

# Aspirin Inhibits Serine Phosphorylation of Insulin Receptor Substrate 1 in Tumor Necrosis Factor-treated Cells through Targeting Multiple Serine Kinases\*

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The hypoglycemic effects of high dose salicylates in the treatment of diabetes were documented before the advent of insulin. However, the molecular mechanisms by which salicylates exert these anti-diabetic effects are not well understood. In this study, we analyzed the effects of aspirin (acetylsalicylic acid) on serine phosphorylation of insulin receptor substrate 1 (IRS-1) in cells treated with tumor necrosis factor (TNF)- $\alpha$ . Phosphorylation of IRS-1 at Ser<sup>307</sup>, Ser<sup>267</sup>, and Ser<sup>612</sup> was monitored by immunoblotting with phospho-specific IRS-1 antibodies. In 3T3-L1 and Hep G2 cells, phosphorylation of IRS-1 at Ser<sup>307</sup> in response to TNF- $\alpha$  treatment correlated with phosphorylation of JNK, c-Jun, and degradation of I $\kappa$ B $\alpha$ . Moreover, phosphorylation of IRS-1 at Ser<sup>307</sup> in embryo fibroblasts derived from either JNK or IKK knockout mice was reduced when compared with that in the wild-type controls. Taken together, these data suggest that serine phosphorylation of IRS-1 in response to TNF- $\alpha$  is mediated, in part, by JNK and IKK. Interestingly, aspirin treatment inhibited the phosphorylation of IRS-1 at Ser<sup>307</sup> as well as the phosphorylation of JNK, c-Jun, and degradation of I $\kappa$ B $\alpha$ . Furthermore, other serine kinases including Akt, extracellular regulated kinase, mammalian target of rapamycin, and PKC $\zeta$  were also activated by TNF- $\alpha$  (as assessed by phospho-specific antibodies). Phosphorylation of IRS-1 at Ser<sup>267</sup> and Ser<sup>612</sup> correlated with the activation of these kinases. Phosphorylation of Akt and the mammalian target of rapamycin (but not extracellular regulated kinase or PKC $\zeta$ ) in response to TNF- $\alpha$  was inhibited by aspirin treatment. Finally, aspirin rescued insulin-induced glucose uptake in 3T3-L1 adipocytes pretreated with TNF- $\alpha$ . We conclude that aspirin may enhance insulin sensitivity by protecting IRS proteins from serine phosphorylation catalyzed by multiple kinases.

It has been known for more than a century that salicylates are able to reduce fasting blood glucose in diabetic patients (1–3). The cellular and molecular mechanism of the hypoglycemic activity of aspirin has not been well elucidated. For the mechanism of salicylates activity, several hypotheses were raised 20 years ago explaining the hypoglycemic action. The extra-pancreatic hypothesis, which suggests salicylates action

through a pancreatic-independent mechanism (4), seems to have become dominant as it has gained substantial support from studies published recently (5–7).

TNF- $\alpha$ ,<sup>1</sup> an inflammatory cytokine, is a major risk factor of insulin resistance in obesity and chronic inflammation (8–10). TNF- $\alpha$  has been reported to inhibit insulin-induced glucose uptake by targeting more than one component in the insulin signaling cascade. These TNF-targeting components include insulin receptor (11), insulin receptor substrate (IRS) (12, 13), and glucose transporter 4 (Glut4) (14). Of these targets, IRS proteins have been studied widely as a major target in the TNF-induced insulin resistance. TNF- $\alpha$  has been consistently reported to inhibit insulin signaling by increasing serine phosphorylation of IRS proteins (11, 15–17). Serine phosphorylation of IRS proteins leads to IRS inhibition through at least two possibilities, which are inhibition of tyrosyl phosphorylation of IRS-1 (13, 16, 17), and proteasome-mediated degradation of IRS-1 (18–21). The first possibility is a result of blocking the interaction of IRS-1 and insulin receptor.

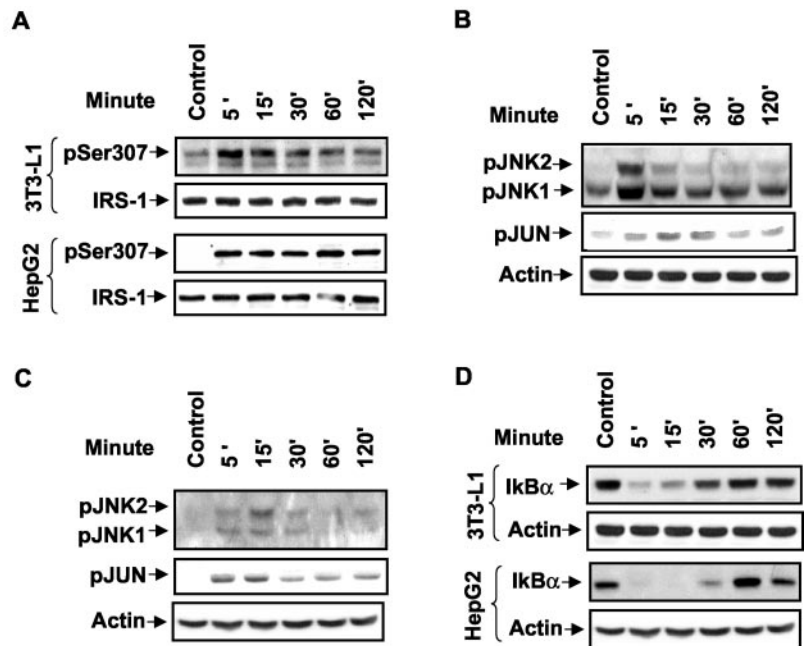
TNF- $\alpha$  can induce IRS-1 serine phosphorylation through activation of several serine kinases. (a) Activation of c-Jun NH<sub>2</sub>-terminal kinase (JNK). It is known that JNK can be activated by TNF- $\alpha$  through induction of ceramide (22). JNK was shown to phosphorylate IRS-1 at serine 307 directly (12, 13). (b) Activation of extracellular signal-regulated kinase (ERK). Several studies indicate that ERK can phosphorylate IRS-1 at serine residues (23–26). (c) Activation of protein kinase C $\zeta$  (PKC $\zeta$ ). In addition to JNK activation, TNF-induced ceramide is also able to activate PKC $\zeta$  (27, 28), an isoform of atypical PKC. PKC $\zeta$  was reported to phosphorylate IRS-1 at serine residues (29, 30). (d) Activation of Akt (PKB). Akt was shown to phosphorylate IRS-1 proteins at four consensus Akt phosphorylation sites (RXRXXS/T) (31). (e) Activation of the mammalian target of rapamycin (mTOR). Like Akt, mTOR is one of these serine kinases located downstream of phosphatidylinositol 3-kinase (32). Several studies suggest that mTOR results in IRS-1 serine phosphorylation (19, 33, 34). (f) Activation of glycogen synthase kinase 3. Glycogen synthase kinase 3 is another serine kinase located downstream of phosphatidylinositol 3-kinase. Glycogen synthase kinase 3 was shown to phosphorylate IRS-1 *in vivo* and *in vitro* (35). (g) Activation of IKK. In our recent study, we showed that IKK phosphorylated IRS-1 at Ser<sup>307</sup> (36). Although these seven kinases have been shown to be involved in

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<sup>1</sup> The abbreviations used are: TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IRS, insulin receptor substrate; Glut4, glucose transporter 4; JNK, c-Jun NH<sub>2</sub>-terminal kinase; ERK, extracellular signal-regulated kinase; PKC, protein kinase C; mTOR, mammalian target for rapamycin; IKK, I $\kappa$ B kinase; PBS, phosphate-buffered saline; MEK, mitogen-activated protein kinase kinase; 15dPGJ<sub>2</sub>, 15-deoxyprostaglandin J<sub>2</sub>.

**FIG. 1. Ser<sup>307</sup> phosphorylation of IRS-1 is associated with activation of JNK and IKK.** 3T3-L1 and Hep G2 cells were treated with TNF- $\alpha$  (20 ng/ml) for the times indicated. Phosphorylation status of IRS-1, JNK, and c-Jun was determined in the whole cell lysate by immunoblotting with phospho-specific antibodies to IRS-1 (Ser<sup>307</sup>), JNK (Thr<sup>183</sup>/Tyr<sup>185</sup>), and c-Jun (Ser<sup>63</sup>). The  $\beta$ -actin protein level is a control of protein loading in the assay. Representative blots are shown from experiments that were repeated independently at least three times with similar results. *A*, Ser<sup>307/312</sup> phosphorylation of IRS-1. *B*, phosphorylation of JNK and c-Jun in 3T3-L1 cells. *C*, phosphorylation of JNK and c-Jun in Hep G2 cells. *D*, I $\kappa$ B $\alpha$  degradation. I $\kappa$ B $\alpha$  protein abundance was determined in the whole cell lysate by immunoblotting.



IRS-1 phosphorylation, the results are derived from different assay systems. Activities of these serine kinases have not been collectively examined in a cell treated with TNF- $\alpha$ . It remains to be verified whether all of the seven kinases are activated under the same condition.

To understand the molecular mechanism of TNF-induced insulin resistance, we examined six of seven serine kinases listed above in 3T3-L1 and Hep G2 cells after TNF treatment. A role of JNK and IKK in IRS-1 serine phosphorylation was a focus in the study. IRS-1 serine phosphorylation was monitored using three phospho-specific IRS-1 antibodies to Ser<sup>307/312</sup>, Ser<sup>267/270</sup>, and Ser<sup>612/616</sup> in rodent/human IRS-1, respectively. Our results demonstrate that all six serine kinases are activated by TNF- $\alpha$  and the activation is correlated to the phosphorylation of the three serine residues. To investigate the molecular events of salicylates-associated insulin sensitivity, aspirin was used to test an influence of salicylates on the IRS-1 serine phosphorylation. The results show that aspirin is able to inhibit IRS-1 serine phosphorylation through targeting multiple serine kinases including JNK, IKK, Akt, and mTOR.

#### EXPERIMENTAL PROCEDURES

**Cells and Reagents**—Cell lines including mouse fibroblast 3T3-L1 (CL-173) and human hepatoma Hep G2 (HB-8065) were purchased from the American Type Culture Collection (ATCC). IKK1/2 double knockout and JNK knockout cell lines were used in previous studies (36, 37). All of the cells are maintained in the Dulbecco's modified Eagle's culture medium supplemented with 10% fetal calf serum. For 3T3-L1 cells, the glutamine concentration was 4 mM in the culture medium. Phospho-IRS-1 (Ser<sup>307</sup>) antibody (number 07-247) was from Upstate Biotechnology (Lake Placid, NY). Phospho-specific antibodies to Akt (Ser<sup>308</sup>), p70S6 (Thr<sup>412</sup>/Ser<sup>424</sup>), and PKC $\zeta$  (Thr<sup>410/403</sup>) were obtained from Cell Signaling (Beverly, MA). Antibodies to phospho-IRS1 at Ser<sup>270</sup> (sc-17192) and Ser<sup>612</sup> (sc-17195), phospho-JNK (sc-6254), phospho-ERK (sc-7383), phospho-JUN (sc-822), IRS-1 (sc-7200), and I $\kappa$ B $\alpha$  (sc-371) were made by Santa Cruz Biotechnology (Santa Cruz, CA).  $\beta$ -Actin antibody (ab6276) was from Abcam (Cambridge, UK). Calyculin A (EI-192) and SP600125 (EI-305) were acquired from Biomol (Plymouth Meeting, PA). 15-Deoxyprostaglandin J<sub>2</sub> (15dPGJ<sub>2</sub>, 538927), PD98059 (513000), and SB203580 (203580) were purchased from Calbiochem (San Diego, CA). Aspirin (acetylsalicylic acid, A-5376) was from Sigma.

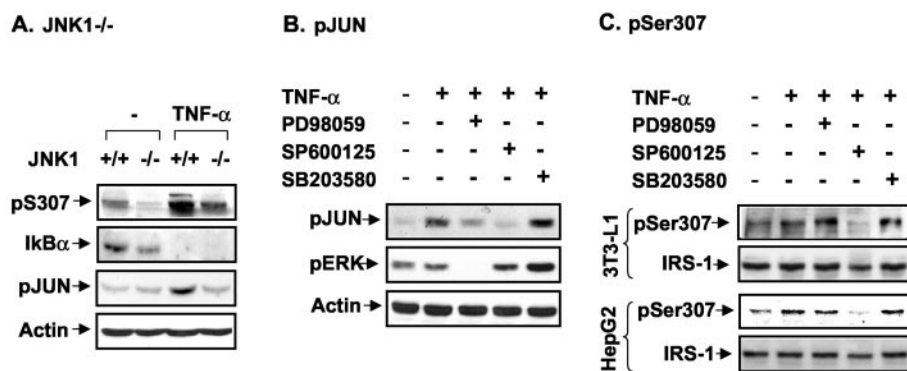
**Western Blotting**—Cells were treated with 20 ng/ml TNF- $\alpha$  for 30 min after overnight serum starvation in 1% bovine serum albumin medium. Whole cell lysate protein was made in lysis buffer (1% Triton X-100, 50 mM KCl, 25 mM Hepes, pH 7.8, 10  $\mu$ g/ml leupeptin, 20  $\mu$ g/ml

aprotinin, 125  $\mu$ M dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate) with sonication. The protein (100  $\mu$ g) in 50  $\mu$ l of reducing sample buffer was boiled for 3 min, and resolved in 6% mini-SDS-PAGE for 90 min at 100 volts. Then, the protein was transferred onto polyvinylidene difluoride membrane (162-0184, Bio-Rad) at 21 volts for 120 min. Blotting of the membrane was conducted in milk buffer. The membrane was pre-blotted in milk buffer for 20 min, blotted with first antibody for 1–24 h and secondary antibody for 30 min. The horseradish peroxidase-conjugated secondary antibodies (NA934V or NA931, Amersham Biosciences) were used with chemiluminescence reagent (NEL-105, PerkinElmer Life Sciences) for generation of the light signal. To detect multiple signals from one membrane, the membrane was treated with a stripping buffer (59 mM Tri-HCl, 2% SDS, 0.75% 2-mercaptoethanol) for 20 min at 37  $^{\circ}$ C after each cycle of blotting to remove the bound antibody. All the experiments were conducted for three or more times. Intensity of the immunoblot signal is analyzed quantitatively using a computer program, PDQuest 7.1, which is made by Bio-Rad. A mean value of results from three experiments were presented.

**Glucose Uptake by 3T3-L1 Adipocytes**—3T3-L1 preadipocytes ( $5 \times 10^5$ /well) were differentiated into adipocytes in a 12-well plate using a standard differentiation mixture (5  $\mu$ g/ml insulin, 0.5 mM isobutylmethylxanthine, and 1  $\mu$ M dexamethasone) for 10 days. After serum starvation in 0.1% bovine serum albumin/Dulbecco's modified Eagle's medium overnight, the cells were pretreated with aspirin (5 mM) for 30 min, and then treated with TNF- $\alpha$  (20 ng/ml) for an additional 5 h before glucose uptake assay. Glucose uptake assay was conducted using a method as reported (38). The cells were incubated in 1 ml/well phosphate-buffered saline containing 200 nM insulin for 30 min at 37  $^{\circ}$ C. After washing in phosphate-buffered saline, the cells were incubated in 1 ml of phosphate-buffered saline containing 0.1 mM 2-deoxyglucose and 1  $\mu$ Ci/ml 2-deoxy-D-[<sup>3</sup>H]glucose for 5 min. Then, the cells were washed three times in ice-cold phosphate-buffered saline, and solubilized in 0.4 ml of 1% SDS. [<sup>3</sup>H]Glucose uptake was detected in 4 ml of scintillant using Beckman LS6500 scintillation counter. Nonspecific deoxyglucose uptake was measured in the presence of 20  $\mu$ M cytochalasin B and is subtracted from the total uptake to get insulin-specific glucose uptake.

#### RESULTS

**Ser<sup>307</sup> Phosphorylation of IRS-1 Is Associated with Activation of JNK and IKK**—IRS-1 represents one of four IRS protein isoforms, which include IRS-1, IRS-2, IRS-3, and IRS-4 (39, 40). Serine phosphorylation of IRS-1 has been proposed as a general mechanism of functional inhibition of the IRS-1 protein. Among the serine residues that become phosphorylated in response to risk factors of insulin resistance, Ser<sup>307</sup> has been studied extensively and Ser<sup>307</sup> phosphorylation has become a molecular indicator of insulin resistance (13, 36, 41, 42). Although it was

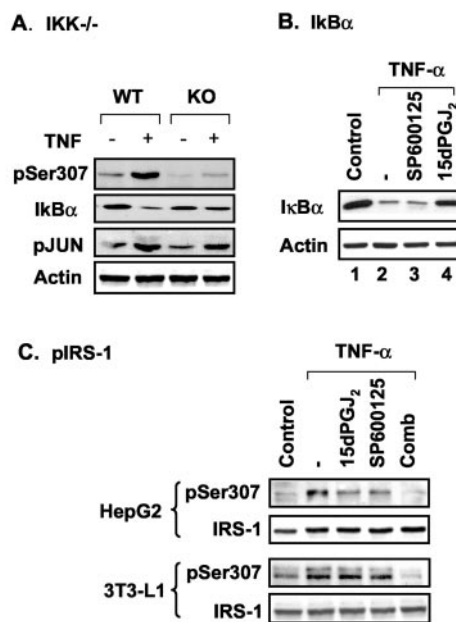


**FIG. 2. Inhibition of JNK results in prevention of Ser<sup>307/312</sup> phosphorylation.** A, JNK1 knockout ( $-/-$ ) and wild type ( $+/+$ ) fibroblasts were derived from JNK1 $^{-/-}$  mice and the control littermate. Ser<sup>307</sup> phosphorylation of IRS-1 was determined after TNF treatment for 30 min. B, SP600125 inhibits JNK activity. After serum starvation, the 3T3-L1 cells were pretreated with PD98059 (40  $\mu$ M), SP600125 (50  $\mu$ M), and SB203580 (2  $\mu$ M) for 30 min. Phosphorylation of Jun and ERK was determined in the cell lysate after TNF treatment for 30 min. Phosphospecific antibodies to c-Jun (Ser<sup>63</sup>) and ERK1 were used. C, SP600125 inhibits IRS-1 Ser<sup>307</sup> phosphorylation in 3T3-L1 and Hep G2 cells. IRS-1 phosphorylation was determined with phospho-specific IRS-1 (Ser<sup>307</sup>) antibody. Representative blots are shown from experiments that were repeated independently at least three times.

reported that Ser<sup>307</sup> is a phosphoacceptor of JNK and IKK (12, 36), activities of these two kinases have not been confirmed simultaneously in cells that are resistant to insulin. To address this issue, we tested Ser<sup>307</sup> phosphorylation in 3T3-L1 preadipocytes and Hep G2 cells after TNF- $\alpha$  treatment. Ser<sup>307</sup> phosphorylation was induced by TNF- $\alpha$  in both cell lines, and the phosphorylation was detected as early as 5 min following TNF treatment (Fig. 1A). 3- and 10-fold increases in Ser<sup>307</sup> phosphorylation were detected at 5 min in 3T3-L1 and Hep G2 cells, respectively. A difference in the dynamics of the phosphorylation was observed in the two cell lines. In 3T3-L1 cells, Ser<sup>307</sup> phosphorylation peaked at 5 min and then reduced in a time-dependent manner (Fig. 1A, 3T3-L1). In Hep G2 cells, the reduction was not observed in Ser<sup>307</sup> phosphorylation up to 2 h (Fig. 1A, HepG2). As 3T3-L1 and Hep G2 are representatives of preadipocytes and hepatocytes, the data suggests a cell type-related difference in Ser<sup>307</sup> phosphorylation.

JNK and IKK were activated when Ser<sup>307</sup> phosphorylation was induced by TNF treatment. However, the time course of JNK and IKK activation is different in the 3T3-L1 and Hep G2 cells. JNK activation was determined by monitoring phosphorylation of JNK (Thr<sup>183</sup> and Tyr<sup>185</sup>) and c-Jun (Ser<sup>63</sup>), which is a substrate of JNK. In 3T3-L1 cells, JNK1 phosphorylation was detected before TNF treatment, but a 3-fold increase was induced by TNF- $\alpha$  at the peak time (Fig. 1B, JNK1). JNK1 phosphorylation then reduced to the basal level by 30 min. JNK2 phosphorylation was not detectable before TNF treatment, and it was induced by 13 times at the peak after TNF treatment, and then decreased immediately (Fig. 1B, JNK2). Consistent with the JNK activation, c-Jun phosphorylation (Ser<sup>63</sup>) was induced by TNF treatment in a time course comparable with the JNK activity (Fig. 1B, pJUN). In Hep G2 cells, JNK activation was also detected at 5 min, but the peak was 15 min after TNF treatment (Fig. 1C, pJNK1 and pJNK2). A peak of c-Jun phosphorylation correlated to the peak of JNK activity at 15 min (Fig. 1C, pJUN). Taken together, these results suggest that JNK activation starts and decays earlier in 3T3-L1 cells.

IKK activity was monitored using I $\kappa$ B $\alpha$  protein abundance as an indicator in the study. I $\kappa$ B $\alpha$  undergoes proteasome-dependent degradation immediately after IKK activation. I $\kappa$ B $\alpha$  protein levels recover within 1 h following the degradation as a result of feedback of NF- $\kappa$ B activation. In both 3T3-L1 and Hep G2 cells, I $\kappa$ B $\alpha$  degradation was induced by TNF- $\alpha$ , however, I $\kappa$ B $\alpha$  degradation was more complete in Hep G2 cells (Fig. 1D, HepG2) than that in 3T3-L1 cells. This suggests that IKK



**FIG. 3. IKK inhibition leads to reduction of Ser<sup>307/312</sup> phosphorylation.** Phosphorylation of IRS-1 Ser<sup>307</sup> in response to TNF- $\alpha$  was examined in IKK1/2 $^{-/-}$  (KO) and wild type (WT) embryo fibroblasts, Hep G2 and 3T3-L1 cells. The cells were treated with 15dPGJ<sub>2</sub> (15  $\mu$ M), SP600125 (50  $\mu$ M), or both for 30 min. I $\kappa$ B $\alpha$  degradation and c-Jun phosphorylation were examined to evaluate specificity of the two inhibitors. Ser<sup>307</sup> phosphorylation of IRS-1 was monitored with the phospho-specific IRS-1 antibody. Immunoblotting was conducted as described under "Experimental Procedures." Representative blots are shown from experiments that were repeated independently at least three times with similar results. A, IRS-1 phosphorylation in IKK1/2 $^{-/-}$  cells. Ser<sup>307</sup> phosphorylation was induced by TNF- $\alpha$  in a treatment of 30 min. B, inhibition of I $\kappa$ B $\alpha$  degradation by 15dPGJ<sub>2</sub> in HepG2 cells. C, inhibition of Ser<sup>307</sup> phosphorylation by 15dPGJ<sub>2</sub> and SP600125 in Hep G2 and 3T3-L1 cells.

activity is higher in Hep G2 cells. The JNK and IKK activation correlates to the induction of Ser<sup>307</sup> phosphorylation in 3T3-L1 and Hep G2 cells.

**Inhibition of JNK Resulted in Reduction of Ser<sup>307</sup> Phosphorylation**—To confirm the role of JNK in phosphorylation of IRS-1, Ser<sup>307</sup> phosphorylation was investigated in two systems in which JNK activity was specifically reduced. In the embryo fibroblasts of JNK1 knockout mice, JNK1 activity was reduced by gene inactivation as indicated by a decreased c-Jun phosphorylation after TNF treatment (Fig. 2A, pJUN). In JNK1 $^{-/-}$

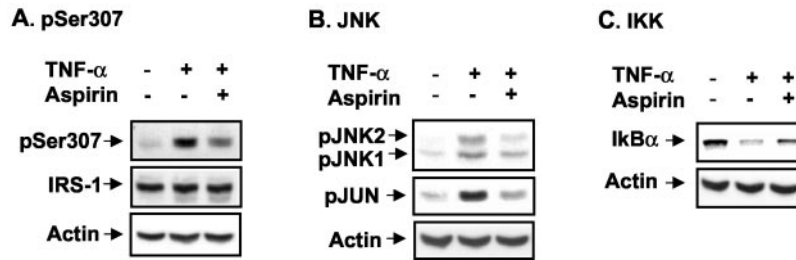


FIG. 4. **Aspirin inhibits Ser<sup>307</sup> phosphorylation in 3T3-L1 cells.** Ser<sup>307</sup> phosphorylation of IRS-1 was induced by TNF- $\alpha$  (20 ng/ml) in 3T3-L1 cells. Freshly made aspirin (5 mM) was used to block TNF- $\alpha$  activity through a 2-h pretreatment of 3T3-L1 cells. Phosphorylation of IRS-1, JNK, Jun, and protein abundance of I $\kappa$ B $\alpha$  was determined by immunoblotting as stated in the legend to Fig. 1. Representative blots are shown from experiments that were repeated independently at least three times with similar results. A, inhibition of IRS-1 Ser<sup>307</sup> phosphorylation by aspirin. B, inhibition of JNK activity by aspirin. C, inhibition of IKK activity by aspirin.

cells, IKK activity remains intact as indicated by degradation of I $\kappa$ B $\alpha$  after TNF treatment. Ser<sup>307</sup> phosphorylation was decreased by 65% in the JNK1<sup>-/-</sup> cells (Fig. 2A, pS307). SP600125 is a JNK-specific inhibitor (43). When tested together with MEK inhibitor PD98059, SP600125 exhibited a specific activity in reducing c-Jun phosphorylation, but not ERK phosphorylation (Fig. 2B). MEK inhibitor expressed a strong inhibitory activity in ERK phosphorylation, but it also reduced c-Jun phosphorylation (Fig. 2B). This suggests that the MEK inhibitor exhibited certain levels of nonspecificity. The nonspecific effect of MEK inhibitor on JNK may not be strong enough to block IRS-1 Ser<sup>307</sup> phosphorylation (Fig. 2C). Both JNK and ERK activities were not influenced by p38 inhibitor SB203580 (Fig. 2B). When applied to 3T3-L1 and Hep G2 cells, only JNK inhibitor SP600125 reduced Ser<sup>307</sup> phosphorylation induced by TNF treatment (Fig. 2C). These results consistently support the role of JNK in Ser<sup>307/312</sup> phosphorylation.

**Inhibition of IKK Reduced Ser<sup>307</sup> Phosphorylation**—To confirm the role of IKK in phosphorylation of IRS-1, a similar strategy was taken as to the study of JNK. In the embryo fibroblasts derived from IKK1 and IKK2 double knockout mice, both IKK1 and IKK2 activities are reduced by gene inactivation, but JNK activity remains intact. These are indicated by I $\kappa$ B $\alpha$  protein degradation and c-Jun phosphorylation after TNF treatment (Fig. 3A). In IKK1/2<sup>-/-</sup> cells, TNF-induced Ser<sup>307</sup> phosphorylation was reduced by 60% as indicated by a result from the quantitative analysis (Fig. 3A). 15dPGJ<sub>2</sub> is an established IKK inhibitor (44). When tested together with the JNK inhibitor, 15dPGJ<sub>2</sub> exhibited a specific activity in the suppression of IKK activity by blocking I $\kappa$ B $\alpha$  degradation (Fig. 3B). In the same condition, JNK inhibitor failed to prevent IKK activation because I $\kappa$ B $\alpha$  reduction was not blocked by SP600125 (Fig. 2B). When the IKK inhibitor was used to treat Hep G2 cells, Ser<sup>307</sup> phosphorylation was significantly reduced (Fig. 3C). The activity of the IKK inhibitor is comparable with that of the JNK inhibitor for inhibition of Ser<sup>307</sup> phosphorylation (Fig. 3C). A similar pattern of inhibition of IRS-1 phosphorylation was observed using 3T3-L1 cells (Fig. 3C). It was noted that both IKK and JNK inhibitors were unable to completely remove Ser<sup>307</sup> phosphorylation when used individually. When combined together, IKK and JNK inhibitors led to a 95% inhibition of Ser<sup>307</sup> phosphorylation (Fig. 3C, *Comb*). These results support the role of IKK and JNK in Ser<sup>307</sup> phosphorylation of IRS-1.

**Aspirin Inhibits Ser<sup>307</sup> Phosphorylation in 3T3-L1 Cells**—It was reported that aspirin could inhibit IRS-1 serine phosphorylation (5), but aspirin-sensitive serine residues were not identified in the study. In a recent study, aspirin was shown to block Ser<sup>307</sup> phosphorylation by reducing JNK activity (45), but IKK activity was not successfully evaluated in the study. In the current study, we investigated an effect of aspirin on Ser<sup>307</sup> phosphorylation through examination of both JNK and IKK

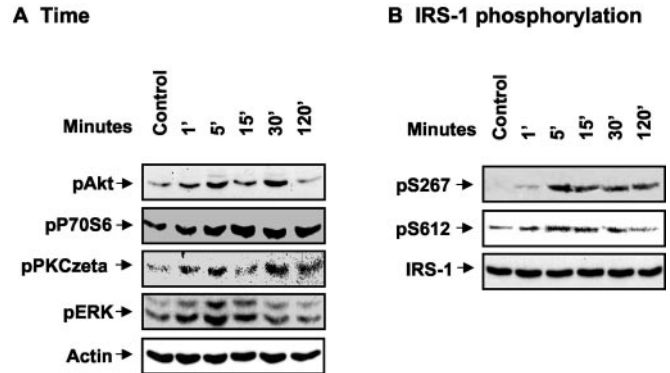
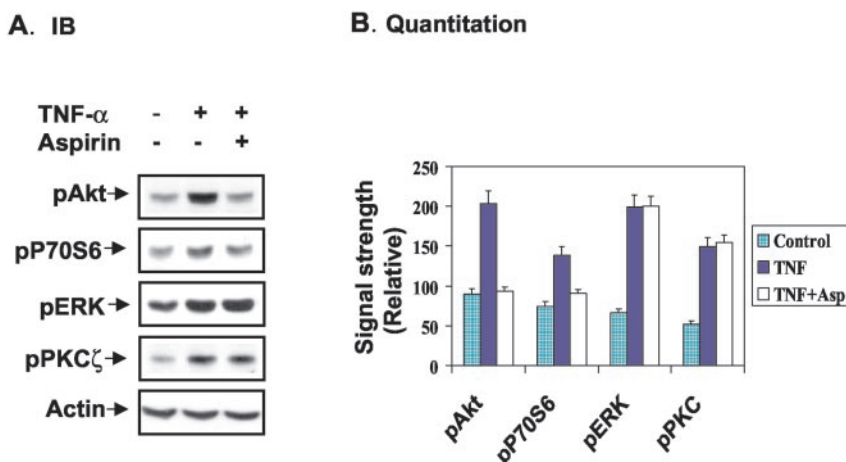


FIG. 5. **Activation of Akt, mTOR, PKC $\zeta$ , and ERK by TNF.** Activation of the four serine kinases and phosphorylation of IRS-1 at Ser<sup>267</sup> and Ser<sup>612</sup> were investigated in 3T3-L1 cells after TNF treatment. The signals were determined in the whole cell lysate by immunoblotting. Representative blots are shown from experiments that were repeated independently three times with similar results. A, phosphorylation of the kinases themselves or its immediate substrate (mTOR substrate is P70S6) was monitored for activation of Akt (Thr<sup>308</sup>), mTOR (pP70S6), PKC $\zeta$  (Thr<sup>410/403</sup>), and ERK (Tyr<sup>204</sup>). B, IRS-1 phosphorylation was induced at Ser<sup>267</sup> and Ser<sup>612</sup> in TNF-treated 3T3-L1 cells.

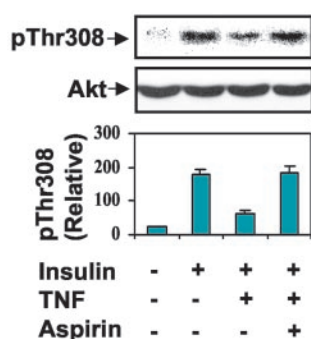
activities. In 3T3-L1 cells, aspirin (5 mM) was found to reduce Ser<sup>307</sup> phosphorylation by 70% (Fig. 4A). This reduction was associated with a reduction in JNK activity as phosphorylation of JNK and c-Jun were both decreased by aspirin (Fig. 4B). IKK activity was also suppressed by aspirin in the same cells by checking I $\kappa$ B $\alpha$  protein abundance (Fig. 4C). These results suggest that aspirin inhibits Ser<sup>307</sup> phosphorylation by blocking both JNK and IKK in 3T3-L1 cells. This observation is consistent with published data that aspirin is able to block both JNK and IKK activity (46, 47).

**TNF- $\alpha$  Activates Other Serine Kinases**—TNF- $\alpha$  is able to induce IRS-1 serine phosphorylation by activating other serine kinases in addition to JNK and IKK. Those serine kinases include Akt (48), ERK (49), mTOR (19, 33, 34), and PKC $\zeta$  (28). In this study, we examined activation of these serine kinases in 3T3-L1 preadipocytes through their phosphorylation status by immunoblotting. The results show that all of these four serine kinases are activated by TNF- $\alpha$  in a time-dependent manner (Fig. 5A). Thr<sup>308</sup> phosphorylation of Akt, Thr<sup>410/403</sup> phosphorylation of PKC $\zeta$ , and Tyr<sup>204</sup> phosphorylation of ERK1 and ERK2 were induced by TNF- $\alpha$ . Activation of mTOR was determined using the phosphorylation status of p70S6 (Thr<sup>412</sup>/Ser<sup>424</sup>), an immediate downstream substrate of mTOR. In terms of time course of activation, Akt, mTOR, and PKC $\zeta$  exhibited a similar pattern, in which activation was detectable at 1 min and peaked at 15–30 min. Although ERK activation was also detectable at 1 min, the activation peak was observed at 5 min. At 2 h after TNF stimulation, activation of all four serine kinases went down as indicated by the phosphorylation

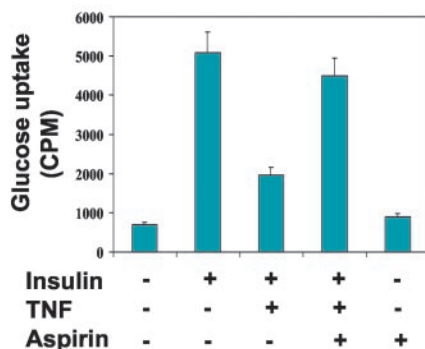
**FIG. 6. Aspirin inhibits Akt and mTOR.** Phosphorylation of Akt (Thr<sup>308</sup>), P70S6, PKC $\zeta$  (Thr<sup>410/403</sup>), and ERK (Tyr<sup>204</sup>) was monitored in 3T3-L1 whole cell lysate after a 2-h aspirin treatment for inhibition of Akt, mTOR, PKC $\zeta$ , and ERK. Freshly made aspirin (5 mM) was added to block TNF activity 30 min before TNF exposure (30 min). The experiment was conducted three times with similar results. *A*, the representative immunoblot. *B*, relative signal strength of three immunoblots. Each bar represents a mean  $\pm$  S.E.



### A. Akt Thr308



### B. Glucose uptake



**FIG. 7. Aspirin reverts inhibition by TNF- $\alpha$ .** *A*, 3T3-L1 cells were pretreated with TNF- $\alpha$  (20 ng/ml) for 4 h to inhibit insulin signaling. After insulin (100 nM) stimulation for 15 min, insulin-induced Akt activation was monitored in the whole cell lysate by immunoblotting with a phospho-specific Akt (Thr<sup>308</sup>) antibody. Freshly made aspirin (5 mM) was added to block TNF activity 30 min before TNF exposure. The signal strength of three experiments is presented in the bar figure. *B*, glucose uptake assay was conducted as described under "Experimental Procedures." Each bar represents a mean  $\pm$  S.E. of three independent experiments.

status. These results suggest that Akt, mTOR, PKC $\zeta$ , and ERK are activated at the same time in cells after TNF treatment, supporting that they may be involved in IRS-1 serine phosphorylation.

It was suggested that in IRS-1, phosphorylation of Ser<sup>267/270</sup> is related to Akt activation (31), and phosphorylation of Ser<sup>612/616</sup> is related to ERK activation (24–26). To test these relationships, phosphorylation of these two serine residues were determined in IRS-1 using phospho-specific IRS-1 antibodies. The result showed that phosphorylation of these two serine residues was induced by TNF- $\alpha$  in a time-dependent manner (Fig. 5B). The time course of the phosphorylation correlates to that of Akt and ERK activation, which peaks at 5 min and then decreases with time.

**Aspirin Inhibits TNF-induced Akt and mTOR**—To our knowledge, it is not clear whether aspirin has an effect on the activity of serine kinases like Akt, mTOR, ERK, and PKC $\zeta$ . To determine the activity of aspirin on these four kinases, TNF-induced activation of these kinases was examined in 3T3-L1 preadipocytes with aspirin pretreatment. The results show that aspirin exhibits different activities on these four kinases. It suppressed activation of Akt and mTOR, but exhibited no significant effect on ERK and PKC $\zeta$  (Fig. 6, A and B). Because both Akt- and mTOR-induced serine phosphorylation of IRS-1 were indicated in the impairment of insulin signaling (19, 33, 34, 50, 51), these results suggest that suppression of Akt and mTOR by aspirin may contribute to insulin sensitization by aspirin.

**Aspirin Prevents TNF-induced Impairment of Insulin Signaling**—In this study, we observed that aspirin prevented activation of four serine kinases that may phosphorylate IRS-1 at serine residues. This represents at least part of the mechanism

by which aspirin enhances insulin signaling. To test the effect of aspirin on insulin signal transduction, insulin-induced activation of Akt was examined in cells with aspirin pretreatment. Thr<sup>308</sup> phosphorylation in Akt is used as an indicator of the activation of the insulin signaling pathway because the phosphorylation is dependent on insulin-induced activation of phosphatidylinositol 3-kinase. The results show that Akt activation was induced by insulin and the induction was inhibited at 70% by TNF- $\alpha$  (Fig. 7A). Pretreatment of the cells with aspirin reverted the inhibition completely (Fig. 7). To confirm the effect, metabolic activity of insulin was evaluated by glucose uptake in 3T3-L1 adipocytes. The result shows that TNF- $\alpha$  represses insulin-induced glucose uptake by 60% (Fig. 7B). The TNF activity was blocked significantly by aspirin. This result supports that aspirin protects cells from insulin resistance induced by TNF- $\alpha$ .

## DISCUSSION

Serine phosphorylation of IRS proteins is believed to be a major mechanism of suppression of IRS-1 activity that contributes to insulin resistance (52, 53). Regulation of serine phosphorylation of IRS proteins has been a focus in the search for the molecular mechanism of insulin resistance. Eight serine kinases have been highlighted for their activities in phosphorylation of IRS-1 at serine residues. These serine kinases include JNK (12, 13, 26), IKK (36), Akt (31), mTOR (32), ERK (23, 24), PKC $\zeta$  (30), glycogen synthase kinase 3 (35), and casein kinase II (54). However, it is not clear how many of these kinases are activated by TNF- $\alpha$  at the same time in cells. In this study, we examined the first six kinases in TNF-treated cells. The results show that TNF- $\alpha$  activates all six kinases within 30 min. We think that there are at least two biological

meanings of activation of these kinases. First, activation of the six serine/threonine kinases may contribute to insulin resistance by induction of IRS-1 serine phosphorylation. Second, activation of Akt and PKC $\zeta$  may account for part of the acute effect of TNF- $\alpha$  in glucose metabolism, such as promoting glucose uptake in the body. It was reported that in rats, TNF administration alone enhanced whole body glucose disposal and stimulated glucose uptake by many organs including the liver and lungs (55, 56). However, prolonged infusion of TNF- $\alpha$  impaired insulin sensitivity as revealed by a decreased glucose tolerance (55, 56). Serine phosphorylation of IRS proteins by the six kinases may be responsible for insulin resistance after prolonged TNF infusion. Additionally, inhibition of serine/threonine phosphatase protein phosphatase 2A (PP2A) by TNF- $\alpha$  may contribute to extension of the serine phosphorylation status of IRS-1. It is known that PP2A activity is inhibited by TNF- $\alpha$  treatment (57). Additionally, calyculin A, a PP2A inhibitor, does enhance IRS-1 serine phosphorylation (36).

Aspirin inhibited four of the six kinases tested in this study. They are JNK, IKK, Akt, and mTOR. Therefore, aspirin may enhance insulin sensitivity by protecting IRS-1 from the four kinases. Three recent studies provide clear evidence that aspirin promotes peripheral glucose disposal through enhancing insulin sensitivity (5–7). In these studies, aspirin was found to improve insulin-stimulated peripheral glucose uptake (5, 7) and to increase tyrosine phosphorylation of IRS-1 (5, 6) or phosphatidylinositol 3-kinase activity associated with IRS-1 (6). These studies provided the first evidence that IKK may be a target of aspirin for the hypoglycemic action (5, 6). Although aspirin was reported to inhibit IKK activity (47), IKK activity was not examined in these studies after aspirin treatment (5, 6). A role of IKK in aspirin-induced insulin sensitivity remains to be evaluated at the molecular level. In this study, we observed that aspirin inhibited TNF-induced IKK activity in 3T3-L1 cells, and this inhibition contributed to prevention of IRS-1 phosphorylation. Our previous observation suggests that the antioxidant activity of aspirin may be responsible for the inhibition of IKK activity (58).

JNK is a serine kinase that is responsible for activation of transcription factor c-Jun and ATF2 by phosphorylating these two proteins (59, 60). Because c-Jun plays an important role in oncogenesis, JNK has been studied extensively for its role in carcinogenesis (61–63). Recently, JNK has been linked to the regulation of insulin signaling by several studies (12, 13, 26, 41, 42). It is suggested that JNK contributes to insulin resistance by phosphorylating IRS-1 at serine 307, and this phosphorylation leads to inhibition of the IRS-1 function (12, 13, 26, 41). In this study, we observed that aspirin inhibited JNK, and this inhibition led to reduction of IRS-1 serine phosphorylation at Ser<sup>307/312</sup>. This result is consistent with our previous observation that aspirin inhibits JNK activity (46, 64).

Although activities of Akt, ERK, mTOR, and PKC $\zeta$  in serine phosphorylation of IRS-1 have been suggested by data from several groups (24, 26, 30, 31, 33), it remains to be studied how these kinases act in insulin resistance induced by TNF- $\alpha$ . Additionally, their roles in insulin sensitization by aspirin have not been reported. In this study, both issues were addressed. We believe that Akt may result in IRS-1 serine phosphorylation through direct and indirect actions. Directly, Akt phosphorylates IRS-1 at Ser<sup>267/270</sup> as reported (31). Functional consequence of Ser<sup>267/270</sup> phosphorylation is controversial. Some studies indicate that the phosphorylation leads to inhibition of IRS-1 through a scaffold protein by the name of 14-3-3 (50, 51). It is also reported that the phosphorylation is required for maintenance of IRS-1 activity (31). Our result supports Akt in phosphorylation of Ser<sup>267/270</sup> because Akt activation was corre-

lated to Ser<sup>267</sup> phosphorylation in 3T3-L1 cells after TNF treatment. Indirectly, Akt may promote IRS-1 phosphorylation by activation of kinases, such as IKK (48) and mTOR (33). It is known that Akt activation may lead to activation of IKK and mTOR (33, 48). Consistently, we observed that Akt, IKK, and mTOR were activated together in cells within 30 min post-TNF exposure.

It is suggested that ERK may mediate PKC activity in phosphorylation of Ser<sup>612</sup> of murine IRS-1 (23, 24). In this study, we observed that Ser<sup>612</sup> phosphorylation and ERK activation had similar dynamics, supporting a role of ERK in the serine phosphorylation. Like Akt, PKC $\zeta$  may also regulate IRS-1 function through direct and indirect approaches (30, 65–67). It is likely that ERK and PKC $\zeta$  contribute to IRS-1 inhibition in response to TNF- $\alpha$ . However, ERK and PKC $\zeta$  alone may not be sufficient to result in inhibition of IRS-1 function because aspirin reverted TNF inhibition without blocking these two kinases. In the absence of TNF- $\alpha$ , our data suggests that aspirin itself has no effect on phosphorylation of IKK, JNK, Akt, and p70S6K (data not shown).

In summary, we examined six IRS-1 serine kinases in TNF-treated cells. The results demonstrate that all the serine kinases are activated by TNF- $\alpha$ . The results confirm that JNK and IKK are involved in IRS-1 (Ser<sup>307/312</sup>) phosphorylation. Additionally, activation of Akt, ERK, mTOR, and PKC $\zeta$  correlates to IRS-1 phosphorylation at Ser<sup>307/312</sup>, Ser<sup>267/270</sup>, and Ser<sup>612/616</sup> in rodent/human IRS-1. Aspirin is able to inhibit four of the six IRS-1 kinases. Because the four kinases (JNK, IKK, Akt, and mTOR) are able to phosphorylate IRS-1 directly or indirectly (12, 13, 26, 31, 32, 35, 36), our data suggests that aspirin enhances insulin sensitivity by blocking multiple IRS-1 serine kinases. This conclusion is supported by the data that aspirin rescued glucose uptake activity of TNF-treated 3T3-L1 adipocytes. It is possible that inhibition of Akt by aspirin is responsible for inactivation of IKK and mTOR.

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