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Salicylate Blocks Lipolytic Actions of Tumor Necrosis Factor-α in Primary Rat Adipocytes

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Running title: Salicylate inhibits adipocyte lipolysis

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The abbreviations used are:

ATGL, adipose triglyceride lipase;
ERK, extracellular signal-related kinase;
FFA, free fatty acid;
HSL, hormone-sensitive lipase;
IKKβ, IkappaB kinase β
LDH, lactate dehydrogenase;
MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;
PCV, packed cell volume;
PDE3B, cyclic-nucleotide phosphodiesterase 3B;
PKA, cAMP-dependent protein kinase A;
TNF-α, tumor necrosis factor-α

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ABSTRACT

Increased systemic free fatty acids (FFA) impair insulin sensitivity. In obese and diabetic subjects, production of a proinflammatory cytokine, tumor necrosis factor-α (TNF-α), is elevated. TNF-α has a variety of effects by inducing inflammation, decreasing glucose utilization, and also stimulating adipocyte lipolysis to release FFA to plasma. High doses of nonsteroidal anti-inflammatory drug salicylates have been long recognized to lower blood FFA and glucose in humans, although the mechanisms are not fully understood. In this report, we show that sodium salicylate at therapeutic concentrations directly blocks TNF-α-stimulated lipolysis and therefore inhibits FFA release from primary rat adipocytes. To elucidate the cellular basis of this action, we show that salicylate suppresses TNF-α-induced extracellular signal-related kinase activation and intracellular cAMP elevation, two early events during the lipolysis response to TNF-α. Further, salicylate prevents the downregulation of cyclic-nucleotide phosphodiesterase 3B, an enzyme responsible for cAMP hydrolysis. Perilipins coat intracellular lipid droplet surface by restricting lipase access to the triacylglycerol substrates. TNF-α downregulates perilipin but promotes its phosphorylation during lipolysis stimulation; these actions are efficiently reversed by salicylate. Salicylate slightly reduces basal but completely inhibits TNF-α-liberated lipase activity. In contrast, neither salicylate nor TNF-α alters the protein levels of hormone-sensitive lipase and adipose triglyceride lipase. In addition, sodium salicylate restricts basal lipolysis simulated by a high concentration of glucose and significantly diminishes the high glucose-enhanced lipolysis response to TNF-α. These results provide novel evidence that salicylate directly blocks TNF-α-mediated FFA efflux from adipocytes, hence reducing plasma FFA levels and increasing insulin sensitivity.

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Introduction

Obesity and type 2 diabetes mellitus are associated with elevated levels of plasma FFA, which directly induce insulin resistance (Bergman and Ader, 2000). The increased systemic FFA is thought to result from dysregulated lipolysis of triacylglycerols in adipose cells. One mechanism that may contribute to the elevated FFA release is an increase of TNF-α production under obese and diabetic conditions (Hotamisligil et al., 1995). TNF-α is a proinflammatory cytokine that has multifunctional effects in inflammatory and metabolic disorders. Recent studies suggest that TNF-α is an important mediator in the development of insulin resistance (Hotamisligil et al., 1995; Uysal et al., 1997). For example, although controversial (Ofei et al., 1996), prolonged TNF-α neutralization by its antibodies effectively improves insulin resistance in diabetic patients (Kiortsis et al., 2005). TNF-α deficient obese mice have lower circulating FFA levels and are protected from obesity-related insulin sensitivity (Uysal et al., 1997). TNF-α has important metabolic actions, which stimulates chronic lipolysis in primary (Green et al., 1994; Ren et al., 2006) and differentiated adipocytes (Green et al., 2004; Ryden et al., 2002; Souza et al., 1998). The lipolytic action of TNF-α governs FFA efflux from adipocytes to plasma, thereby elevating systemic FFA levels and causing insulin resistance.

The nonsteroidal anti-inflammatory drugs sodium salicylate and acetylsalicylic acid (aspirin) are widely used to control pain, fever, and rheumatic arthritis. Aspirin is standard care for diabetic patients with cardiovascular disease. Early in 1877, Ebstein found that high doses of sodium salicylate dramatically reduced glucosuria in diabetic patients (Ebstein, 1877). Further early studies showed that high doses of salicylates also lowered blood glucose concentrations in rodents (Bizzi et al., 1965) and in diabetic humans (Carlson and Ostman, 1961; Reid et al., 1957). In contrast, conflicting results demonstrate that lower doses of aspirin (3 g/d for 3 days) may not improve glucose utilization in normal (Newman and Brodows, 1983) and diabetic subjects

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(Bratusch-Marrain et al., 1985). Important discrepancies between these studies included lower salicylate dosages (<3 g/d) and therapeutic duration (a few days) in the more recent studies than in the earlier studies (6–9 g/d for 1–3 weeks). Although salicylate reduces TNF-α production in rat macrophages (Vittimberga et al., 1999), administration of low dose aspirin (325 mg/d) may result in a rebound increase in cytokine-induced synthesis of interleukin-1β and TNF-α in human (Endres et al., 1996), which can be expected to impair insulin sensitivity. Most recently, several studies indicate that high doses of salicylates attenuate deleterious effects of lipids and therefore improve lipid-induced insulin resistance both in rodents (Kim et al., 2001; Yuan et al., 2001) and humans (Hundal et al., 2002; Mohlig et al., 2006).

Early studies indicated that salicylates lower serum FFA concentrations in normal and diabetic subjects, which possibly contributes to their hypoglycemic effects. The FFA-lowering action may result from the suppression of FFA release from adipose tissue to plasma (Bizzi et al., 1965; Carlson and Ostman, 1961; Reid et al., 1957), because salicylates seem not affect FFA esterification and turnover. The FFA mobilization to plasma is governed by lipolytic reaction of adipocytes physiologically stimulated by catecholamines. Although the mechanism is yet unidentified, salicylates can reduce catecholamine-stimulated lipolysis in isolated adipocytes (Schonhofer et al., 1973; Stone et al., 1969).

Under obese conditions elevated TNF-α acts as a strong lipolytic stimulator (Hotamisligil et al., 1995). We (Ren et al., 2006) and others (Ryden et al., 2002) have indicated that activation of extracellular signal-related kinase (ERK) is a critical factor mediating TNF-α-induced lipolysis of adipocytes, whereas suppression of ERK signaling inhibits the lipolysis. Salicylates are able to inhibit ERK activation in neutrophils (Pillinger et al., 1998) and in TNF-α-stimulated fibroblasts (Schwenger et al., 1996). We hypothesize that salicylate may directly attenuate TNF-α-stimulated lipolysis response in adipocytes, which could be one of cellular basis for salicylate to reduce plasma FFA concentrations and thus improve insulin resistance.

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In this report, we show that sodium salicylate at therapeutic concentrations directly inhibits adipocyte lipolysis response to TNF-α. Salicylate attenuates TNF-α-induced ERK activation, cellular cAMP elevation, and the downregulation of cyclic-nucleotide phosphodiesterase 3B (PDE3B), thus restricting lipolysis. Further, salicylate corrects TNF-α-dysregulated protein levels and phosphorylation state of perilipins on adipocyte lipid droplet surface, inhibits TNF-α-liberated lipase activity, but does not alter the protein expression of hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL). This study provides novel evidence that salicylate directly antagonizes TNF-α-stimulated FFA efflux from adipocytes to plasma, thus lowering systemic FFA levels and increasing insulin sensitivity.

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Materials and Methods

Materials. Recombinant rat TNF-α was purchased from PeproTech EC (London, UK). Sodium salicylate was from Beijing Chemical Reagents Co. (Beijing, China). Phenol red-free Dulbecco's modified Eagle's medium (DMEM) containing glucose (5 mmol/L) and PKA inhibitor H89 were from Sigma Chemical (St. Lous, MO, USA). Enzyme materials used for enzymatic assays were products of Totobo Co. (Tokyo, Japan). Antibodies against ERK-1 (sc-93), phospho-ERK1/2 (sc-7383), PDE3B (sc-11835), anti-Gi1α (sc-391), actin (sc-1616R), and horseradish peroxidase (HRP)-conjugated second antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit antibodies against rat perilipin and rat HSL (He et al., 2006) were generous gifts from Dr. Londos at the U.S. National Institutes of Health. Antibody against phosphorylated protein kinase A (PKA) substrate (RRXS/T motif, #100G7) was from Cell Signaling (Boston, MA, USA). Rat anti-ATGL antibody was from Cayman Chemical (Michigan, USA). Nitrocellulose blot membrane, prestained protein molecular weight marker, and ultrasensitive enhanced chemiluminescence (ECL) detection reagents were from Applygen Technologies Inc. (Beijing, China).

Isolation and culture of primary rat adipocytes. Adipocytes were isolated from epididymal fat pads of Sprague-Dawley rats (150-180 g) according to our laboratory method (He et al., 2006; Jiang et al., 2007). The fat pads were minced and digested in 5 ml Krebs-Ringer solution containing 0.75 mg/ml type I collagenase, 200 nM adenosine, 25 mM Hepes, pH 7.4, and 1% defatted bovine serum albumin. After incubation for 40 min at 37 ºC in a water bath with shaking at 100 cycles/min, cells were filtered through a nylon mesh and washed 3 times with warmed DMEM containing 200 nM adenosine. Adipocytes floating on the top of the tube were packed by centrifuging at 200 × g for 3 min. Every 25 µl of packed adipocytes was resuspended in 500 µl phenol red- and serum-free DMEM containing 2% defatted bovine serum albumin and preincubated at 37 ºC for 1 h prior to treatments (He et al., 2006). Next, adipocytes were incubated in the presence or absence of the tested agents, followed by the assays described below.

Fatty acid assay. The concentration of FFA in the culture medium was determined by colorimetric assay as described (Itaya, 1977) with some modifications. Briefly, 50 µl of culture medium was mixed with 120 µl isooctane and 80 µl cupric acetate-pyridine. The mixture was vortexed and centrifuged for 10 min at 12,000 × g at room temperature. The upper organic phase (80 µl) was transferred to a clean tube. An amount of 180 µl of the color development reagent consisting of diphenylcarbazone and diphenylcarbazide in methanol was then added to the tube. The mixture was vortexed for 5 s, and the color of the reaction was developed immediately. The absorbance of the color reaction at 540 nm was spectrophotometrically measured in a 96-well plate.

Glycerol assay. Glycerol content released in culture medium of adipocytes served as an index of lipolysis and was determined at the absorption at 490 nm (He et al., 2006; Ren et al., 2006), by use of a colorimetric assay (GPO Trinder reaction) kit from Applygen Technologies Inc. (Beijing, China). Lipolysis data was expressed as micromoles of glycerol or FFA per milliliter of packed cell volume (PCV) of adipocytes.

Western blot. Adipocytes were packed and lysed in sample buffer containing 62 mM Tris-HCl, pH 6.8, 5% SDS, 0.1 mM sodium orthovanadate, and 50 mM sodium fluoride (He et al., 2006). After centrifuged at 12,000 × g for 10 min at 4 ºC, the lysate was transferred to a new tube and heated at 95 ºC for 5 min. Protein content in the extracts was determined by use of a bicinchoninc acid protein assay kit from Applygen Technologies Inc. (Beijing, China). Equal amounts of proteins were loaded and separated by 10% SDS-PAGE, then transferred to a nitrocellulose membrane. The membranes were blocked for 1 h in 5% non-fat milk in TBS-T buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.05% Tween-20) (Xu et al., 2006; Xu et al., 2005), then incubated with primary antibodies overnight at 4 ºC, followed by incubation for 1 h with HRP-conjugated secondary antibodies. The blots were developed by use of an enhanced

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chemiluminescence (ECL) detection kit. If required, the antibodies bound to membranes were removed by a commercial Stripping Solution from Applygen Technologies Inc. (Beijing, China). Blots were then reprobed with use of other antibodies and developed as described above. Densitometric analysis of protein bands involved use of NIHimage software.

cAMP immunoradioassay. Adipocytes were lysed in 150 µl of ice-cold buffer containing 50 mM Tris-HCl, pH 7.4, and 1 mM EDTA. To solidify the fat cake-enriched oil from lysed adipocytes, the lysate was incubated on ice for 15 min, vortexed vigorously, and centrifuged at 12,000 × g for 15 min at 4 ºC. The cytosolic fraction was collected from below the solidified fat cake in the tube (He et al., 2006; Jiang et al., 2007). The protein content in the cytosol fraction was determined. Then, 90 µl of cytosol fraction was mixed with 30 µl of 40% trichloroacetic acid. The tubes were vortexed and centrifuged at 12,000 × g for 5 min at 4 ºC. The supernatant was collected and used for cAMP assay according to the manufacturer-provided protocol from a commercial ^{125}I radioimmunoassay kit (Isotope Laboratory of Shanghai University of Chinese Medicine, Shanghai, China). The value of cAMP concentrations was normalized and expressed as pmol per mg of cytosolic proteins.

Assay of adipose lipase activity. After the treatments, adipocytes were washed twice with warmed PBS buffer and packed by centrifugation. The 50 µl packed adipocytes was lysed in 120 µl buffer containing 50 mM Tris-HCl, pH 7.4, and 1 mM EDTA. After being vortexed vigorously, the lysate was centrifuged at 12,000 × g for 15 min at 4 ºC. The infranatant phase below the fat cake fraction was transferred to a new tube, then centrifuged at 12,000 × g for 5 min at 4 ºC. The supernatant was used for the determination of cellular lipase activity against emulsified triolein substrate (Peled and Krenz, 1981). The mixture was incubated for 30 min at 37 ºC, when the lipases hydrolyze emulsified triolein to produce glycerol. The release of glycerol from triolein hydrolysis represented the activity of adipose lipase and was assayed as described above.

Cell viability assays. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and lactate dehydrogenase (LDH) assay were performed according to the manufacturer

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instructions of two commercial kits available from Applygen Technologies Inc. (Beijing, China). The MTT assay was based on the reduction of tetrazolium salt to colored formazan product by mitochondrial enzymes present only in living cells. Cell viability rates by MTT assay were directly proportional to the absorbance values of optical density measured at 570 nm in 96-well plate, and were presented as percentage of the control values. LDH activity was measured based on the conversion of lactate to pyruvate in the presence of LDH with parallel reduction of NAD^+, and the further reaction of pyruvate with 2,4-dinitrophenylhydrazine to form a colored hydrazone product that has a high optical density in the 400-500 nm wavelength range. The absorbance at 440 nm was determined and used for calculating LDH activity. The cell viability index of LDH was presented as percentage of LDH leakage in medium compared with total LDH activity.

Statistical analysis. Data are expressed as means ± S.E.M.. One-way ANOVA Tukey's test involved use of GraphPad Prism version 4.0. $p < 0.05$ was considered significant.

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Results

Sodium salicylate blocks TNF-α-induced lipolysis of primary adipocytes. Triacylglycerol lipolysis results in the release of FFA and glycerol from fat cells. To determine the effect of sodium salicylate on TNF-α-induced lipolysis, primary rat adipocytes were pretreated at 37 ºC for 1 h with 0.5, 1.0, or 5.0 mM sodium salicylate, then incubated for 24 h with 50 ng/ml TNF-α, sodium salicylate, or both. The levels of glycerol and FFA released in the culture media were determined as indexes of lipolysis. TNF-α promoted the levels of glycerol (Fig. 1A) and FFA (Fig. 1B) released in the culture media, which indicated that TNF-α stimulated remarkable lipolysis. The TNF-α-mediated lipolytic action was readily inhibited by sodium salicylate at 0.5 mM ($p <$ 0.05), and completely blocked at 5 mM ($p < 0.01$), so the antilipolytic effect of sodium salicylate was concentration dependent.

Next, adipocytes were pretreated with 5 mM sodium salicylate for 1 h, then incubated for 6, 12, or 24 h with 50 ng/ml TNF-α, 5 mM sodium salicylate, or both. TNF-α-stimulated releases of glycerol (Fig. 2A) and FFA (Fig. 2B) were increased at 6 h after treatment and further enhanced at 12 or 24 h. The lipolytic action of TNF-α was completely suppressed by the addition of 5 mM sodium salicylate in the media. The basal releases of FFA and glycerol from unstimulated adipocytes were also slightly inhibited by sodium salicylate. Partial oxidation and/or reesterification of fatty acids within adipocytes may account for the ratio of fatty acid to glycerol being less than the theoretical 3:l proportion.

In contrast to the above experiments during which salicylate was pre-administrated by 1 h prior to the TNF-α challenge, we next determined whether sodium salicylate was also effective in reducing lipolysis in the cells pretreated with TNF-α. Adipocytes were pre-incubated with 25- or 50 ng/ml TNF-α for 24 h, followed by another 24-h incubation in the freshly changed media in

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the presence of TNF-α or/and 5 mM salicylate. Salicylate also greatly inhibited the lipolysis in adipocytes that had been pre-stimulated by TNF-α (Table 1). Therefore, the salicylate antagonized the lipolysis in adipocytes pre- and post-stimulated by TNF-α.

Evaluation of adipocyte viability by LDH assay indicated no significant changes of LDH leakage into the culture media from adipocytes incubated for 24 h in the presence of sodium salicylate, TNF-α, or both (Table 2). Further, the MTT assay revealed that no obvious cytotoxicity of adipocytes was observed under same conditions (Table 2). These data suggest that the tested agents did not affect adipocyte viability.

Salicylate inhibits TNF-α-stimulated ERK phosphorylation. Activation of ERK may be one of the major mechanisms by which TNF-α induces adipocyte lipolysis (Ren et al., 2006; Ryden et al., 2002). We next examined whether sodium salicylate affects TNF-α-induced activation of ERK1/2. Primary rat adipocytes were incubated for 0.5, 6, and 24 h with 50 ng/ml TNF-α, 5 mM sodium salicylate, or both. Proteins extracted from adipocytes underwent immunoblotting. Neither TNF-α nor sodium salicylate altered the level of total ERK-1 proteins, but TNF-α induced a notable phosphorylation of ERK1/2 at 30 min after treatment. ERK1/2 activation was sustained at high levels 6 and 24 h after TNF-α stimulation (Fig. 3); these alterations paralleled the elevated lipolysis of adipocytes (Fig. 2). Sodium salicylate at 5 mM slightly attenuated basal level of the ERK1/2 phosphorylation in unstimulated adipocytes but completely eliminated the promoted ERK phosphorylation in TNF-α-stimulated adipocytes (Fig. 3). These effects were concomitant with the inhibitory action of sodium salicylate on basal- and TNF-α-stimulated lipolysis (Fig. 2). In addition, we also examined the phosphorylation of two other mitogen-activated protein kinases (MAPKs), p38 and JNK (c-Jun-NH2-terminal kinase); phosphorylation of JNK was rarely detected, but that of p38 was not affected by TNF-α or sodium salicylate (data not shown).

Sodium salicylate abrogates PDE3B downregulation and cAMP elevation mediated by

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TNF-α. Elevation of cellular cAMP is an important mediator of the lipolytic response. The lipolytic effect of TNF-α in adipocytes involves the down-regulation of PDE3B enzyme (Rahn Landstrom et al., 2000), which might lead to elevated intracellular cAMP levels and therefore activated PKA. To inspect the molecular basis of the antilipolytic effect of sodium salicylate, we examined the changes in level of PDE3B protein and intracellular cAMP content in adipocytes by immunoblotting analysis. PDE3B level was decreased by 2-fold in TNF-α-stimulated adipocytes as compared with in unstimulated cells. Treatment with 5 mM sodium salicylate reversed the TNF-α-mediated downregulation of PDE3B (Fig. 4A,B). We next assayed the adipocyte cAMP content and lipolysis response. TNF-α increased the level of intracellular cAMP by 1.6-fold; in contrast, 5 mM sodium salicylate significantly suppressed the basal cAMP level and abrogated the TNF-α-mediated cAMP elevation (Table 3). In parallel to these effects, sodium salicylate completely inhibited the lipolytic action stimulated by TNF-α (Table 3).

Sodium salicylate inhibits TNF-α-induced phosphorylation and downregulation of perilipin. Perilipin coats the surface of intracellular lipid droplets (Xu et al., 2006) and is a major PKA substrate in adipocytes. Down-regulation or phosphorylation of perilipins facilitates lipase-catalyzed hydrolysis of triacylglycerols (He et al., 2006; Ren et al., 2006; Sztalryd et al., 2003). TNF-α-elevated cellular cAMP, which can result in PKA activation, might result in perilipin phosphorylation. First, we determined whether the PKA might manipulate the phosphorylation of perilipins in TNF-α-stimulated adipocytes. Following the glycerol assays, the adipocyte extracts were underwent immunoblotting with use of the specific antibody against a phospho-PKA motif, and then the blots were reprobed with the anti-perilipin antibody. Stimulation with TNF-α for 6 h significantly increased perilipin phosphorylation (Fig. 5A) and elevated early lipolysis by 30.3% (Fig. 5B), which can be suppressed by a PKA inhibitor H89, indicating that the PKA activation accounted for those effects. Next, we evaluated the protein level and phosphorylation of perilipin in the adipocytes treated with TNF-α and/or sodium

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salicylate. TNF-α did not alter perilipin levels but greatly promoted its phosphorylation by 6 h (Fig. 5A,C,D). Perilipins were significantly downregulated by 24 h, but the ratio of phosphorylated perilipins to total species remained at a high level (Fig. 5C,D), which reveals that the long-term lipolytic action of TNF-α is related to multiple dysregulation of perilipin proteins. Coincubation with sodium salicylate prevented TNF-α-mediated phosphorylation and downregulation of perilipin proteins (Fig. 5C,D), therefore attenuating the lipolytic action of TNF-α.

Effects of TNF-α and sodium salicylate on the activity and protein level of adipose lipases. HSL and ATGL are two major lipases that coordinately hydrolyze triacylglycerols stored in adipocytes (Zimmermann et al., 2004). We examined the activity and protein level of cellular lipases in the adipocytes incubated with TNF-α, sodium salicylate, or both. TNF-α moderately promoted the total activity of adipose lipases ($p < 0.05$; Fig. 6A), an effect eliminated by sodium salicylate ($p < 0.05$). We further determined whether the increased lipase activity was associated with changes in protein expression. Immunoblotting results suggested that TNF-α and/or sodium salicylate did not affect the protein level of HSL and ATGL in adipocytes (Fig. 6B), so the levels of HSL and ATGL were less important than their activities, and sodium salicylate inhibited the TNF-α-stimulated lipase activity without altering their protein level.

Salicylate restricts lipolysis stimulated by TNF-α in a high-glucose environment. A high concentration of glucose has been indicated to increase basal lipolysis and enhance TNF-α-induced lipolysis in adipocytes (Green et al., 2004; Ren et al., 2006). We examined the antilipolytic action of sodium salicylate under a high-glucose environment. Incubation with a high concentration of glucose (25 mM) increased basal lipolysis by 41.6% ($p < 0.01$), whereas TNF-α alone elevated the glycerol release by 87.9% (Fig. 7). Further, the coincubation of the adipocytes with 25 mM glucose plus TNF-α promoted the lipolysis by 254.9%, which indicates that high glucose significantly enhanced the basal- and TNF-α stimulated lipolysis. Sodium

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salicylate attenuated the basal glycerol release with excess glucose ($p < 0.05$) and completely inhibited the lipolytic response with TNF-α ($p < 0.01$) by 25 mM glucose (Fig. 7).

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Discussion

A major goal of the present study was to investigate the antilipolytic effects of sodium salicylate on TNF-α-stimulated lipolysis in primary adipocytes. The bloodstream concentrations of salicylate for anti-inflammatory therapy are usually between 120 and 350 µg/ml in humans (Insel, 1996), equivalent to 0.75~2.2 mmol/L. We observed that sodium salicylate at 0.5 mmol/L already significantly inhibits the lipolytic action of TNF-α, and at 5 mmol/L achieves maximal antilipolytic effects.

The mechanisms by which TNF-α stimulates adipocyte lipolysis are multifactoral. ERK activation is a major and early signal in the regulation of TNF-α-stimulated lipolysis (Ren et al., 2006; Ryden et al., 2002). Our data indicate that TNF-α promotes significant phosphorylation of ERK1/2 in primary adipocytes at 30 min after stimulation, and the activation remains high at 6 and 24 h. Under the same conditions, phosphorylation of the two other MAPKs, p38 and JNK, was undetectable or unchanged, which is consistent with our previous observation (Ren et al., 2006). In parallel to the ERK activation mediated by TNF-α, the release of FFA and glycerol from adipocytes was increased at 6 h. Salicylates can inhibit ERK activation in neutrophils (Pillinger et al., 1998) and in TNF-α-stimulated fibroblasts (Schwenger et al., 1996); they may therefore limit lipolysis response to ERK activation in adipose cells. Consistent with this hypothesis, sodium salicylate attenuated TNF-α-activated ERK phosphorylation in adipocytes, thereby abrogating the lipolytic action of TNF-α. Salicylate also slightly inhibited basal ERK phosphorylation in unstimulated adipocytes, which may account for its inhibitory effect on basal lipolysis.

Alterations in proteins expression are also involved in the regulation of the chronic lipolytic response to TNF-α. Our study confirms that PDE3B, responsible for cellular cAMP hydrolysis, can be downregulated by TNF-α (Rahn Landstrom et al., 2000), an effect associated with

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promoted adipocyte cAMP levels. When the TNF-α-mediated downregulation of PDE3B is prevented by sodium salicylate, the elevation of cellular cAMP falls and the lipolytic action of TNF-α is consequently eliminated. Previously, other investigators indicated that TNF-α downregulates inhibitory G_i-proteins, leading to a withdrawal of endogenous inhibition in adenylyl caclase, hence elevating cAMP concentrations and stimulating lipolysis in adipocytes (Gasic et al., 1999). We observed that salicylate prevented TNF-α-mediated decrease of $G_i1\alpha$ isoforms (data now shown); which might also account for the antilipolytic effects of salicylate.

During lipolysis, catecholamine or TNF-α elevates cellular cAMP to activate PKA. PKA phosphorylates downstream HSL and perilipin, which cooperatively confer a full lipolytic reaction (Sztalryd et al., 2003). Perilipins are localized at intracellular lipid droplet surface, by functioning as a barrier to restrict lipase access to the triacylglycerol core stored within the lipid droplets (Sztalryd et al., 2003). When perilipin is downregulated (Ren et al., 2006) or its phosphorylation state is upregulated (He et al., 2006; Sztalryd et al., 2003), its barrier function could be impaired, thus leading to increased lipolysis (Sztalryd et al., 2003). By using two separate antibodies against phosphorylated PKA substrate and perilipin, we confirmed that the activation of PKA is at least partly responsible for TNF-α-induced perilipin phosphorylation and early (6 h) lipolytic elevation, because these effects can be blunted by a PKA inhibitor H89. Further, we observed a two-stage alteration of perilipin protein levels in response to TNF-α and sodium salicylate. First, the phosphorylation of perilipin was accelerated but total perilipin level unchanged on 6-h stimulation with TNF-α, when glycerol release started to increase. The addition of sodium salicylate suppressed perilipin phosphorylation and therefore inhibited lipolysis stimulated by TNF-α. Then, 24-h stimulation with TNF-α caused a significant decrease in total perilipin levels, but the strength of perilipin phosphorylation remained high. Clearly, salicylate efficiently prevents TNF-α-mediated early phosphorylation or subsequent loss of perilipin. Perilipin phosphorylation is essential in governing the full lipolysis response to various

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stimulators (He et al., 2006; Sztalryd et al., 2003). Also, we (Ren et al., 2006) and other investigators (Souza et al., 1998) have previously demonstrated the lipolytic action of TNF-α associated with perilipin downregulation, whose effects can be restored by the thiazolidinedione antidiabetic agent BRL 49653 (Souza et al., 1998) or the classic antihyperglycemic drug metformin (Ren et al., 2006). Obviously, the amelioration of TNF-α-dysregulated perilipin and its phosphorylation state is an important basis for the antilipolytic effects of salicylate.

HSL and ATGL are two major lipases for adipose triacylglycerol hydrolysis. With 24-h stimulation, TNF-α causes a moderate increase in total lipase activity in adipocytes. Sodium salicylate slightly reduces basal lipase activity but completely inhibits TNF-α-liberated lipase activity. Surprisingly, TNF-α (as well as sodium salicylate) did not alter the protein levels of HSL and ATGL in primary rat adipocytes. TNF-α is reported to downregulate HSL and ATGL mRNA (Kralisch et al., 2005) and HSL protein (Souza et al., 1998) in differentiated 3T3-L1 adipocytes. However, Green et al. did not find TNF-α affecting HSL protein expression during the lipolytic process in a study also involving primary rat adipocytes (Green et al., 1994). These discrepancies in lipase expression may be due to the different responses of TNF-α in fibroblast-derived differentiating 3T3-L1 adipocytes and primary rat adipocytes. HSL is phosphorylated on PKA activation and translocated from the cytosol to the lipid droplet surface to initiate lipolysis in adipocytes; this translocation requires fully phosphorylatable perilipin (Sztalryd et al., 2003). In contrast, ATGL is not a target for PKA phosphorylation and lacks the translocation reaction during lipolytic stimulation (Zimmermann et al., 2004). Whether sodium salicylate and TNF-α influence HSL phosphorylation remains to be further clarified.

A high concentration of glucose is also an effective lipolytic stimulator for adipocytes, as observed in the present and prior studies (Green et al., 2004; Ren et al., 2006). TNF-α-mediated lipolysis can be further enhanced by a high glucose supplied in the adipocyte culture; the salicylate restricts the basal lipolysis simulated alone by a high concentration of glucose at 25

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mM and also diminishes the high glucose-enhanced lipolysis response to TNF-α. This antilipolytic characteristic of salicylate could be particularly beneficial in limiting FFA efflux from adipose tissue and reducing systemic FFA concentrations under high glucose conditions.

High doses of salicylates have long been recognized to concurrently lower blood FFA and glucose levels in normal and diabetic subjects (Bizzi et al., 1964; Bizzi et al., 1965; Carlson and Ostman, 1961; Reid et al., 1957). Although salicylates can also inhibit catecholamine-stimulated lipolysis (Schonhofer et al., 1973; Stone et al., 1969), they remain effective in lowering plasma FFA level in adrenalectomized or hypothyroid animals (Bizzi et al., 1965), which implies that the FFA-lowering effects probably relate more to the antilipolytic actions against non-catecholamine and non-thyroxine-mediated lipolysis. Obesity is considered a chronic inflammatory state. TNF-α production is greatly increased in adipose and non-adipose tissues of obese and diabetic individuals (Hotamisligil et al., 1995). TNF-α stimulates lipolysis to liberate FFA flux from adipocytes and promotes plasma FFA concentrations, hence impairing insulin sensitivity. Thus, direct suppression of TNF-α-mediated lipolysis by salicylates provides a novel explanation for salicylates lowering plasma FFA levels.

Recent studies suggest that salicylate can reverse obesity- and diet-induced insulin resistance by inactivating IkappaB kinase β (IKKβ) (Hundal et al., 2002; Kim et al., 2001; Yuan et al., 2001). However, other study has indicated that conditional abrogation of IKKβ fails to prevent obesity-induced insulin resistance in vivo (Rohl et al., 2004); which argues against a basis of the salicylate to improve insulin resistance via inhibiting IKKβ. Interestingly, in the former studies, the amelioration of insulin sensitivity by salicylates via inactivating IKKβ is virtually accompanied by a ~50% decrease of plasma FFA levels in obese and diabetic animals (Yuan et al., 2001) and humans (Hundal et al., 2002). Therefore, it may be rational to speculate that the FFA-lowering effects via limiting FFA efflux from adipocytes could be more critical than the IKKβ-inhibition to contribute to the hypoglycemic actions of the salicylates.

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In conclusion, we have presented novel evidence showing that salicylate at therapeutic concentrations directly antagonizes adipocyte lipolysis response to TNF-α through multiple mechanisms. This antilipolytic action definitely restricts FFA mobilization from adipocytes to plasma, which could be a cellular basis for the roles of salicylates in reducing systemic FFA levels and improving insulin sensitivity. Future studies are necessary to determine whether the antilipolytic effect of salicylates contributes to their hypoglycemic actions in vivo.

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Legends for figures

Fig. 1. Sodium salicylate inhibition of TNF-α-stimulated lipolysis in rat adipocytes is concentration dependent. 25 µl of packed primary rat adipocytes was resuspended and pretreated at 37 ºC for 1 h with 0.5, 1.0, or 5.0 mM sodium salicylate (NaSal), then incubated for 24 h in the presence of 50 ng/ml TNF-α, sodium salicylate, or both. Glycerol (A) and FFA (B) released in the culture media were lipolysis indexes. The data are expressed as micromoles per milliliter packed cell volume (PCV) of adipocytes. Results are means ± S.E.M. of 3 separate experiments performed in triplicate. *, $p < 0.01$ *versus* control; †, $p < 0.05$ and ††, $p < 0.01$ *versus* TNF-α.

Fig. 2. Salicylate inhibition of TNF-α-stimulated lipolysis is time dependent. Rat adipocytes were pretreated at 37 ºC for 1 h with 5 mM sodium salicylate (NaSal), then stimulated for 6, 12, or 24 h with 50 ng/ml TNF-α, sodium salicylate, or both. Glycerol (A) and FFA (B) released in the culture media were lipolysis indexes. The data are means ± S.E.M. of 3 separate experiments performed in triplicate.

Fig. 3. Inhibition of ERK activation by salicylate during TNF-α-stimulated lipolysis. Adipocytes were preincubated for 1 h with 5 mM sodium salicylate (NaSal), then incubated for 0.5 h, 6 h, or 24 h with 50 ng/ml TNF-α in the presence or absence of 5 mM sodium salicylate. The culture media were collected and used for glycerol assay (data not shown). The adipocytes were lysed and the equivalent amounts of proteins underwent immunoblotting with use of primary antibodies against phosphorylated-ERK1/2 (p-ERK). After incubation with HRP-conjugated secondary antibodies, the blots were developed by use of an enhanced chemiluminescence detection kit. To detect total ERK-1, the blots were stripped and reprobed

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with primary anti-ERK-1 antibodies (ERK). The phosphorylated-ERK1/2 levels were quantitated densitometrically. The data of 3 separate experiments expressed as percentage of the control. *, $p < 0.05$ and **, $p < 0.01$ *versus* control; †, $p < 0.01$ *versus* TNF-α.

Fig. 4. Sodium salicylate reverses TNF-α-downreglulated PDE3B. Adipocytes were pretreated for 1 h with 5 mM sodium salicylate (NaSal), then incubated for 24 h with or without 50 ng/ml TNF-α. A, the cells were lysed and equivalent amounts of proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were immunoblotted with anti-PDE3B antibodies and peroxidase-conjugated secondary antibodies. After the development of the PDE3B bands, the blots were reprobed with use of anti-actin antibodies. B, the bands were quantitated densitometrically. The PDE3B/actin ratios are the mean ± S.E.M. of 3 separate experiments. *, $p < 0.05$ *versus* control; †, $p < 0.05$ *versus* TNF-α.

Fig. 5. Effects of sodium salicylate on perilipin phosphorylation and downregulation stimulated by TNF-α. Following glycerol assays, adipocyte extracts were underwent immunoblotting analysis. Phosphorylated perilipin (P-peri) was detected by use of an antibody against a specific motif (RRXS/T) of the PKA phosphosubstrate. Next, to assess total perilipin proteins, the blots were stripped and reprobed with rabbit anti-perilipin antibodies. The patterns of the protein bands recognized by the specific antibodies separately against PKA phosphosubstrate or perilipin are completely overlapped on the developed X-ray film. In final steps, the blots were stripped again and reprobed with anti-actin antibodies. A, immunoblot. Adipocytes were pretreated for 1 h with a PKA inhibitor H89, then incubated for 6 h with 50 ng/ml TNF-α. B, lipolysis assay in parallel to the tests of panel A. C, immunoblots. Adipocytes were pretreated for 1 h with 5 mM sodium salicylate (NaSal), then incubated for 6 h or 24 h with 50 ng/ml TNF-α, sodium salicylate, or both. D, densitometric analysis of protein bands showed in

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panel C. The ratios of phosphorylated and total perilipins (P-Peri/Peri) are the mean ± S.E.M. of 3 experiments. Peri, total perilipin; P-Peri, phosphorylated perilipin; NaSal, sodium salicylate; *, $p < 0.05$ *versus* control; †, $p < 0.05$ *versus* TNF-α.

Fig. 6. Effects of sodium salicylate and TNF-α on activity and protein level of adipose lipases. A, lipase activity. After incubation for 24 h, primary adipocytes were collected, washed, and lysed. The supernatant was used for the determination of cellular lipase activity. Results are expressed as percentage of the control values and are means ± S.E.M. of 3 individual experiments performed in triplicate. *, $p < 0.05$ *versus* control; †, $p < 0.05$ *versus* TNF-α. B, immunoblotting. Adipocytes were pretreated for 1 h with 5 mM sodium salicylate, then incubated with 50 ng/ml TNF-α for 6 or 24 h. Adipocyte extracts underwent immunoblotting with use of the antibodies against HSL or ATGL.

Fig. 7. Sodium salicylate restricts the lipolytic actions of TNF-α enhanced by a high concentration of glucose. The culture media were supplied with normal- (5 mM) or high-concentration (25 mM) of glucose. Adipocytes were preincubated with 5 mM sodium salicylate for 1 h, then incubated for 24 h with or without 50 ng/ml TNF-α. Glycerol released in the culture media was determined as an index of lipolysis. The data are means ± S.E.M. of 3 experiments performed in triplicate. **, $p < 0.01$ compared with no 25 mM glucose; †, $p < 0.05$ *versus* 25 mM glucose alone; ††, $p < 0.01$ *versus* TNF-α plus 25 mM glucose.

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Table 1. Sodium salicylate reduces lipolysis in adipocytes pre-stimulated with TNF-α.

Primary adipocytes were pre-stimulated with TNF-α (25 and 50 ng/ml) for 24 h, and then the incubation media were freshly replaced. The cells were incubated for another 24 h in the presence of TNF-α or/and 5 mM sodium salicylate (NaSal). Glycerol released in the culture media was then assayed as an index of lipolysis. Data are means ± S.E.M. of 3 individual experiments each assayed in triplicate. **, $p < 0.01$ *versus* control, ††, $p < 0.01$ *versus* related TNF-α.

	Lipolysis level (μmol glycerol/ ml PCV)
Control	2.3 ± 0.3
25 ng/ml TNF-α	3.8 ± 0.4**
50 ng/ml TNF-α	5.1 ± 0.8**
25 ng/ml TNF-α + 5 mM NaSal	2.6 ± 0.4††
50 ng/ml TNF-α + 5 mM NaSal	3.3 ± 0.5††

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Table 2. Effects of TNF-α and sodium salicylate on cell viability of adipocytes

Primary rat adipocytes were pretreated at 37 ºC for 1 h with 5 mM sodium salicylate (NaSal), then incubated for 24 h in the presence of 50 ng/ml TNF-α, sodium salicylate, or both. The MTT assay and LDH leakage assay were performed as described in *Materials and Methods.* Cell viability data by MTT assay were represented as percentage of the control values. LDH leakage data were expressed as percentage of LDH activity in the culture media compared to total LDH activity present both in the culture media and in whole adipocyte extracts. Results are means ± S.E.M. of three individual experiments performed in triplicate.

	MTT assay	LDH assay
	Cell viability (% control)	LDH leakage (% total)
Control	100.0 ± 0.0	8.8 ± 3.1
NaSal (5 mM)	96.6 ± 1.2	9.1 ± 3.4
TNF-α (50 ng/ml)	97.1 ± 3.6	10.7 ± 3.7
TNF-α + NaSal	98.7 ± 1.0	10.5 ± 4.0

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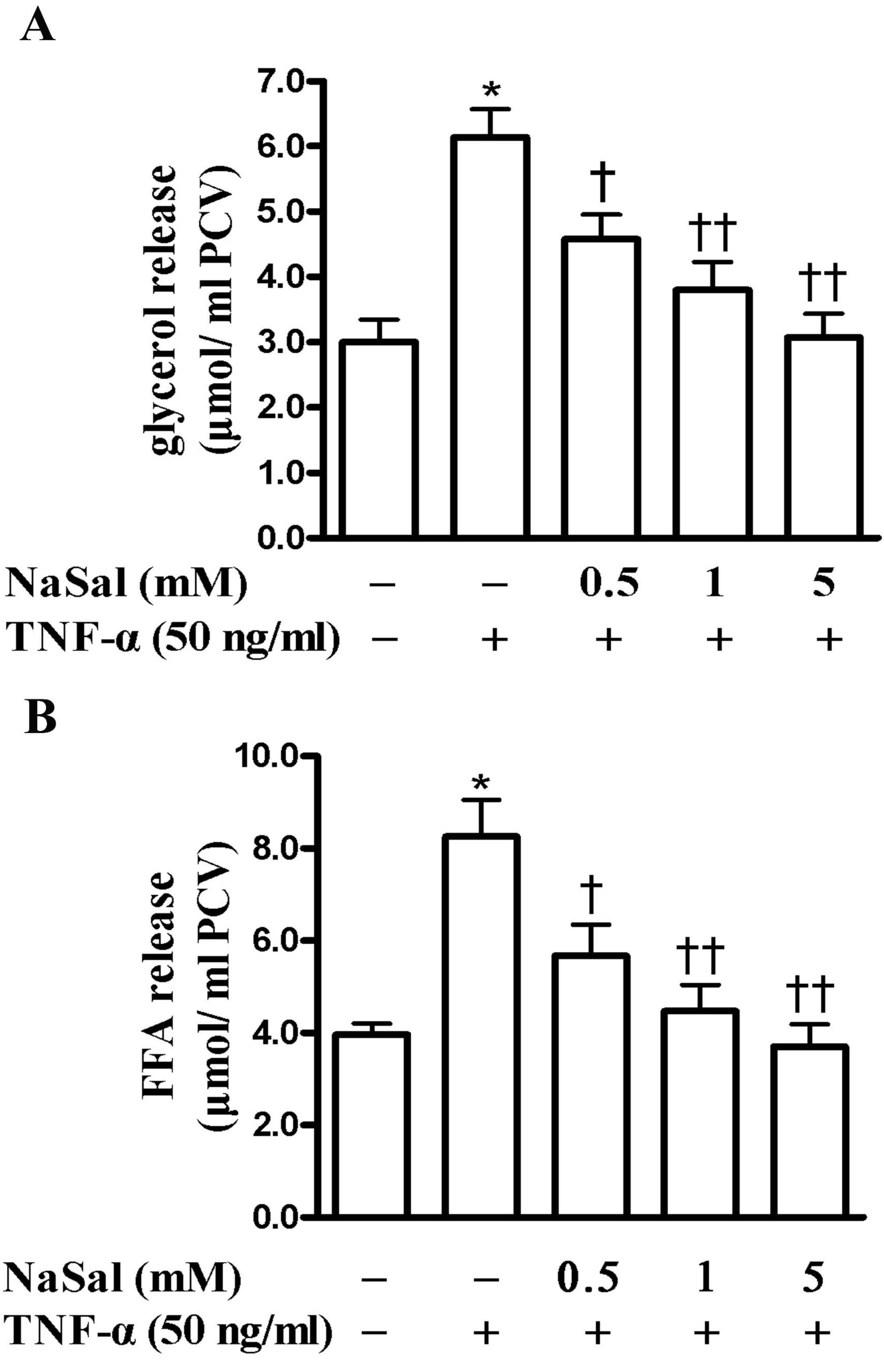
Table 3. Effects of TNF-α and sodium salicylate on intracellular cAMP levels.

Primary rat adipocytes were pretreated at 37 ºC for 1 h with 5 mM sodium salicylate (NaSal), then incubated for 24 h in the presence of 50 ng/ml TNF-α, sodium salicylate, or both. The culture media were collected and assayed for glycerol (µmol/ml PCV) as an index of lipolysis. Intracellular cAMP (pmol/mg protein) content was determined by use of a commercial ^{125}I radioimmunoassay kit. Results (means ± S.E.M.) are representative of three separate experiments each performed in sextuple. **, $p < 0.05$, **, $p < 0.01$, versus control; ††, $p < 0.01$ versus TNF-α.*

	cAMP level	*glycerol level*
Control	6.1 ± 0.9	2.7 ± 0.1
NaSal (5 mM)	3.6 ± 1.1 *	2.0 ± 0.3 *
TNF-α (50 ng/ml)	10.1 ± 3.4 **	5.4 ± 1.0 **
TNF-α + NaSal	3.8 ± 0.9 ††	2.8 ± 0.3 ††

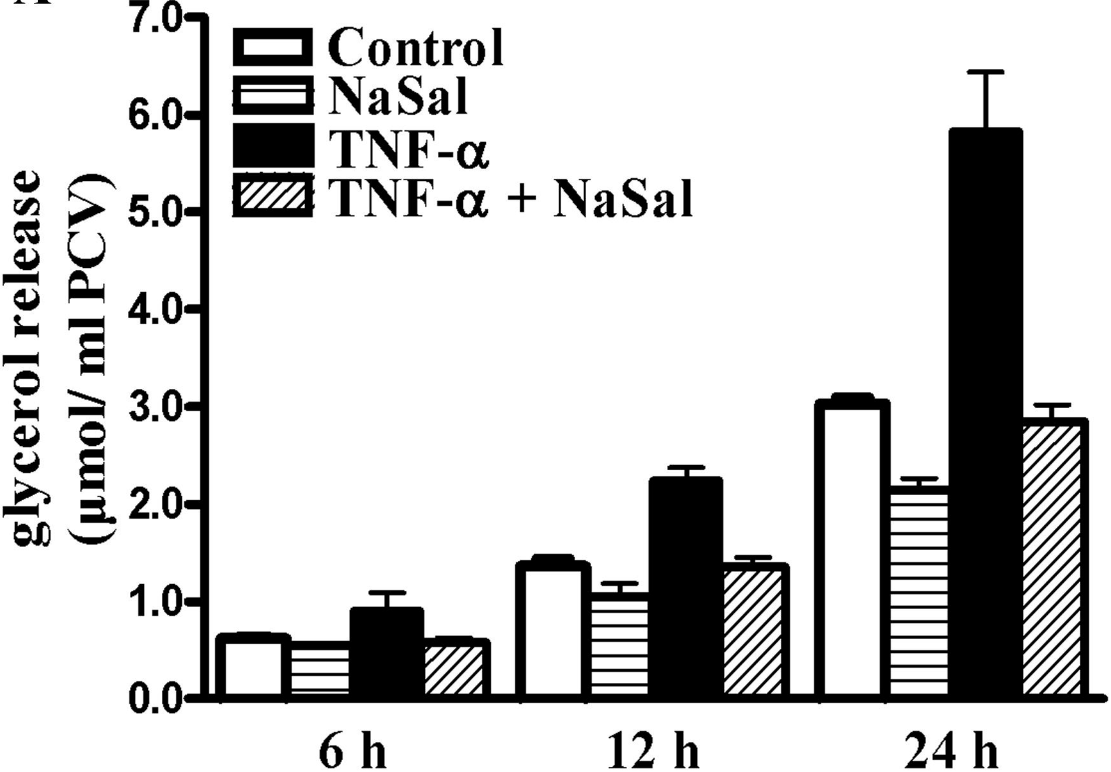
Fig. 1



A
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
glycerol release
(µmol/ ml PCV)
*
†
††
††
NaSal (mM) – – 0.5 1 5
TNF-α (50 ng/ml) – + + + +
B
10.0
8.0
6.0
4.0
2.0
0.0
FFA release
(µmol/ ml PCV)
*
†
††
††
NaSal (mM) – – 0.5 1 5
TNF-α (50 ng/ml) – + + + +

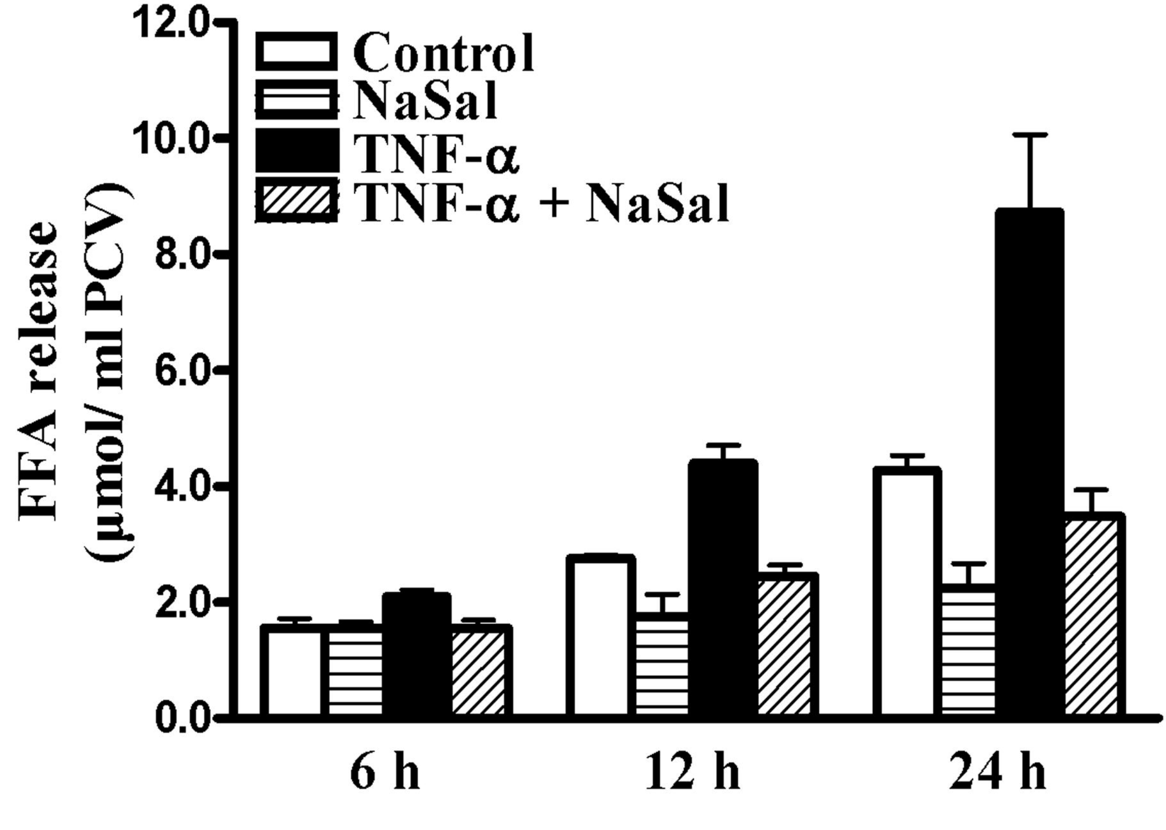
Fig. 2

A



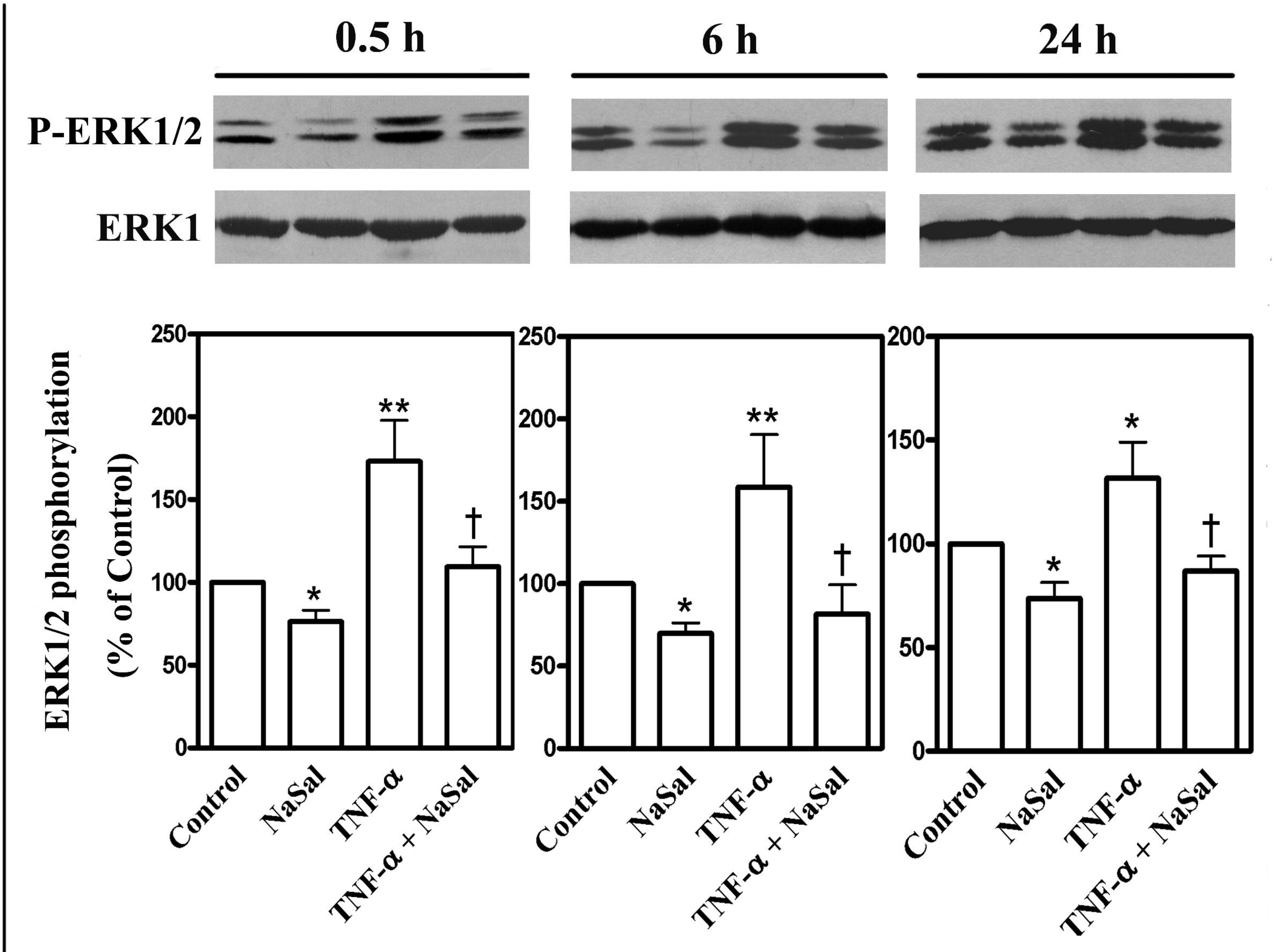
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
glycerol release
(μmol/ ml PCV)
Control
NaSal
TNF-α
TNF-α + NaSal
6 h
12 h
24 h

B



12.0
10.0
8.0
6.0
4.0
2.0
0.0
FFA release
(μmol/ ml PCV)
Control
NaSal
TNF-α
TNF-α + NaSal
6 h
12 h
24 h

Fig. 3



0.5 h
6 h
24 h
P-ERK1/2
ERK1
ERK1/2 phosphorylation (% of Control)
250
200
150
100
50
0
Control
NaSal
TNF-α
TNF-α + NaSal
*
**
†

Fig. 4

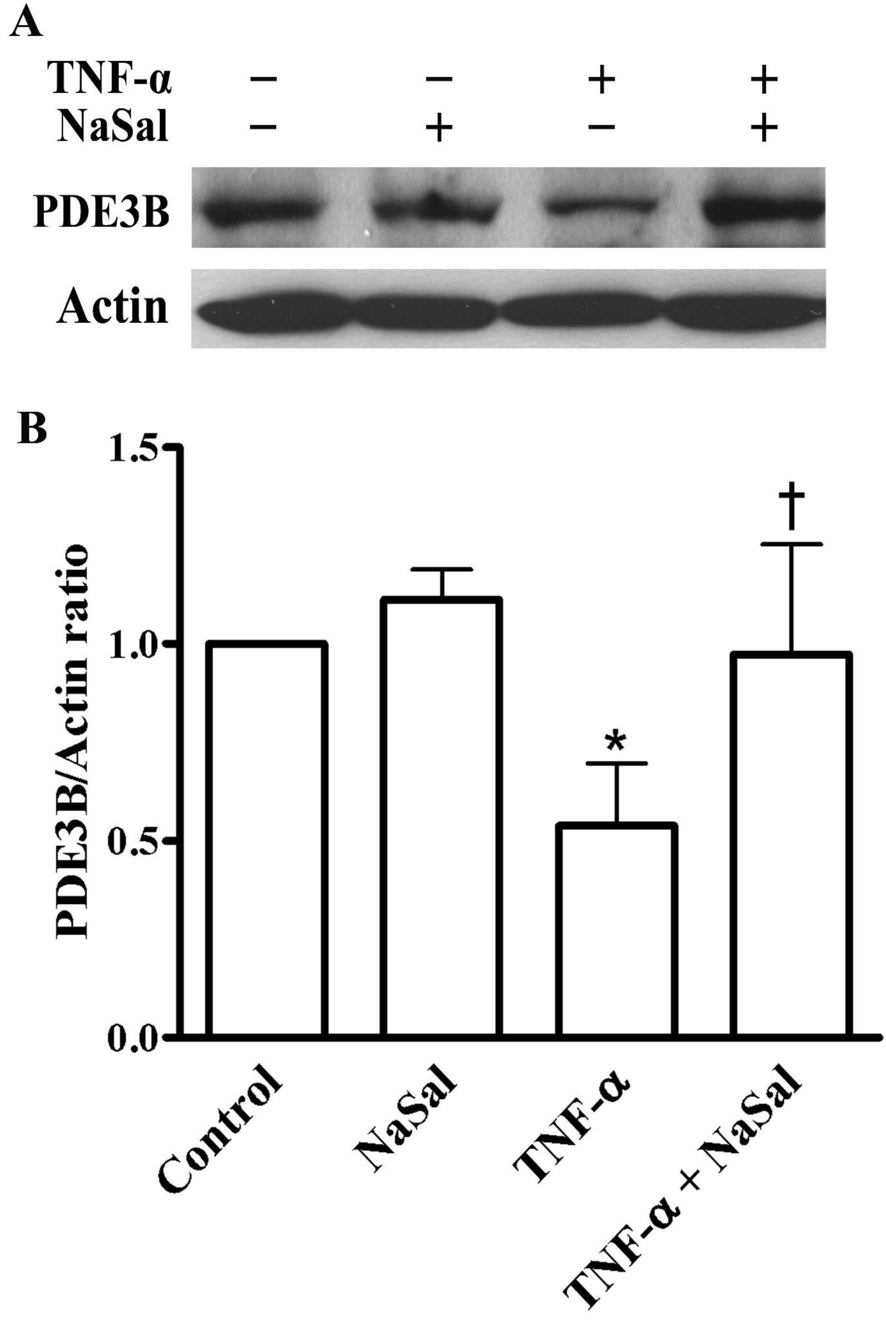

A
TNF-α − − + +
NaSal − + − +
PDE3B
Actin
B
1.5
1.0
0.5
0.0
PDE3B/Actin ratio
*
†
Control
NaSal
TNF-α
TNF-α + NaSal

Fig. 5

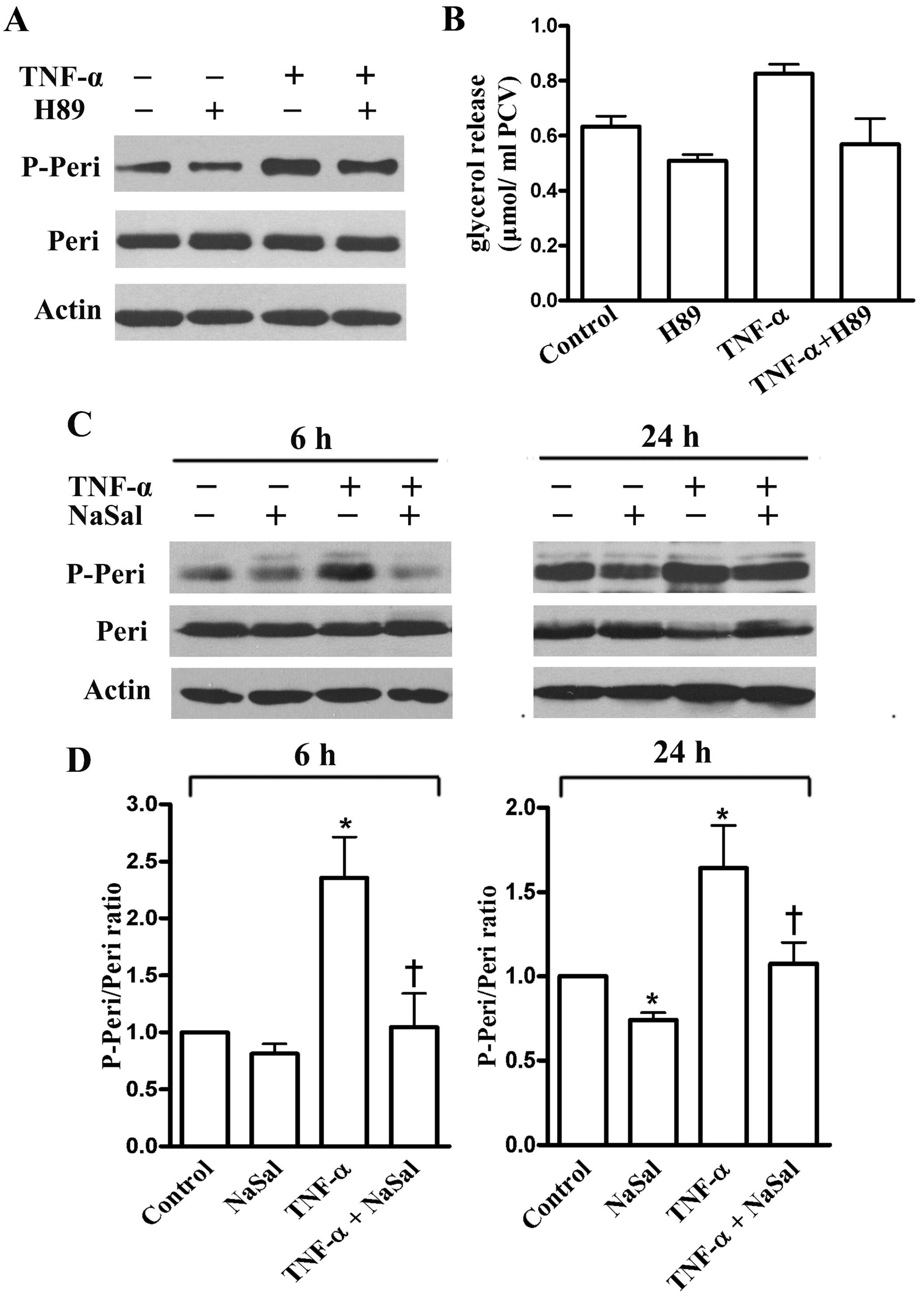

A
TNF-α − − + +
H89 − + − +
P-Peri
Peri
Actin
B
glycerol release (µmol/ ml PCV)
1.0
0.8
0.6
0.4
0.2
0.0
Control
H89
TNF-α
TNF-α+H89
C
6 h
24 h
TNF-α − − + +
NaSal − + − +
P-Peri
Peri
Actin
D
6 h
24 h
P-Peri/Peri ratio
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
†
Control
NaSal
TNF-α
TNF-α + NaSal

Fig. 6

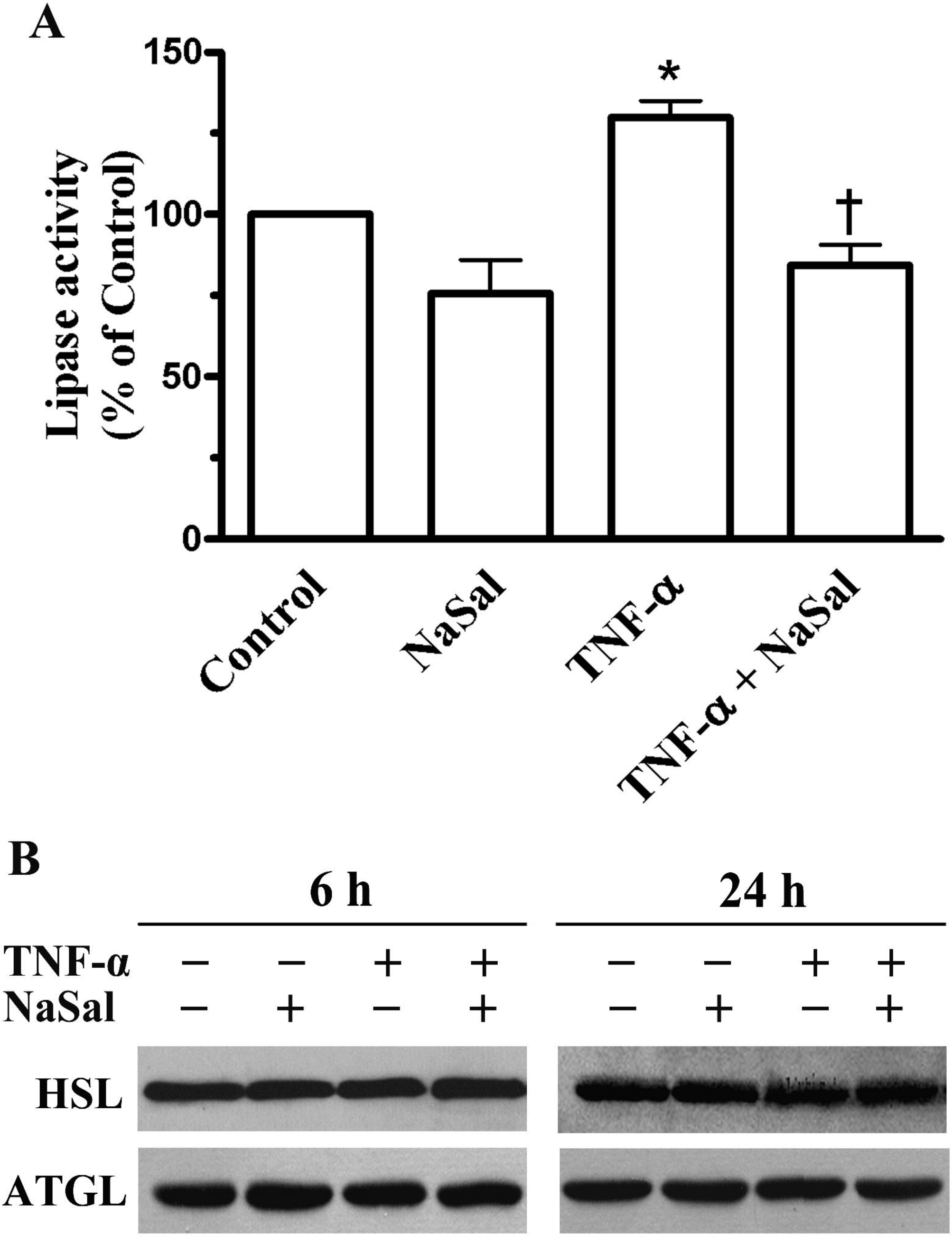
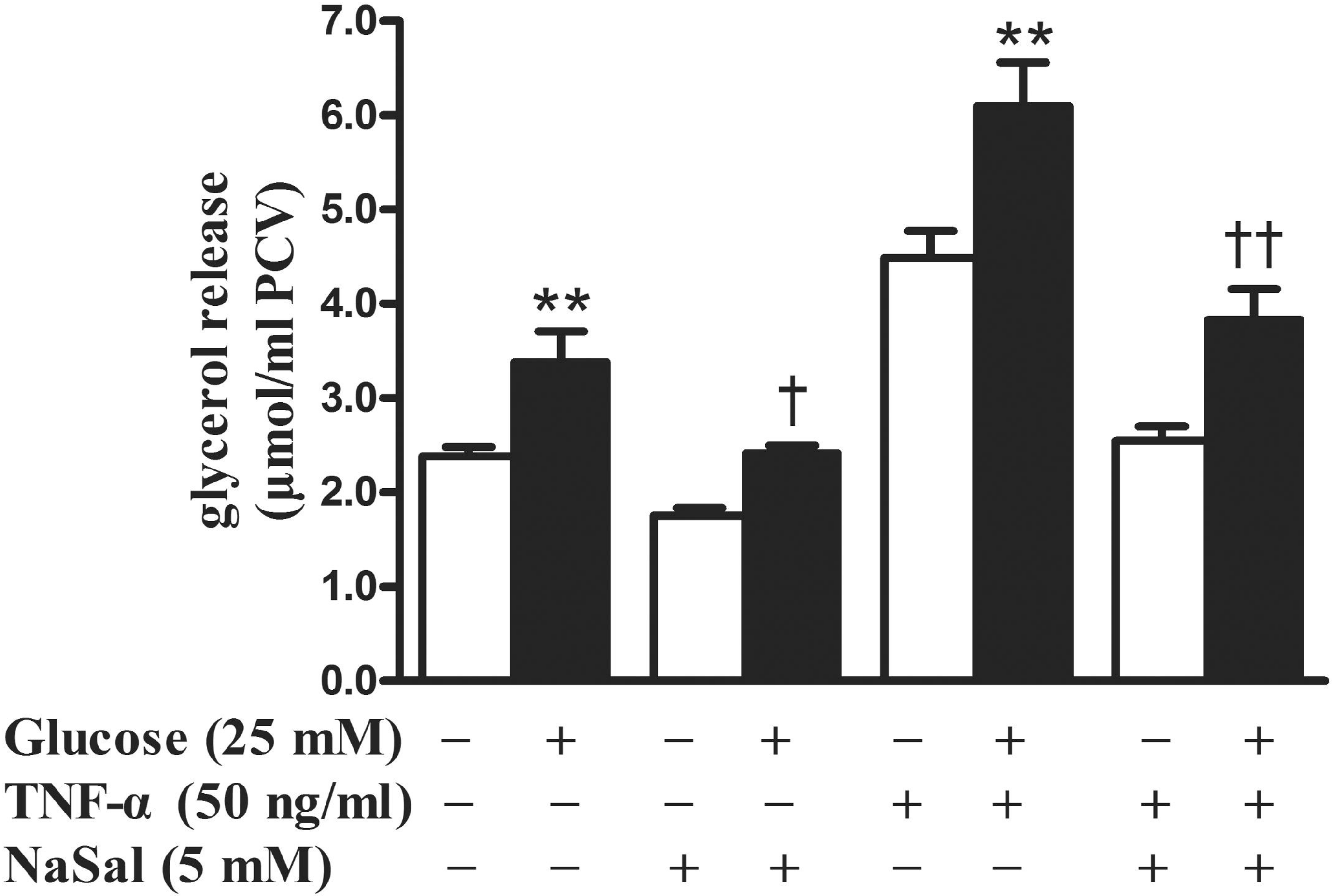

A
Lipase activity
(% of Control)
150
100
50
0
*
†
Control
NaSal
TNF-α
TNF-α + NaSal
B
6 h
24 h
TNF-α
NaSal
HSL
ATGL

Fig. 7



glycerol release (μmol/ml PCV)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
**
†
**
††
Glucose (25 mM) − + − + − + − +
TNF-α (50 ng/ml) − − − − + + + +
NaSal (5 mM) − − + + − − + +