis (PAGE), and proteins were transferred to a polyvinilidene difluoride (PVDF) membrane. The antibodies used in this study were anti-phospho-ERK1/2 (Thr202/Tyr204), anti-ERK1/2, anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-JNK (Thr183/Tyr185), anti-JNK, anti-phospho-p38, and anti-p38. Antibodies were detected by means of a horseradish peroxidase-linked secondary antibody. Immunoreactive bands were visualized using a LumiGLO Reserve Chemiluminescent Substrate Kit and quantified by densitometry in the linear range of NIH image 1.60.

2.6. Statistics

Values are expressed as means ± S.E.M. Statistical comparisons were performed by ANOVA followed by Fisher's protected least significance difference (PLSD) test. A probability value <0.05 was considered significant.

3. Results

3.1. Effect of LY117018 on endothelial cell apoptosis

On the basis of concentration- and time-response experiments (data not shown), H_2O_2 (100 μM) was added to BCEC for 1 h to induce apoptosis. BCEC apoptosis induced by H_2O_2 was significantly attenuated by treatment with LY117018 in a concentration-dependent manner (Fig. 1), while LY117018 per se did not show any effect on apoptosis (data not shown).

3.2. Involvement of MEK/ERK pathway in anti-apoptotic action of LY 110718

Phosphorylation levels of p38, JNK, ERK1/2, and Akt were examined because these kinases have been shown to regulate apoptosis (Xia et al.,

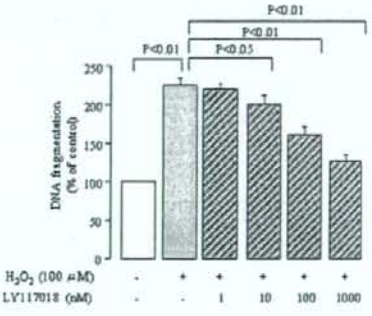

Fig. 1. Effect of LY117018 on H_2O_2-induced endothelial cells apoptosis. At 70–80% confluence, BCEC were starved and exposed to 100 μM H_2O_2 for 1 h as described in Materials and methods. Various concentrations of LY117018 (1 nM–1 μM) were added to the culture medium 30 min before H_2O_2 stimulation in the apoptosis assay. After a 24-h incubation, cell apoptosis was evaluated by means of DNA fragmentation (with a Cell Death Detection ELISA ^{plus} kit) as described in Materials and methods. Data are expressed as means ± S.E.M. Differences with a value of $P<0.05$ were considered statistically significant ($n=6$).

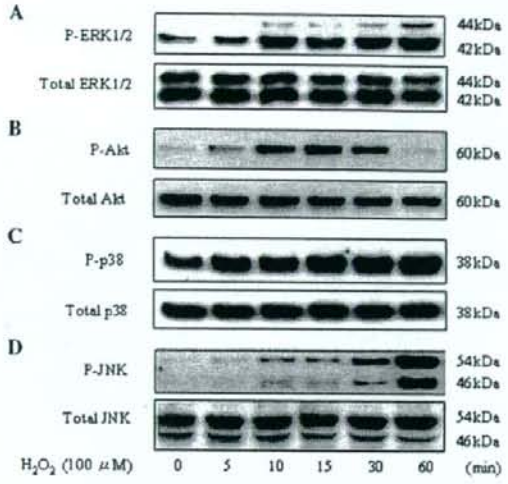

Fig. 2. Phosphorylation of p38, JNK, ERK1/2 and Akt induced by H_2O_2. Serum-starved cells were stimulated with H_2O_2 (100 μM) and harvested at the times indicated for Western blot analysis. Antibodies against (A) phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, (B) phospho-Akt (Ser473), Akt, (C) phopho-p38, p38, (D) phospho-JNK (Thr183/Tyr185) and JNK were used as described in Materials and methods.

1995; Matsuzaki et al., 1999; Uchiyama et al., 2004). Phosphorylation levels of p38 (D-8), JNK (Thr183/Tyr185), ERK1/2 (Thr202/Tyr204), and Akt (Ser473) were elevated after exposure to H_2O_2, with no significant change in the total protein level (Fig. 2A, B, C, D). Maximal phosphorylation was observed at 15 min for Akt (Fig. 2B) and at 60 min for ERK, p38 and JNK (Fig. 2A, C and D).

We examined the effects of a p38 inhibitor, SB203580, and a JNK inhibitor, SP600125, on BCEC apoptosis. BCEC apoptosis was significantly decreased by the inhibitors of p38 and JNK (data not shown). We also examined the effects of a MEK1 (MEK is the immediate upstream regulator of ERK) inhibitor, PD98059, and a phosphatidylinositol-3 OH (PI3) kinase inhibitor, wortmannin, on BCEC apoptosis. PD98059 and wortmannin significantly enhanced H_2O_2-induced BCEC apoptosis (data not shown). These results suggest that p38 and JNK act as cell death signals, whereas ERK1/2 and PI3-kinase/Akt act as survival signals in the process of BCEC apoptosis. The induction of apoptosis by H_2O_2 may be regulated by the balance between death signaling and survival signaling.

Next, we examined the effects of LY117018 on the phosphorylation levels of p38, JNK, ERK1/2, and Akt. On the basis of time-response experiments (Fig. 2A, B, C, D), cells were stimulated with 100 μM H_2O_2 for 15 min for examination of Akt activity and for 60 min for examination of ERK1/2, p38 and JNK activity. Cells were pretreated with LY117018 for 30 min prior to exposure to H_2O_2. LY117018 significantly enhanced the phosphorylation level of ERK1/2 (Fig. 3A). However, no change in the phosphorylation of Akt (Fig. 3B), p38 (Fig. 3C), and JNK (Fig. 3D) was induced by LY117018.

We examined the effects of PD98059 on the anti-apoptotic activity of LY117018. The anti-apoptotic effect of LY117018 was prevented by PD98059 (Fig. 3E), while PD98059 alone did not induce BCEC apoptosis. These results suggest that the anti-apoptotic effect of LY117018 was not exerted by inhibition of cell death signals such as p38 or JNK, or by activation of a survival signal, PI3-kianse/Akt, but

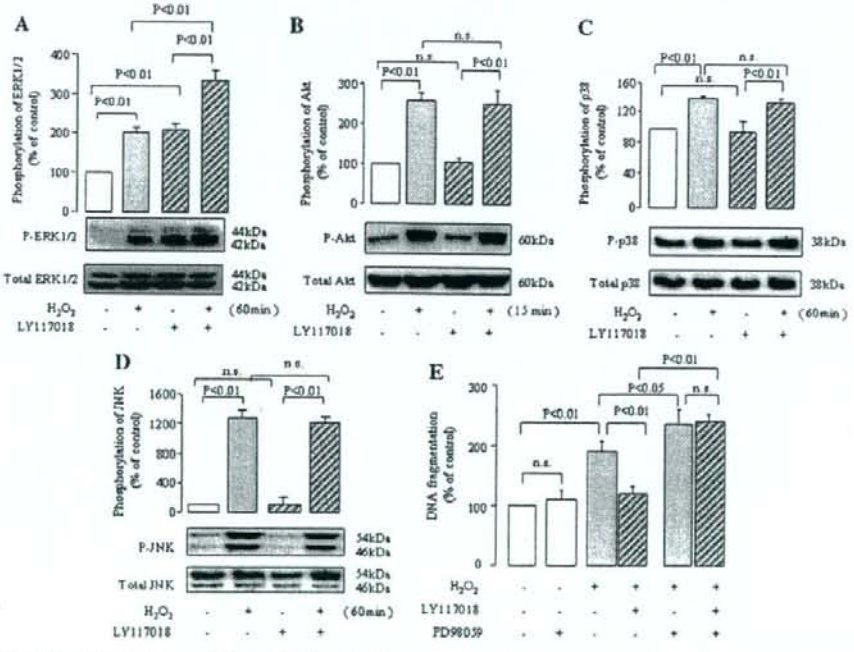

Fig. 3. Effect of LY117018 on H_2O_2-induced activation of p38, JNK, ERK1/2 and Akt. Serum-starved cells were pretreated with 1 μM LY117018 for 30 min. Then cells were stimulated with H_2O_2 (100 μM) for 15 min for determination of Akt activity (B) and for 60 min for determination of ERK1/2, p38 and JNK (A, C and D) activity. Cells were harvested, lysed and used for Western blot analysis. The activities of ERK1/2 (Thr202/Thr204), Akt (Ser473), p38 (D-8) and JNK (Thr183/Tyr185) were measured as described in Materials and methods. Representative blots and quantitative data evaluated by densitometry are shown ($n=3$). The data are expressed as means±S.E.M. Differences with a value of $P<0.05$ were considered statistically significant. (E) In the PD98059 experiment, cells were pretreated with PD98059 (10 μM) for 1 h before addition of LY117018 (1 μM, 30 min), then stimulated with 100 μM H_2O_2 for 1 h. After a 24-h incubation, cell apoptosis was evaluated by means of DNA fragmentation as described in Materials and methods. Values are expressed as means±S.E.M. Differences with a value of $P<0.05$ were considered statistically significant ($n=6$).

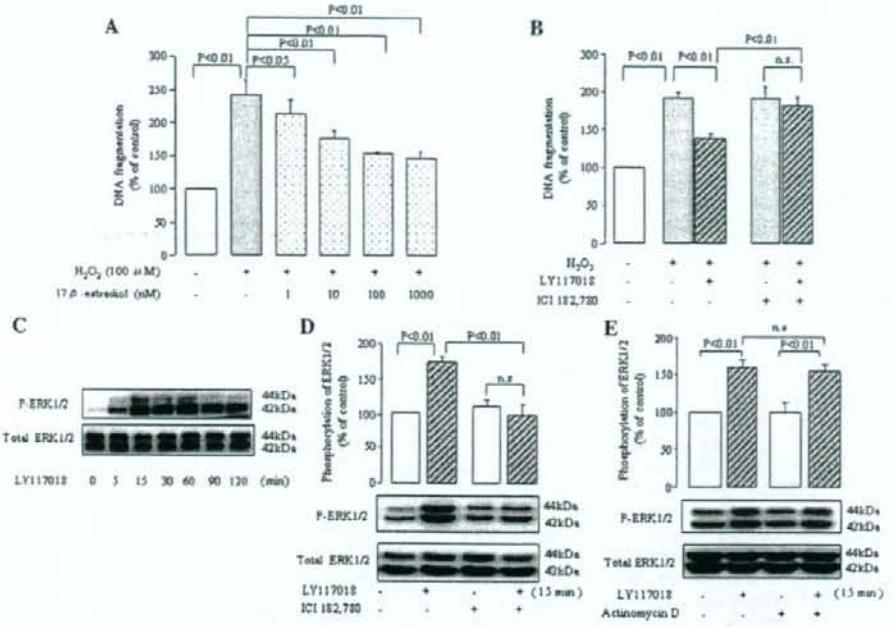

Fig. 4. Involvement of estrogen receptors in anti-apoptotic action of LY117018. At 70–80% confluence, BCEC were starved and exposed to H_2O_2 (100 μM) for 1 h as described in Materials and methods (A and B). (A) Various concentrations of 17β-estradiol (1 nM–1 μM) were added to the culture medium 30 min prior to exposure to H_2O_2 in the apoptosis assay. (B) In the estrogen receptor antagonist experiment, cells were pretreated with ICI 182,780 (10 μM) for 1 h before addition of 1 μM LY117018. Apoptosis was evaluated after 24 h of H_2O_2 treatment by means of DNA fragmentation (with a Cell Death Detection ELISA ^{plus} kit). Data are expressed as means ± S.E.M. Differences with a value of $P<0.05$ were considered statistically significant ($n=6$). (C, D and E) Serum-starved cells were stimulated with 1 μM LY117018 and harvested at the times indicated (C). In some groups, cells were pretreated with 10 μM ICI 182,780 (D) or 5 μg/ml actinomycin D (E) for 1 h before addition of LY117018 (1 μM, 30 min). Cell lysates were analyzed by Western blot as described in Materials and methods using a specific antibody against phospho-ERK1/2 (Thr202/Tyr204) or total ERK1/2. Representative blots and quantitative data evaluated by densitometry are shown ($n=3$). Values are expressed as means ± S.E.M. Differences with a value of $P<0.05$ were considered statistically significant.

was mediated through activation of another survival signal, the ERK1/2 pathway.

3.3. Involvement of estrogen receptors in anti-apoptotic action of LY117018

17β-Estradiol, an endogenous ligand for estrogen receptors, inhibited BCEC apoptosis in a concentration-dependent manner (Fig. 4A). 17β-Estradiol exerted an anti-apoptotic action at 1 nM, while LY117018 at 10 nM protected endothelial cells from apoptosis induced by H_2O_2 (Fig. 1). ICI 182,780, an estrogen receptor antagonist, significantly diminished the inhibitory effect of LY117018 on BCEC apoptosis (Fig. 4B). In addition, LY117018 per se rapidly increased the phosphorylation of ERK1/2 more than 5 min after its addition. Maximal phosphorylation was attained after 15 min of incubation (Fig. 4C). The LY117018-induced increase in ERK1/2 phosphorylation was significantly suppressed by ICI 182,780 (Fig. 4D). These results suggest that estrogen receptors are involved in the increased phosphorylation of ERK1/2 by LY117018.

To examine whether the LY117018-induced increase in ERK1/2 phosphorylation is due to a genomic or non-genomic action, the transcription inhibitor, actinomycin D, was added to BCEC prior to treatment with LY117018. The activation of ERK1/2 was not inhibited by actinomycin D (Fig. 4E). These results suggest that the anti-apoptotic activity of LY117018 is exerted through a non-genomic action.

4. Discussion

In the present study, we found that the raloxifene analogue, LY117018, inhibited BCEC apoptosis induced by H_2O_2. This inhibitory effect of LY117018 was concentration dependent. LY117018 at 10 nM protected endothelial cells from apoptosis by H_2O_2, while 17β-estradiol exerted an anti-apoptotic action at 1 nM. This may be explained by the difference in receptor ligand affinity between 17β-estradiol and LY117018. Indeed, the relative binding affinity of 17β-estradiol to estrogen receptor alpha is about 10 times higher than that of raloxifene in estrogen receptor-positive MCF-7 cells (Wijayaratne et al., 1999). The lower affinity of raloxifene for the estrogen receptor may be attributable to a structural difference. In addition, the concentrations of LY117018 used in our study might be relevant, because if we consider that the dose of raloxifene used in clinical settings is 120 mg/day, the serum concentration found in women treated with raloxifene is about 6 nM (Eli-Lilly, Indianapolis, IN, USA, unpublished data, 2003), which is close to the effective concentration of LY117018 in our experiments.

It has been reported that the inhibitory effect of raloxifene on bone absorption is mediated by direct binding with estrogen receptors. Endothelial cells express both estrogen receptor alpha (ER-alpha) and beta (ER-beta). In order to examine whether the anti-apoptotic effects of LY117018 are mediated by estrogen receptors, we examined the effects of a specific estrogen receptor antagonist, ICI 182,780. The anti-apoptotic effect of LY117018 was abolished by ICI 182,780. In addition, 17β-estradiol, an endogenous ligand for estrogen receptors, significantly inhibited apoptosis in BCEC. These observations suggest that LY117018 acts as an estrogen receptor agonist in endothelial cells, leading to endothelial cell survival. It has been reported that steroid hormones cause rapid responses, in minutes, through their membrane receptors. In recent years, several studies regarding the non-genomic actions of estradiol through estrogen receptors have been reported

(Razandi et al., 2003). In vascular cells, the roles of membrane estrogen receptors have been extensively investigated. Estrogen receptors mainly exist in the nucleus as ligand-dependent transcriptional factors, whereas a small amount of estrogen receptors in the cytoplasm do not enter the nucleus upon ligand stimulation and induce rapid signaling events (Pedram et al., 2002). LY117018 rapidly increased the phosphorylation of ERK1/2 after 5 min, and maximal phosphorylation was attained after 15 min of incubation. In addition, the increase in ERK1/2 phosphorylation was not inhibited by actinomycin D. These results suggest that the anti-apoptotic activity of LY117018 is exerted through a non-genomic action.

Recent studies support the idea that the induction of apoptosis by H_2O_2 is regulated by the balance between death signaling (p38, and JNK) and survival signaling (MEK/ERK1/2, and PI3-kinase/Akt) (Xia et al., 1995; Matsuzaki et al., 1999; Uchiyama et al., 2004). Indeed, in our study, H_2O_2 induced the phosphorylation of Akt, ERK1/2, JNK and p38. The p38 inhibitor, SB203580, and JNK inhibitor, SP600125, significantly decreased BCEC apoptosis induced by H_2O_2, whereas the PI3-kinase inhibitor, wortmannin, and MEK1 inhibitor, PD98059, significantly enhanced it. These results suggest that p38 and JNK act as cell death signals, whereas ERK1/2 and PI3-kinase/Akt act as survival signals in the process of BCEC apoptosis. Then we investigated the signaling pathways responsible for the anti-apoptotic effect of LY117018. Interestingly, LY117018 enhanced the phosphorylation level of ERK1/2 only, while it did not enhance the phosphorylation level of Akt or decrease that of p38 and JNK. In addition, PD98059 completely abolished the anti-apoptotic effect of LY117018, suggesting that the anti-apoptotic effect of LY117018 is mediated through enhancement of ERK1/2 signaling in vascular endothelial cells.

In conclusion, LY117018, an analogue of raloxifene, inhibits H_2O_2-induced endothelial apoptosis by activating ERK1/2, which is a non-genomic action via estrogen receptors. This study provides experimental evidence to support a novel therapeutic approach to pathological vascular conditions such as atherosclerosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejphar.2008.04.052.

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Analysis of factors influencing pancreatic β-cell function in Japanese patients with type 2 diabetes: Association with body mass index and duration of diabetic exposure

Shogo Funakoshi [a], *Shimpei Fujimoto* [a,*], *Akihiro Hamasaki* [a], *Hideya Fujiwara* [a], *Yoshihito Fujita* [a], *Kaori Ikeda* [a], *Yoshiyuki Hamamoto* [a], *Masaya Hosokawa* [a], *Yutaka Seino* [b], *Nobuya Inagaki* [a]

[a] *Department of Diabetes and Clinical Nutrition, Graduate School of Medicine, Kyoto University, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan*
[b] *Kansai Electric Power Hospital, Osaka, Japan*

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ABSTRACT

Aims: To elucidate the clinical factors affecting β-cell function, serum C-peptide immunoreactivity (CPR) levels of patients with type 2 diabetes were analyzed.

Methods: Seven hundred Japanese patients with type 2 diabetes were enrolled. β-Cell function was evaluated by fasting CPR (FCPR), 6 min after intravenous injection of 1 mg glucagon (CPR-6 min), and the increment of CPR (ΔCPR). Simple regression analysis between FCPR, CPR-6 min, and ΔCPR and measures of variables and stepwise multiple regression analysis were carried out.

Results: Years from diagnosis and BMI were the major independent variables predicting β-cell function. Years from diagnosis was negatively correlated with CPR-6 min ($P < 0.0001$, $r = -0.271$), and decrease in CPR-6 min was 0.050 ng/(ml year). BMI was positively correlated with CPR-6 min ($P < 0.0001$, $r = 0.369$). When subjects were divided according to BMI, the decrease in CPR-6 min per year in the high-BMI group (0.068 ng/(ml year)) was greater than that in the low-BMI group (0.035 ng/(ml year)).

Conclusion: A linear decline in endogenous insulin secretion over more than several decades of diabetes was confirmed by this cross-sectional study. The duration of diabetes exposure and BMI are thus major factors in β-cell function in Japanese patients with type 2 diabetes.

1. Introduction

Type 2 diabetes is a heterogeneous disease characterized by insulin resistance and defective insulin secretion [1], and is progressive in that therapy must be altered over time. Initially upon diagnosis, diet and exercise are generally adequate to achieve glycemic control. Oral hypoglycemic agents (OHA) are later required when patients cannot achieve glycemic control with diet and exercise. Daily insulin injection is finally indicated when patients are unable to achieve glycemic control with a combination of oral agents, diet, and exercise [2,3]. These requirements may well be, at least in part, due to progressive loss of pancreatic β-cell function. The results of the United Kingdom Prospective Diabetes Study (UKPDS) shows that pancreatic β-cell function (%β), assessed by Homeostasis Model Assessment (HOMA) in patients allocated

* *Corresponding author.* Tel.: +81 75 751 3560; fax: +81 75 751 4244.
E-mail address: fujimoto@metab.kuhp.kyoto-u.ac.jp (S. Fujimoto).
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to diet or OHA, decreased approximately 25% in 5 years [4]. However, the effect of more prolonged duration of diabetic exposure on β-cell function including the insulin-requiring stage has not been determined. It has been proposed that the β-cell secrets additional insulin to compensate for increasing insulin resistance to maintain normal glucose tolerance [5]. However, the effect of insulin resistance on β-cell function in diabetes remains largely unknown.

In the present study, to evaluate the clinical factors affecting β-cell function by cross-sectional study, serum C-peptide immunoreactivity levels of patients with type 2 diabetes including insulin-requiring patients were analyzed.

2. Subjects and methods

2.1. Study subjects

Seven hundred ninety-eight Japanese patients with type 2 diabetes admitted between 1997 and 2007 to Kyoto University Hospital for poor glycemic control were enrolled in the study. Type 2 diabetes mellitus was diagnosed based on the criteria of the American Diabetes Association [6]. Patients with pancreatic disease, liver disease, or those taking diabetogenic medication were excluded. Patients with serum creatinine ≥1.3 mg/dl were excluded, as serum C-peptide immunoreactivity (CPR) is elevated by decreased renal function [7]. Seven hundred patients were enrolled. Age and BMI (mean ± S.E.) were 62.2 ± 0.5 years and 24.1 ± 0.1 kg/m^2, respectively. Years from diagnosis was 11.1 ± 0.4; HbA_{1C} at admission was 9.3 ± 0.1%. Systolic and diastolic blood pressure were 122.6 ± 0.5 and 73.3 ± 0.4 mmHg, respectively. The number of patients treated with diet alone, oral hypoglycemic agents (OHA), insulin, and insulin plus OHA was 71, 225, 274, and 130, respectively.

2.2. Methods

On the first or second day in hospital, medical history, physical examination, and laboratory evaluation including glycosylated hemoglobin were performed. β-Cell function was examined within 1 week. β-Cell function was evaluated after overnight fast by glucagon test measuring CPR before [fasting CPR (FCPR)] and 6 min after intravenous injection of 1 mg glucagon (CPR-6 min) [8], as the test is valid in patients taking insulin therapy. Increment of CPR (ΔCPR) was obtained by subtracting FCPR from CPR-6 min. Serum CPR was measured by radioimmunoassay (RIA) (samples of 299 patients from 1997 to 2002 using Daiichi III, Daiichi Radioisotope Laboratories, Japan); by immunoenzymometric assay (EIA) (samples of 401 patients from 2003 to 2007 using ST AIA-PACK C-Peptide, Toso corporation, Japan). The same samples were measured by two kits, and a formula to convert the value (EIA value = 0.98 × RIA value + 0.41, $n = 161$, $r = 0.99$) was obtained. In patients taking OHA, medication was stopped for the glucagon test, but was maintained until 1 day before to prevent hyperglycemia during the test. Fasting plasma glucose was measured by glucose oxidase method when the glucagon test was performed. The date of diagnosis for patients was determined from their medical record, medical history, and previous clinical data by the criteria for the diagnosis of diabetes proposed by the American Diabetes Association [6].

2.3. Statistical analysis

Statistical analysis was performed with the StatView 5.0 system (SAS institute Inc., Cary, NC). Data are presented as mean ± S.E. The relationship between the parametric clinical data and CPR values was investigated by Pearson analysis. The relationship between the nonparametric clinical data and CPR values was investigated by Spearman analysis. Stepwise multiple regression analysis was performed. Clinical parameters among the three groups above were compared by analysis of variance (ANOVA). For comparison of two groups, Scheffe's test was performed as post hoc analysis. P values <0.05 were considered statistically significant.

3. Results

Simple correlation coefficients between FCPR, CPR-6 min, and ΔCPR and measures of variables (age, years from diagnosis, BMI, HbA_{1C}, systolic and diastolic blood pressure, serum creatinine, sex, and fasting plasma glucose) were calculated and are indicated in Table 1. Stepwise multiple regression analysis was carried out using independent variables in Table 1 to predict indexes of endogenous insulin secretion as a dependent variable (Table 2). Stepwise multiple regression analysis is indicated in Table 2. FCPR was independently predicted by years from diagnosis, BMI, and serum creatinine,

Table 1 – P value of simple correlation between indexes of endogenous insulin secretion and measures of variables.

	FCPR (ng/ml)	CPR-6 min (ng/ml)	ΔCPR (ng/ml)
Age (year)	0.0326	<0.0001	<0.0001
Years from diagnosis	<0.0001	<0.0001	<0.0001
BMI (kg/m^2)	<0.0001	<0.0001	<0.0001
Systolic blood pressure (mmHg)	0.9481	0.2091	0.0593
Diastolic blood pressure (mmHg)	0.1139	0.0044	0.0016
FPG (mg/dl)	0.0783	0.0010	0.0002
HbA_{1C} (%)	0.0375	0.2152	0.7699
sCre (mg/dl)	<0.0001	0.0836	0.3917
Sex	0.8978	0.7958	0.8206

FPG: fasting plasma glucose; sCre: serum creatinine.

Table 2 – Stepwise multiple regression analysis for predictors of indexes of endogenous insulin secretion.

	F value	Partial regression coefficient	Standard partial regression coefficient	R^2 (IRI)
FCPR (ng/ml)				
Years from diagnosis	41.4	−0.020	−0.219	0.217 (0.469)
BMI (kg/m^2)	114.4	0.087	0.362	
sCre (mg/dl)	27.7	0.877	0.179	
CPR-6 min (ng/ml)				
Years from diagnosis	45.8	−0.043	−0.233	0.198 (0.449)
BMI (kg/m^2)	102.8	0.167	0.348	
FPG (mg/dl)	10.8	0.004	0.113	
ΔCPR (ng/ml)				
Years from diagnosis	34.7	−0.025	−0.212	0.129 (0.365)
BMI (kg/m^2)	47.9	0.077	0.248	
FPG (mg/dl)	13.2	0.003	0.130	

FPG: fasting plasma glucose; sCre: serum creatinine.

accounting for 21.7% of the variability of FCPR. CPR-6 min and ΔCPR were independently predicted by years from diagnosis, BMI, and fasting plasma glucose (FPG), accounting for 19.8% and 12.9% of the variability of the dependent variables, respectively.

Scattered plots of linear regression, simple regression coefficient, and formula between indexes of endogenous insulin secretion and years from diagnosis, BMI, FPG, and serum creatinine are shown in Figs. 1–4, respectively.

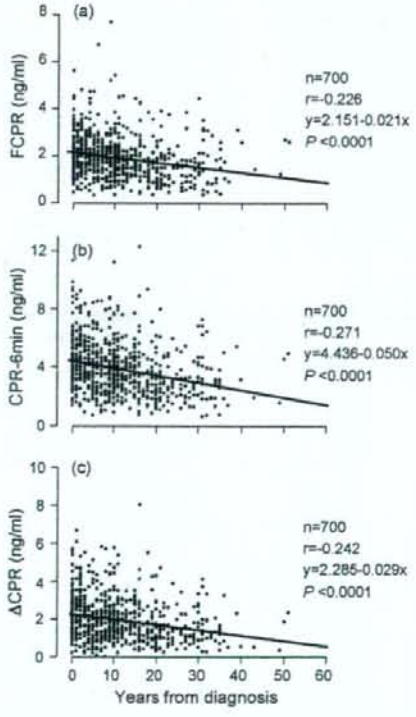

Fig. 1 – The relationship between years from diagnosis and FCPR (a), CPR-6 min (b), and ΔCPR (c).

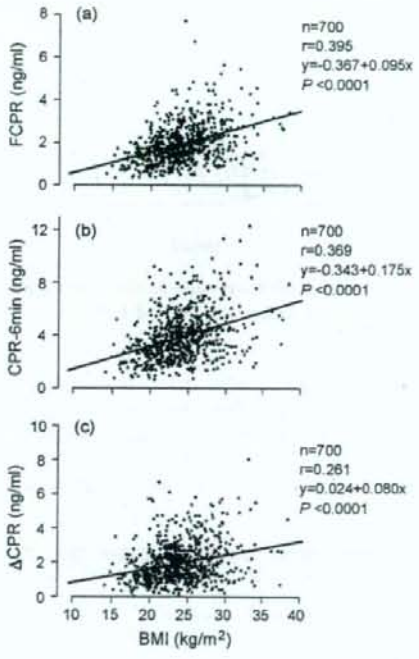

Fig. 2 – The relationship between BMI and FCPR (a), CPR-6 min (b), and ΔCPR (c).

Years from diagnosis was negatively correlated with FCPR ($P < 0.0001$, $r = -0.226$), CPR-6 min ($P < 0.0001$, $r = -0.271$), and ΔCPR ($P < 0.0001$, $r = -0.242$) (Fig. 1). The decrease in FCCP, CPR-6 min, and ΔCPR was 0.021, 0.050, and 0.029 ng/(ml year), respectively (Fig. 1). BMI was positively correlated with FCPR ($P < 0.0001$, $r = 0.395$), CPR-6 min ($P < 0.0001$, $r = 0.369$), and ΔCPR ($P < 0.0001$, $r = 0.261$) (Fig. 2). FPG was weakly correlated with CPR-6 min ($P = 0.001$, $r = 0.125$) and ΔCPR ($P = 0.0002$, $r = 0.141$) (Fig. 3). Serum creatinine was weakly correlated with FCPR ($P < 0.0001$, $r = 0.171$) (Fig. 4).

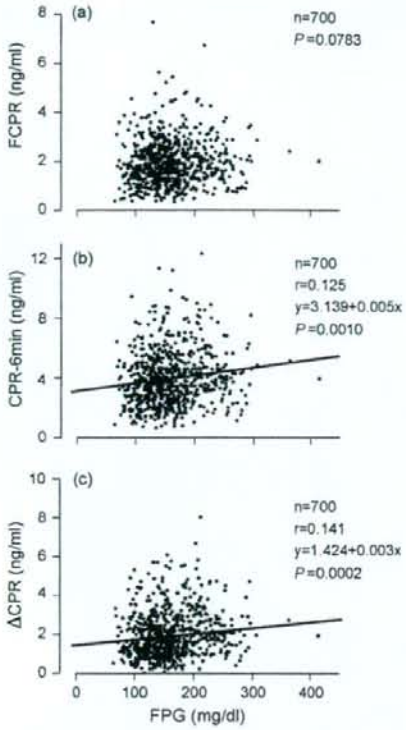

Fig. 3 – The relationship between fasting plasma glucose (FPG) and FCPR (a), CPR-6 min (b), and ΔCPR (c).

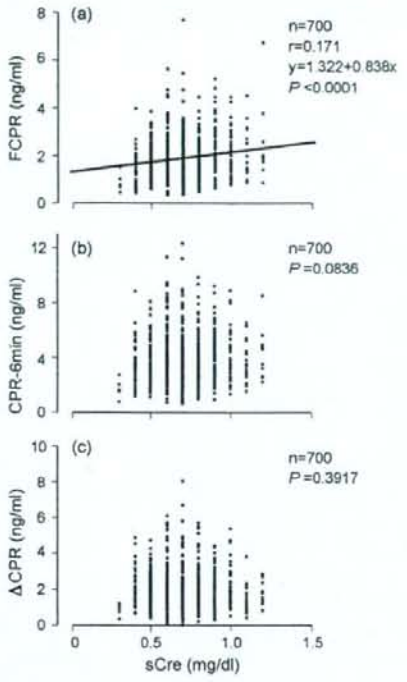

Fig. 4 – The relationship between serum creatinine (sCre) and FCPR (a), CPR-6 min (b), and ΔCPR (c).

Because BMI is the other major independent variable associated with indexes of endogenous insulin secretion, we compared the subjects divided at BMI 25.0. Single regression analysis between CPR-6 min and years from diagnosis was performed on each group. Years from diagnosis was negatively correlated with CPR-6 min in both groups (BMI < 25: $n = 430$, $P < 0.0001$, $r = -0.233$, $y = 3.876 - 0.035x$; BMI ≥ 25.0: $n = 270$, $P < 0.0001$, $r = -0.309$, $y = 5.229 - 0.067x$). The decrease in CPR-6 min per year in the high-BMI (27.9 ± 0.2 kg/m^2) group (0.067 ng/(ml year)) was greater than that in the low-BMI (21.8 ± 0.1 kg/m^2) group (0.035 ng/(ml year)). Comparison of the clinical data among four groups of increasing years from

Table 3 – Comparison of clinical characteristics and clinical profile among groups according to years from diagnosis.

Groups (years from diagnosis)	I (–9.9)	II (10.0–19.9)	III (20.0–29.9)	IV (30–)	P
Years from diagnosis	3.6 ± 0.2	13.3 ± 0.2[a]	23.4 ± 0.3[a,b]	34.0 ± 0.7[a,b,c]	<0.0001
Number of subjects	355	204	94	47	
Sex (M/F)	215/140	114/90	50/44	31/16	
Age (year)	58.4 ± 0.7	63.7 ± 0.8[a]	68.8 ± 0.9[a,b]	70.9 ± 1.2[a,b]	<0.0001
BMI (kg/m^2)	24.4 ± 0.2	24.0 ± 0.3	23.3 ± 0.3	23.9 ± 0.5	0.0912
HbA_{1c} (%)	9.5 ± 0.1	9.1 ± 0.1	9.4 ± 0.2	8.9 ± 0.2	0.0823
SBP (mmHg)	122.0 ± 0.7	122.1 ± 0.9	124.8 ± 1.5	124.8 ± 2.1	0.2163
DBP (mmHg)	75.3 ± 0.5	72.0 ± 0.6[a]	69.9 ± 0.9[a]	70.8 ± 1.3[a]	<0.0001
sCre (mg/dl)	0.70 ± 0.01	0.72 ± 0.01	0.73 ± 0.02	0.76 ± 0.03	0.0650
FPG (mg/dl)	162.9 ± 2.6	159.7 ± 3.4	154.6 ± 4.9	156.1 ± 7.5	0.4498
FCPR (ng/ml)	2.12 ± 0.05	1.83 ± 0.06[a]	1.46 ± 0.08[a,b]	1.73 ± 0.14[a]	<0.0001
CPR-6 min (ng/ml)	4.30 ± 0.10	3.75 ± 0.12[a]	2.87 ± 0.14[a,b]	3.29 ± 0.24[a]	<0.0001
ΔCPR (ng/ml)	2.18 ± 0.07	1.93 ± 0.08	1.42 ± 0.09[a,b]	1.56 ± 0.15[a]	<0.0001

FPG: fasting plasma glucose; sCre: serum creatinine; SBP: systolic blood pressure; DBP: diastolic blood pressure.
[a] $P < 0.05$ vs. group I.
[b] $P < 0.05$ vs. group II.
[c] $P < 0.05$ vs. group III.

Table 4 – Comparison of indexes of endogenous insulin secretion between low-BMI and high-BMI subjects according to years from diagnosis.

Group (year)	I (–9.9)		II (10.0–19.9)		III (20.0–29.9)		IV (30.0–)	
BMI	<25	≥25	<25	≥25	<25	≥25	<25	≥25
Number of subjects	202	153	130	74	66	28	32	15
FCPR (ng/ml)	1.82 ± 0.06	2.52 ± 0.07*	1.66 ± 0.06	2.12 ± 0.12*	1.35 ± 0.09	1.72 ± 0.14	1.67 ± 0.15	1.85 ± 0.27
CPR-6 min (ng/ml)	3.83 ± 0.12	4.99 ± 0.15*	3.41 ± 0.12	4.33 ± 0.25*	2.70 ± 0.16	3.15 ± 0.26	3.20 ± 0.27	3.48 ± 0.50
ΔCPR (ng/ml)	2.00 ± 0.09	2.46 ± 0.11*	1.76 ± 0.08	2.21 ± 0.16*	1.38 ± 0.10	1.50 ± 0.17	1.54 ± 0.18	1.63 ± 0.27

P values were analyzed by unpaired Student *t* test.
* $P < 0.01$ vs. low-BMI patients.

diagnosis (–9.9 years, 10.0–19.9 years, 20.0–29.9 years, and 30.0– years) is shown in Table 3. Age, diastolic blood pressure, FCPR, CPR-6 min, and ΔCPR were significantly different among the groups. Comparison between the high-BMI and low-BMI subjects in the four groups of increasing years from diagnosis shows that FCPR, CPR-6 min, and ΔCPR were significantly increased in high-BMI subjects with years from diagnosis less than 19.9, while this was not the case when years from diagnosis was more than 20.0 (Table 4).

4. Discussion

While the present study should be interpreted carefully since it is a cross-sectional rather than a longitudinal study, we have shown that endogenous insulin secretory capacity in Japanese patients with type 2 diabetes is more closely associated with variables including years from diagnosis which reflects longer term diabetes exposure, than with variables including HbA_{1c} that reflect shorter term diabetes exposure. A decline of indexes of endogenous insulin secretion during more than several decades of diabetes was confirmed in this cross-sectional study, as was found in the longitudinal 5-year UKPDS study, although the decline became gradual. BMI is also an important independent variable to predict CPR level in type 2 diabetes.

The key role of pancreatic β-cell function in the pathogenesis of type 2 diabetes is increasingly apparent. Weyer et al. has established that impaired insulin secretion is associated with conversion from normal glucose tolerance (NGT) to impaired glucose tolerance (IGT) in a longitudinal study of Pima Indians [5]. In other studies, it has been reported that insulin secretory defect is the predominant abnormality even at an early stage of decreasing glucose tolerance [9–13]. The UKPDS findings show that pancreatic β-cell function (%β), assessed by HOMA in patients with type 2 diabetes, decreased approximately 25% in 5 years [4]. Thus, pancreatic β-cell function may continue to deteriorate as glucose intolerance progresses from NGT via IGT and non-insulin-requiring diabetes progresses to insulin-requiring diabetes [14–16].

Progressive loss of pancreatic β-cell function in patients with type 2 diabetes may be derived, at least in part, from reduced pancreatic β-cell mass, which has been found in examination of pancreatic tissue at autopsy [17–19]. One study suggests that increased apoptosis is more important in reduced β-cell mass than impaired neogenesis and proliferation in type 2 diabetes [17]. Factors in deteriorating pancreatic β-cell function during diabetic exposure including hyperglycemia, hyperlipidemia, cytokines secreted by adipocytes, immune response, and medication have been proposed based mainly on *in vitro* studies [20–22], but clinical evidence of the influence of these factors on β-cell deterioration remains to be elucidated. Assessment of insulin secretory function by HOMA in UKPDS indicated that the rates of loss of insulin secretory function were similar among groups of diet-treated, sulfonylurea-treated, and metformin-treated patients [4], which may imply that progressive β-cell deterioration occurs independent of the mode of therapy. Oxidative stress in islets of patients with type 2 diabetes, which is related to hyperglycemia and causes tissue damage, is found to be increased at autopsy [18], but clinical evidence of long-term concentration-dependent effects of hyperglycemia on β-cell deterioration is lacking.

β-Cell function increases to compensate for increased insulin resistance in subjects yet maintaining normal glucose tolerance [5]. The increase in β-cell function is brought about, at least in part, by expansion of β-cell mass, which is derived from promotion of proliferation and neogenesis and from prevention of apoptosis mediated by increased activity of growth factor signaling pathways, glucose metabolism, GLP-1 signaling, and free fatty acid metabolism and signaling [22]. Since BMI is also correlated with insulin resistance estimated by homeostasis model assessment (HOMA-IR) in Japanese patients with type 2 diabetes [23], the relationship between BMI and β-cell function is of special concern. In autopsy of subjects with normal glucose tolerance, β-cell mass is found to be greater in obese than in lean subjects [17]. These findings suggest that increased BMI may be associated with increased β-cell mass and function in subjects maintaining normal glucose tolerance. The present study also suggests that BMI is positively associated with β-cell function in patients with type 2 diabetes. This result is compatible with autopsy findings of good correlation of β-cell mass with BMI [19]. Interestingly, the time-dependent decline of CPR level was steeper in obese than in lean subjects in the present study, which is compatible with the autopsy finding that the difference in β-cell volume between non-diabetic and type 2 diabetes subjects is greater in obese than in lean subjects [17]. While a decline of β-cell function of about 5% (HOMA-β%)/year was observed in subjects with average BMI of about 30 in UKPDS, the figure might well be lower in Japanese patients whose average BMI is under 25. However, direct comparison of our measure of β-cell function using CPR in a cross-sectional study with β-cell function assessed by HOMA-β in a longitudinal UKPDS study is problematic. In the present study, larger values of endogenous insulin secretion in high-BMI subjects compared with low-BMI

subjects were observed in groups with fewer years from diagnosis, suggesting that the influence of BMI on β-cell secretory function is more prominent in disease of shorter duration. One possible explanation for this phenomenon is that the compensatory β-cell mass increase with obesity is gradually lessened by long-term diabetic exposure.

The mean of indexes of endogenous insulin secretion was higher in patients with years from diagnosis more than 30 than in patients with years from diagnosis from 20 to 29.9 (Table 3), but the difference was not significant. This result might be linked to the exclusion of patients with serum creatinine above 1.3 mg/dl, who have diabetic nephropathy with renal insufficiency most likely due to the more severe and prolonged diabetic exposure.

FCPR, but not CPR-6 min and ΔCPR, is positively correlated with serum creatinine (Fig. 4). These results indicate that in the normal range of creatinine, the serum CPR level in the fasting stable state, but not the stimulated CPR level in the dynamic state, is affected by renal function, which determines CPR clearance.

In conclusion, a linear decline in endogenous insulin secretion over more than several decades of diabetes was confirmed by this cross-sectional study. The duration of diabetes exposure and BMI are major factors in β-cell function in Japanese patients with type 2 diabetes.

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Conflict of interest

There are no conflicts of interest.

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Factors responsible for elevation of 1-h postchallenge plasma glucose levels in Japanese men

Norio Harada [a], *Mitsuo Fukushima* [a,b,*], *Kentaro Toyoda* [a], *Rie Mitsui* [a], *Tomoyo Izuka* [b], *Ataru Taniguchi* [c], *Yoshikatsu Nakai* [d], *Yuichiro Yamada* [a], *Yutaka Seino* [a,c], *Nobuya Inagaki* [a]

[a] *Department of Diabetes and Clinical Nutrition, Graduate School of Medicine, Kyoto University, Kyoto, Japan*
[b] *Health Informatics Research Group, Foundation for Biomedical Research and Innovation, 1-5-4 Minatojima-minami-machi, Chuo-ku, Kobe, Hyogo 650-0047, Japan*
[c] *Division of Diabetes and Clinical nutrition, Kansai Electric Power Hospital, Osaka, Japan*
[d] *Karasuma-Oike Nakai Clinic, Kyoto, Japan*

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ABSTRACT

The 1-h postchallenge plasma glucose (1-h PG) level is considered to be a good index of the development of glucose intolerance and type 2 diabetes as well as of diabetic complications. In some cases, in Japanese, 1-h PG is elevated despite normal fasting glucose during oral glucose tolerance test (OGTT), but the factors responsible remain unclear. In the present study, subjects with normal glucose tolerance (NGT), isolated impaired fasting glucose (IFG), and isolated impaired glucose tolerance (IGT) were divided into subgroups at 1-h PG of 10.0 mM, and the four indices of insulin secretion and insulin sensitivity were compared. In all three categories, the insulinogenic index in subjects with elevated 1-h PG was remarkably lower than in those without elevated 1-h PG. In addition, the insulinogenic index was the strongest factor in elevated 1-h PG according to the multiple regression analysis. Interestingly, one third of the NGT subjects enrolled in this study had elevated 1-h PG. These subjects showed significantly elevated area under the curve of glucose (G-AUC) compared to NGT subjects without 1-h PG elevation. Thus, elevated 1-h PG in Japanese subjects indicates mildly impaired glucose tolerance due to decreased early-phase insulin secretion.

1. Introduction

Type 2 diabetes is characterized by both decreased insulin secretion and reduced insulin sensitivity [1–3]. Some patients with glucose intolerance leading to type 2 diabetes show elevated postchallenge plasma glucose without elevated fasting glucose during oral glucose tolerance test (OGTT) [4–6]. Although elevated 1-h postchallenge plasma glucose involves a different regulatory mechanism than 2-h postchallenge plasma glucose (2-h PG), 1-h postchallenge plasma glucose (1-h PG) is also as reliable an index of glucose tolerance as 2-h PG generally [7,8]. However, the relevance of 1-h PG and 2-h PG for diabetes screening is controversial [9,10]. It has been reported that subjects with 1-h PG higher than 10.0 mmol/l show higher risk of developing diabetes than subjects with lower 1-h PG [11]. In addition, 1-h PG higher than 11.2 mmol/l was found to be an independent risk factor for mortality in cardiovascular disease [12–14]. It was recommended in a

* *Corresponding author at:* Health Informatics Research Group, Foundation for Biomedical Research and Innovation, 1-5-4 Minatojima-minami-machi, Chuo-ku, Kobe, Hyogo 650-0047, Japan. Tel.: +81 78 304 5988; fax: +81 78 304 5989.
E-mail address: fukum@tri-kobe.org (M. Fukushima).
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number of studies that subjects having normal fasting plasma glucose at OGTT together with high 1-h PG are followed as carefully as IGT subjects in cases of higher frequency of elevated HbA1c, hypertension, family history of diabetes, or peripheral vascular involvement [15]. In addition, 1-h PG is used in diagnosis of gestational diabetes mellitus (GDM) and risk of macrosomia and other perinatal complications [16,17].

In the present study, the insulin secretion and insulin sensitivity indices of Japanese subjects undergoing OGTT in three WHO categories, normal glucose tolerance (NGT), isolated impaired fasting glucose (IFG) and isolated impaired glucose tolerance (IGT), subdivided at 1-h PG of 10.0 mmol/l were evaluated and compared.

2. Subjects and methods

2.1. Subjects

We recruited subjects undergoing OGTT because of positive urine glucose test, >5.0% HbA1c level, >5.6 mmol/l fasting plasma glucose level, and family history of diabetes at initial examination for medical check-up at Kyoto University Hospital, Ikeda Hospital, Kansai Electric Power Hospital, Kansai Health Management Center, and Kyoto Preventive Medical Center from 1993 to 2005. Subjects in the three categories of glucose tolerance, NGT ($n = 179$: fasting plasma glucose (FPG) level < 6.1 mmol/l and 2-h PG level < 7.8 mmol/l), isolated IFG ($n = 44$: FPG level of 6.1–7.0 mmol/l and 2-h PG < 7.8 mmol/l), and isolated IGT ($n = 103$: FPG level < 6.1 mmol/l and 2-h PG level of 7.8–11.1 mmol/l) according to the diagnostic criteria of World Health Organization in 1998 [18] were enrolled in the study. All subjects were men with no signs of hypertension, hepatic or renal dysfunction, endocrine or malignant disease, engaging in heavy exercise, history of gastrectomy, or history of medication known to affect glucose metabolism. The study was designed in compliance with the ethics regulations of the Helsinki Declaration. After the subjects fasted overnight for 10–16 h, standard OGTT with 75 g glucose was administered according to the National Diabetes Data Group recommendations [16].

The three WHO categories of glucose tolerance were divided into subgroups at 1-h PG of 10.0 mmol/l in this study: NGT with higher 1-h plasma glucose (NGT-HG: NGT criteria and 1-h PG ≥10.0 mmol/l), NGT with lower 1-h plasma glucose (NGT-LG: NGT criteria and 1-h PG <10.0 mmol/l), isolated IFG with higher 1-h plasma glucose (IFG-HG: IFG criteria and 1-h PG ≥10.0 mmol/l), isolated IFG with lower 1-h plasma glucose (IFG-LG: IFG criteria and 1-h PG <10.0 mmol/l), isolated IGT with higher 1-h plasma glucose (IGT-HG: IGT criteria and 1-h PG ≥10.0 mmol/l), and isolated IGT with lower 1-h plasma glucose (IGT-LG: IGT criteria and 1-h PG <10.0 mmol/l).

2.2. Laboratory examination

Blood samples were collected at 0, 30, 60, and 120 min after OGTT, and plasma glucose and serum insulin levels were measured for all subjects. Plasma glucose and serum insulin levels at 90 min were measured for 75 NGT subjects. Blood samples for measurements of HbA1c, total cholesterol, HDL cholesterol, and triglycerides were drawn after an overnight fast.

The plasma glucose level was measured by glucose oxidase method using the Hitachi Automatic Clinical Analyzer 7170 (Hitachi, Tokyo, Japan). Serum insulin was measured by two-site radioimmunoassay (Insulin Riabead II, Dainabot, Tokyo, Japan) as reported previously [19]. Serum total cholesterol and triglycerides levels were measured as reported previously [20].

2.3. Measurement

Basal insulin secretion and sensitivity were evaluated by HOMA β-cell and HOMA-IR [21,22], respectively. Early-phase insulin secretion and systemic insulin sensitivity during OGTT were evaluated by insulinogenic index [23] and ISI composite [24,25]. The calculations were as follows:

$$\text{HOMA } \beta\text{-cell} = \frac{20 \times \text{fasting serum insulin level (FI) (mU/l)}}{\text{fasting plasma glucose level (FPG) (mmol/l)} - 3.5}$$

$$\text{HOMA-IR} = \frac{\text{FI (mU/l)} \times \text{FPG (mmol/l)}}{22.5}$$

$$\text{Insulinogenic index} = \frac{\text{30-min insulin} - \text{FI (pmol/l)}}{\text{30-min plasma glucose} - \text{FPG (mmol/l)}}$$

$$\text{ISI composite} = \frac{10{,}000}{\left[\begin{array}{l}\text{FPG (mg/dl)}\ \text{FI (mU/ml)} \times \text{mean OGTT glucose (mg/dl)} \\ \times \text{mean OGTT serum insulin (mU/ml)}\end{array}\right]^{0.5}}$$

2.4. Statistical analysis

All analyses were performed using STATVIEW 5 system (Stat View, Berkeley, CA). Differences between two groups were assessed by unpaired t-test in terms of age, BMI, plasma glucose level, serum insulin level, HbA1c, triglyceride, total cholesterol, insulinogenic index, ISI composite, HOMA-IR, and HOMA β-cell. We used simple regression analysis and multiple regression analysis for comparison of the relationship between 1-h PG and the indices of insulin secretion and sensitivity. Probability (p) values less than 0.05 were considered statistically significant. Data are presented as mean ± S.E.

3. Results

Table 1 shows the clinical and metabolic characteristics of the six subgroups. NGT-HG had higher average age, BMI, FPG, 2-h PG and HbA1c than NGT-LG. IFG-HG had higher BMI than IFG-LG. IGT-HG had higher BMI, FPG, 2-h PG, 1-h insulin and HbA1c than IGT-LG. There were no significant differences in insulin (fasting and 2-h), triglycerides, total cholesterol and HDL-cholesterol levels between the two subgroups of NGT, isolated IFG, and isolated IGT.

The insulin secretion indices of insulinogenic index and HOMA β-cell indices in the three WHO categories are shown in

Table 1 – Clinical characteristics of the subjects in six subgroups of three WHO categories at 10.0 mmol/l

	NGT		Isolated IFG		Isolated IGT	
	NGT-LG	NGT-HG	IFG-LG	IFG-HG	IGT-LG	IGT-HG
Total N	179		44		103	
N	121	58	14	30	32	71
Age (years)	47.2 ± 1.1	53.1 ± 1.3‴	54.4 ± 2.1	51.5 ± 1.9	52.0 ± 1.6	52.2 ± 1.1
BMI (kg/m2)	23.1 ± 1	24.2 ± 0.4*	22.6 ± 0.8	24.2 ± 0.6*	22.8 ± 0.5	24.0 ± 0.3*
FPG (mmol/l)	5.2 ± 0.0	5.6 ± 0.0‴	6.3 ± 0.1	6.3 ± 0.0	5.2 ± 0.1	5.6 ± 0.0‴
1-h PG(mmol/l)	7.7 ± 0.1	11.5 ± 0.2‴	8.2 ± 0.4	12.5 ± 0.4‴	7.9 ± 0.3	12.2 ± 0.2‴
2-h PG(mmol/l)	5.7 ± 0.1	6.2 ± 0.1*	6.0 ± 0.4	6.5 ± 0.1	8.6 ± 0.1	9.2 ± 0.1‴
Fasting insulin(pmol/l)	31 ± 1	30 ± 2	26 ± 2	33 ± 3	34 ± 4	34 ± 2
1-h insulin(pmol/l)	250 ± 18	287 ± 23	287 ± 68	238 ± 29	146 ± 16	221 ± 18*
2-h insulin(pmol/l)	191 ± 16	196 ± 16	133 ± 17	192 ± 27	211 ± 22	254 ± 20
HbA1c(%)	5.1 ± 0.1	5.4 ± 0.1‴	5.6 ± 0.1	5.6 ± 0.1	5.2 ± 0.1	5.6 ± 0.1*
Triglycerides(mmol/l)	1.28 ± 0.08	1.47 ± 0.16	1.14 ± 0.17	1.31 ± 0.15	1.74 ± 0.29	1.85 ± 0.25
Total cholesterol(mmol/l)	5.33 ± 0.1	5.31 ± 0.11	5.01 ± 0.3	5.41 ± 0.14	5.3 ± 0.16	5.41 ± 0.1
HDL-cholesterol(mmol/l)	1.45 ± 0.05	1.47 ± 0.07	1.45 ± 0.11	1.51 ± 0.11	1.37 ± 0.10	1.39 ± 0.06

* $p < 0.05$, ‾ $p < 0.01$, ‾‾ $p < 0.001$ vs. LG. Data are mean ± S.E.

Fig. 1A and B. The insulinogenic index in the HG groups was remarkably lower than in the LG groups. The insulinogenic index values were 25.6 ± 0.3 vs. 75.9 ± 1.6 (NGT-HG vs. NGT-LG: $p < 0.01$), 23.1 ± 0.5 vs. 67.0 ± 3.5 (IFG-HG vs. IFG-LG: $p < 0.05$) and 22.6 ± 0.3 vs. 56.4 ± 1.9 (IGT-HG vs. IGT-LG: $p < 0.01$). The HOMA-β cell index of the HG group was significantly lower than that of the LG group in NGT and isolated IGT. There was no difference between IFG-HG and IFG-LG in HOMA β-cell index. We also estimated the insulin sensitivity indices using ISI composite and HOMA-IR in the three categories. The ISI composite index represents insulin sensitivity during OGTT, while HOMA-IR represents insulin resistance at fasting state. The ISI composite and the HOMA-IR values were similar in the HG group and the LG group in all three WHO categories. ISI composite values were 7.5 ± 0.6 vs. 9.2 ± 0.4 (NGT-HG vs. NGT-LG: n.s.), 6.9 ± 0.6 vs. 8.0 ± 0.8 (IFG-HG vs. IFG-LG: n.s.) and 7.0 ± 0.4 vs. 7.8 ± 0.7 (IGT-HG vs. IGT-LG: n.s.). HOMA-IR values were 1.2 ± 0.1 vs. 1.2 ± 0.1 (NGT-HG vs. NGT-LG: n.s.), 1.4 ± 0.1 vs. 1.2 ± 0.1 (IFG-HG vs. IFG-LG: n.s.) and 1.4 ± 0.1 vs. 1.4 ± 0.1 (IGT-HG vs. IGT-LG: n.s.).

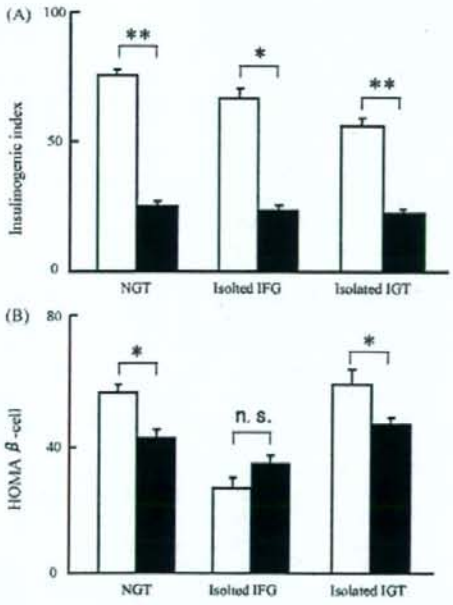

Fig. 1 – Indices of insulin secretion in six subgroups of three WHO categories: (A) insulinogenic index, (B) HOMA β-cell; light bars indicate subjects without elevated 1-h PG, dark bars indicate subjects with elevated 1-h PG. $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. LG, data are mean ± S.E.

In addition, we then analyzed the relationship between 1-h PG and the indices of insulin secretion and insulin sensitivity. Scattered plots of simple regression analysis between 1-hPG and the four indices are shown in Fig. 2. 1-h PG had a significant correlation with the insulinogenic index, HOMA β-cell, and ISI composite. Multiple regression analysis revealed that HOMA β-cell, ISI composite, and insulinogenic index were the independent factors in the variation of 30.0% in 1-h PG ($p < 0.001$). The correlation coefficients of these indices with 1-h PG in simple regression analysis, and the β values and p-values of multiple regression analysis are shown in Table 2. The insulinogenic index was the strongest factor to explain the 1-h PG levels.

4. Discussion

In the present study, we found that elevated 1-h PG is strongly associated with decreased insulinogenic index, indicating reduced capacity of early-phase insulin secretion [24,25]. The insulinogenic index in NGT-HG became remarkably lower than in NGT-LG at about 20, declining to the absolute levels of IFG-HG and IGT-HG. Multiple regression analysis showed that the insulinogenic index was the strongest factor among the four indices in elevated 1-h PG. These results indicate that decreased insulinogenic index is the major factor in elevated 1-h PG during oral glucose tolerance test. Since NGT-HG showed significantly higher area under the curve of glucose

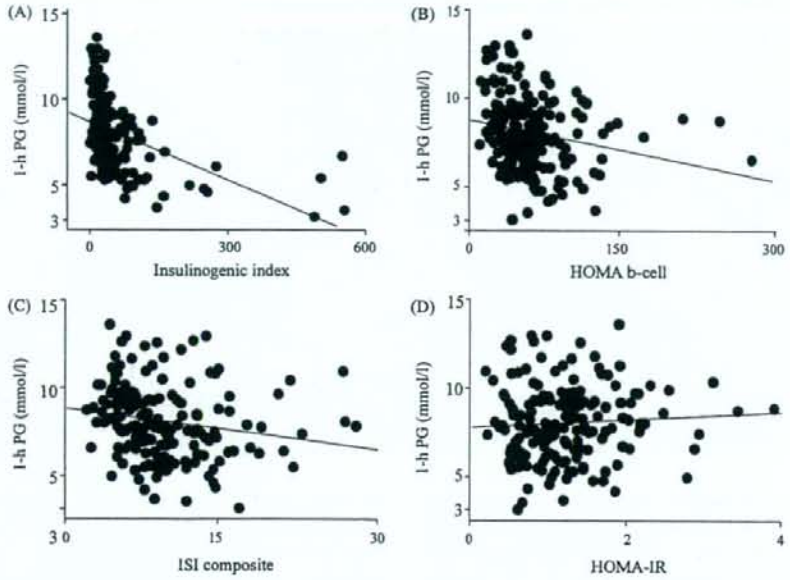

Fig. 2 – Relationship between 1-h PG and indices of insulin secretion and insulin sensitivity; (A) insulinogenic index, (B) HOMA β-cell, (C) ISI composite, (D) HOMA-IR; in insulin secretion, insulinogenic index and HOMA β-cell had significant relationships with 1-h PG ($r = -0.46$, $p < 0.001$, and $r = -0.2$, $p < 0.01$, respectively) In insulin sensitivity, there was a only mild significant relationship between 1-h PG and ISI composite ($r = -0.18$, $p < 0.05$). HOMA-IR had no significant relationship with 1-h PG ($r = 0.06$).

(G-AUC) compared to NGT-LG (19930 ± 256 vs. 15131 ± 181: $p < 0.05$), mildly impaired glucose tolerance due to reduced early-phase insulin secretion may already be present in NGT-HG. In addition, NGT-HG showed normal 2-h PG despite the elevated 1-h PG in the present study. Regarding the serum insulin level during OGTT, the 90 min insulin level in NGT-HG was significantly higher than in NGT-LG subjects (56.4 ± 7.3 vs. 40.3 ± 4.1: $p < 0.05$) in the cases we could analyze. Since late-phase insulin secretion in NGT-HG was sufficient to normalize 2-h PG, the regulatory mechanisms of elevated 1-h PG and 2-h PG are at least partly distinct.

HOMA β-cell measures insulin secretion capacity in the fasting state. HOMA β-cell values in NGT-HG and IGT-HG were significantly lower than those in NGT-LG and IGT-LG, but were similar to those in IFG-LG and IFG-HG. The values of HOMA β-cell is influenced with fasting PG per se. Isolated IFG subjects whose fasting PG levels are higher than those of NGT and isolated IGT had already low HOMA β-cell. It may explain for no significant difference between IFG-HG and IFG-LG in HOMA β-cell. Further studies are necessary to elucidate the other factors to influence HOMA β-cell in isolated IFG subjects.

Table 2 – Relationship of indices of insulin secretion and insulin sensitivity with 1-h PG

	Correlation coefficients	Standardized β	p-Value
Insulinogenic index	−0.47	0.42	<0.001
HOMA β-cell	−0.2	−0.31	<0.05
ISI composite	−0.18	−0.23	<0.05
HOMA-IR	0.06	0.13	n.s.

ISI composite and HOMA-IR are used to estimate insulin sensitivity [23]. We found both indices in LG and HG subjects to be similar in all three WHO categories. However, the insulin sensitivity of these subjects was higher than in Mexican Americans and Caucasians, as previously reported [26–28]. Since Japanese diabetes subjects are less obese than Caucasians, and insulin secretion rather than insulin sensitivity is the more important factor in the progression from NGT to diabetes in Japanese, it is likely that elevated 1-h PG in these subjects is mainly due to decreased early-phase insulin secretion rather than to impaired insulin sensitivity [19,29–31].

The ratio of NGT-HG subjects to total NGT subjects was 33% (58/121), while it was 69% (14/30) and 68% (32/71) for isolated IFG and isolated IGT subjects, respectively. The fact that the ratios increased similarly and progressively from NGT to isolated IFG and isolated IGT also suggests the use of 1-h PG as a marker to detect early stages of impaired glucose tolerance.

In conclusion, we have elucidated that impaired early-phase insulin secretion is strongly associated with an elevated 1-h PG level in Japanese subjects, suggesting that elevated 1-h PG may be a convenient marker to screen for decreased early-phase insulin secretion in early stage glucose intolerance.

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Conflict of interest

There are no conflict of interest.

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