



Title	Endothelin-1 suppresses insulin-stimulated Akt phosphorylation and glucose uptake via GPCR kinase 2 in skeletal muscle cells
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Supplemental data.

Title:

Endothelin-1 suppresses insulin-stimulated Akt phosphorylation and glucose uptake via G protein-coupled receptor kinase 2 in skeletal muscle cells.

Short running title:

Mechanisms for ET-1-induced insulin resistance.

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Supplementary METHODS

Measurement of mRNA expression levels for ET_AR, G_q protein, G₁₁ protein, GRK2, IR, Akt2 and GLUT4 by semiquantitative RT-PCR

Total RNA of L6 cells was extracted at Days 0 and 7 (Figure 1) and purified by using total RNA purification kit (RNeasy Mini Kit, QIAGEN, Tokyo, Japan) following the instructions of the manufacturer. The isolated RNA was reverse transcribed using SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific Inc.), and the resultant first-strand cDNA was applied to PCR which was performed using a PfuUltra II fusion HS DNA polymerase (Agilent Technologies, Inc., Santa Clara, CA, U.S.A.). A negative control without reverse transcriptase was run in parallel to verify that amplification did not proceed from residual genomic DNA (data not shown). The sequences of the forward and reverse primers for ET_AR, G_q protein, G₁₁ protein, GRK2, IR, Akt2 and GLUT4 were summarized in Table 1. cDNA was heated for 5 min at 95°C, then amplified by indicated cycles (Supplementary Figure S1) [95°C for 30 s, 50°C (except GRK2 at 60°C) for 30 s, 72°C for 60 s] followed by 10 min of extension at 72°C. The PCR products were confirmed by electrophoresis with 2.0% ethidium bromide stained agarose gels (Supplementary Figure S1).

Legends for supplemental data.

Supplementary Figure S1.

Changes in mRNA expression level for ET_AR (A), G_q protein (B), G₁₁ protein (C), GRK2 (D), IR (E), Akt2 (F) and GLUT4 (G) during differentiation of myoblasts into myotubes for 7 days. Semiquantitative RT-PCR experiments were carried out using total RNA prepared from 5 individual cell populations. Ordinate represents relative expression levels at Day 7 against Day 0. Data are presented as means ± S.E.M of the results obtained from 5 experiments. **P* < 0.05, ***P* < 0.01, significantly different from unity.

Supplementary Figure S2.

ET-1 induces phosphorylation of ERK1/2 but not Akt in L6 myotubes. The cells were

treated with 30 nM ET-1 for indicated times. Representative immunoblots were obtained using antibodies to phospho-Akt at Thr³⁰⁸ [p-Akt (T308)], phospho-Akt at Ser⁴⁷³ [p-Akt (S473)], total Akt (t-Akt), phospho-ERK1/2 (p-ERK1/2), and total ERK1/2 (t-ERK1/2).

Supplementary Figure S3.

No interaction of endogenous IRS-1 with FLAG-GRK2 is detected in L6 myotubes. Upper panel for immunoprecipitated samples (IP: α -normal IgG or FLAG): FLAG-tagged GRK2 proteins were immunoprecipitated with anti-FLAG antibody. Immunoprecipitates were probed with the anti-IRS-1 antibody to estimate coimmunoprecipitated endogenous IRS-1. Lower panel for IP: FLAG-tagged GRK2 proteins were immunoprecipitated with anti-FLAG antibody, and the immunoprecipitates were probed with the anti-FLAG-HRP antibody to normalize the quantity of coimmunoprecipitated endogenous Akt. Upper panel for whole cell lysate (WCL): the expressed IRS-1 was detected with the anti-IRS-1 antibody. Lower panel for WCL: the expressed FLAG-GRK2 was detected with the anti-FLAG-HRP antibody.

Supplementary Figure S4.

Effects of YM-254890 on Akt phosphorylation in response to 5 min exposure to 300 nM insulin in L6 myotubes. The cells were treated with 300 nM YM-254890 for 30 min before stimulation with insulin. Representative immunoblots were obtained using antibodies to phospho-Akt at Thr³⁰⁸ [p-Akt (T308)], phospho-Akt at Ser⁴⁷³ [p-Akt (S473)], and total Akt (t-Akt). Ordinate represents Akt phosphorylation responses which are normalized to the level of insulin-stimulated Akt phosphorylation in L6 myotubes without treatment with YM-254890.

Data are presented as means $\pm$ S.E.M of the results obtained from 5 experiments.

Supplementary Movie S1.

Spontaneous contraction of L6 myotubes (Day 7).