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Metabolism Clinical and Experimental 54 (2005) 370 – 375

Metabolism
Clinical and Experimental

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Cellular insulin resistance in Epstein-Barr virus-transformed lymphoblasts from young insulin-resistant Japanese men☆,☆☆

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Received 22 June 2004; accepted 30 October 2004

Abstract

The metabolic syndrome is characterized by a blunted insulin-mediated glucose uptake in various cell types. We compared the glucose uptake characteristics of Epstein-Barr virus (EBV)–transformed lymphoblasts obtained from young men with vs without metabolic and cardiovascular evidence of metabolic syndrome. From a population of 218 men, 20- to 25-year-old, 10 men with a systolic blood pressure (BP) $\geq$130 mm Hg and family history of hypertension were assigned to a high BP (HBP) group, and 10 with a BP $\leq$110 mm Hg, and no family history of hypertension was assigned to a low BP (LBP) group. Multiple clinical and metabolic characteristics were examined in both groups and compared. Peripheral lymphocytes from HBP and LBP subjects were EBV-transformed, and the glucose transporter (Glut)–mediated glucose uptake from each group was compared in lymphoblasts. Body mass index, fasting glucose, immunoreactive insulin, insulin resistance index based on a homeostasis model assessment (HOMA-R), and total and low-density lipoprotein cholesterol were significantly higher in the HBP than the LBP subgroup (whole-body insulin resistance). Baseline Glut-mediated and Glut-mediated insulin-stimulated glucose uptake by lymphoblasts from the HBP group were significantly lower than by lymphoblasts from the LBP group (cellular insulin resistance). The net increment in Glut-mediated glucose uptake by insulin was inversely correlated with HOMA-R. In conclusion, cellular insulin resistance in EBV-transformed lymphoblasts is associated with young Japanese subjects with HBP. The net increment in Glut-mediated glucose uptake by insulin in lymphoblasts may be a useful intermediate phenotype to study genetic aspects of the metabolic syndrome.

1. Introduction

Abnormal metabolism of glucose, insulin, and lipid is more prevalent in untreated hypertensive patients than in normotensive control subjects [1]. The cluster of these metabolic abnormalities associated with cardiovascular disease (CVD) has been named the metabolic syndrome. Insulin resistance, a highly prevalent pathophysiological disorder implicated in the development of type 2 diabetes, obesity, hyperlipidemia, and hypertension, is expressed as a blunted biologic response to normal concentrations of circulating insulin. Compensatory hyperinsulinemia is one of its consequences.

The etiology of this syndrome, which promotes the development of premature atherosclerosis and increases significantly the risk of CVD early in life [2], remains controversial. Candidate mechanisms by which hypertension may develop include sodium retention, vascular hyperresponsiveness, arteriolar smooth muscle cell proliferation, abnormal cellular electrolyte transport and composition, stimulation of sympathoadrenergic activity, and

☆ This work was supported by a grant-in-aid for Scientific Research on Priority Areas (C) Medical Genome Science from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

☆☆ The authors have no conflict of interest to disclose.

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0026-0495/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.metabol.2004.10.003

growth-promoting effects [3]. Offspring studies have confirmed that insulin resistance associated with essential hypertension is inherited, though ambient environmental factors play an important role [4,5]. Measurements of insulin sensitivity by the glucose clamp technique have identified skeletal muscle and adipose tissue as major targets in the metabolic syndrome. However, these tissues are difficult to obtain and study in vitro. We have, therefore, created cell lines of Epstein-Barr virus (EBV)–transformed lymphoblasts. These lymphoblasts represent a useful cellular model with which to compare primary and secondary factors because (a) they reproduce the genetic characteristics of the donor, (b) the cells removed from the in vivo environment of the donor can be cultured under controlled conditions for an unlimited number of generations [6], (c) they enable repetitive measurements with minimally invasive sampling techniques.

Sesti et al [7] have studied the recycling of the insulin receptor and processing of insulin in EBV-transformed lymphoblasts from insulin-resistant patients, though they did not examine their glucose uptake. If in insulin-resistant patients, the blunted glucose uptake is due to an abnormality common to various cell types, this abnormality should be detectable in lymphoblasts. To clarify the mechanisms of cellular insulin resistance, we have focused our experiments on the glucose transport system. The glucose transporters are the main mediators of glucose entry into the cells. The glucose transporters, Glut1, abundant in various cells, and Glut4, mainly expressed in skeletal muscle and adipocytes, are implicated in insulin resistance [8].

In this study, we investigated the association of whole-body insulin resistance in individuals with high blood pressure (HBP) and a family history of hypertension, identified among a population participating in an annual health maintenance program. We then examined the cellular insulin resistance in their EBV-transformed lymphoblasts and the relationship between whole-body and cellular insulin resistance.

2. Materials and methods

2.1. Study population, physical findings, and laboratory measurements

A population of 218 men between the ages of 20 and 25 years was recruited from an annual health maintenance program at Keio University, Tokyo, Japan. This study was approved by the Institutional Ethic Committee of Keio University, and written informed consent was obtained from all participants who underwent (a) a physical examination, including measurements of body mass index (BMI), systolic and diastolic BP, and heart rate; (b) fasting blood chemistry, including fasting blood glucose (FBG), immunoreactive insulin (IRI), insulin resistance index based on a homeostasis model assessment (HOMA-R, calculated as FBG [mg/dL] × IRI [μU/mL]/405), total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides, free fatty acids, and uric acid; and (c) a medical questionnaire probing into the personal and family history.

High BP and low BP (LBP) groups were selected from the study population. A study participant was included in the HBP group if he had (1) a systolic BP $\geq$130 mm Hg and (2) a family history of hypertension or CVD among second-degree relatives. He was included in the LBP group if he had (1) a systolic BP $\leq$110 mm Hg and (2) no family history of hypertension or CVD among second-degree relatives.

2.2. Immortalization of lymphocytes and culture of lymphoblasts

A 7-mL blood sample was drawn from a peripheral vein, and the circulating lymphocytes were isolated on a Ficoll conray (IBL, Gunma, Japan). B lymphocytes were immortalized by incubation with EBV-containing supernatant. Lymphocytes from peripheral blood of HBP and LBP subjects were transformed with EBV by a modification of the method of Sesti et al [9]. Cells were grown and routinely cultured in Rapid Prototyping and Manufacturing Institute 1640 (RPMI) medium (Manufacturing Research Institute, Georgia Institute of Technology, Atlanta, Ga) supplemented with 100 μg/mL kanamycin and 10% heat-inactivated fetal calf serum (FCS; Gibco, Grand Island, NY). When the lymphoblasts had become confluent, cyclosporine A, 200 ng/mL, was added to the RPMI medium. Passages were performed at least twice a week. The same lot of FCS was used throughout the experiments. After immortalization, the lymphoblasts were grown for 4 to 8 weeks and stocked frozen. Fresh cultures were thawed from the frozen stocks for each experiment. Cells were grown in RPMI medium supplemented with 10% FCS and subcultured for 3 days before the experiments.

2.3. Uptake of 2-deoxyglucose into lymphoblasts

The uptake of 2-deoxyglucose (DG) into lymphoblasts was measured by a modified method of Daneman et al [10]. At first, to determine the optimal insulin concentration, preliminary experiments were performed using lymphoblasts derived from randomly selected subjects (n = 5 in each group). We resuspended 500 μL of lymphoblasts in concentration of 2×10^6 cells/mL in *N*-2-hydroxyethylpiperazine-*N*′ -2-ethanesulfonic acid (HEPES)–buffered RPMI medium (glucose concentration, 100 mg/dL), and preincubated for 1 hour with insulin in concentrations of 0, 0.3, 1.0, 3.0, and 10 nmol/L, and with 100-μmol/L concentration of cytochalasin B, a fungal metabolite inhibitor of the insulin-regulated transport of glucose [11-13]. The lymphoblasts were incubated in presence of 100-mg/dL concentration of [^{3}H]-2-DG (40 μL) at 37°C and gently agitated for 20 minutes. The cells were thoroughly rinsed 3 times with glucose-free HEPES-buffered saline solution and centrifuged. The cell pellets were lysed by

adding 400 μL of 0.05N NaOH, and the cell-associated radioactivity was measured by a scintillation counter. Each measurement was made at least in triplicate in absence (Glut-mediated baseline glucose uptake) and presence (Glut-mediated insulin-stimulated glucose uptake) of insulin. The Glut-mediated glucose uptake was maximally increased by 1-nmol/L concentration of insulin. We then compared the Glut-mediated glucose uptake at the 1-nmol/L concentration of insulin (n = 10 in each group).

2.4. Determination of cell proliferation

Epstein-Barr virus–immortalized lymphoblasts from the HBP and LBP groups were seeded on day 0 at a concentration of 1×10^6 cells/mL. The cell concentration was measured daily with a cell counter (Fukuda Denshi, Tokyo, Japan) combined with visual counting of cells stained with trypan blue. Experiments were performed in triplicate, in absence and presence of 0.25-, 0.5-, and 1.0-nmol/L concentration of insulin.

2.5. Statistical analysis

The data were analyzed with the StatView software, version 5.0 (SAS Institute Japan Ltd, Tokyo, Japan). All results are expressed as means ± SD. The results of clinical measurements were compared by analysis of variance, followed by Scheffe's F test. A Bonferroni correction was made for multiple testing. Glucose uptake and clinical parameters were compared by Pearson's correlation analysis. Statistical significance was defined as $P < .05$.

3. Results

3.1. Characteristics of the study populations

Important characteristics of the entire study population, and of the HBP and LBP subgroups, are summarized in Table 1. Heart rate, BMI, IRI, FBG, HOMA-R, mean total, and LDL-C were significantly higher in the HBP than in the LBP group. In addition, a trend was observed toward higher triglycerides and lower HDL-C in the HBP group, though these differences did not reach statistical significance.

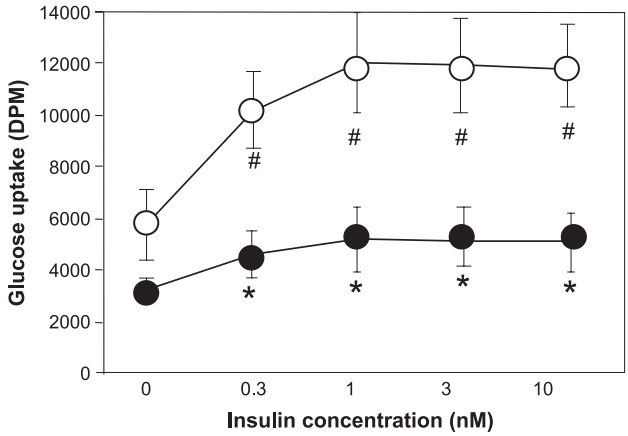

Fig. 1. Dose-response curve of Glut-mediated insulin-stimulated glucose uptake into lymphoblasts from HBP and LBP groups. Filled circle indicates HBP group (n = 5); open circle, LBP group (n = 5). Asterisk and number sign indicates $P < .001$ and $P < .0001$, respectively, compared to 0-nmol/L concentration of insulin in the same group. Significance was retained when corrected with Bonferroni for multiple tests.

3.2. Glut-mediated glucose uptake in lymphoblasts at various concentrations of insulin in hypertensive vs normotensive study participants

We first compared the Glut-mediated, insulin-stimulated glucose uptake in the HBP (n = 5) vs LBP (n = 5) groups at 0-, 1-, 3-, and 10-nmol/L concentrations of insulin. Insulin significantly stimulated the Glut-mediated glucose uptake in both groups (Fig. 1). The significance was retained Bonferroni correction for multiple tests. Repeated-measures analysis of variance revealed that the insulin dose-response curve was significantly lower in the HBP than in the LBP group. However, the patterns of response to insulin were similar, such that a plateau was reached at a concentration of 1 nmol/L in both groups. Therefore, we

Table 1
Characteristics of entire study population and of HBP and LBP subgroups

	All subjects (N = 218)	HBP (n = 10)	LBP (n = 10)	P
Age (y)	23 ± 2	23 ± 2	24 ± 1	NS
Systolic BP (mm Hg)	122 ± 15	142 ± 11	103 ± 5	<.0001
Diastolic BP (mm Hg)	71 ± 9	85 ± 7	61 ± 8	<.0001
Heart rate (beats/min)	75 ± 13	86 ± 9	71 ± 12	.007
BMI (kg/m^2)	21.2 ± 2.7	23.3 ± 4.1	19.9 ± 1.1	.020
Immunoreactive insulin (μU/mL)	6.3 ± 4.00	8.5 ± 3.5	3.8 ± 2.0	.003
FBG (mg/dL)	84 ± 7	88 ± 8	81 ± 6	.045
HOMA-R index	1.3 ± 0.8	1.9 ± 0.9	0.8 ± 0.4	.042
Total cholesterol (mg/dL)	176 ± 32	191 ± 37	160 ± 17	.028
Triglyceride (mg/dL)	96 ± 54	115 ± 65	71 ± 28	.064
LDL-C (mg/dL)	100 ± 28	115 ± 32	85 ± 17	.016
HDL-C (mg/dL)	62 ± 12	54 ± 9	64 ± 13	.068
Free fatty acid (mEq/L)	0.42 ± 0.19	0.54 ± 0.23	0.41 ± 0.22	NS
Uric acid (mg/dL)	5.8 ± 0.9	6.0 ± 1.2	5.9 ± 1.0	NS

P values reflect comparisons between HBP and LBP groups.

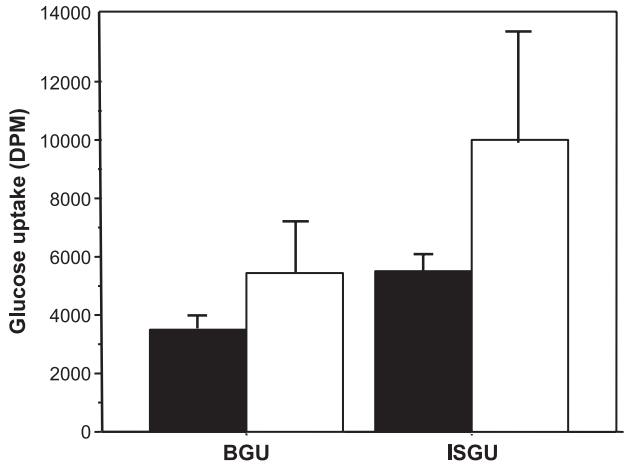

Fig. 2. Glut-mediated baseline (left) and insulin-stimulated (right) glucose uptake in HBP (filled bars) vs LBP (clear bars) group. N = 10 in each group. Glut-mediated glucose uptake was significantly lower in the lymphoblasts of the HBP group than in the LBP group ($P < .001$). Insulin stimulation synergistically enhanced Glut-mediated glucose uptake only in the LBP group. The net increment stimulated by insulin was significantly greater in the LBP group than in the HBP group ($P < .05$). BGU indicates baseline glucose uptake; ISGU, insulin-stimulated (1 nmol/L) glucose uptake.

chose a 1-nmol/L concentration of insulin for the insulin-stimulated glucose uptake.

3.3. Glut-mediated glucose uptake in lymphoblasts from HBP vs LBP subjects

Comparison of the baseline Glut-mediated glucose uptake revealed a significantly lower uptake in lymphoblasts from the HBP (n = 10) than in lymphoblasts from the LBP group (n = 10). Insulin stimulated a prominent response in the cells from both groups ($P < .001$, Fig. 2). Glut-mediated glucose uptake was significantly lower in lymphoblasts from the HBP group than in lymphoblasts from the LBP group ($P < .001$, Fig. 2). An interaction between insulin stimulation and group was observed. Insulin stimulation enhanced the Glut-mediated glucose uptake synergistically

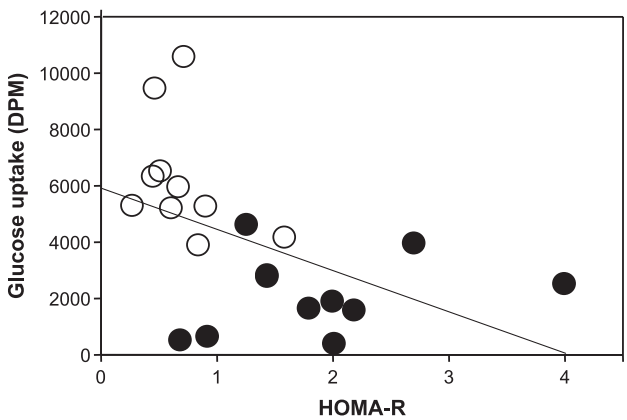

Fig. 3. Significant inverse correlation between net increment in Glut-mediated glucose uptake and HOMA-R index (R = −0.334, $P < .05$). Filled circle indicates HBP group (n = 10); open circle, LBP group (n = 10).

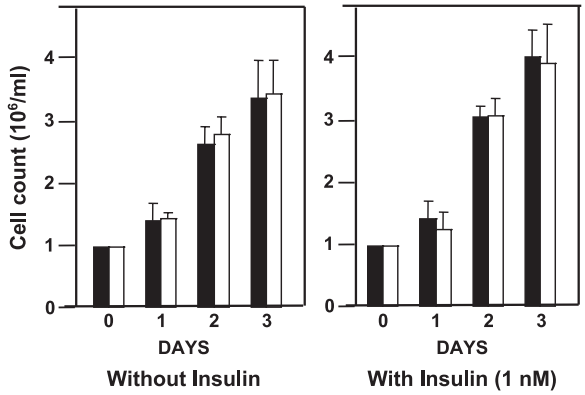

Fig. 4. Lymphoblast proliferation in absence (left) and in presence (right) of insulin (1 nmol/L) in HBP (filled bars) vs LBP (clear bars) group. n = 5 in each group.

only in the LBP group. The net increment stimulated by insulin was significantly greater in the LBP than in the HBP group ($P < .05$).

3.4. Correlation between glucose uptake and clinical characteristics

Immunoreactive insulin ($R = -0.380$, $P < .05$) and the HOMA-R index ($R = -0.334$, $P < .05$) were both inversely correlated with the net increment in glucose uptake stimulated by insulin in EBV-transformed lymphoblasts (Fig. 3).

3.5. Cell growth

We cultured lymphoblasts from the HBP and LBP groups for 3 days. The cell lines derived from both groups proliferated similarly in absence or presence of insulin (Fig. 4).

4. Discussion

The main findings of our study are compared to the LBP subgroup; (1) subjects with HBP had metabolic findings consistent with whole-body insulin resistance, (2) lymphoblasts from HBP subjects had an abnormal baseline and insulin-stimulated Glut-mediated glucose uptake, and (3) Glut-mediated insulin-stimulated glucose uptake was inversely correlated with whole-body insulin resistance. These observations indicate that cellular insulin resistance associated with a BP ≥130 mm Hg is a functional marker of the metabolic syndrome in young Japanese men.

The phenomenon of insulin resistance in lymphoblasts may represent an intermediate phenotype for further genetic research, because EBV-transformed lymphoblasts can be stored and widely distributed after proliferation and because their cellular function can be measured repetitively. Attempts have been made to measure glucose transport, focusing on circulating mononuclear cells [14]. However, observations in mononuclear cells are not proof of a primary defect in cellular insulin resistance, because the glucose uptake by these cells is markedly influenced by the extracellular environment. Epstein-Barr virus–transformed

lymphoblasts are a valuable tool to investigate genetic aspects of cellular insulin resistance.

In this study, the mean BMI of the HBP subjects was 23.3. This value is lower than in western populations. The degree of obesity of Asians is less than in Caucasians [15]. Our results suggest that even relatively mild obesity is associated with insulin-resistant characteristics in young Japanese men. In an earlier study, we found that slight differences in BMI greatly influenced insulin resistance and BP in young, lean, normotensive, Japanese subjects [16]. Another recent study suggests that Japanese with BMI in a range of 23.0 to 24.9 are at higher risk of developing the metabolic syndrome [15].

Hyperinsulinemia is believed to contribute in the pathogenesis of atherosclerosis [17,18]. An abnormal growth pattern of vascular cells has been proposed as a developmental factor in experimental genetic hypertension [19,20]. However, we did not observe a difference in the rates of cellular proliferation among the study groups. Rosskopf et al [21] reported a distinctly faster proliferation of EBV-transformed lymphoblasts with high sodium-proton exchange activity from hypertensive patients than lymphoblasts from normotensive controls. In their study, the mean BMI of hypertensive and normotensive patients was 27.5 and 24.3, respectively. The value of IRI was not reported. In this study, the mean BMI of HBP and LBP subjects was 23.3 and 21.2, respectively. We believe that the whole-body insulin resistance in our study participants was relatively mild compared with that observed in the patient population of Rosskopf et al. Therefore, in our study subgroups, we did not detect a difference in cellular proliferative activity.

The selection criteria for our insulin-resistant group were (1) a systolic BP $\geq$130 mm Hg and (2) a family history of hypertension or CVD among second-degree relatives. These criteria were useful to distinguish subjects with metabolic syndrome from healthy young Japanese men. When we arbitrarily set the criteria for HBP at $\geq$130 mm Hg and for LBP at $\leq$110 mm Hg, approximately 10% of the whole population was classified as HBP or LBP. After additional screening for family history of hypertension, only 5% fulfilled the criteria. We could not apply the systolic BP $\geq$140-mm Hg criterion for hypertension, defined by the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure 7 [22], because young men with systolic BP $\geq$140 mm Hg were few in this study. However, another prospective longitudinal study, tracking BP in a large similar population of Japanese students, supports our postulate that our own subjects with systolic BP $\geq$130 mm Hg and a positive family history of hypertension are at high risk of developing CVD [23]. A rise in BP may occur at a young age in insulin-resistant Japanese, probably because they consume greater quantities of salt than in other populations [24].

Glucose transport into most tissues is mediated by members of a family of transport proteins (Glut1-7), which facilitate the diffusion of glucose [25]. Under both baseline and insulin-stimulated conditions, Glut1 is responsible for glucose uptake by various cells [26], and it is insulin-responsive in adipocytes [27]. In cell cultures that predominantly express Glut1 transporters (3T3-L1 fibroblasts and BC3H1 muscle cells in culture), this isoform is responsive to insulin [28,29]. However, its expression in lymphoblasts has not been examined. It has been suggested that the decrease in 2-DG uptake by lymphoblasts, both at baseline and in the insulin-stimulated state, is due, at least in part, to a decrease in the number and in the intrinsic activity of Glut1. We plan to investigate whether the expression of Glut1 is decreased in the metabolic syndrome to clarify these issues.

Glut4 is insulin-sensitive and is the predominant glucose transporter in skeletal muscle and adipose tissue. An increase in muscle Glut4 content is associated with an increased insulin-stimulated glucose disposal (insulin sensitivity) [30]. Although there is evidence that the insulin receptor is expressed in human lymphoblasts, the expression of Glut4 in lymphocytes and lymphoblasts is negligible [7,31]. Therefore, it is unlikely to play a key role in glucose transport in lymphoblasts. The potential contribution of other glucose transporter isoforms, for instance, Glut3, cannot be excluded.

We propose that the Glut-mediated insulin-stimulated glucose uptake in lymphoblasts is a useful marker of whole-body insulin sensitivity. Two plausible explanations may be considered: (1) cellular insulin resistance contributes to whole-body insulin resistance, and (2) compensatory hyperinsulinemia impairs the Glut-mediated insulin-stimulated glucose uptake in lymphoblasts. The latter is unlikely, because (a) compensatory hyperinsulinemia in vivo is negligible in cultured lymphoblasts, and (b) the insulin concentration was fixed at 1-nmol/L concentration in cultured lymphoblasts. Because the correlation was weak, Glut-mediated insulin-stimulated glucose uptake in lymphoblasts is not an ideal surrogate for whole-body insulin resistance. However, it is a useful functional phenotype to study the mechanisms of cellular insulin resistance.

Young women were not included in this study because their BP is lower than that of men due to ovarian hormonal effects [32,33]. Furthermore, insulin resistance could be masked by estrogen, and we could not time the annual health examination with the menstrual cycle of potential women candidates for our study. Therefore, the differences in BP and HOMA-R between women in the HBP vs LBP groups would have been less prominent than in men. Excluding women increased the sensitivity and specificity of the relationship between cellular and whole-body insulin resistance in this pilot study.

Though the euglycemic hyperinsulinemic clamp technique is the gold standard, we chose the HOMA-R method to measure insulin sensitivity [34]. However, the correlation between intravenous glucose tolerance test, or a euglycemic clamp, and HOMA-R was high in epidemiological studies, except in severe diabetes or older subjects, because of glucose toxicity and diminished intrinsic insulin secretion activity [35]. Because the subjects in this study are healthy

young male, they have a relatively high intrinsic insulin secretion activity. The HBP group was confirmed to be insulin-resistant by their lipid profile.

In conclusion, our study clarifies the cellular insulin resistance in EBV-transformed lymphoblasts harvested from young insulin-resistant Japanese men. Cellular insulin resistance was significantly correlated with IRI and HOMA-R, that is, whole-body insulin resistance. We propose the methods reported here for the development of lymphocyte cell line banks for the study of cellular insulin resistance in men with high BP and other clinical characteristics associated with the metabolic syndrome. This system may serve as a model to study defective cellular mechanisms in insulin-resistant individuals.

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