2-Aminopurine inhibits leptin receptor signal transduction

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ABSTRACT

Leptin is an important circulating signal for regulation of food intake and body weight. In the present study, we investigated the effect of 2-aminopurine (2-AP), an inhibitor of double-strand RNA-activated protein kinase (PKR), on leptin signal transduction. 2-AP dose-dependently inhibited leptin-induced phosporylation of signal transducer and activator of transcription 3 (STAT3), extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) in HEK293 cells stably transfected with the Ob-Rb leptin receptor. On the other hand, we observed only slight inhibition of leptin-induced STAT3 activation by purine treatment, indicating that the inhibitory effect will be dramatically enhanced in the presence of an amino group. 2-AP did not inhibit PMA-induced ERK activation, indicating that the effect may be leptin-signal specific. The inhibitory effect of 2-AP was not mediated by newly synthesized protein because the inhibitory effect of 2-AP on leptin-induced STAT3 activation was not abrogated in the presence of the protein synthesis inhibitor cycloheximide. Interestingly, leptin did not induce PKR activation, suggesting that the effect of 2-AP on leptin signal may be independent of PKR. Finally, 2-AP inhibited leptin-induced phosphorylation of the Ob-Rb leptin receptor. These results provide evidence of a novel action of 2-AP, i.e., inhibition of activation of leptin signal transduction at the level of the Ob-Rb leptin receptor.

Keywords: leptin, 2-aminopurine, signal transducer and activator of transcription 3 (STAT3), extracellular signal-regulated kinase (ERK), double-strand RNA-activated

protein kinase (PKR), Ob-Rb leptin receptor

1. **INTRODUCTION**

Leptin, a 16-kDa protein encoded by the *ob* gene (Zhang et al., 1994), is known to be an important regulator of energy balance through its actions in the brain on appetite and energy expenditure (Campfield et al., 1995; Pelleymounter et al., 1995). Furthermore, recent studies have suggested that leptin is involved in cancer progression (Garofalo et al., 2006). Leptin is mainly secreted by adipose tissue and is released into circulation to act both in the peripherally and the brain (Auwerx et al., 1998; Hosoi et al., 2002a, Hosoi et al., 2002b, Hosoi et al., 2002c). Leptin receptors (Ob-Rs) are found in many tissues in several alternatively spliced forms (Tartaglia et al., 1995; Fei et al., 1997; Hosoi et al., 2000; Hosoi et al., 2002d). Ob-Rb, one form of the receptor, has a long cytoplasmic region with consensus amino acid sequences involved in receptor binding to JAK-STAT tyrosine kinases (Lee et al., 1997), and it activates STAT3, which is responsible for leptin signaling (Vaisse et al., 1996; Ghilardi et al., 1996; McCowen et al., 1998; Bjørbæk et al., 1997; Hosoi et al., 2002c).

The double-stranded RNA (dsRNA)-activated protein kinase (PKR) is an interferon (IFN)-induced enzyme that controls protein synthesis through phosphorylation of eukaryotic initiation factor 2α (eIF-2α). Recently, it has been reported that PKR physically interacts with STAT3 and is required for platelet-derived growth factor (PDGF)-induced phosphorylation of STAT3 at Tyr705 and at Ser727, resulting in DNA binding and transcriptional activation (Deb et al., 2001). Using 2-aminopurine (2-AP), which is known to inhibit PKR activation, we observed inhibition of leptin-induced activation of STAT3, ERK and JNK. However, we did not observe activation of PKR in

response to leptin. These results indicate that 2-AP has new pharmacological properties, which exerts its action independently through PKR activation. Thus, in the present study, we investigated the novel site of action of 2-AP downstream of leptin signal transduction. In addition, we discuss the possibility of therapeutic application of 2-AP for leptin-induced cancer progression.

2. MATERIALS AND METHODS

2.1. Materials

Human leptin, cycloheximide, puromycin, purine and 2-aminopurine were obtained from SIGMA. Phorbol 12-Myristate 13-Acetate (PMA) and dithiothreitol were obtained from Wako Pure Chemical Ltd. (Japan).

2.2. Cell Culture

Human neuroblastoma SH-SY5Y cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum at 37°C in humidified 5% CO2 and 95% air. Human HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum and antibiotics (100 units/ml penicillin G, and 100 μg/ml streptomycin; GIBCO BRL) at 37°C in humidified 5% CO2 and 95% air.

2.3. Generation of Ob-Rb leptin receptor-stable transfectant cell line

A human Ob-Rb leptin receptor construct was a kind gift from Genetech Inc. (Luoh et al., 1997). The Ob-Rb leptin receptor construct was transfected into SH-SY5Y and HEK293 cells using LipofectAMINE PLUS Reagent (Life Technologies Inc.) according to the manufacture’s instructions. After the transfection, stable transfectants were obtained by selection with the antibiotic G418.

2.4. Western blotting

Western blotting was performed as described previously (Hosoi et al., 2003). Cells were washed with ice-cold PBS and lysed in a buffer containing 10 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1 mM Na_3VO_4, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM Phenylmethylsulfonyl fluoride (PMSF) and 1% NP-40 for 20 min. The lysates were centrifuged at 15,000 rpm for 20 min at 4 °C, and the supernatants were collected. The samples were boiled with laemmli buffer for 3 min, fractionated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred at 4°C to nitrocellulose membranes. The membranes were incubated with anti-pSTAT3 (Tyr705: Cell Signaling; diluted to 1:1,000), anti-pERK (Thr202/Tyr204: Cell Signaling; diluted to 1:1,000), anti-pJNK (Thr183/Tyr185: Cell Signaling; diluted to 1:1,000), anti-pPKR (Thr451: Cell Signaling; diluted to 1:1,000) and anti-pLeptin Receptor (Tyr1138 and Tyr985: Upstate Biotechnology, diluted to 1:1,000) antibody followed by incubation with anti-horseradish peroxidase-linked antibody. Peroxidase was detected by chemiluminescence using an enhanced chemiluminrscence system.

2.5. Statistics

The results were expressed as means ± S.E.M. of three to five experiments. Statistical analysis was performed with Jonckheere-Terpstra test (Fig. 1), Steel test (Fig. 2) and a paired Student's t test (Figs. 3, 5 and 6). Significant values were those with $p<0.05$ (*).

3. RESULTS

3.1. 2-AP inhibited leptin-induced STAT3, ERK and JNK activation.

To determine the role of PKR in leptin-induced signaling, we investigated the effects of 2-AP, an inhibitor of double-strand RNA-activated protein kinase (PKR), on leptin-induced STAT3, ERK and JNK activation. In HEK293 cells stably transfected with the Ob-Rb leptin receptor (HEK293-Ob-Rb cells), we observed STAT3, ERK and JNK phosphorylation 10 min after leptin (1 μg/ml) application (Fig. 1). The control bands of the phosphorylation were due to high level of leptin-independent phosphorylation derived from serum. We next pretreated the cells with 2-AP and then analyzed leptin-induced STAT3, ERK and JNK phosphorylation. The doses of 2-AP used in the present study was determined based on the previous reports (Zinn et al., 1988; Jarrous N et al., 1996; Osman et al., 1999; Ben-Asouli et al., 2002). 2-AP at doses used in mM order can efficiently inhibit PKR activation. Thus, we used 2-AP at mM order to determine the effect against leptin signaling. 2-AP (1-10 mM) alone did not affect phosphorylation of STAT3, ERK and JNK (Fig. 1). On the other hand, as shown in Figure 1, 2-AP dose-dependently (1-10 mM) inhibited leptin-induced phosphorylation of STAT3, ERK and JNK. We observed clear complete inhibition of the STAT3, ERK and JNK phosphorylation at 10 mM. No cytotoxicity due to 2-AP was apparent in the present experimental conditions.

2-AP contains a purine skeleton. Thus, to determine whether the effect is 2-AP-specific or not, we investigated the effect of purine on leptin-induced STAT3 phosphorylation. Cells were treated with purine (10 mM) 1 h prior to stimulation with

leptin (0.5 μg/ml). Interestingly, as shown in Figure 2, purine showed a tendency but not significant difference to inhibit leptin-induced STAT3 phosphorylation. Thus, these results indicate that purine might inhibit leptin-induced STAT3 phosphorylation and that the inhibitory effect will be dramatically enhanced in the presence of an amino group.

To determine whether the effect was specific for leptin signaling, we examined the effect of 2-AP on PMA-induced ERK phosphorylation. Ten minutes after PMA (30 nM) application, we observed an increase in ERK phosphorylation in HEK293-Ob-Rb cells (Fig. 3). However, 2-AP (10 mM, 1-h pretreatment) did not inhibit PMA-induced ERK phosphorylation (Fig. 3). The results suggest that the inhibitory effect of 2-AP on ERK activation was specific for the leptin signal.

3.2. Leptin did not induce PKR phosphorylation.

Since 2-AP inhibited leptin-induced STAT3, ERK and JNK activation, we next investigated whether leptin activates PKR in HEK 293-Ob-Rb cells. We stimulated the cells with leptin (1 μg/ml) for the indicated times and then analyzed STAT3 and PKR activation. As a positive control for PKR phosphorylation, we stimulated the cells with dithiothreitol (3 mM, 30 min) and observed an increase in PKR phosphorylation in the cells (Fig. 4). Although leptin increased STAT3 phosphorylation, leptin did not induce PKR phosphorylation at nether time point (2-360 min) determined (Fig. 4). Thus, the results suggest that leptin did not induce PKR phosphorylation.

3.3. 2-AP inhibited leptin-induced Ob-Rb leptin receptor phosphorylation.

Although leptin did not activate PKR, we observed inhibition of leptin-induced

STAT3, ERK and JNK activation by 2-AP. These results suggest that 2-AP has a novel site of action against leptin signal transduction. Thus, we next investigated a possible target site of 2-AP action. We investigated the effect of 2-AP on phosphorylation status of the Ob-Rb leptin receptor. We observed phosphorylation of the leptin receptor Ob-Rb (Tyr 1138, Tyr 985) 10 min after leptin (1 μg/ml) application in HEK 293 cells stably transfected with the Ob-Rb leptin receptor (Fig. 5). At this condition, as shown in Fig. 5, 2-AP (10 mM, 1-h pretreatment) showed significant inhibition of leptin-induced Ob-Rb phosphorylation at Tyr 1138, and showed a tendency but not significant difference to inhibit leptin-induced Ob-Rb phosphorylation at Tyr 985. Thus, it was suggested that 2-AP inhibited leptin-induced Ob-Rb receptor activation.

3.4. The inhibitory effect of 2-AP on leptin signaling was not mediated by newly synthesized protein.

To determine whether 2-AP inhibits leptin-induced signal transduction mediated by some newly synthesized protein, we pretreated the cells with a protein synthesis inhibitor, and investigated the effect of 2-AP on leptin-induced STAT3. As shown in Fig. 6, the protein synthesis inhibitor did not abrogate the inhibitory effect of 2-AP on leptin-induced STAT3 activation. We obtained similar results using phoshpo-ERK antibody (data not shown). The inhibitory effects of 2-AP in the presence and absence of protein synthesis inhibitor were similar. Thus, the inhibitory effect of 2-AP was not mediated by newly synthesized protein. 2-AP may act directly to inhibit leptin-induced signal transduction.

4. **DISCUSSION**

In the present study we found that 1) 2-AP, an inhibitor of PKR inhibited leptin-induced STAT3. 2) However leptin did not activate PKR. 3) 2-AP may inhibited activation of leptin signal transduction at the level of the Ob-Rb leptin receptor.

2-AP inhibited leptin-induced signaling (STAT3, ERK and JNK activation) independently of PKR. PKR physically interacts with STAT3 and is required for PDGF-induced phosphorylation of STAT3, resulting in DNA binding and transcriptional activation (Deb et al., 2001). In the present study, however leptin did not activate PKR. Thus, the mechanisms by which PDGF and leptin activate STAT3 may be different.

We found that 2-AP inhibited leptin-induced Ob-Rb receptor phosphorylation at Tyr 1138 and Tyr 985. Thus, Ob-Rb tyrosine residues 1138 and 985, which are phosphorylated during receptor activation, may be the target of 2-AP. Tyr 1138 is an important site for leptin-induced STAT3 activation and Tyr 985 is an important site for leptin-induced ERK activation (Banks et al., 2000). Recent *in vivo* data indicate the physiological importance of Tyr 1138-induced STAT3 signaling for mediating energy balance (Bates et al., 2003). Therefore, the effect of 2-AP on leptin signaling may be due to direct inhibition of Ob-Rb Tyr 1138 and subsequent activation of STAT3. On the other hand, the inhibitory effect of 2-AP on leptin-induced ERK activation may be due to the inhibition of Ob-Rb Tyr 985.

It appears that the effect of 2-AP is not mediated by newly synthesized protein because pretreatment with a protein synthesis inhibitor did not abrogate the inhibitory effect of 2-AP (Fig. 6). Moreover, the effect of 2-AP may be specific to the leptin signal

because 2-AP did not inhibit PMA-induced ERK activation (Fig. 3). Purine slightly inhibited leptin-induced STAT3 phosphorylation. Thus, the effects may be dramatically enhanced in the presence of an amino group on the purine skeleton (Fig. 2). We have done dose-response study for leptin-induced STAT3 activation. We observed leptin-induced STAT3 activation at 0.03, 0.1 and 0.3 μg/ml (data not shown). Thus, physiological dose of leptin functions effectively at present experimental conditions. However, the concentration of 2-AP is still high for the therapeutic use. We think that 2-AP may be used as a lead compound, which may improve in the future studies.

Leptin plays an important role in regulation of food intake, body weight and fat mass. On the other hand, these critical parameters are associated with an increased risk for development of digestive and mammary gland cancer, and it has been reported that leptin promotes invasiveness of kidney and colonic epithelial cells (Attoub et al., 2000). Expressions of leptin and its receptor were shown to be enhanced in human breast cancer (Ishikawa et al., 2004). Leptin induces activation of ERK and STAT3 and increases proliferation of MCF-7 breast carcinoma cells (Dieudonne et al., 2002; Yin et al., 2004). Furthermore, JNK activation has been suggested to be involved in leptin-mediated, androgen-independent prostate cancer cell proliferation (Onuma et al., 2003). These observations indicate that leptin is involved in cancer progression in some tissues or cells. In the present study, we found that 2-AP inhibited leptin-induced activation of STAT3, ERK and JNK. Thus, 2-AP might be useful as a drug for leptin-mediated cancer therapy, and our basic findings may be important from a pharmaceutical point of view.

FIGURE LEGENDS

Figure 1. 2-AP inhibited leptin-induced STAT3 ERK and JNK activation. (A, C, E) HEK 293-Ob-Rb cells were pretreated with 2-AP for 1 h at the indicated doses and stimulated with leptin (Lep: 1 μg/ml, 10 min). Control cells (Cont) were stimulated with sterilized water. Western blotting was performed using phospho-specific antibodies. (B, D, F) Densitometric analysis of phospho-STAT3, phospho-ERK and phospho-JNK using image analysis software. As for Fig. 1 CD and Fig. 1EF same samples were used for Fig. 1AB. Loading control data of Fig. 1CD and Fig. 1EF was indicated in Fig. 1A (total STAT3 protein). The leptin-induced increase in the phosphorylation of STAT3 was highly significantly inhibited by 1 to 10 mM of 2-AP ($p<0.001$). The increases in the phosphorylation of ERK and JNK were also significantly inhibited by 1 to 10 mM of 2-AP ($p<0.05$). Values are presented as means ± S.E.M. (n=3-4 per group).

Figure 2. Effect of purine on leptin-induced STAT3 phosphorylation. (A) HEK 293-Ob-Rb cells were pretreated with 2-AP or purine (10 mM) for 1 h and stimulated with leptin (Lep: 0.5 μg/ml, 10 min). Control cells (Cont) were stimulated with sterilized water. Western blotting was performed using a phospho-STAT3-specific antibody. (B) Densitometric analysis of phospho-STAT3 using image analysis software. Values are presented as means ± S.E.M. (n=5 per group). *: $p<0.05$ significantly different from the group with leptin alone.

Figure 3. 2-AP did not inhibit PMA-induced ERK phosphorylation. (A) HEK 293-Ob-Rb cells were pretreated with 2-AP for 1 h and stimulated with PMA (30 nM, 10 min). Control cells (Cont) were stimulated with sterilized water. Western blotting was performed using a phospho-ERK-specific antibody. (B) Densitometric analysis of phospho-ERK using image analysis software. Values are presented as means ± S.E.M. (n=3 per group).

Figure 4. Leptin did not induce PKR activation. Cells were treated with leptin (1 μg/ml) for the indicated times and then PKR and STAT3 phosphorylation was analyzed by Western blotting. Control cells (Cont) were stimulated with sterilized water. Although leptin induced STAT3 phosphorylation, leptin did not induce PKR phosphorylation at an ether time (up to 6 h) examined. Cells were treated with dithiothreitol (DTT: 3 mM, for 30 min) as a positive control.

Figure 5. Figure 5. 2-AP inhibited leptin-induced Ob-Rb phosphorylation. (A, C) 293-Ob-Rb cells were pretreated with 2-AP (10 mM) for 1 h and stimulated with leptin (1 μg/ml, 10 min). Control cells were stimulated with sterilized water. Western blotting was performed using a specific antibody for Ob-Rb Tyr 1138 or Ob-Rb Tyr 985 phosphorylation. (B, D) Densitometric analysis of phospho-Ob-Rb using image analysis software. Values are presented as means ± S.E.M. (n=3-4 per group). *: $p<0.05$ significantly different from the group with leptin alone.

Figure 6. 2-AP inhibited leptin-induced STAT3 phosphorylation in the presence of protein synthesis inhibitor. (A) HEK 293-Ob-Rb cells were pretreated (1 h) with cycloheximide (5 μg/ml), and then the inhibitory effect of 2-AP on leptin (1 μg/ml, 10 min)-induced STAT3 activation was analyzed by Western blotting. Control cells were stimulated with sterilized water. (B) Densitometric analysis of phospho-STAT3 using image analysis software. Values are presented as means ± S.E.M. (n=4 per group). *: $p<0.05$ significantly different from the group with leptin alone.

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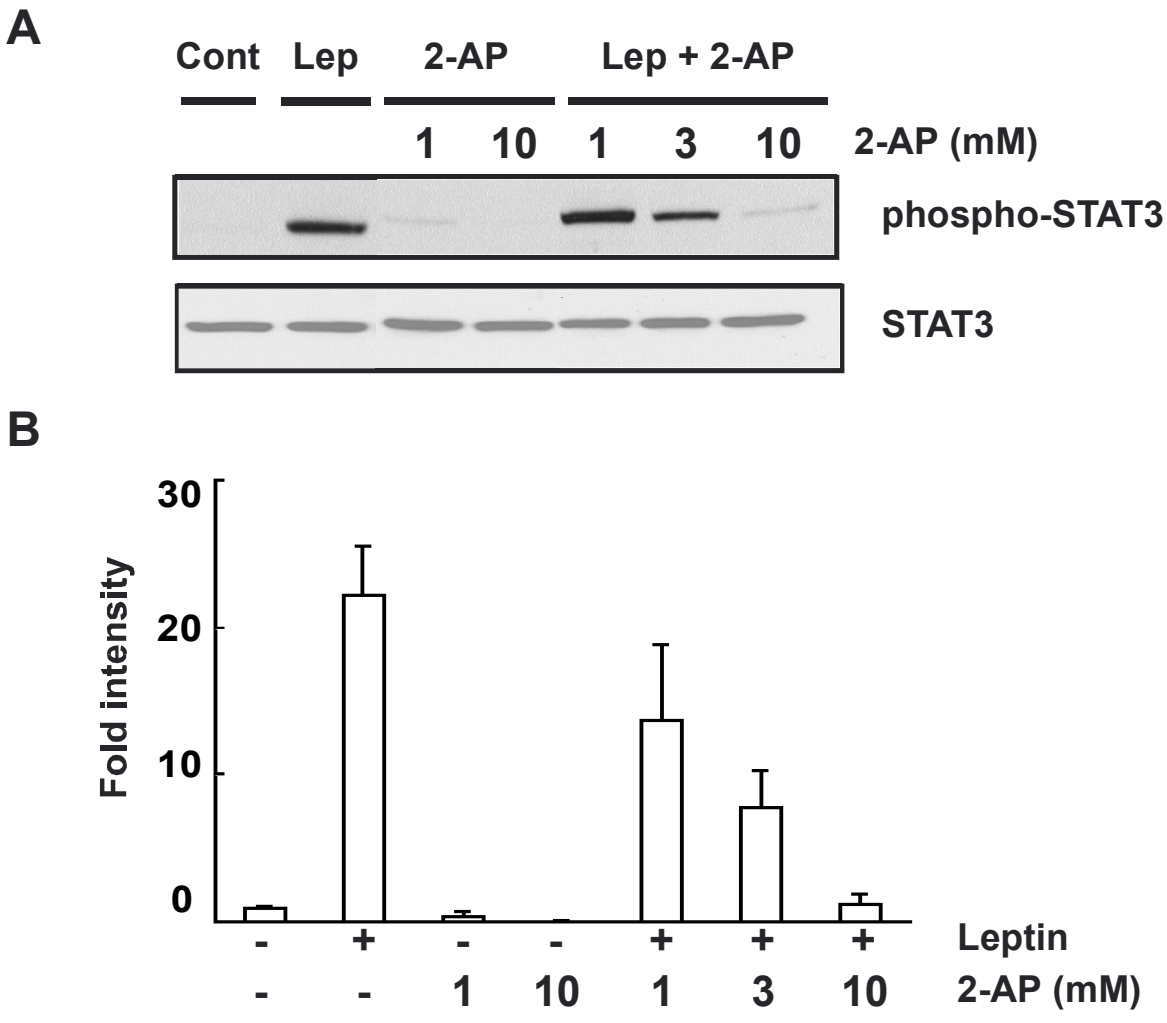

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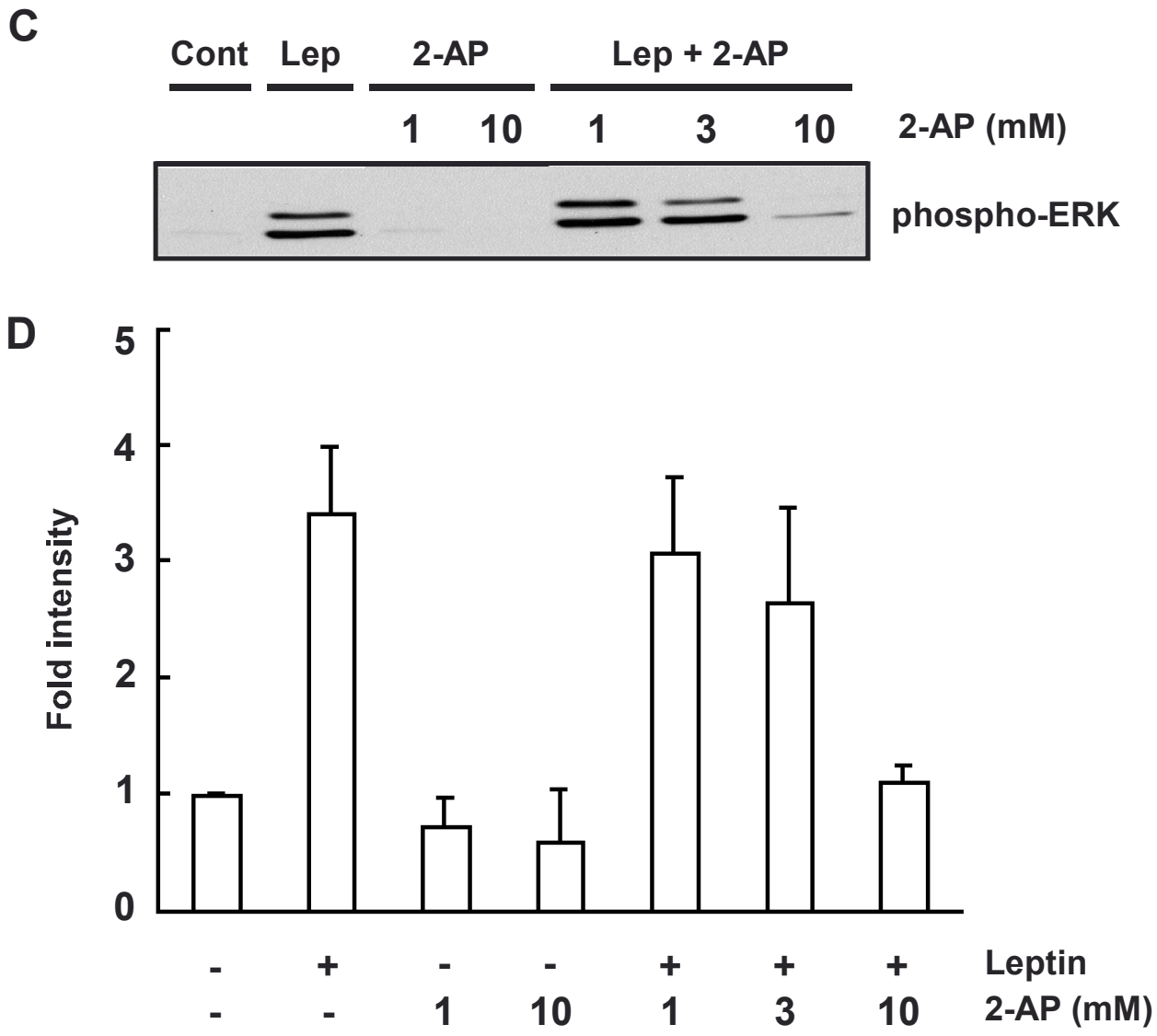

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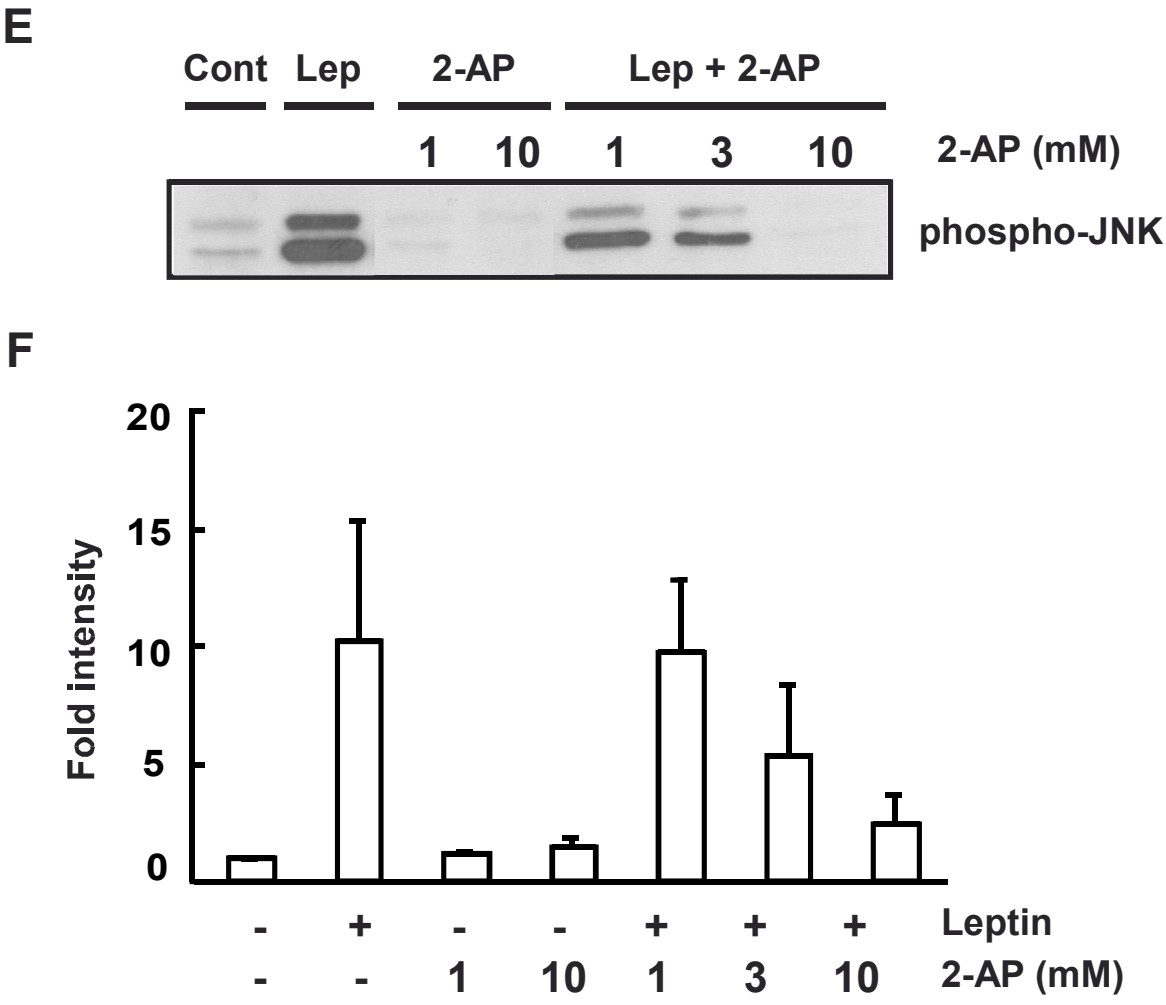

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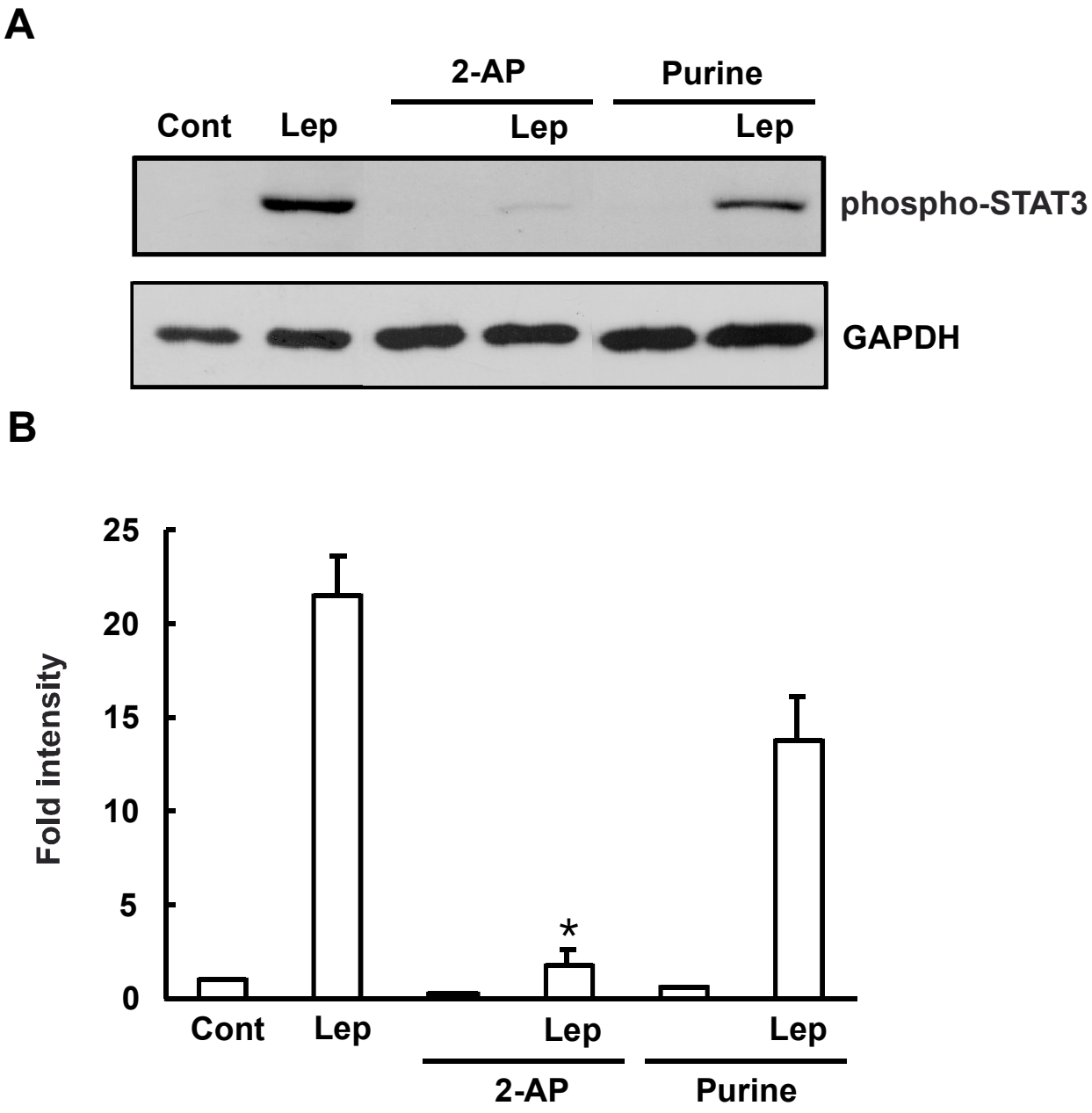

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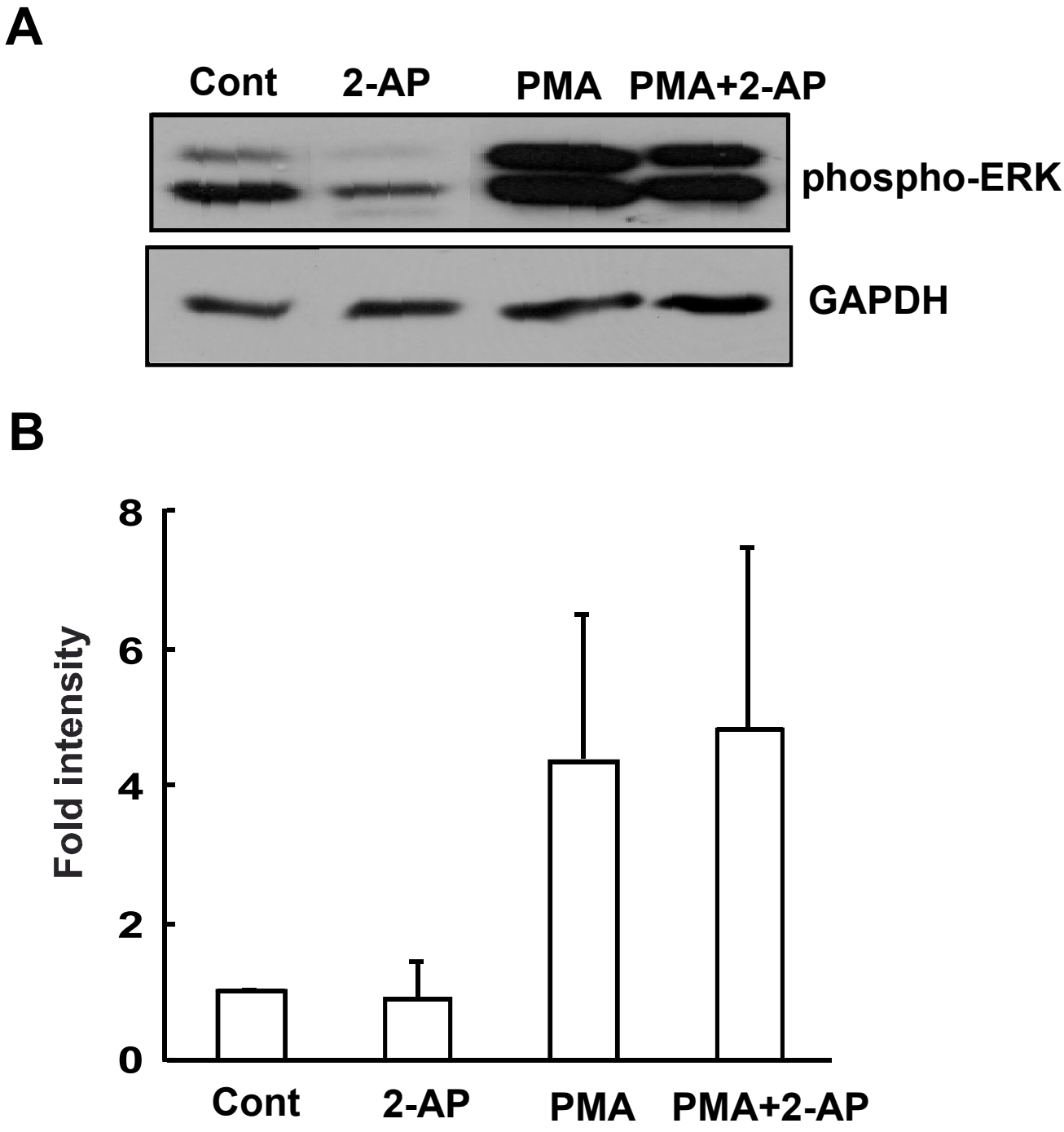

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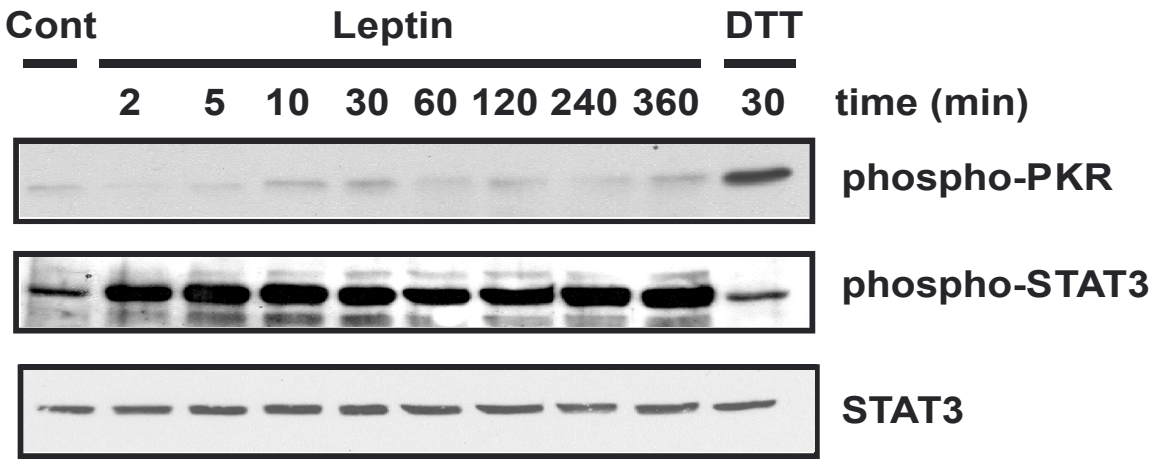

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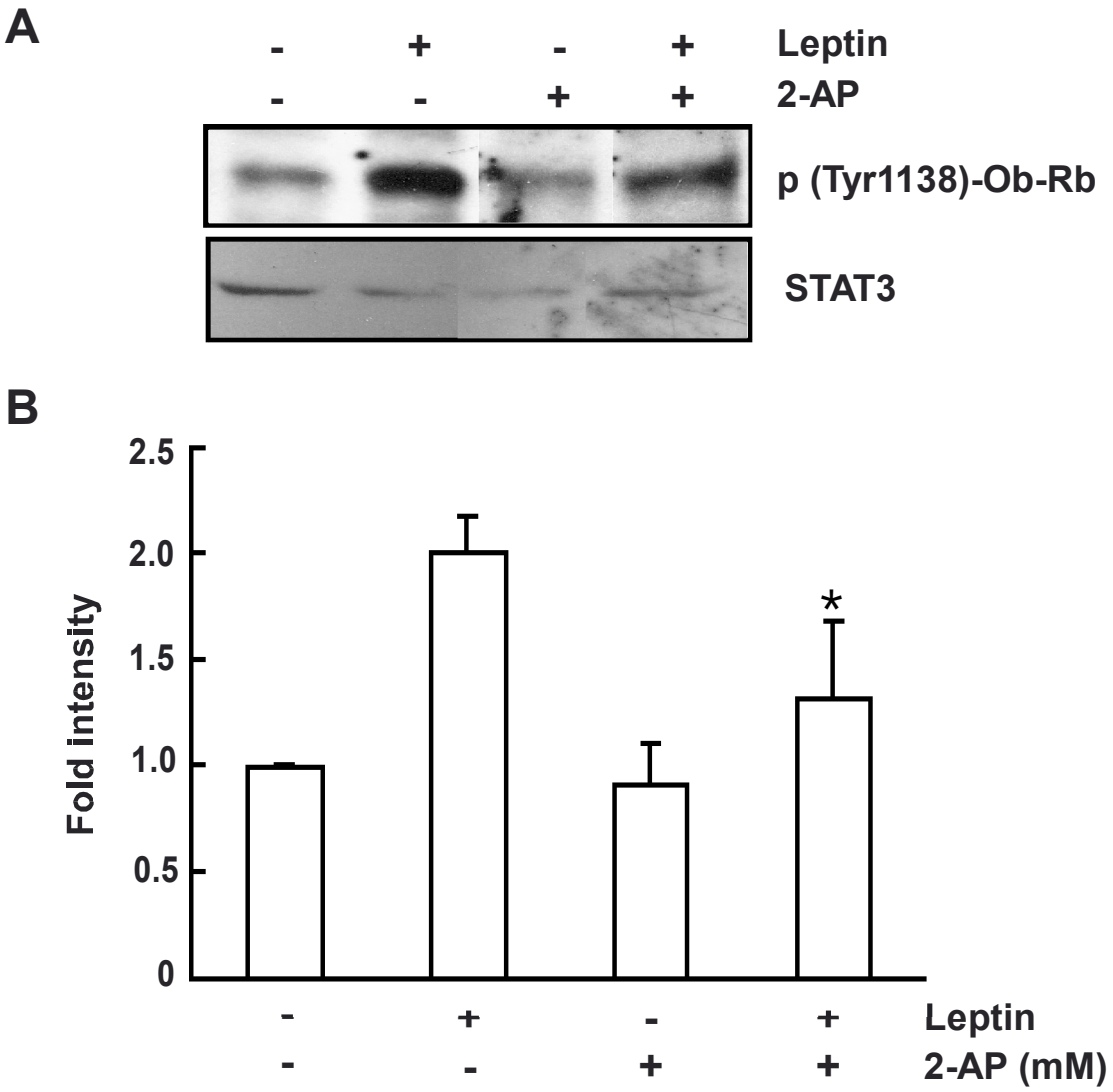

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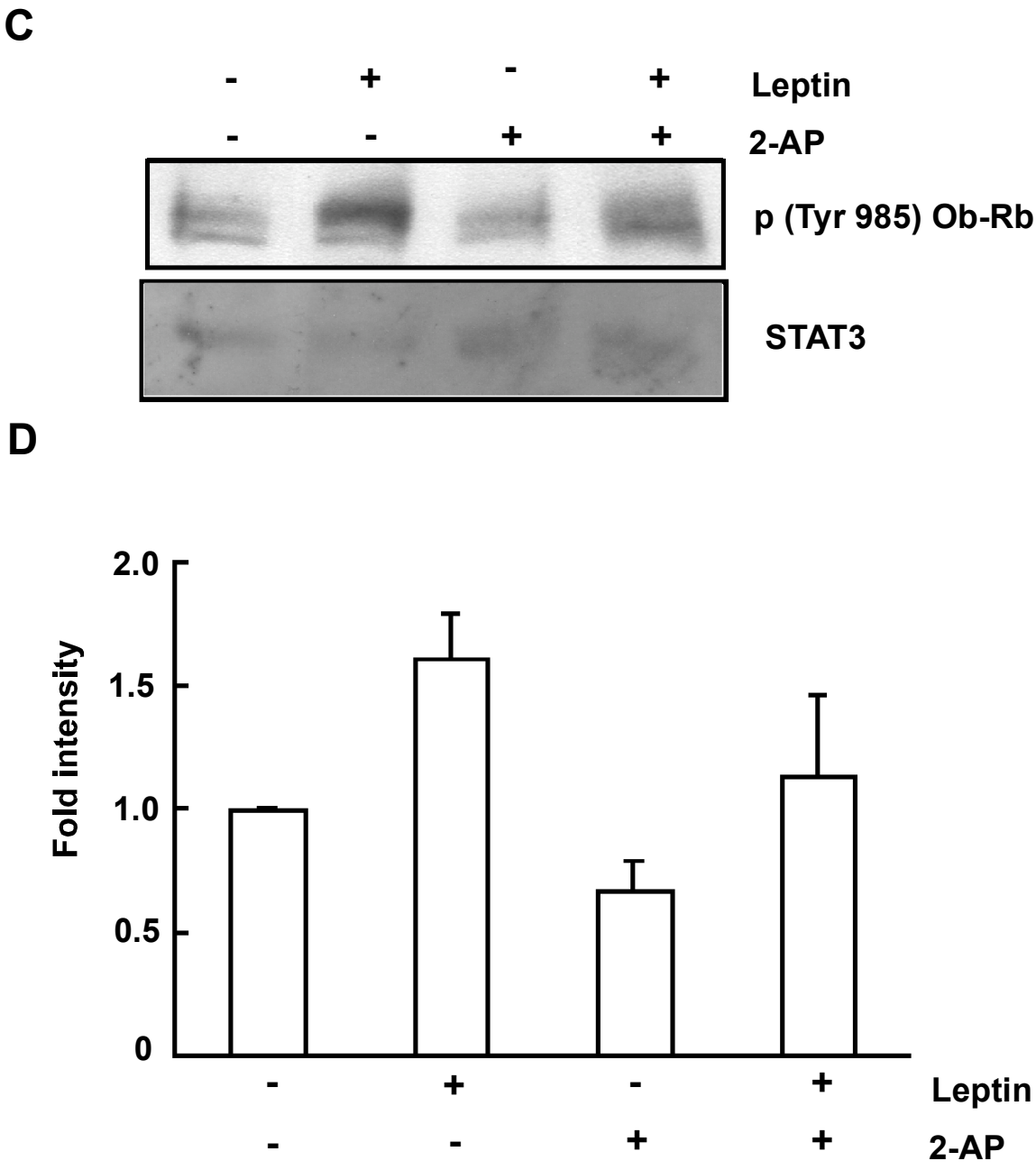

Figure 5 (cd) Hosoi T et al

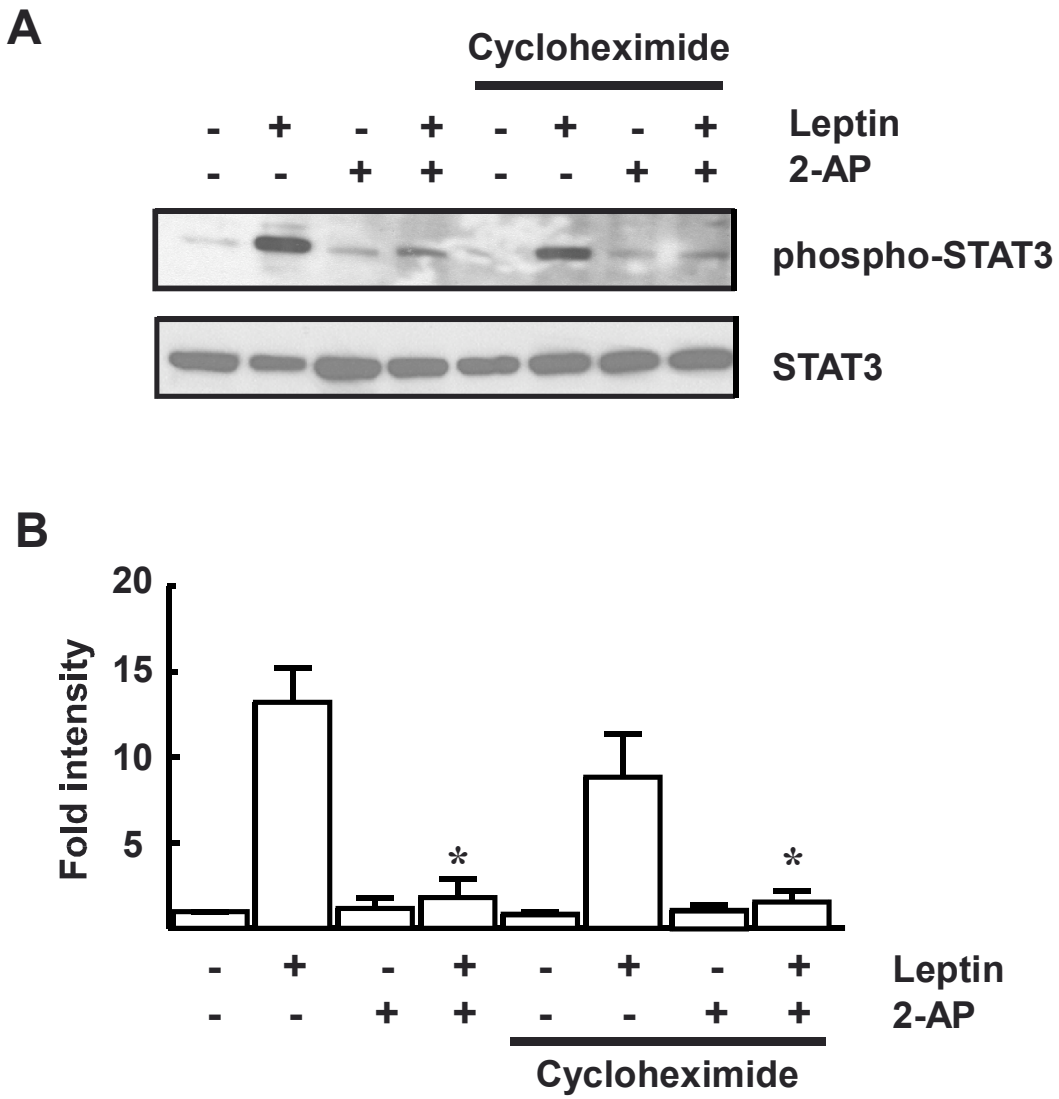

Figure 6 Hosoi T et al.