TABLE 2
Magnitude of LD (D′ and r^2) between *ABCC8* and *KCNJ11* variants

D′/r^2	ABCC8_SNP1	ABCC8_SNP2	ABCC8_SNP3	KCNJ11_SNP1
ABCC8_SNP1 rs1799854	—	0.0012	0.0177	0.0151
ABCC8_SNP2 rs4148643	0.0867	—	0.0919	0.0808
ABCC8_SNP3 rs757110	0.1708	0.9867	—	0.8703
KCNJ11_SNP1 rs5219	0.1653	0.9711	0.9794	—

rs2688, and *NKX2-2*_SNP1 (+856 TGA) and in controls at *NKX6-1*_SNP1 rs1017560 and *ABCC8*_SNP1 rs1799854 (online appendix Table 1). Although none of these are significant with correction for multiple comparisons, we reanalyzed several of the variants, including *NKX2-2*_SNP1 (+856 TGA) and *NKX6-1*_SNP1 rs1017560, and confirmed that there was no typing error for these variants. We also tested whether the observed departures were consistent with the genotype frequencies expected for a genetic disease model (21). The genotype distributions for *TCF2*_SNP5 rs2689, *TCF2*_SNP6 rs2688, *NKX2-2*_SNP1 (+856 TGA), and *ABCC8*_SNP1 rs1799854 are consistent with genetic models that best fit these data. In contrast, the departure from HWE observed in the control samples for *NKX6-1*_SNP1 rs1017560 is not consistent with any genetic model for disease. Thus, the observed departure from HWE in controls at *NKX6-1*_SNP1 rs1017560 is likely to be a chance observation. The remaining departures are unlikely to be attributable to genotyping errors and are consistent with the possibility that the selection of case and control samples from a population in HWE at a susceptibility locus (at the test marker or a polymorphism in strong linkage disequilibrium [LD]) has generated genotype distributions with the observed departures from HWE.

Among the 33 variants of 12 genes, 6 variants (*TCF2*_SNP4 rs1016991, *TCF2*_SNP6 rs2688, *HNF4A*_SNP3 rs745975, *NKX2-2*_SNP2 rs3746741, *NKX6-1*_SNP1 rs1017560, and *ABCC8*_SNP1 rs1799854) showed at least nominally significant associations ($P < 0.05$) with type 2 diabetes (Table 1 and online appendix Table 2). *ABCC8*_SNP1 rs1799854 showed the strongest association ($P = 0.0073$) with diabetes among the SNPs examined in this study. By further analysis of the variant, the T/T genotype was found in 454 (28.6%) and 298 (24.0%) subjects in the diabetic and control groups, respectively, a significant difference in the frequency of individuals with the T/T genotype between the two groups (C/C + C/T vs. T/T, $P = 0.0068$) (online appendix Table 2). The odds ratio (OR) for the T/T genotype was 1.27 (95% CI 1.07–1.50; C/C + C/T vs. T/T), indicating that the T/T genotype in *ABCC8*_SNP1 rs1799854 is associated with type 2 diabetes in Japanese subjects.

There was no association of other variants in *ABCC8* and *KCNJ11* with diabetes (Table 1 and online appendix Table 2). These include the E23K variant in *KCNJ11* (*KCNJ11*_SNP1 rs5219: for the K allele, OR 1.08 [95% CI 0.97–1.21], $P = 0.15$). To determine the extent of LD between the four variants in *ABCC8* and *KCNJ11*, we calculated D′ and r^2 (Table 2). There was modest LD between *ABCC8*_SNP2 rs4148643 and *ABCC8*_SNP3 rs757110. Strong LD was found between *ABCC8*_SNP3 rs757110 and *KCNJ11*_SNP1 rs5219. In the latter, we tested two-locus haplotypes having a frequency of >5% for association with diabetes and found no association of any of the haplotypes with diabetes (data not shown).

We examined the genes involved in pancreatic β-cell function (transcription factors and K_{ATP} channel subunits) in relation to type 2 diabetes in a large cohort of Japanese subjects. The study included 2,834 subjects, the largest case-control study so far conducted on these variants in a Japanese population. For disease susceptibility allele frequencies in the range of 0.3–0.5, our sample had >99% power to detect a susceptibility gene with a genotype relative risk in the range of 1.5–1.85 (for any genetic model of inheritance). For allele frequencies in this range, we had >80% power to detect susceptibility genes with genotype relative risk in the range of 1.25–1.55. Power was similarly good for dominant models with lower susceptibility allele frequencies (0.1–0.3) or recessive models with higher susceptibility allele frequencies (0.5–0.9). The sample was reasonably powered (>90%) to detect recessive susceptibility alleles at low frequencies (0.1–0.3) for higher genotype relative risks (2.1–4.0) but was not sufficiently powered to detect very common (>0.7) dominant susceptibility genes (genotype relative risk >100).

*ABCC8*_SNP1 rs1799854 (exon 16 −3c/t variant) was significantly associated with type 2 diabetes, primarily due to increased frequency of T/T homozygotes among patients. Since this variant is located in the 3′ splice site, it might impair normal splicing. Alternatively, the variant could be in strong LD with an unidentified functional variant in the unscreened region harboring the *ABCC8* gene. There have been two case-control association studies (22,23) conducted for the variant in Japanese populations, both of which found no association of this variant with type 2 diabetes. However, because these studies were based on a relatively small number of subjects (167 subjects in 22; 456 subjects in 23), their power to detect associations is limited. In Caucasians, several studies (7,8,11–13,17) have reported association of the variant with type 2 diabetes, although other studies found no association of the variant with type 2 diabetes (14–16). On the other hand, several studies have reported an association of the E23K variant in *KCNJ11* (*KCNJ11*_SNP1 rs5219 in this study) with type 2 diabetes in Caucasians (9,10,16,18). Recent meta-analyses (19,20) of the variant support this association. Although our present study finds no association of the E23K variant with diabetes in Japanese subjects (for the K allele, OR 1.08 [95% CI 0.97–1.21], $P = 0.15$), 95% CI around the OR overlaps in meta-analysis of European populations, suggesting that our results are not inconsistent with the previous studies on the E23K variant in *KCNJ11*.

The International HapMap Project aims to determine the common patterns of DNA sequence variation in the human genome (24). In the initial phase of the project, genetic data are being gathered from four populations with African, Chinese, Japanese, and European ancestry. Twenty of 33 SNPs used in this study were genotyped on Japanese subjects in the HapMap project, providing important information for determining whether the genes of interest are associated with type 2 diabetes in a Japanese cohort. To

clarify the relationships between our SNPs and those of the HapMap, the patterns of LD between SNPs for each gene are shown in online appendix Fig. 1. Among nine genes (*TCF1*, *TCF2*, *HNF4A*, *ISL1*, *PAX6*, *NKX6-1*, *NEUROD1*, *ABCC8*, and *KCNJ11*) that have a relatively large number of genotyped SNPs in the HapMap, four genes (*ISL1*, *NKX6-1*, *NEUROD1*, and *KCNJ11*) show a relatively strong LD, while five genes (*TCF1*, *TCF2*, *HNF4A*, *PAX6*, and *ABCC8*) show a weak LD across each gene. For the former genes, our data provide considerable information on the association of genes of interest with type 2 diabetes. However, for the latter genes, we could provide only partial information on their association with type 2 diabetes. In contrast, there are none or few genotyped SNPs in the HapMap for three genes (*IPF1*, *NEUROG3*, and *NKX2-2*). For these genes, our data provide valuable information on both the SNPs and their association with type 2 diabetes.

Associations of *HNF4A* variants in the upstream promoter region with type 2 diabetes have recently been reported in several populations (25–27). Using Japanese samples in the HapMap data, we calculated LD between our SNPs (rs717247 and rs745975) and those (rs1884614, rs2144908, and rs4810424) showing association with type 2 diabetes. However, LD was not found (online appendix Fig. 1), indicating that the association of our SNPs with type 2 diabetes is different from that of other studies. In this study, modest associations ($P < 0.05$) with type 2 diabetes were also detected for several of the candidate genes examined (*TCF2*_SNP4 rs1016991, *TCF2*_SNP6 rs2688, *NKX2-2*_SNP2 rs3746741, and *NKX6-1*_SNP1 rs1017560). As there has been no large association study for these variants, these associations need to be confirmed by further replication studies. Nevertheless, this is the largest association study so far conducted on these genes in Japanese subjects, providing valuable information not only for this population, but also for comparison with other ethnic groups.

RESEARCH DESIGN AND METHODS

We examined 1,590 unrelated Japanese type 2 diabetic subjects recruited from nine university hospitals and affiliated hospitals located in seven prefectures in Japan. Type 2 diabetes was diagnosed using World Health Organization criteria. The clinical data on these type 2 diabetic subjects are as follows (continuous data are given as median [interquartile range]): male 54.2%, age at diagnosis 49 years (40–57), and BMI 22.9 kg/m^2 (21.1–25.2). We also examined 1,244 nondiabetic control subjects matched for geographic region under the following criteria: aged ≥60 years, no past history of diagnosis of diabetes, HbA_{1c} <5.6%, and no diabetes within third-degree relatives. Analyses were performed on the whole population of subjects. Genetic analysis of human subjects was approved by the ethics committee at each university. Appropriate informed consent was obtained from all of the subjects examined.

Selection of SNPs and genotyping. We resequenced several target genes using DNA samples of Japanese subjects and selected SNPs for an association study mainly based on a minor allele frequency >0.10 and the possibilities of haplotype construction with the SNPs used in this study. We also selected several SNPs from previous publications (9,10,22).

Genomic DNA was extracted from peripheral blood samples by standard procedures. Genotyping of SNPs was performed by MassARRAY system (Sequenom, San Diego, CA), chip-based matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of primer extension products following the PCR amplification. Extension primers, extended across the SNP site, were designed using SpectroDESIGNER software (Sequenom, San Diego, CA). The extension reaction is controlled by a mixture of dideoxy-terminated nucleotides, such that one single-base extension product is created and one double-base extension product is created corresponding to an SNP allele. This scheme creates two peaks in the mass spectrometer that are separated by ~300 Da. The primer extension reaction products were loaded onto SpectroCHIPs preloaded with matrix. SpectroCHIPs were analyzed in fully automated mode by MassARRAY mass spectrometer (Bruker-Sequenom). Quality values are attached to each genotyping result, and samples with low quality value were reanalyzed.

Statistical analyses. Differences in distribution of allele or genotype frequencies between type 2 diabetic and control subjects were assessed using χ^2 tests. The extent of LD and haplotype frequencies were estimated using the Hitagene software (Hitachi Europe, Dublin, Ireland) and PowerMarker software (Kejun Liu and Spencer Muse, PowerMarker: new genetic data analysis software, version 3.0; free program distributed over the internet available from http://www.powermarker.net). Power calculations were completed using the Genetics Power Calculator (28). The pairwise r^2 values for SNPs in the HapMap were calculated by the Haploview (29).

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Estrogen Receptor α Regulates Insulin Sensitivity through IRS-1 Tyrosine Phosphorylation in Mature 3T3-L1 Adipocytes

KAZUHIKO MURAKI, SHIGERU OKUYA AND YUKIO TANIZAWA

Division of Endocrinology, Metabolism, Hematological Sciences and Therapeutics, Department of Bio-Signal Analysis, Yamaguchi University Graduate School of Medicine, 1-1-1 Minami-Kogushi, Ube, Yamaguchi 755-8505, Japan

Abstract. There are many clinical and experimental reports demonstrating that estrogens and insulin interact when affecting their target organs. Estrogen receptors consist of two isoforms, estrogen receptors-alpha (ER-α) and -beta (ER-β), but their roles in insulin-induced glucose uptake in mature adipose tissue have yet to be clarified. To evaluate the roles of ER-α, expressed predominantly in adipocytes, we have investigated the effects of estradiol (E2), an ER-α selective agonist (PPT), and its selective antagonist (MPP) on glucose uptake and insulin action in 3T3-L1 adipocytes. 3T3-L1 adipocytes were exposed to E2 or PPT and/or MPP at different concentrations. The cells were then subjected to 2-deoxy-D-glucose transport assay, western blot analysis, or RT-PCR analysis. Treatment of these cells with E2 or PPT resulted in biphasic effects on glucose transport, that is high (10^{-5} M or 3×10^{-6} M each) and low (10^{-8} M) doses produced inhibition and stimulation, respectively. The favorable effect observed at 10^{-8} M of E2 was diminished by treatment with MPP. Western bolt analysis revealed that these effects of E2, PPT and MPP paralleled the level of IRS-1 tyrosine phosphorylation. However, IRS-1 serine phosphorylation, suppressor of cytokine signaling (SOCS)-1,-2,-3 and protein tyrosine phosphatase 1B (PTP1B) expression did not change compaired to control subjects. Our data clearly show that ER-α contributes to insulin stimulated glucose uptake through regulation of the tyrosine phosphorylation of IRS-1 protein.

Key words: ER-α, Estradiol, IRS-1, Tyrosine phosphorylation, Adipocyte

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THERE is a great deal of clinical and experimental evidence demonstrating that sex steroids and insulin interact in exerting their effects on insulin targeting organs [1]. In clinical studies, high concentrations of estrogens, as in pregnancy [2, 3], trans-sexuality [4] and during menstrual cycles [5], appear to contribute to the development of insulin resistance. On the other hand, there is considerable evidence against adverse effects of estrogens on glucose metabolism and some for beneficial actions of estrogen. In male mice with complete aromatase deficiency, which are unable to catalyze the formation of estrogens from androgens, progressive insulin resistance is observed [6]. Ovary-ectomized (OVX) mice are more insulin-resistant than sham controls and treatment of these OVX mice with estrogen normalizes both glucose tolerance and insulin sensitivity [7]. These results suggest the necessity for appropriate estrogen concentrations to maintain insulin sensitivity, while excessively high concentrations lead to deterioration of insulin sensitivity.

Estrogens mediate their effects through two receptor isoforms, estrogen receptor alpha (ER-α) and estrogen receptor beta (ER-β), each of which is responsible for different biological functions as reflected by their tissue-specific expression patterns. Different roles have been assigned to ER-α and ER-β in mediating the effects of estrogens in adipose tissue. This was suggested by the observations that ER-α gene knockout mice become obese, whereas ER-β knockout mice have a normal amount of adipose tissue [8–10]. Furthermore,

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Correspondence to: Dr. Yukio TANIZAWA, Division of Endocrinology, Metabolism, Hematological Sciences and Therapeutics, Department of Bio-Signal Analysis, Yamaguchi University Graduate School of Medicine, 1-1-1 Minami-Kogushi, Ube, Yamaguchi 755-8505, Japan

in human adipocytes, the level of ER-β mRNA is much lower than that of ER-α mRNA [11]. These findings indicate that ER-α might play a major role in mediating the effects of estrogens on adipocytes.

In recent years, ER subtype-selective agonists and antagonists contributing to the functional study of these ER subtypes have been developed. Katzenellenbogen *et al.* [12, 13] have developed compounds that are capable of stimulating ER-α very selectively. Propylpyrazole triol (PPT), a member of the triarylpyrazole class, is more than 10,000-fold more potent on ER-α than on ER-β [12, 13]. Recently, Katzenellenbogen *et al.* developed a compound known to antagonize ER-α, but not ER-β, by adding basic side-chains typically found in non steroidal anti-estrogens to pyrazole compounds, and this specific antagonist was named methylpiperidino-pyrazole (MPP) [14]. These compounds would presumably be useful for studying the functions of ER-α in adipocytes.

Although evidence that very low and high concentrations of estrogen can cause insulin resistance has been obtained in clinical studies, relatively little information is available on the molecular basis of this mechanism. *In vitro*, culture of 3T3-L1 adipocytes with high concentrations of estrogens (estrone, estradiol or estriol) resulted in a reduced ability of insulin to stimulate glucose uptake by attenuating insulin signaling events, leading to GLUT4 translocation to the plasma membrane [15]. This study suggests that these estrogens can modulate insulin sensitivity and contribute to the development of insulin resistance by acting directly on adipocytes, but the precise role of ER subtypes was not examined. With the use of an ER-α selective agonist (PPT) and a selective antagonist (MPP), we investigated the role of ER-α in insulin sensitivity in mature 3T3-L1 adipocytes. Furthermore, we examined mechanisms of insulin sensitivity associated with estrogen.

Materials and Methods

Materials and antibodies

We obtained E2 (1,3,5[10]-Estratriene-3,17(-diol), cell culture tested) and PPT (1,3,5-Tris (4-hydroxyphenyl)-4-propyl-1H-pyrazole) from Sigma (St. Louis, MO, USA). MPP (1,3-Bis (4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride) was from Tocris (St. Louis, MO, USA). Anti IRS-1 rabbit polyclonal, anti-Akt1 and anti-GLUT4 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho Akt (serine 473), phospho IRS-1 (serine 307), phospho IRS-1 (serine 612) and phospho Erk antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-insulin receptor beta subunit (IRβ) antibody was obtained from BD Transduction Laboratories (San Diego, CA, USA). Anti-Erk antibody was purchased from New England Biolabs (Beverly, MA, USA). Anti-GAPDH antibody was from CHEMICON (Temecula, CA, USA). Other reagents are described below.

Cell culture and differentiation

We grew 3T3-L1 fibroblasts in DMEM-HG (Dulbecco's Modified Eagle Medium-high glucose), 10% bovine calf serum at 37°C in 10% CO_2 and split the cultures at about 30% confluence. Cells for use in experiments were grown in the same medium until 2 days after confluence, and then differentiated into adipocytes by treatment with induction medium (0.7 μM insulin, 0.1 μg/ml dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine) [17]. The cells treated with induction medium for 3 days were incubated in DMEM-HG and 10% fetal bovine serum (FBS). The cells were used for experiments at least 8 days after differentiation. After 6 days' induction, our 3T3-L1 fibroblasts are fully differentiated. Because the differentiated cells tend to be dislodged from the culture dishes or plates after 14 days' culture, we completed the experiments before 11 days after the induction. Phenol red-free medium and charcoal-stripped FBS was used while incubating the cells with E2, PPT or MPP to eliminate estrogenic effects of the medium. Phenol red is known to exert weak estrogenic effects [18].

2-deoxy-D-glucose transport assays

3T3-L1 adipocytes on 24-well plates were pretreated in DMEM-HG, 10% FBS containing E2 or other compounds (PPT or MPP) for 24 h, then deprived of serum by incubation for 3 h in KRH buffer (25 mM HEPES, pH 7.4, 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$ and 1.3 mM KH_2PO_4) containing E2, PPT or MPP. E2 and PPT were dissolved in ethanol, MPP in water. The cells were then stimulated with 100 nM insulin for 15 min. Glucose transport assay

was initiated by the addition of 2-[^{3}H]deoxy-D-glucose to a final concentration of 100 μM 2-deoxy-D-glucose (20 μCi/mmol). After 5 minutes, the incubation transport assay was terminated with rapid washing in ice-cold KRH buffer three times. The cells were recovered by adding 0.5 ml/well of 0.1 N NaOH, and radioactivity was measured by liquid scintillation counting.

Immunoprecipitation

3T3-L1 adipocytes grown in 6-well plates were pre-incubated in DMEM-HG, 10% FBS containing E2 or chemical compounds (PPT or MPP) for 12 h, then deprived of serum by incubation for 12 h in serum-free DMEM-HG containing E2, PPT or MPP. Cells were solubilized with lysis buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1% NP-40, 25 mM NaF, 1 mM sodium orthovanadate, 1 mM benzamidine, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM DTT and 1 mM phenylmethylsulfonyl fluoride (PMSF)). To examine IRS-1 phosphorylation, 500 μg of total cell lysates were subjected to immunoprecipitation for 1 h at 4°C with polyclonal antibody to IRS-1 (1 μg).

SDS-PAGE and immunoblotting

Total cell lysates and immunoprecipitates were resolved by SDS-PAGE on 8% gels and transferred to PVDF (Amersham, Piscataway, NJ, USA) membrane. Immunolabelled proteins were visualized using HRP-conjugated secondary antibodies and the ECL system (Amersham, Piscataway, NJ, USA). Bands were analyzed and quantified using NIH Image version 1.62.

Isolation of RNA and cDNA synthesis

3T3-L1 adipocytes on 6-well plates were incubated in DMEM-HG, 10% FBS for 12 h, then in serum free, DMEM-HG medium containing E2 or PPT for 12 h, and finally washed twice with ice-cold PBS. Cells were homogenized in ISOGEN (NIPPON GENE, Tokyo, Japan), and total RNA was extracted following the manufacturer's protocol.

Total RNA (1 μg) was converted to cDNA utilizing oligo-dT primer and Super Script II (Invitrogen, Carlsbad, CA, USA) in a total reaction volume of 20 μl containing 1 × RT buffer, 0.5 mM dNTP, 0.01 M DTT, and 40 units of ribonuclease inhibitor (RNAsin, Promega, Madison, WI, USA). The reaction mixture was incubated at 65°C for 5 min, 42°C for 50 min and terminated by increasing the temperature to 70°C for 15 min in a thermal cycler as described by the manufacturer.

PCR and Semi quantitative RT-PCR

PCR and semi quantitative RT-PCR were performed in a total reaction volume of 20 μl containing oligonucleotide primers (0.2 μM), 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 0.5 units of Taq DNA polymerase (Roche, Basel, Switzerland).

Primers for ER-α, -β (16), suppressor of cytokine signaling (SOCS)-1, SOCS-2, SOCS-3, PTP1B and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were as follows, ER-α: sense 5'-GGCCTGACTCTG CAGCAGCAG-3', anti-sense 5'-GTTGGGGAAGCC CTCTGCTTC-3' (annealing temp. 65°C, 300-bp product), ER-β: sense 5'-GGCATTCTACAGTCCTGCTG-3', antisense 5'-TCTGCATAGAGAAGCGATGA-3' (annealing temp. 60°C 165-bp product), SOCS-1: sense 5'-CAGACACAAGCTGCTACAACC-3', antisense 5'-CGTGACTACCTGAGTTCCTTCC-3' (annealing temp. 58°C, 274-bp product), SOCS-2: sense 5'-CGCGTCTGGCGAAAGCCCTGC-3', antisense 5'-CATTAACAGTCATACTTCCCCAGTACC-3' (annealing temp. 68°C, 69-bp product), SOCS-3: sense 5'-CACAGCAAGTTTCCCGCCGCC-3', antisense 5'-GTGCACCAGCTTGAGTACACAGTC-3' (annealing temp. 68°C, 365-bp product), PTP1B: sense 5'-CTCCTGAACAGGTTAAGGCC-3', antisense CTG GTCAATGTGTGCATGG-3' (annealing temp. 56°C, 269-bp product), GAPDH: sense CAGACACAAGC TGCTACAACC-3', antisense CGTGACTACCTGAG TTCCTTCC-3' (annealing temp. 68°C, 100-bp product). Exactly 4% of the first strand cDNA of each sample was used for PCR amplification by the specific primers indicated above. The reaction mixtures were incubated in a thermal cycler for 40 (ER-α, ER-β), 35 (SOCS-1, SOCS-2, SOCS-3), 26 (PTP1B) and 23 cycles (GAPDH) as follows: at 94°C for 60 sec, at annealing temperatures indicated for 60 sec and at 72°C for 60 sec. In semiquantitative RT-PCR for SOCS-1, SOCS-2, SOCS-3, PTP1B and GAPDH, the cycles were terminated during the phase in which there was exponential generation of PCR products before reaching a plateau. After the cycles had been completed, the tubes were maintained at 72°C for 10 min. Twenty microliters of the PCR product were analyzed on 2%

agarose gel in the presence of ethidium bromide. Bands were analyzed and quantified using NIH Image version 1.62.

Statistical analysis

The statistical significance of differences in measured quantities was determined with Student's *t*-test. A P value less than 0.05 was considered statistically significant. Average values were expressed as means ± standard error of the mean (SEM).

Results

PCR for ER-α and -β in mature 3T3-L1 adipocytes

To determine the functional role of ER-α in mature adipocytes, we studied 3T3-L1 adipocytes that had been cultured for more than 8 days after the induction of differentiation. 3T3-L1 cells subjected to such long-term culture are considered to be representative of mature adipocytes, given that the expression of proteins linked to adipocyte differentiation does not change significantly after 8 days of such treatment [19]. To confirm the expressions of ER-α and -β, mRNA expressions of both ERs on fully differentiated 3T3-L1 adipocytes were examined by the RT-PCR method. ER-α and -β were both expressed in the mature 3T3-L1 adipocytes used in our present experiments (Fig. 1).

2-deoxy-D-glucose transport assay during treatment with E2, PPT and MPP

The 3T3-L1 adipocytes we used in the series of experiments were fully differentiated, and no apparent change was observed by the E2 or PPT and/or MPP treatment on inspection.

Fig. 1. RT-PCR analysis for ER-α and -β mRNA expressions in mature 3T3-L1 adipocytes.
PCR products were applied to 2% agarose gel (left: ER-α, right: ER-β). The sizes of PCR products for ER-α and -β are 300 bp and 165 bp, respectively. The RT-PCR results shown are representative of three separate experiments with the same protocol.

We first examined the effects of several concentrations of E2 on 2-deoxy-D-glucose transport. 3T3-L1 adipocytes pretreated with E2 for 24 h were compared with cells exposed to the same concentration of vehicle (ethanol: EtOH).

We consistently observed that insulin-stimulated deoxyglucose transport was significantly reduced (29% reduction compared to control samples) by treatment with E2 at 10^{-5} M for 24 h ($P<0.01$) (Fig. 2A). On the other hand, E2 treatment at 10^{-8} M for 24 h significantly increased insulin-stimulated deoxyglucose transport ($P<0.05$) (Fig. 2A).

To investigate whether ER-α contributes to the effects observed with E2 treatment, we next examined deoxyglucose transport using PPT, a selective ligand for ER-α. Treatment of cells with PPT at concentrations of 10^{-6} and 3×10^{-6} M decreased insulin-stimulated deoxyglucose uptake by 15% and 39%, respectively, as compared to vehicle treated controls ($P<0.01$) (Fig. 2B). The increase in deoxyglucose uptake seen with E2 at 10^{-8} M was also seen at the PPT concentration of 10^{-8} M ($P<0.05$) (Fig. 2B).

To confirm that the effects observed with E2 treatment occurred via ER-α, we compared the E2 10^{-8} M treated cells with those to which MPP at 10^{-6} M had been added. Increased insulin-stimulated deoxyglucose uptake observed with E2 treatment at 10^{-8} M was not recognized when the cells were co-incubated with MPP (Fig. 2C). Incubation of the cells in DMEM containing MPP only had no effect on deoxyglucose transport (data not shown). Unfortunately, we could not test whether the inhibitory effect of a high E2 concentration on insulin stimulated glucose uptake also occurs through ER-α, because this would require a very high concentration of MPP.

Biphasic changes of insulin-stimulated glucose uptake were not associated with the amount of GLUT4 protein expression (Fig. 2D).

Effects of E2 (10^{-5} M) or PPT (3×10^{-6} M) treatment on protein expression and insulin-stimulated phosphorylation of insulin signaling molecules

We next investigated the effects of a high concentration of E2 or PPT on the molecules involved in insulin signaling for glucose transport. Cells pretreated for 24 h with a high concentration of E2 (10^{-5} M) or PPT (3×10^{-6} M) were incubated in the presence or absence

of 100 nM insulin for 3 minutes at 37°C. Incubation in a high concentration of PPT (3×10^{-6} M) or E2 (10^{-5} M) resulted in significant inhibition of insulin-induced tyrosine phosphorylation of IRS-1 (Fig. 3A (b), Fig. 3B (b)) and serine phosphorylation of Akt, a signaling molecule downstream from IRS-1 (Fig. 3A (c), Fig. 3B (c)) (P<0.05). However, high concentrations of E2 and PPT had no effect on the abundance of IRβ (Fig. 3A (a), Fig. 3B (a)), IRS-1 (Fig. 3A (b), Fig. 3B (b)), Akt proteins (Fig. 3A (c), Fig. 3B (c)), or the insulin-stimulated tyrosine phosphorylation of IRβ (Fig. 3A (a), Fig. 3B (a)). These findings indicate that high concentrations of E2 and PPT produce insulin resistance at the IRS-1 level of the insulin signaling pathway. To evaluate the IR-Erk pathway, we simultaneously analyzed Erk protein expression and phosphorylation of Erk. However, neither changed and these results indicate E2 and PPT to specifically impair IRS-1 tyrosine phosphorylation (Fig. 3A (d), Fig. 3B (d)).

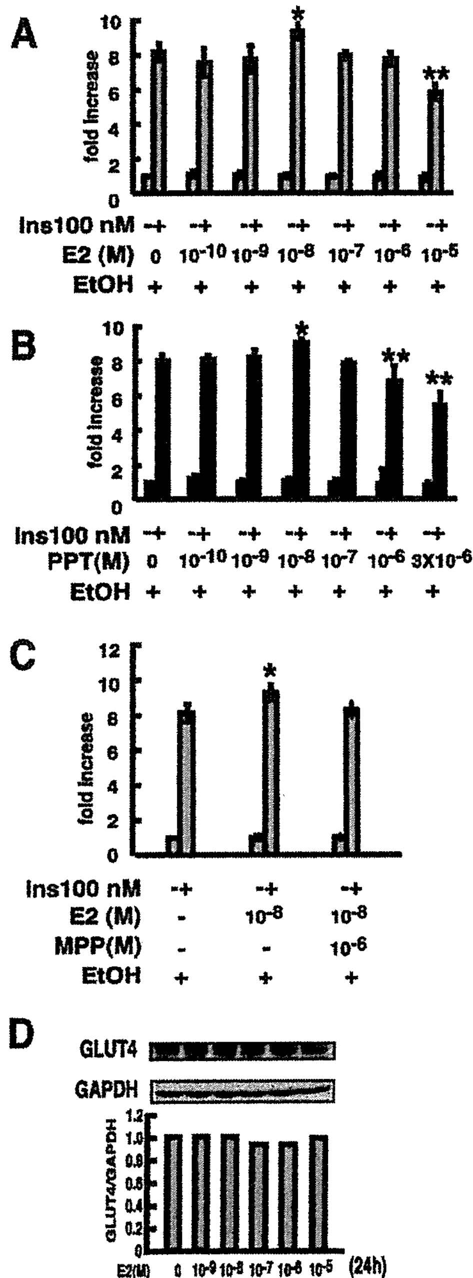

Fig. 2

Effects of E2 (10^{-8} M) plus MPP (10^{-6} M) treatment on insulin stimulated phosphorylation of insulin signaling molecules

Next, we investigated whether the regulations by

Fig. 2. Measurement of 2-deoxy-D-glucose transport after treatment with E2, PPT and MPP.
(A) Effects of E2 on 2-deoxy-D-glucose transport in 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated with vehicle (EtOH) or E2 at the indicated concentrations for 24 h. (B) Effects of PPT on 2-deoxy-D-glucose transport in 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated with vehicle (EtOH) or PPT at the range of indicated concentrations for 24 h. After serum starvation, the cells were incubated for an additional 15 min with or without 100 nM insulin. Data are means ± standard error of the mean (SEM) for five independent experiments, each performed in triplicate, and are expressed relative to the value for control cells without 100 nM insulin. Significant differences in comparison with the control (100 nM insulin without E2) are indicated: *P<0.05, **P<0.01. (C) Effects of MPP treatment on 2-deoxy-D-glucose uptake in 3T3-L1 adipocytes incubated with E2 at 10^{-8} M for 24 h. 3T3-L1 adipocytes were treated with EtOH, E2 at 10^{-8} M or E2 at 10^{-8} M plus MPP at 10^{-6} M for 24 h, and then incubated with or without 100 nM insulin for 15 min. Significant differences in comparison with the control (100 nM insulin without E2) are indicated: *P<0.05. (D) 3T3-L1 adipocytes treated with indicated concentrations of E2 for 24 h were analyzed by western blotting. The cells were solubilized and immunoblotted as described in Materials and Methods, and each band was then quantified by NIH image version 1.62. GLUT4 protein was detected using anti-GLUT4 antibody, and the same membrane was then stripped and re-blotted with anti-GAPDH antibody. The amount of GLUT4 protein was normalized by the amount of GAPDH protein, and expressed relative to that of the E2 (–) control. Data are representative of three independent experiments.

tyrosine phosphorylation of IRS-1 observed in E2 and PPT at high concentrations also occur at a relatively low concentration of E2 (10^{-8} M). After all, tyrosine phosphorylation of IRβ did not change, while tyrosine phosphorylation of IRS-1 was increased (Fig. 4A, Fig. 4B). Furthermore, we used an ER-α selective antagonist, MPP, to examine whether the regulation of IRS-1 tyrosine phosphorylation was through ER-α or ER-β activation. With simultaneous addition of MPP and E2, the tyrosine phosphorylation level of IRS-1 returned to the control level (Fig. 4B).

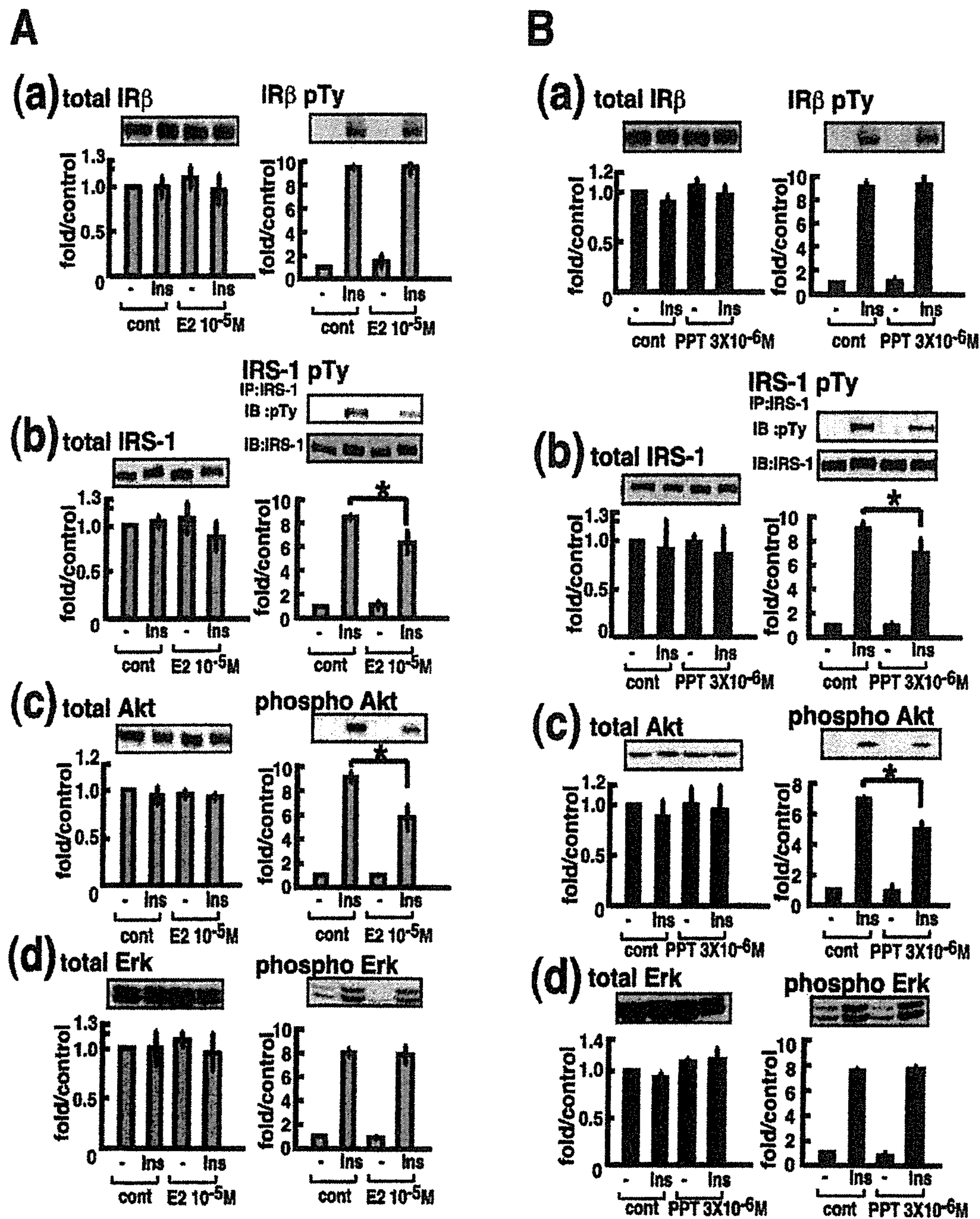

Fig. 3

Effects of E2 on IRS-1 protein serine phosphorylation and on mRNA expression of SOCS1-3 and PTP1B in mature 3T3-L1 adipocytes

Finally, we reviewed serine phosphorylation of IRS-1 protein and expression of the mRNAs of SOCS-1, 2, 3 and PTP1B to examine how E2 regulates IRS-1 tyrosine phosphorylation.

Though IRS-1 has many serine phosphorylation sites, we selected serine 307 and 612 as representative serine phosphorylation sites [20]. A high concentration of E2 (10^{-5} M), which decreased IRS-1 tyrosine phosphorylation, did not increase IRS-1 serine phosphorylation (serine 307 and 612) (Fig. 5A). We also attempted to detect JNK activation using phospho-JNK specific antibody, but found none (data not shown).

Fig. 3. Effects of a high concentration of E2 or PPT on protein expression and phosphorylations of IRβ, IRS-1, Akt and Erk.
3T3-L1 adipocytes were treated with or without E2 (10^{-5} M) or PPT (3×10^{-6} M) for 24 h and then incubated for an additional 3 minutes with or without 100 nM insulin (Ins) at 37°C. The cells were solubilized, immunoprecipitated and immunoblotted as described in Materials and Methods, and each band was then quantified by NIH image version 1.62. A: 3T3-L1 adipocytes treated with a high concentration of E2 were analyzed by western blotting. (a) IRβ protein was detected using anti-IRβ antibody (left panel and graph) and the same membrane was then stripped and re-blotted with an anti-phosphotyrosine antibody (4G10) (pTy) (right panel and graph). (b) IRS-1 protein was detected using anti-IRS-1 antibody (left panel and graph). To analyze tyrosine phosphorylation of IRS-1, total cell lysates were subjected to immunoprecipitation (IP) with antibody against IRS-1. Resulting precipitates were subjected to immunoblot analysis (IB) with antibody to IRS-1, and the same membrane was then stripped and re-blotted with anti-phosphotyrosine antibody (4G10) (right panel and graph). (c) Akt protein was detected using anti-Akt antibody (left panel and graph), and the same membrane was then stripped and re-blotted with an anti-phospho Akt antibody (right panel and graph). (d) Erk protein was detected using anti-Erk antibody (left panel and graph), and the same membrane was then stripped and re-blotted with an anti-phospho Erk antibody (right panel and graph). B: 3T3-L1 adipocytes treated with a high concentration of PPT were analyzed by western blotting. Experiments in Figure 3B-(a) (b) (c) (d) were the same as those in 3A (a) (b) (c) (d). Data are means ± SEM of at least three independent experiments, and are expressed relative to the value for control cells without insulin. Significant differences in comparison with the control (100 nM insulin without E2 or PPT) are indicated: *$P<0.05$.
pTy, tyrosine phosphorylation.

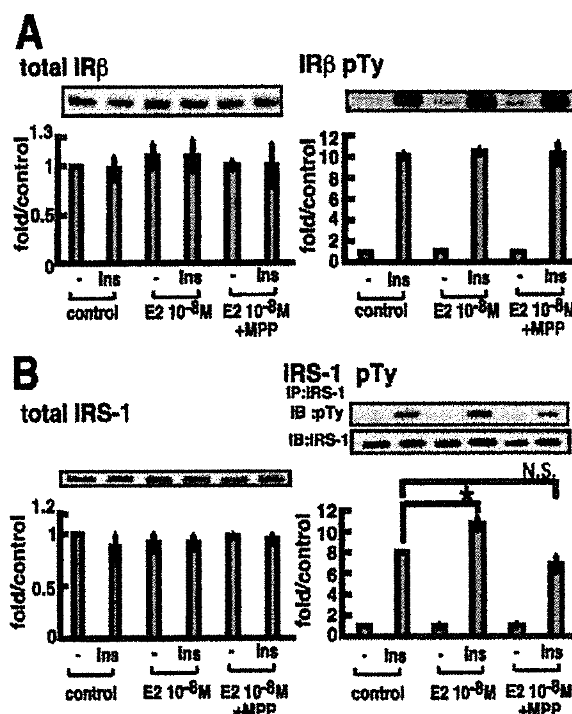

Fig. 4. Effects of E2 at 10^{-8} M plus MPP treatment on IRβ and IRS-1 protein expression and tyrosine phosphorylation.
3T3-L1 adipocytes were treated with EtOH, E2 (10^{-8} M) and E2 (10^{-8} M) plus MPP (10^{-6} M) for 24 h and then incubated for an additional 3 minutes with or without 100 nM insulin (Ins) at 37°C. The cells were solubilized, immunoprecipitated and immunoblotted as described in Materials and Methods. Then, each band was quantified by NIH image version 1.62. A: IRβ protein was detected using anti-IRβ antibody (left panel and graph), and the same membrane was then stripped and re-blotted with an anti-phosphotyrosine antibody (4G10) (pTy) (right panel and graph). B: IRS-1 protein was detected using anti-IRS-1 antibody (left panel and graph). To analyze tyrosine phosphorylation of IRS-1, total cell lysates were subjected to immunoprecipitation (IP) with antibody to IRS-1. Resulting precipitates were subjected to immunoblot analysis (IB) with antibody to IRS-1. Then, the same membrane was stripped and re-blotted with anti-phosphotyrosine antibody (4G10) (right panel and graph). Data are means ± SEM of three independent experiments, and are expressed relative to the value for control cells without 100 nM insulin. Significant differences in comparison with the control (100 nM insulin without E2) are indicated: *$P<0.05$.

We next speculated that increased expression of SOCS-1, 2, 3 or PTP1B might be involved in the regulation of IRS-1 tyrosine phosphorylation. We therefore analyzed these mRNA expressions in cells treated for 24 h with or without E2, at concentrations of 10^{-9}–10^{-5} M, by the semi-quantitative RT-PCR method. There were no changes in the expressions of these mRNAs (Fig. 5B, C).

Discussion

In our present study on mature 3T3-L1 adipocytes, we demonstrated that exposure to high doses of E2 or PPT (from 3×10^{-6} M to 10^{-5} M) inhibits insulin-stimulated glucose transport, and also that cells treated with a relatively low concentration of E2 or PPT (10^{-8} M) show increased glucose uptake, without affecting GLUT4 protein expression.

These results are consistent with clinical and experimental observations that estrogen excesses, as in pregnancy [2, 3], trans-sexuality [4], and during menstrual cycles [5], or estrogen deficiencies as in aromatase deficient [6] and OVX animals [7], cause insulin resistance. Moreover, a selective ER-α antagonist, MPP, diminished the beneficial effect of E2 at 10^{-8} M on glucose uptake. This observation suggests that ER-α plays a crucial role in maintaining insulin-stimulated glucose uptake during treatment with a relatively low concentration of E2 in mature adipocytes. Unfortunately, the effect of MPP on cells treated with a high concentration of E2 could not be examined, because such an experiment would require a very high concentration of MPP, a competitive antagonist of ER-α [14]. In our 3T3-L1 adipocytes, treatment with PPT produced biphasic effects on insulin stimulated glucose uptake as was observed with E2 treatment. This result again suggests ER-α to be involved in the modulation

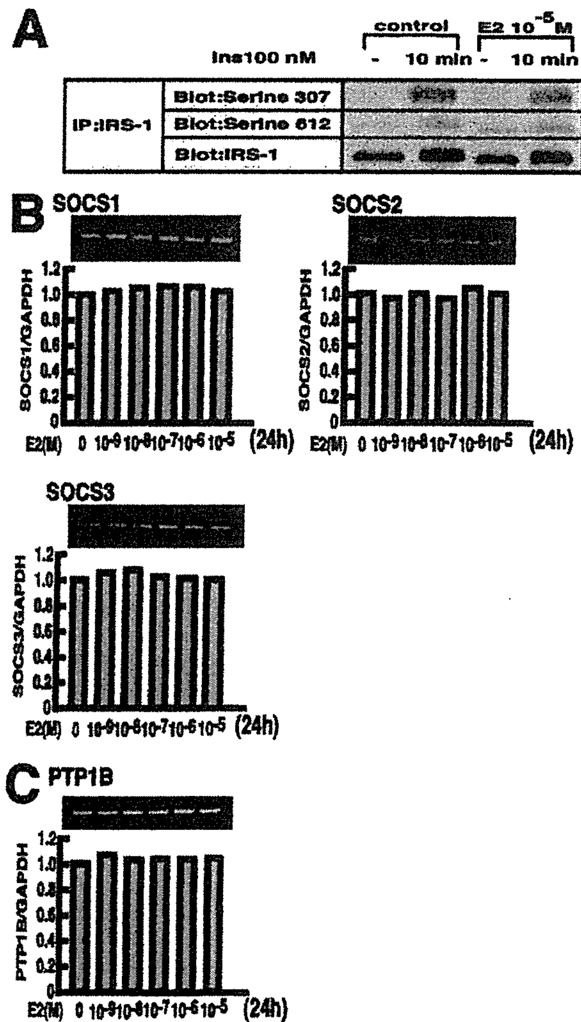

Fig. 5

Fig. 5. Effects of E2 on IRS-1 serine phosphorylation and SOCS-1, 2, 3 and PTP1B mRNA expressions in mature 3T3-L1 adipocytes.
A: Effects of E2 on IRS-1 protein serine phosphorylation. 3T3-L1 adipocytes were treated with EtOH or E2 (10^{-5} M) for 24 h and then incubated for an additional 10 minutes with or without 100 nM insulin (Ins) at 37°C. The cells were solubilized, immunoprecipitated and immunoblotted as described in Materials and Methods. To analyze IRS-1 serine phosphorylation, total cell lysates were subjected to immunoprecipitation (IP) with antibody to IRS-1. Resulting precipitates were subjected to immunoblot analysis (IB) with antibody to IRS-1 and the same membrane was then stripped and reblotted with antibodies to anti-IRS-1(phosphoserine 307) and anti-IRS-1(phosphoserine 612). Data are representative of three independent experiments. B and C: Effects of E2 on SOCS-1, 2, 3 (B) and PTP1B (C) mRNA expressions. The amounts of these mRNAs were determined by semi-quantitative RT-PCR after incubation of cells for 24 h with E2 at the concentrations indicated in the figure. PCR products were visualized by ethidium bromide staining after agarose gel electrophoresis and were quantified using NIH image. The amount of each target mRNA was normalized by the amount of GAPDH mRNA, and expressed relative to that of the E2 (–) control. Data are representative of three independent experiments.

of insulin-stimulated glucose uptake by E2.

The molecular mechanisms by which estrogen modulates glucose uptake in mature adipocytes have not been well established. Collison *et al.* previously showed, using fully differentiated 3T3-L1 adipocytes, that the decreased glucose uptake associated with estrogens (estrone, estradiol or estriol) was due at least in part to decreased cellular contents and an altered subcellular distribution of IRS proteins, in turn resulting in a reduction in proximal insulin signaling cascades [15]. However, under our experimental conditions, decreased tyrosine phosphorylation of IRS-1 protein occurred without IRS-1 protein degradation even during treatment with high doses of E2 or PPT. Moreover, according to our data, the increased or decreased insulin-stimulated glucose transport in response to E2 or PPT is associated with biphasic changes in tyrosine phosphorylation levels of IRS-1, that is consistent with the very recent report [31], and these phenomena appear to be mediated mainly through the ER-α isoform.

Herein, we considered the question of how this modulation of insulin signaling was brought about at the level of IRS-1. The involvement of serine phosphorylation of IRS-1 in the desensitization of insulin action has been pointed out [20–22]. Serine phosphorylation of IRS-1 could induce conformational changes, steric hindrance and cellular re-localization, leading to the decrease in tyrosine phosphorylation by IR. We first examined the possibility that the reduction of IRS-1 tyrosine phosphorylation by estrogen was due to serine phosphorylation of IRS-1. However, IRS-1 serine phosphorylation did not increase. Given that serine phosphorylation (307 or 612) was not increased, it is unlikely that activation of serine kinases for IRS-1 proteins, such as JNK, Erk, mTOR and S6K [20], were involved, at least on our experimental conditions. However, a very recent report suggested that high concentrations of E2 decrease IRS-1 tyrosine phosphorylation by modulating serine phosphorylation of IRS-1 at serine 307 [31]. The difference in IRS-1 serine phosphorylation results may be explained by the difference in cell clone, or experimental conditions. In addition, we used 3T3-L1 adipocytes 8 to 11 days after differentiation, while they used cells 14 to 16 days after differentiation. As to the decreased IRS-1 tyrosine phosphorylation, other mechanisms such as association of SOCSs with IRS-1 could be involved [23, 24]. Because ERs are transcriptional factors, we speculated that they might modulate the mRNA levels of these target molecules, and regulate IRS-1 tyrosine phosphorylation. In fact, estrogen reportedly up-regulates SOCS-2 and -3 in hepatocytes via ER-α through mechanisms not involving the estrogen response element [25]. However, in adipocytes, E2 treatment did not up-regulate SOCS-1, -2 or -3 mRNA. This discrepancy is probably attributable to estrogen action depending on intracellular machinery that differs among various cell types [26, 27]. PTP1B is an important protein phosphatase that is known to regulate IRS-1 tyrosine phosphorylation [28–30]. We also examined PTP1B expression, but it did not change after E2 treatments.

In summary, our data suggest that estrogen, through ER-α, modulates insulin-stimulated glucose uptake by regulating tyrosine phosphorylation of IRS-1 protein in mature adipocytes. Further studies are needed to elucidate the mechanisms by which estrogen regulates IRS-1 tyrosine phosphorylation via ER-α, as well as to understand the mechanism of glucose intolerance associated with estrogen excess and deficiencies.

Acknowledgements

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Physiological Significance of Resistin and Resistin-Like Molecules in the Inflammatory Process and Insulin Resistance

Tomoichiro Asano[1,4]¶, Hideyuki Sakosda[2], Midori Fujishiro[2], Motonobu Anai[3], Akifumi Kushiyama[2], Nanao Horike[1], Hideaki Kamata[4], Hiroki Kurihara[1], Yasunobu Uchijima[1]

[1]*Department of Physiological Chemistry and Metabolism, Graduate School of Medicine, University of Tokyo,* [2]*Department of Internal Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan 113-0033,* [3]*Institute for Adult Diseases, Asahi Life Foundation, 1-9-14 Nishishinjuku, Shinjuku-ku, Tokyo 116, and* [4]*Department of Medical Science, Graduate School of Medicine, University of Hiroshima, 1-2-3 Kasumi, Minami-ku, Hiroshima City, Hiroshima Japan 734-8551*

Abstract: Resistin was initially identified as a protein, secreted by adipocytes, which inhibits insulin action and adipose differentiation. The three proteins homologous to resistin were termed resistin-like molecules (RELM) α, β and γ. Resistin and RELMα are abundantly expressed in adipose, but RELMβ and RELMγ are secreted mainly from the gut.

Recently, resistin and RELMs were reported to be associated with inflammation. For example, RELMα, viewed as an inflammation-related protein, was originally identified in broncho-alveolar lavage fluid obtained from animals with experimentally induced pulmonary inflammation. RELMβ is also related to bacterial colonization, but RELMβ injection or hepatic overexpression of RELMβ induced insulin resistance. RELMγ isolated from rat nasal respiratory epithelium was found to be altered by cigarette smoke. Thus, resistin and RELMs could be useful for assessing the inflammatory condition *in vivo*.

On the other hand, whether the serum resistin or RELM concentration is strongly related to insulin resistance remains unclear. However, taking recent studies showing a close relationship between inflammation and insulin resistance in diabetes into consideration, these proteins may have interactive roles linking inflammation and insulin resistance, both of which major involvement in the progression of atherosclerosis. If so, the serum resistin or RELM concentration may be a good marker of atherosclerotic risk. In addition, these proteins or unidentified receptors are potential therapeutic targets for the treatment of diabetes and prevention of atherosclerosis. These possibilities merit further study.

INTRODUCTION

Type 2 diabetes is characterized by insulin resistance of peripheral tissues such as liver, muscle and adipose. Insulin resistance is reportedly induced by many acquired factors, but obesity with excessive caloric intake has been regarded as the most common cause. Regarding the molecular mechanism underlying obesity-associated insulin resistance, much attention has recently been focused on the involvement of adipocytes. Namely, adipose tissue was described not only as a site of lipid storage, but also as an endocrine organ producing hormones, cytokines and other substances [1-2]. To elucidate how adipocytes regulate insulin sensitivity, the proteins secreted by adipocytes, of which the expressions were increased with enlargement of adipocytes and/or decreased by treatment with a PPARγ agonist, have been intensively studied by many investigators. Resistin was identified as one of the adipocyte-secreted proteins of which expression was suppressed by PPARγ agonist treatment [3].

*Address correspondence to these authors at the Department of Physiological Chemistry and Metabolism, Graduate School of Medicine, University of Tokyo; Tel: 81-3-5841-3603: Fax: 81-3-5803-1874: E-mail: asano-tky@umin.ac.jp

On the other hand, a significant relationship between inflammation and insulin resistance has been reported. Since the inflammation in vessels leads to atherosclerosis, the relationships among inflammation, abnormal glucose, lipid metabolism and the resultant atherosclerosis may be key event to the development of metabolic syndrome. In this review, we discuss reports suggesting the involvement of resistin and its isoform proteins in insulin resistance and other disorders, and also discuss their possible diagnostic usage and/or therapeutic significance.

STRUCTURES AND TISSUE DISTRIBUTIONS OF RESISTIN AND THREE RESISTIN-LIKE MOLECULES (RELMS)

As discussed above, resistin is a newly identified adipocyte-secreted protein, which induces insulin resistance. In addition, there are three proteins homologous to resistin, termed resistin-like molecules (RELMs), indicating that resistin belongs to a novel family of cysteine-rich secreted proteins. RELMα [4] is expressed mainly in white adipose tissue and the lungs. Expression of the third isoform, RELMβ, is restricted to the intestine and colon [5,6]. Finally, RELMγ, a fourth member of the RELM family, was identified as a gene showing decreased expression in rat

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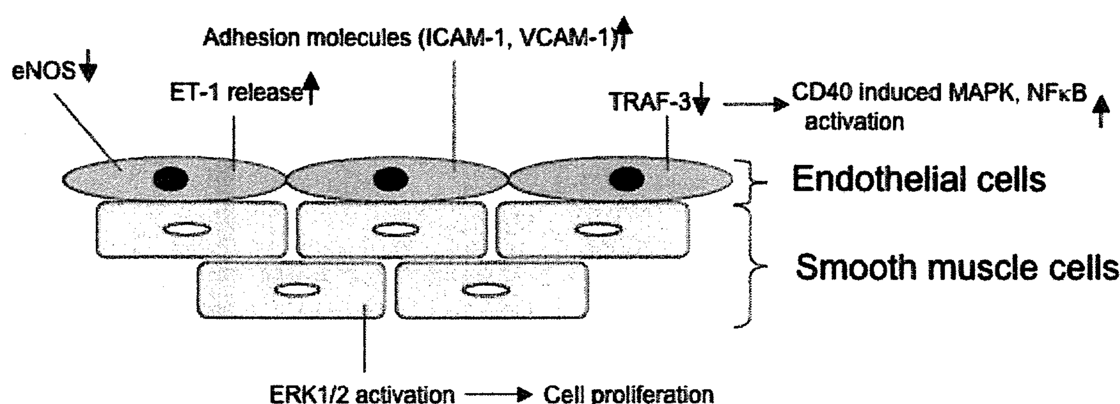

Fig. (1). Possible mechanisms of atherosclerosis-enhancing effects of resistin and RELMs.

nasal respiratory epithelium exposed to cigarette smoke [7]. RELMγ, expressed in the bone marrow, spleen, pancreas and colon, was revealed to play the role of a cytokine in hematopoiesis [8,9]. Mice have all 4 isoforms, but a gene search revealed that humans have neither RELMα nor RELMγ and that rats lack RELMβ.

The RELM structure is composed of two domains; one half being the N-terminal, including an N-terminal signal sequence and a variable middle portion, and the other half the C-terminal domain, which has a highly conserved C-terminal signature sequence containing a unique spacing of cysteine residues. The N-terminal domains of mouse RELMα, RELMβ and RELMγ are 15, 35 and 17% identical, whereas those of the C-terminal are 47, 54 and 52% identical, respectively, to resistin. The RELMγ C-terminus is highly homologous (84%) with RELMβ. Recently, resistin and RELMβ were revealed to have a unique multimeric structure [10]. Each protomer is comprised of a "head" and "tail" segment and circulates as an assembly of hexamers and trimers, reflecting the activation of resistin.

RESISTIN INDUCES INSULIN RESISTANCE AND VASCULAR DYSFUNCTION *IN VITRO* AND *IN VIVO*.

Adipocytes secrete resistin, which has been demonstrated to antagonize insulin actions such as insulin-stimulated glucose transport in skeletal myocytes, hepatocytes and even adipocytes, of rodents [3]. Indeed, giving resistin to mice diminishes glucose tolerance, and serum resistin levels were reportedly elevated in genetically obese mice and down-regulated by administration of thiazolidinediones, Peroxisome Proliferator-Activated Receptor γ (PPARγ) agonists [11,12], although contradictory data have also been presented [13]. Resistin also exerts an inhibitory effect on adipose differentiation [14], suggesting that, in addition to being an important regulator of insulin sensitivity, resistin may be involved in the regulation of adipose mass. In addition, resistin knock-out mice had lower blood glucose levels, associated with reduced hepatic glucose production [15]. Thus, resistin was suggested to be among the important adipokines causing insulin resistance.

Although it is not clear whether resistin contributes directly to vascular disease formation, several studies have recently demonstrated the effects of resistin on vascular cells. Furthermore, in humans peripheral blood mononuclear cells (PBMC) are apparently a major source of resistin [16]. Resistin increases expressions of the adhesion molecules vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), up-regulates the monocyte chemoattractant chemokine-1, and promotes endothelial cell activation via release of endothelin-1 [17,18]. Furthermore, resistin downregulates tumor necrosis factor receptor-associated factor 3 (TRAF-3), which inhibits CD40 ligand signaling [19], thereby causing endothelial dysfunction in porcine coronary arteries through oxidative stress and down-regulation of eNOS [20]. Resistin also reportedly induces vascular smooth muscle cell proliferation via activation of ERK 1/2 and Akt signaling pathways [21]. These findings raise the possibility of a mechanistic link between cardiovascular disease and the metabolic syndrome. These hypotheses are presented in Fig. 1.

ANIMAL STUDIES SUGGEST NOT ONLY RESISTIN BUT ALSO RELMS TO BE INDUCERS OF INSULIN RESISTANCE

RELMβ is expressed specifically in the intestinal tract, especially abundantly in the colon [5]. The other gut-derived RELM isoform, RELMγ, is also expressed in the colon, as well as in the bone marrow, spleen and lungs [7-9]. Serum RELMβ and RELMγ concentrations are significantly elevated in high-fat diet-induced and obese diabetic mice, and these increases are attributable to increased production in the gut [22]. This link may reflect the total caloric and/or nutrient content of the food ingested.

Injecting RELMβ reportedly induces insulin resistance in rats, similar to that observed with resistin injection [23]. We have generated transgenic mice overexpressing hepatic RELMβ, and demonstrated these RELMβ transgenic mice to have significantly increased fasting blood glucose, hyperinsulinemia and elevated serum and hepatic triglyceride contents while on a high fat diet [24]. In terms of the insulin signaling pathway, insulin receptor substrate-1/2 (IRS-1/2) protein contents of both the liver and muscle were markedly reduced in RELMβ transgenic mice, with resultant decreases in IRS protein phosphorylation, PI3-kinase activation and Akt phosphorylation. Thus, it was reasonable to speculate that RELMs have an effect similar to that of resistin [23],

although the regulation of expression of these proteins differs in terms of not only the organs in which they are expressed but also the stimuli increasing expression.

As yet, the specific receptor(s) for resistin or RELMs is/are not known. However, activated signal transductions resulting from treatment with resistin or RELMs have been reported. Resistin reportedly induces a transient phosphorylation of p42/44 mitogen-activated protein kinase (ERK 1/2) in vascular smooth muscle cells [25]. We also found ERK, p38 and JNK to be activated in hepatocytes in response to RELMβ stimulation. Taken together, these observations indicate that resistin and RELMs activate ERK and possibly p38 and JNK as well, although whether these activations take place irrespective of cell types or differences among tissues remains unknown. JNK activation leads to NF-κB activation, which promotes inflammatory processes including secretion of a variety of cytokines. In addition, JNK was shown to induce insulin resistance by phosphorylating IRS-1, the major substrate of the insulin receptor, at serine 307, thereby diminishing tyrosine phsophosphorylation of IRS-1 [26,27]. Thus, JNK activation, which can induce both inflammatory and insulin responses, may be involved in the molecular mechanism linking inflammation and insulin resistance.

ERK activation also produces insulin resistance via a mechanism different from that of JNK activation. The MEK1-ERK pathway leads to upregulation of GLUT1 expression and downregulation of GLUT4 expression, significantly increasing basal glucose transport while dec- reasing insulin-induced transport [28]. In addition, activation of the MEK1-ERK pathway was shown to downregulate both IRS-1 and IRS-2 expression [29-31].

THE CONTROVERSIAL RELATIONSHIP BETWEEN SERUM RESISTIN CONCENTRATION AND OBESITY OR INSULIN RESISTANCE IN HUMANS

Based on the resistin findings obtained using cultured cells and rodent models, subsequent studies were undertaken to identify possible links between resistin and obesity, insulin resistance and/or diabetes. The plasma resistin level is reportedly increased in obese subjects, and serum resistin correlates positively with obesity [25], visceral fat area [32] and type 2 diabetes mellitus [33,34]. In addition, the serum resistin concentration correlates positively with CRP, endothelin-1 and TNFα, and negatively with HDL. Interestingly, in humans, resistin gene expression effects in adipocytes are not related to insulin resistance [35], suggesting that resistin expression in mononuclear cells, but not adipocytes, may be uniquely controlled in obesity. It was shown that rosiglitazone treatment decreased plasma resistin while metformin treatment increased these levels in patients with type 2 diabetes mellitus [36]. Resistin was also reported to be unaffected by a similar PPAR agonist, pioglitazone [37].

However, some studies showed serum resistin to not correlate with any markers of adiposity, blood pressure, fasting plasma glucose or insulin sensitivity, according to HOMA, or to plasma insulin [35,38-41]. Likewise, only a weak relationship was seen between serum resistin and insulin sensitivity employing the insulin clamp technique [42].

As to resistin gene polymorphisms, four non-coding single-nucleotide polymorphisms (SNPs) are present: -420C>G from the promoter region, +156C>T and +298G>A from intron 2, and +1084G>A from the 3' untranslated region. The -420G and the -537A alleles were reportedly associated with higher plasma resistin concentrations, and individuals with haplotypes A-G (-537A and -420G) did, in fact, have significantly higher plasma resistin concentrations [43] and higher BMI [44] than the other subjects. In addition, SNPs -420C>G, +156C>T and +298G>A may be associated with obesity and high diastolic blood pressure, and the +1084G allele with relatively low HDL [45]. Furthermore, among men but not women, -420G/G homozygotes were reported to have less visceral fat and to be more insulin sensitive than carriers of the C allele [46]. Another study found the -420G/G genotype to be a primary variant determining Type 2 diabetes mellitus susceptibility, suggesting this polymorphism to increase promoter activity, raising serum resistin levels and thereby inducing diabetes mellitus [47,48].

In viewing this range of controversial reports, it appears that the serum resistin concentration is probably only weakly associated with insulin resistance, obesity or diabetes mellitus in humans. Further studies are needed to reach a definitive conclusion.

SERUM RESISTIN CONCENTRATION MAY BE A GOOD INFLAMMATION MARKER

In humans, resistin is highly expressed in bone marrow as compared to other tissues, and rosiglitazone decreases resistin expression in human primary monocyte-derived macrophages *in vitro* [49]. In addition, resistin reportedly exerts potent proinflammatory effects by up-regulating IL-6 and TNF-α and enhancing its own activity via positive feedback [50]. Proinflammatory properties of resistin were blunted by an NF-κB inhibitor indicating the importance of the NF-κB signaling pathway in resistin-induced inflammation. Resistin was also shown to specifically accumulate in the inflamed joints of rheumatoid arthritis patients, at levels correlating with other markers of inflammation. These results indicate that resistin is an important, newly-identified member of the cytokine family with potent regulatory functions. Importantly, the identified properties of resistin make it a promising therapeutic target in chronic inflammatory diseases such as rheumatoid arthritis.

Another study raised the possibility that plasma resistin levels correlate with markers of inflammation including CRP [51,52] and can predict human coronary atherosclerosis, independently of CRP. In addition, it was found that resistin mRNA in human peripheral blood mononuclear cells is strongly increased by proinflammatory cytokines such as interleukin (IL)-1, IL-6 and TNF-α, as well as by lipopolysaccharides (LPS) [53]. Thus, resistin may be a novel link among metabolic signals, inflammation, and atherosclerosis.

THE POSSIBILITY OF RELMS AS A MARKER OF INSULIN RESISTANCE OR INFLAMMATORY DISEASE

Similar close associations with inflammation have been suggested for other RELM isoforms. RELMα was originally identified in broncho-alveolar lavage fluid obtained during

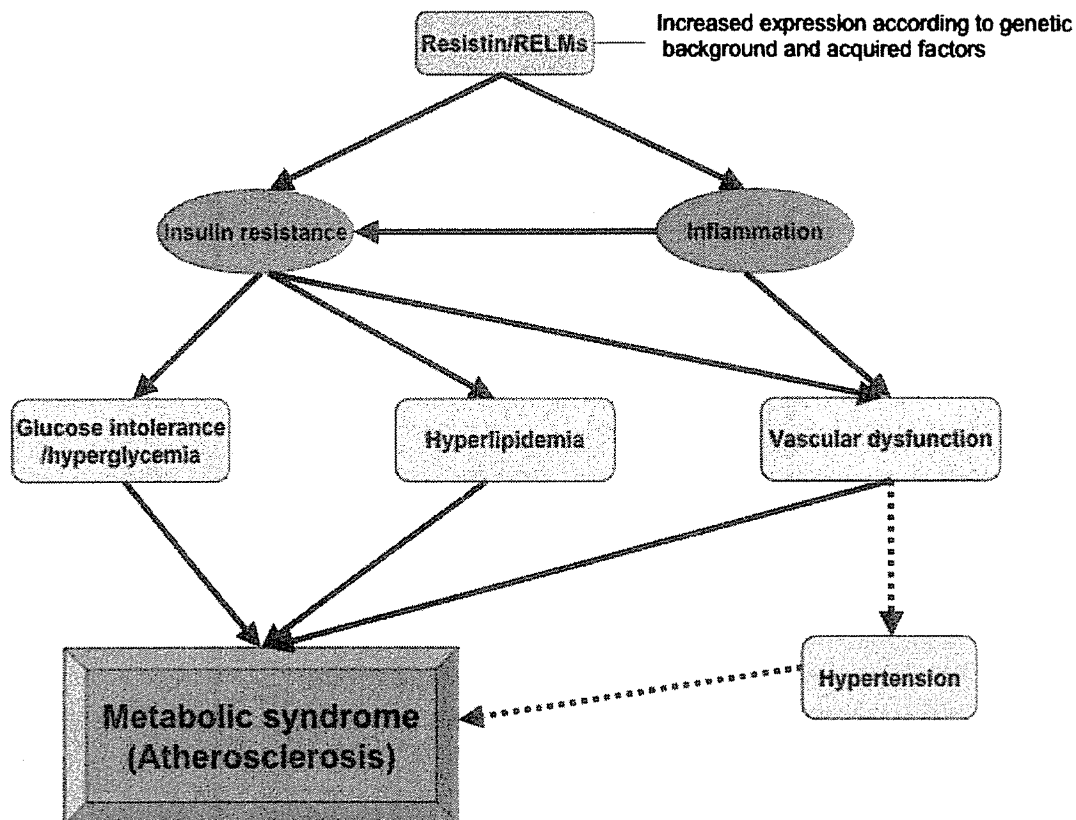

Fig. (2). Hypothetical involvement of resistin and RELMs in metabolic syndrome.

experimentally induced pulmonary inflammation, but is now known to be most abundant in adipose tissues [4]. RELMβ in the colon has been suggested to be involved in bacterial colonization [54]. RELMγ is also expressed in rat nasal respiratory epithelium, and is altered by cigarette smoke [7]. A transcriptional factor may be involved in the inflammation-induced increases in RELMβ and RELMγ expressions. Indeed, the RELMβ promoter region contains a binding sequence for NF-κB and STAT6 [54] and RELMγ is reportedly a target gene of C/EBPε which mediates the role of promyelocytic cell development [9]. These findings suggest an interaction between RELMs and inflammation, although it is unclear whether an elevated RELM concentration causes or is a consequence of inflammation.

RESISTIN AND RELMS MAY LINK INSULIN RESISTANCE AND INFLAMMATION, AND PROMOTE ATHEROSCLEROSIS

The metabolic syndrome is a cluster of pathologies including diabetes, atherosclerosis, hypertension and dyslipidemia. Recently, obesity has been described as a 'low-grade' inflammatory condition, based on elevated serum concentrations of acute phase proteins, pro--inflammatory cytokines and soluble cell adhesion molecules [55] as well as the total leukocyte count. Correspondingly, weight loss was shown to normalize these inflammatory mediator levels.

The contribution of systemic inflammation to the pathogenesis of atherosclerosis has increasingly been recognized, and is associated with inflammatory markers. Such observations have led to the hypothesis that a systemic low-grade inflammatory state plays a key role in the development of insulin resistance and endothelial dysfunction, ultimately leading to diabetes and atherosclerosis. As noted above, resistin and RELMs are likely to be upregulated in association with obesity and to cause insulin resistance and vascular dysfunction. Thus, it is reasonable to speculate that resistin and/or RELMs are among the important molecules involved in the metabolic syndrome, which is characterized by the simultaneous development of diabetes, hyperlipidemia, obesity, and possibly hypertension, and the resultant acceleration of atherosclerosis (Fig. **2**). Measured levels of these molecules may prove to be useful markers for assessing the risk of future atherosclerosis-related disorders. Future studies will focus on whether serum RELMβ and RELMγ concentrations are also increased in obese or insulin resistant diabetic human subjects. If this is the case in humans, measured serum RELMβ and RELMγ concentrations may be diagnostically useful markers.

SUMMARY

No functional differences between resistin and RELMs have as yet been identified, while these proteins have been demonstrated to similarly induce insulin resistance in both

cultured cells and animals. While previous studies have suggested the serum resistin concentration or resistin gene polymorphisms to possibly not be sufficiently reliable for clinical use as markers of insulin sensitivity, RELMβ has potential, since the serum RELMβ concentration is elevated in insulin resistant rodent models. To clarify this issue, a system, such as an RIA kit, allowing serum concentrations of human RELMβ to be measured easily, needs to be developed.

Contrary to confusing clinical reports describing interactions with insulin resistance, the relation with inflammation is quite obvious. Resistin and RELMs impair vascular cell functions, showing a close correlation with the degree of inflammation. Taking into consideration that insulin resistance and concomitant low level inflammation are major risk factors for developing atherosclerosis, resistin and RELMβ are potentially useful markers for the prediction of atherosclerosis. Furthermore, although no specific receptor(s) have as yet been identified, blockers for such receptors might effectively suppress inflammation and insulin resistance, and thereby prevent atherosclerosis. Future translational research should focus on these proteins.

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