Original Article

Tumor Necrosis Factor-α Induces Intestinal Insulin Resistance and Stimulates the Overproduction of Intestinal Apolipoprotein B48-Containing Lipoproteins

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There is growing evidence suggesting intestinal insulin resistance and overproduction of apolipoprotein (apo) B48–containing chylomicrons in insulin-resistant states. In the current study, we investigated the potential role of the inflammatory cytokine tumor necrosis factor-α (TNF-α) in the development of insulin resistance and aberrant lipoprotein metabolism in the small intestine in a Syrian golden hamster model. TNF-α infusion decreased whole-body insulin sensitivity, based on in vivo euglycemic clamp studies in chow-fed hamsters. Analysis of intestinal tissue in TNF-α–treated hamsters indicated impaired phosphorylation of insulin receptor-β, insulin receptor substrate-1, Akt, and Shc and increased phosphorylation of p38, extracellular signal–related kinase-1/2, and Jun NH_2-terminal kinase. TNF-α infusion also increased intestinal production of total apoB48, triglyceride-rich lipoprotein apoB48, and serum triglyceride levels in both fasting and postprandial (fat load) states. The effects of TNF-α on plasma apoB48 levels could be blocked by the p38 inhibitor SB203580. Ex vivo experiments using freshly isolated enterocytes also showed TNF-α–induced p38 phosphorylation and intestinal apoB48 overproduction, effects that could be blocked by SB203580. Interestingly, TNF-α increased the mRNA and protein mass of intestinal microsomal triglyceride transfer protein without altering apoB mRNA levels. Enterocytes were found to have detectable levels of both TNF-α receptor types (p55 and p75), and antibodies against either of the two TNF-α receptors partially blocked the stimulatory effect of TNF-α on apoB48 production and p38 phosphorylation. In summary, these data suggest that intestinal insulin resistance can be induced in hamsters by TNF-α infusion, and it is accompanied by intestinal overproduction of apoB48-containing lipoproteins. TNF-α–induced stimulation of intestinal lipoprotein production appears to be mediated via TNF-α receptors and the p38 mitogen-activated protein kinase pathway. *Diabetes* 56: 450–461, 2007

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apo, apolipoprotein; ERK, extracellular signal–related kinase; IRS, insulin receptor substrate; JNK, Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; MTP, microsomal triglyceride transfer protein; TNF, tumor necrosis factor.

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Substantial evidence has accumulated over the past decade that the atherosclerotic process is regulated by inflammatory mechanisms (1,2). In all phases of the atherosclerotic disease process, from lesion initiation, to progression, to plaque rupture and the ensuing thrombotic complications of cardiovascular disease, inflammatory factors play a central role (1). Epidemiological and clinical studies have shown strong and consistent relationships between markers of inflammation and risk of cardiovascular disease events (3). Insulin resistance has also been increasingly recognized as having an important role in inflammatory pathways (4–7). Thus, inflammatory mechanisms are likely to underlie the pathophysiological mechanisms leading to both atherosclerosis and insulin resistance (5,8). A major proinflammatory cytokine, tumor necrosis factor-α (TNF-α), has been implicated in this process (9).

TNF-α is one of the most potent proinflammatory cytokines. A number of studies have provided evidence that TNF-α can directly cause loss of insulin sensitivity (10,11). Furthermore, numerous studies have shown TNF-α–induced perturbations in insulin signaling cascades in insulin-sensitive tissues such as the liver, muscle, and adipose tissue (1,2,10). TNF-α can block tyrosine phosphorylation of the insulin receptor, interfere with insulin signal transduction (12), and activate the mitogen-activated protein kinase (MAPK) pathway (13). TNF-α exerts its effects by binding to at least two specific membrane receptors expressed ubiquitously: p55 (TNF receptor-1) and p75 (TNF receptor-2) (14).

TNF-α is a cytokine produced by different cell types that is involved in the regulation of several biological processes, including immunoinflammatory and metabolic homeostasis (15). Previous studies demonstrated that administration of TNF-α rapidly increases triglyceride-rich lipoprotein levels and stimulates hepatic fatty acid synthesis in diabetic animals (16,17), suggesting its potential role in the prominent disturbances in intermediary metabolism that occur during infections and inflammation. TNF-α profoundly affected hepatic lipid metabolism, increasing de novo fatty acid synthesis with a time course consistent with its ability to raise plasma triglycerides (17,18). The activation of the TNF-α/TNF receptor pathway, due to either increased levels of TNF-α or reduced shedding, has been shown to be associated with several metabolic abnormalities that are commonly found in hyperlipidemic patients, such as insulin resistance, increased release of VLDL by the liver, and increased free fatty acid (FFA)

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output by the adipose tissue (19,20). Furthermore, TNF-α at doses found in pathophysiological states significantly stimulated LDL receptor function and gene expression (21). Although much attention has been paid to the roles of TNF-α in insulin resistance and lipid metabolism in liver, adipose, and muscle tissues (9), little is known of the effects of TNF-α on intestinal insulin sensitivity and lipoprotein metabolism. To our knowledge, there are no direct studies of the link between TNF-α, intestinal insulin sensitivity, and intestinal lipoprotein metabolism.

Among the many risk factors identified by epidemiological studies, elevated levels of lipoproteins containing apolipoprotein (apo)B can lead to the development of atherosclerosis in human subjects and experimental animals even in the absence of additional risk factors (22). There is increasing evidence that triglyceride-rich lipoproteins, including intestinally derived apoB48-containing lipoproteins, may be particularly atherogenic (23–25), and a significant relationship exists between intestinally derived apoB48 and the development of atherosclerosis in nondiabetic (26) and diabetic patients (25). Our laboratory has previously used a fructose-fed hamster model to investigate the role of intestinal lipoprotein production in the development of metabolic dyslipidemia (27). This insulin-resistant hamster model exhibited increased overproduction of apoB48-containing particles and showed evidence of intestinal insulin resistance (28). In the current report, we examined the effects of TNF-α on intestinal insulin signaling and intestinally derived lipoprotein production, using the Syrian golden hamster model (29). We present both in vivo and ex vivo evidence that the inflammatory cytokine TNF-α not only induces intestinal insulin resistance, but also stimulates fasting and postprandial overproduction of intestinally derived lipoproteins. We further demonstrate that the intestinal effects of TNF-α are at least partially mediated by TNF-α receptors and the p38 MAPK cascade.

RESEARCH DESIGN AND METHODS

Male Syrian golden hamsters (*Mesocriceus auratus*) weighing 130–150 g were obtained from Charles River (Montreal). All animals were housed individually and given free access to food and water. After a 1-week acclimatization period, the animals either underwent the in vivo protocol or were killed for isolation of enterocytes for the ex vivo protocols described below. All animal protocols were approved by the animal ethics committee of the Hospital for Sick Children, University of Toronto.

In vivo euglycemic clamp study. The euglycemic clamp study was performed essentially as previously described, with some modifications (30). In brief, the right jugular vein and the left carotid artery were catheterized under isoflurane anesthesia, and catheters were exteriorized at the back of the neck, encased in silastic tubing. After overnight recovery and a 16-h overnight fast, a 4-h infusion of 0.9% normal saline or TNF-α (0.5 $\mu g \cdot kg^{-1} \cdot h^{-1}$) was given by the venous catheter, and with the infusion of saline or TNF-α continuing, a baseline blood sample was drawn. The hamster was then submitted to a euglycemic clamp procedure for the final 2 h to assess whole-body insulin sensitivity. The venous catheter was then used for the infusion of glucose (20%) and insulin (3 $mU \cdot kg^{-1} \cdot min^{-1}$). TNF-α was dissolved in saline and administered at a dosage shown to be sufficient to effectively inhibit insulin action (31). During the euglycemic clamp, blood glucose concentrations were measured every 10 min, and euglycemia was maintained at ~4.2 ± 0.1 mmol/l of baseline. The glucose infusion rate was calculated every 10 min as reported previously (32). Finally, the intestine was excised under isoflurane anesthesia and stored at −80°C until analysis.

In vivo Triton WR-1339 infusion and determination of total and triglyceride-rich lipoprotein apoB48 production. Hamsters were fasted overnight for 16 h, and the catheters were inserted as previously described (30). After a 4-h recovery period, hamsters were infused with saline or TNF-α (0.5 $\mu g \cdot kg^{-1} \cdot h^{-1}$) for 4 h. After the first 2 h and 20 min of infusion, an intravenous bolus of Triton-WR1339 (0.5 g/kg) was administered. After Triton-WR1339 administration, 300 μl of blood was collected as a baseline reading. Blood collections (300 μl) continued every 30 min for a total of 90 min with 50 μl of 0.5 mol/l EDTA added to inhibit clotting. The intestine was excised under isoflurane anesthesia after the 90-min period. Studies performed in the postprandial state were as described above except that hamsters were manually administered a 200-μl olive oil load via oral gavage. The bolus of Triton-WR1339 was given after feeding, and blood samples were drawn as described above.

Immunoprecipitation and immunoblotting. Enterocytes were lysed, and immunoprecipitation/immunoblotting protocols were performed as previously described (27,28,33).

Preparation of triglyceride-rich lipoprotein and assessment of total apoB. To isolate the triglyceride-rich lipoprotein fraction, blood samples were centrifuged at 4°C for 15 min at 5,000 rpm, and the plasma layer was separated. Then, 150 μl of plasma was centrifuged at 122,000*g* for 70 min at 15°C, using a SW 55 Ti rotor (Beckman Coulter, Mississauga, ON, Canada), with the sample overlaid with 4 ml KBr solution (density = 1.006 g/ml). The top layer (300 μl) containing the triglyceride-rich lipoprotein was removed. Samples were resolved in 5% SDS-PAGE and subjected to immunoblotting using hamster apoB antiserum, and apoB bands were visualized and quantified using an imaging densitometer. To determine the total plasma apoB48, the plasma samples were diluted (1:200 fold dilution) and then treated as described above.

Metabolic labeling of intact primary enterocytes. Primary enterocytes were isolated from hamsters and were used for pulse-chase experiments as previously described (27). Enterocytes were first treated with TNF-α (10 ng/ml) in methionine-free minimal essential medium at 37°C for 45 min and then used for pulse-chase experiments as previously described (27,28).

ApoB and microsomal triglyceride transfer protein mRNA abundance. Enterocytes were treated with TNF-α (10 ng/ml) at 37°C for 4 h. Total RNA was isolated using Trizol reagent (Invitrogen, Gaithersburg, MD). The RNA concentration was calculated from the absorbance at 260 nm. The primers used for PCR were: apoB 5′-AGATGCCAACCTGGATTTCTTA-3′ and 5′-CCATATG GAGAAATCCTTCAGC-3′, microsomal triglyceride transfer protein (MTP) 5′-GTCAGGAAGCTGTGTCAGAATG-3′ and 5′-CTCCTTTTTCTCTGGCTTT TCA-3′, and 18S 5′-TAAGTCCCTGCCCTTTGTACACA-3′ and 5′-GATC CGAGGGCCTCACTAAAC-3′. mRNA levels were assessed by real-time quantitative RT-PCR using an ABI Prism 7700 sequence detector. All PCRs were performed in a total volume of 50 μl and included the following components: cDNA derived from 10 ng of total RNA, 400 nmol/l of each primer, RNase-free water, and 25 μl of SYBR Green PCR Master Mix (ABI), an optimized buffer system containing AmpliTaq Gold DNA polymerase and dNTPs. All PCRs were performed in duplicate, and cycling parameters were as follows: after an initial denaturation step for 10 min at 95°C, 40 subsequent cycles were performed in which samples were denatured for 15 s at 95°C followed by primer annealing and elongation at 60°C for 1 min. Relative quantities of mRNA were normalized by 18S rRNA content.

Effects of anti–TNF receptor-1/2 on TNF-α–induced apoB48 secretion ex vivo. Enterocytes were pulse-labeled as previously described (27) with minor alterations. Briefly, enterocytes were pretreated with antibodies against p55 TNF-α receptor-1 and p75 TNF-α receptor-2 for 30 min and then stimulated with TNF-α (10 ng/ml) for 30 min in methionine-free Dulbecco's modified Eagle's medium at 37°C. Cells were [^{35}S]methionine-labeled for 60 min. The cells and media were harvested and lysed, and apoB48 was immunoprecipitated as previously described (27).

Enterocyte TNF-α and SB203580 treatment. Enterocytes were cultured in Dulbecco's modified Eagle's medium at 37°C and then treated in the absence or presence of TNF-α without or with the p38 MAPK inhibitor SB203580. Dose and treatment time are shown in the figure legends. The phosphorylation of p38 MAPK was evaluated by immunoblotting and analyzed as described above.

Other laboratory methods. Glucose was determined on whole blood using a Glucometer (Sure Step; One Touch LifeScan, Milpitas, CA). Plasma insulin concentrations were determined by radioimmunoassay using a rat insulin kit from LincoResearch (St. Louis, MO). Plasma triglyceride and cholesterol levels were measured enzymatically using kits and following the manufacturer's recommendations (Boehringer Mannheim Diagnostica, Montreal).

Statistical analysis. Statistical significance was calculated using two-tailed paired Student's *t* test analysis or one-way ANOVA. *P* values <0.05 were considered significant.

RESULTS

TNF-α infusion impairs glucose infusion rate and affects intestinal insulin signaling. We investigated the effect of TNF-α on whole-body insulin sensitivity during euglycemic-hyperinsulinemic clamp. There was no significant difference in the blood concentration of glucose between vehicle and TNF-α treatment (the steady-state

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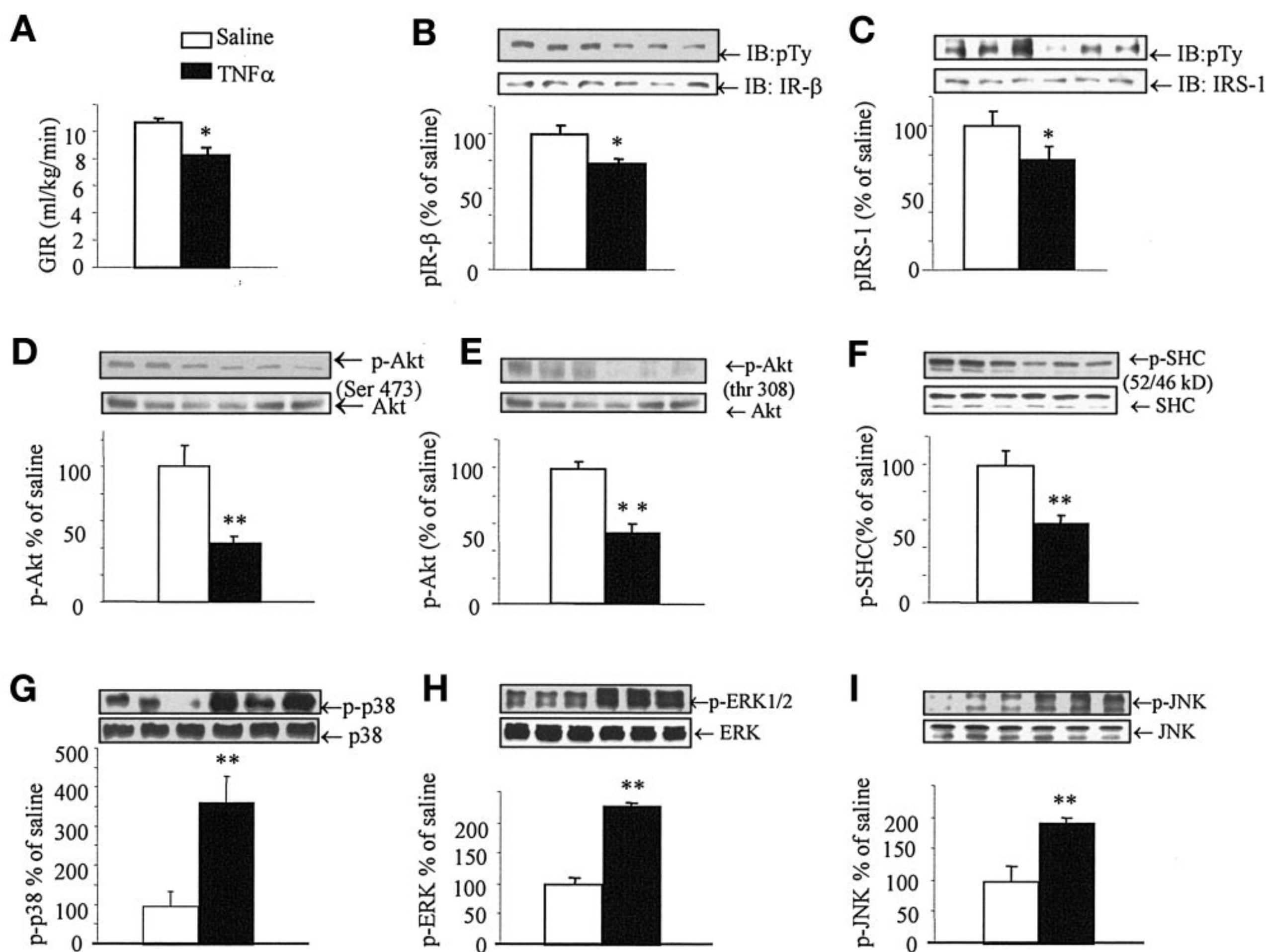

FIG. 1. Effect of TNF-α infusion on in vivo glucose infusion rate and intestinal insulin signaling cascade during euglycemic-hyperinsulinemic clamp. *A*: Effect of TNF-α infusion on in vivo glucose infusion rate during the euglycemic clamp ($n = 4$ for saline and $n = 5$ for the TNF-α group). *B–I*: Effect of TNF-α infusion on the mass and phosphorylation of insulin receptor-β, IRS-1, Akt (S473 and Thr308), Shc, and MAPKs in the intestine of hamsters from the euglycemic clamp study. Tissue lysates were analyzed by immunoblotting with the corresponding antibodies against phosphorylation or total mass. Data are the means ± SE ($n = 3$). *$P < 0.05$ and **$P < 0.01$ vs. control. □, saline; ■, TNF-α. GIR, glucose infusion rate; IP, immunoprecipitation; IB, immunoblotting; IR, insulin receptor; pTy, phosphotyrosine; p, phosphorylation.

insulin levels were 1,400 ± 249 pmol/l for saline vs. 1,320 ± 175 pmol/l for TNF-α). A plateau glucose infusion rate was achieved during the last 30 min of euglycemic clamp. Under these conditions, TNF-α infusion induced a marked decrease in the glucose infusion rate compared with saline-treated controls (8.1 ± 0.4 vs. 10.4 ± 0.3 ml · kg^{-1} · min^{-1}, $P < 0.05$) (Fig. 1*A*).

We also examined the effects of TNF-α on intestinal (small intestine) insulin signaling after euglycemic clamp (Fig. 1*B–I*). The total protein mass of intestinal insulin receptor-β, insulin receptor substrate (IRS)-1 Shc (src homology–containing protein), Akt, p38, extracellular signal–related kinase (ERK)-1/2, and Jun NH_2-terminal kinase (JNK) were not significantly different between saline and TNF-α–treated hamsters. The tyrosine phosphorylation levels of insulin receptor-β and IRS-1 in the intestine of TNF-α–treated hamsters were significantly lower compared with controls (78 ± 2 and 76 ± 6% of saline, respectively; $P < 0.05$) (Fig. 1*B* and *C*). Likewise, decreased phosphorylation of Akt (at residues Ser473 and Thr308) and Shc (52/46 kDa) were observed in TNF-α–treated groups (44 ± 5, 52 ± 7, and 58 ± 5% of saline, respectively; $P < 0.05$) (Fig. 1*D*, *E*, and *F*). Additionally, we examined the effects of TNF-α on MAPKs, including p38, ERK-1/2 (44/42 kDa), and stress-activated protein kinase/JNK (55/46 kDa), all of which have been linked to insulin resistance. Interestingly, the phosphorylation of p38, ERK-1/2, and JNK were all significantly increased (365 ± 68, 225 ± 9, and 192 ± 11% of saline, respectively; $P < 0.01$) (Fig. 1*G*, *H*, and *I*) after TNF-α treatment.

TNF-α infusion increases the accumulation of apoB48-containing lipoproteins in fasted hamsters. To investigate the effect of TNF-α on circulating apoB48-containing lipoproteins, fasted hamsters were infused 4 h with either saline or TNF-α, and plasma was collected at times 0, 30, 60, and 90 min after an intravenous administration of Triton-WR1339, a nonionic detergent that strips triglyceride-rich lipoprotein particles of cofactors and prevents them from being catabolized (34). After detergent treatment, triglyceride-rich lipoprotein particles accumulate in the plasma in a linear fashion, allowing quantitation of the triglyceride-rich lipoprotein production rate in vivo. Plasma cholesterol and triglyceride levels were assessed in the fasting state and after 90-min Triton WR-1339 treatment. The cholesterol and triglyceride levels were significantly increased 1.8- and 7.0-fold, respectively, by 90-min treatment with Triton WR-1339 compared with baseline in control hamsters ($P < 0.001$) (Fig. 2*A*). There was a strong trend for TNF-α–treated hamsters to have higher cholesterol and triglyceride levels, but, perhaps

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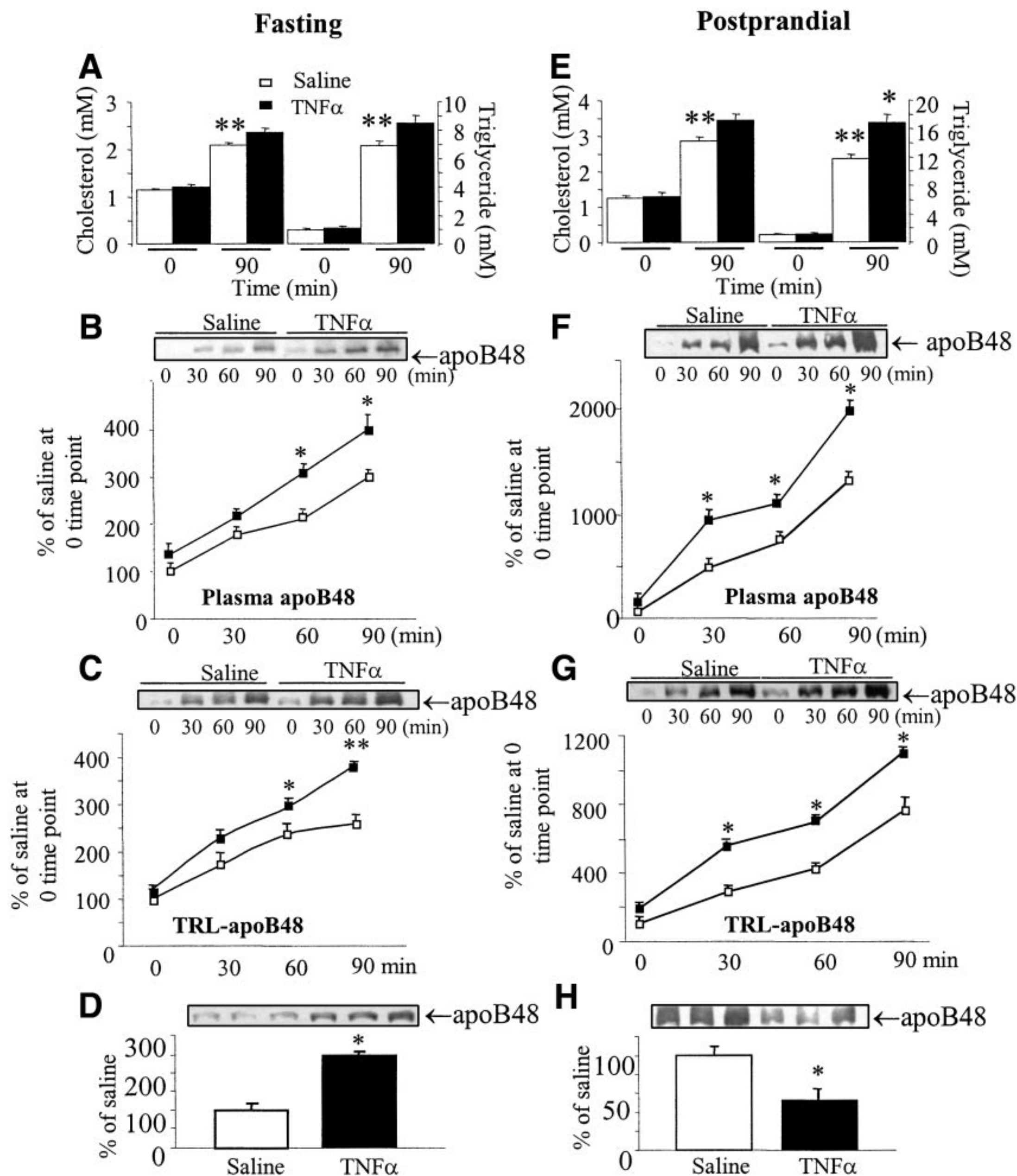

FIG. 2. Effect of TNF-α infusion on plasma cholesterol, triglycerides, and circulating apoB48-containing lipoproteins in the fasting and postprandial state. *A* and *E*: Levels of cholesterol and triglyceride in the fasting and postprandial state, by Triton WR-1339 treatment. Values are the means ± SE (n = 6 for both). *P < 0.05 vs. saline (90-min point); **P < 0.001 vs. 0 time point. The plasma and triglyceride-rich lipoprotein apoB48 were immunoblotted using an anti-hamster apoB primary antibody followed by anti-rabbit horseradish peroxidase secondary antibody. Immunoblots were analyzed using densitometry and expressed as a percentage of time 0 (means ± SE, n = 3, respectively). *B*, *C*, *F*, and *G*: Graphical representation of apoB48 in plasma and the triglyceride-rich lipoprotein fraction of the fasting (*B* and *C*) and postprandial (*F* and *G*) state. *P < 0.05 and **P < 0.001 vs. saline. *D* and *H*: Intestinal tissue apoB48 mass in the fasting and postprandial state. *P < 0.05 vs. saline. □, saline; ■, TNF-α.

because of high interanimal variability, the differences were not statistically significant.

The amount of plasma and apoB48-containing lipoproteins continued to increase throughout the 90-min time period in both groups compared with baseline. There was a significantly higher accumulation of plasma (Fig. 2*B*) and triglyceride-rich lipoprotein apoB48 (Fig. 2*C*) in the TNF-α group compared with controls at the 60- and 90-min time points (P < 0.05 for both). Moreover, TNF-α infusion markedly increased the total apoB48 mass in the intestinal tissue compared with the saline group (250 ± 6% of saline, P < 0.05) (Fig. 2*D*).

TNF-α infusion increases postprandial triglyceride levels and the accumulation of apoB48-containing lipoproteins in hamsters. In vivo postprandial intestinal lipoprotein production was assessed in TNF-α–treated hamsters. Plasma cholesterol and triglyceride levels were significantly increased 2.2- and 11.2-fold after a 90-min treatment with Triton WR-1339 compared with baseline in control hamsters (P < 0.001) (Fig. 2*E*). There was a strong trend for TNF-α infusion–treated hamsters to have higher cholesterol levels, but the difference was not significant. However, TNF-α treatment significantly increased plasma triglyceride levels (P < 0.05) compared with controls. The

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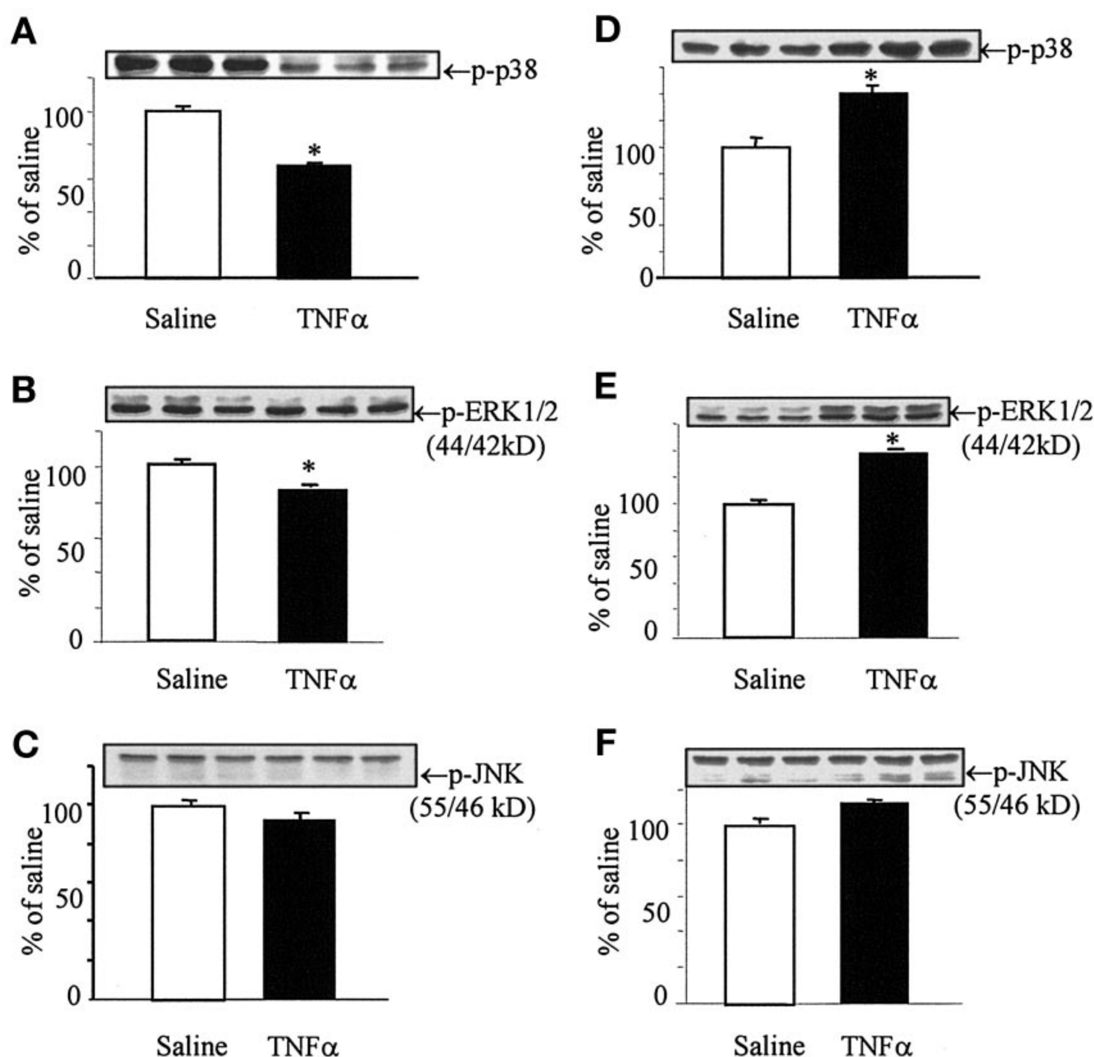

FIG. 3. Effect of TNF-α on the phosphorylation levels of intestinal MAPKs in the fasting and postprandial states. The intestinal tissue from saline- and TNF-α–treated hamsters was immunoblotted using phosphorylation-specific antibodies for p38, ERK-1/2, or JNK. Data are the means ± SE (n = 3, respectively). *P < 0.05 vs. saline. *A–C*: Fasting state. *D–F*: Postprandial state.

levels of total plasma apoB48 (Fig. 2*F*) and triglyceride-rich lipoprotein apoB48 (Fig. 2*G*) were significantly higher in the TNF-α group at the 30-, 60-, and 90-min time points ($P < 0.05$). However, in contrast to the fasting state, we observed a significantly decreased mass of total apoB48 in the intestinal tissue of the TNF-α group (51 ± 12% of saline, $P < 0.05$) (Fig. 2*H*).

Effect of TNF-α infusion on the phosphorylation levels of MAPKs in fasting and postprandial states. MAPK activation is known to be an important step in inflammatory bowel diseases (35) and insulin-resistant states (36,37). However, induction of the MAPK pathway by TNF-α in intestinal tissue has not been previously demonstrated. Here, we investigated the effects of TNF-α on MAPKs in the hamster small intestine. After a 4-h TNF-α infusion and after Triton-WR1339 treatment, the phosphorylation status of p38, ERK-1/2, and JNK were assessed by immunoblotting. In the fasting state, the phosphorylation levels of p38 and ERK-1/2 were significantly decreased by TNF-α (65 ± 3 and 86 ± 2% of saline, respectively; $P < 0.05$) (Fig. 3*A* and *B*). In contrast, in the postprandial state, the phosphorylation levels of p38 and ERK-1/2 were markedly increased by TNF-α (140 ± 9 and 137 ± 3% of saline, respectively; $P < 0.05$) (Fig. 3*D* and *E*), with no significant difference in JNK phosphorylation in either the fasting or postprandial states (Fig. 3*C* and *F*). Additionally, TNF-α infusion did not affect the total mass of MAPKs in either the fasting or postprandial states (data not shown).

In vivo treatment with SB203580 blocks the stimulatory effects of TNF-α on intestinal lipoprotein production. We also examined whether SB203580, an inhibitor of the p38 MAPK pathway, affects TNF-α–induced intestinal lipoprotein overproduction in fasting or postprandial states. We examined the effect of SB203580 infusion on TNF-α–treated hamsters. We observed that SB203580 infusion did not affect the levels of plasma cholesterol, triglycerides (Fig. 4), total plasma apoB48, or triglyceride-rich lipoprotein apoB48 (data not shown) in the saline-treated hamsters in the fasting or postprandial state. Interestingly, SB203580 infusion appeared to inhibit the higher cholesterol and triglyceride levels induced by TNF-α in the fasting state, but not significantly (Fig. 4*A*). Postprandially, SB203580 infusion significantly decreased postprandial triglyceride levels in TNF-α–treated hamsters

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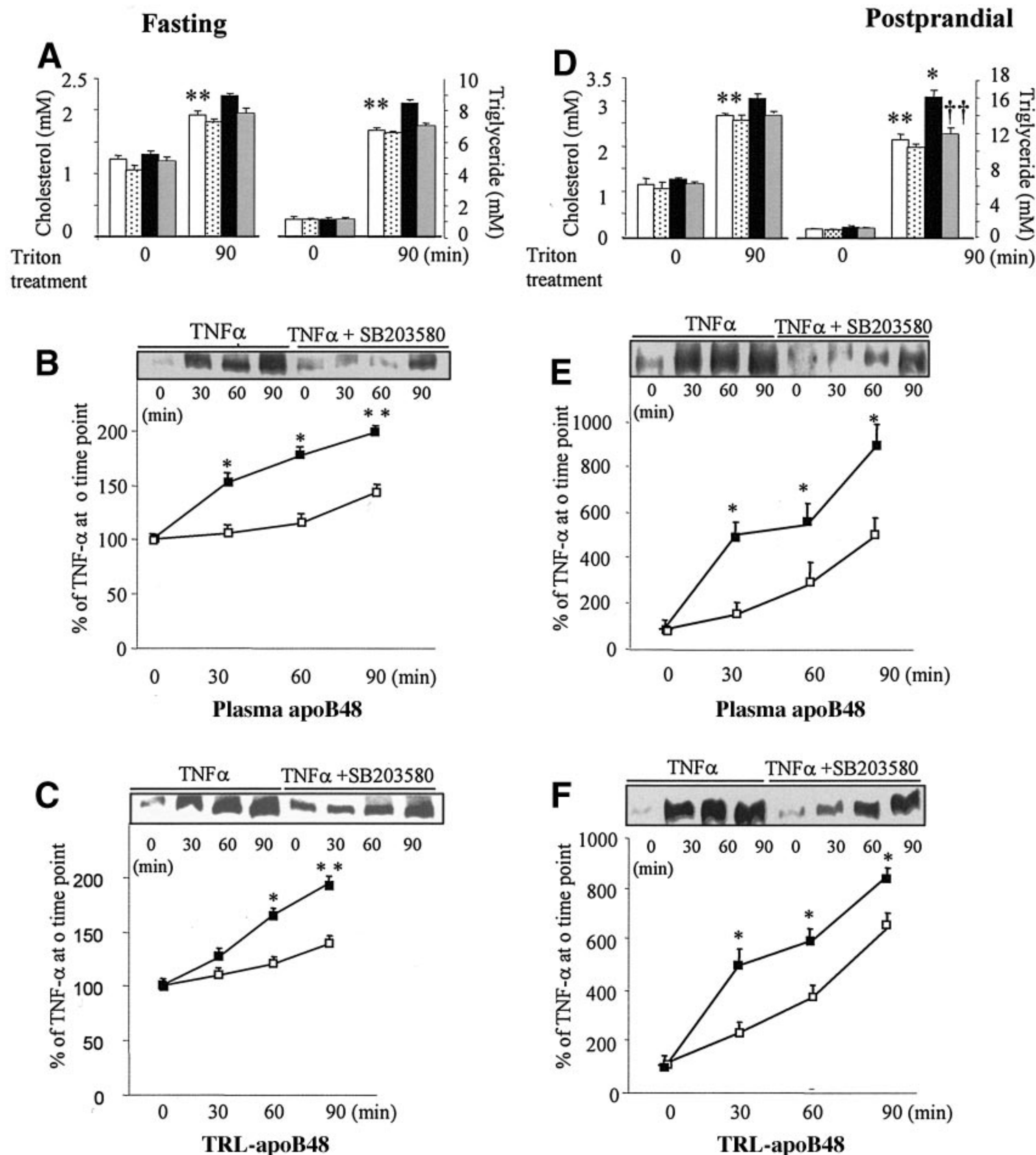

FIG. 4. The p38 MAPK inhibitor SB203580 blocks the stimulatory effects of TNF-α on intestinal apoB48 lipoprotein production in the fasting and postprandial states. Hamsters were manually infused with SB203580 (1.2 ng · kg^{-1} · min^{-1} in saline) or DMSO as control for 4 h. *A* and *D*: Levels of cholesterol and triglyceride in the fasting and postprandial after Triton WR-1339 treatment. Data are the means ± SE (n = 3 for both). Plasma and triglyceride-rich lipoprotein samples were then processed as described in Fig. 2. *B* and *C*: apoB48 levels in the fasting state. *E* and *F*: apoB48 levels in the postprandial state. Data are the means ± SE (n = 3, respectively). *P < 0.05 vs. control (90-min point); ††P < 0.05 vs. TNF-α (90-min point); **P < 0.001 vs. time 0 point.

(Fig. 4*D*); however, the effect on cholesterol was not significant.

SB203580 infusion markedly decreased the overproduction of apoB48 induced by TNF-α in both fasting and postprandial states, as shown in Fig. 4*B–F*. After the 30-min time point, SB203580 showed a significant inhibitory effect on the accumulation of both total plasma apoB48 and triglyceride-rich lipoprotein apoB48. SB203474, an inactive analog of p38 MAPK, did not have any observable effects (data not shown).

TNF-α stimulates intestinal apoB48 secretion ex vivo in primary hamster enterocytes. To investigate whether TNF-α directly affects the production of apoB48-containing lipoprotein, primary enterocytes were isolated from chow-fed hamsters and then incubated ex vivo with TNF-α. Cells were then subjected to pulse-chase labeling experiments to assess the stability and secretion of apoB in control and TNF-α–treated enterocytes. Figure 5*E–G* shows the intracellular turnover (Fig. 5*E*), extracellular secretion (Fig. 5*F*), and total apoB remaining (Fig. 5*G*) of apoB48 in saline- and TNF-α–treated enterocytes. Intracellular apoB levels were significantly higher at the 90-min time point in cells treated with TNF-α compared with both solvent control as well as TNF-α plus inhibitor conditions. As shown in Fig. 5*F*, TNF-α–treated enterocytes secreted newly synthesized apoB48 at significantly higher levels compared with control cells. Additionally, SB203580 markedly inhibited apoB48 oversecretion induced by TNF-α, but SB203580 did not affect the stability or secretion of apoB48 in vehicle-treated enterocytes.

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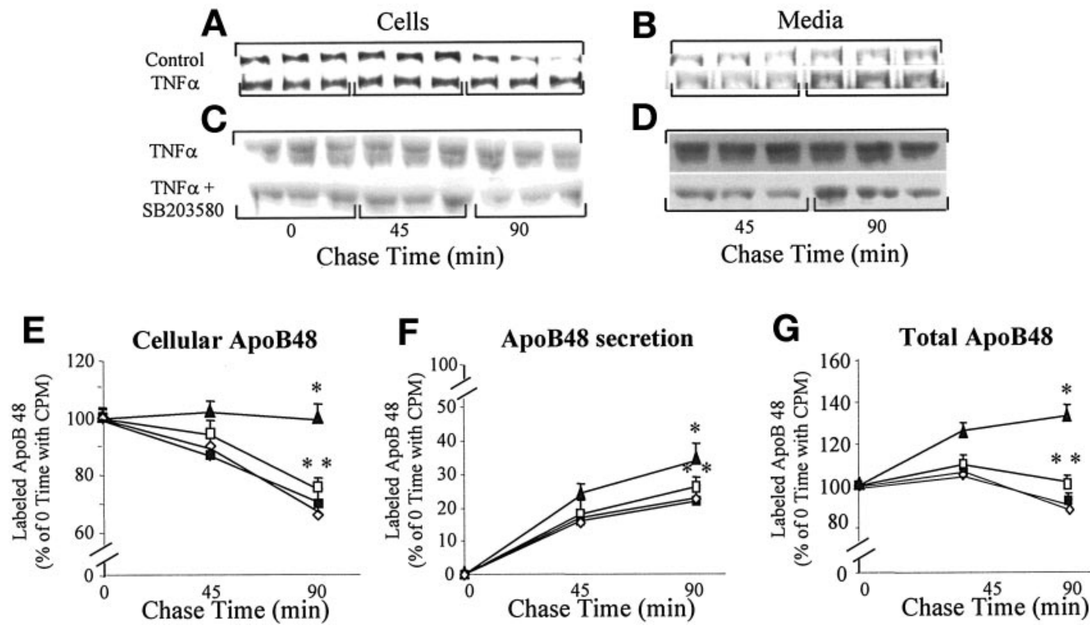

FIG. 5. Ex vivo effect of TNF-α and SB203580 on the synthesis and secretion of apoB48 in primary enterocytes. Primary enterocytes were pretreated with saline or TNF-α (10 ng/ml) for 45 min at 37°C, then were pulsed with [^{35}S]methionine and chased for 0, 45, and 90 min. The media samples and cell lysates collected at each chase time point were subjected to immunoprecipitation and then analyzed by SDS-PAGE. ApoB48 was visualized by autoradiography and quantified by liquid scintillation counting. *A* and *B*: Representative experiment for newly synthesized apoB48 in cells (*A*) and media (*B*) for control vs. TNF-α. *C* and *D*: TNF-α + SB203580 in cells (*C*) and media (*D*). *E*, *F*, and *G*: Distribution of labeled apoB48 counts per minute (CPM) percentage in cells (*E*), media (*F*), and cells plus media (total apoB48) (*G*). Data are the means ± SE. **P* < 0.05 vs. saline; ***P* < 0.05 vs. TNF-α. ◇, control; ■, control + SB203580; ♦, TNF-α; □, TNF-α + SB203580.

When total apoB48 recovery was examined, it appeared that the total labeled apoB recovered under TNF-α–treated conditions was >100% (almost 130%). This may have potentially resulted from some incorporation of radiolabel into newly synthesized apoB48 even during the chase period. TNF-α treatment may have induced continued radiolabel incorporation during the chase period. ApoB48 recovery was almost 100% under TNF-α plus inhibitor conditions, whereas it was slightly lower under saline-treated conditions (~10% lower with saline alone). The data appear to suggest that some intracellular degradation of apoB48 may occur in basal conditions with little or no degradation in cells treated with TNF.

Ex vivo effect of TNF-α on MTP. MTP is an important enabler for secretion of chylomicrons by the intestine (38). Our previous study (34) showed that in the fructose-fed insulin-resistant hamster, an increase in the protein mass of MTP was associated with oversecretion of intestinally derived apoB48 lipoproteins. Furthermore, intestinal MTP mRNA, which reflects MTP activity, is elevated in animal models of both insulin resistance and diabetes (39–41). Here, we investigated the mRNA levels and mass of MTP using RT-PCR and immunoblotting. Our results show that the MTP mRNA and protein mass were markedly increased by TNF-α (144 ± 5 and 140 ± 10% of saline, respectively; $P < 0.05$) (Fig. 6*A* and *B*). In addition, we also found that TNF-α treatment did not affect apoB mRNA levels in enterocytes (data not shown). This observation is well supported by Davidson et al. (42), who found that apoB48 is required for the normal assembly and secretion of triglycerides by the intestine, and the secretion of apoB48 is increased without alterations in its gene expression.

Ex vivo TNF-α treatment increases the activation of intestinal p38 MAPK. To confirm our in vivo findings that SB203580 inhibited TNF-α–induced apoB48 overproduction, we examined the ex vivo effect of TNF-α on p38 MAPK activation. First, we performed a time course of MAPK induction by TNF-α (Fig. 7*A*). TNF-α induced a transient activation of p38 phosphorylation, peaking at 60 min and returning to baseline values within 180 min. Next, we determined the dose-response effect of TNF-α on p38 activation. Stimulation with TNF-α for 30 min induced p38 MAPK activation in a dose-dependent manner, resulting in a 4.4 ± 0.5-fold increase in phosphorylated activated p38 compared with saline-treated controls (Fig. 7*B*). Moreover, treatment with SB203580 blocked TNF-α–induced p38 activation in a concentration-dependent manner (Fig. 7*C*). TNF-α–induced p38 activation was inhibited by 55 ± 8% (an average of two experiments) and completely abolished at 10 and 20 μmol/l, $P < 0.05$ and $P < 0.01$, respectively. Figure 7*D* shows the dose-dependent effect of TNF-α on apoB48 secretion in primary enterocytes.

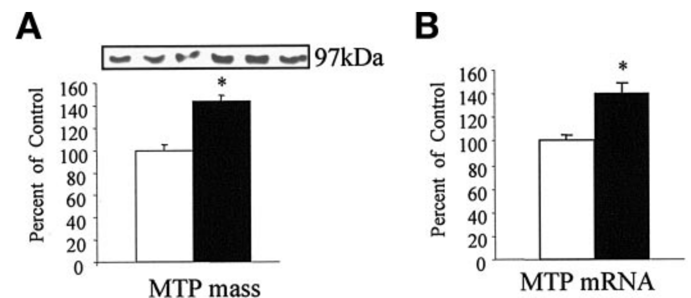

FIG. 6. Effect of TNF-α on the mass and mRNA levels of MTP in primary hamster enterocytes. The cell lysates were prepared from freshly isolated enterocytes, subjected to SDS-PAGE, and immunoblotted with an anti-mouse MTP antibody. The MTP mRNA levels were determined with real-time PCR using cDNA made from 10 ng total RNA as template. The mRNA levels were normalized using the 18s rRNA level in each sample. *A* and *B*: MTP protein mass (*A*) and MTP mRNA (*B*). Data are the means ± SE and are presented as a percentage of control (*n* = 3). **P* < 0.05 vs. control.

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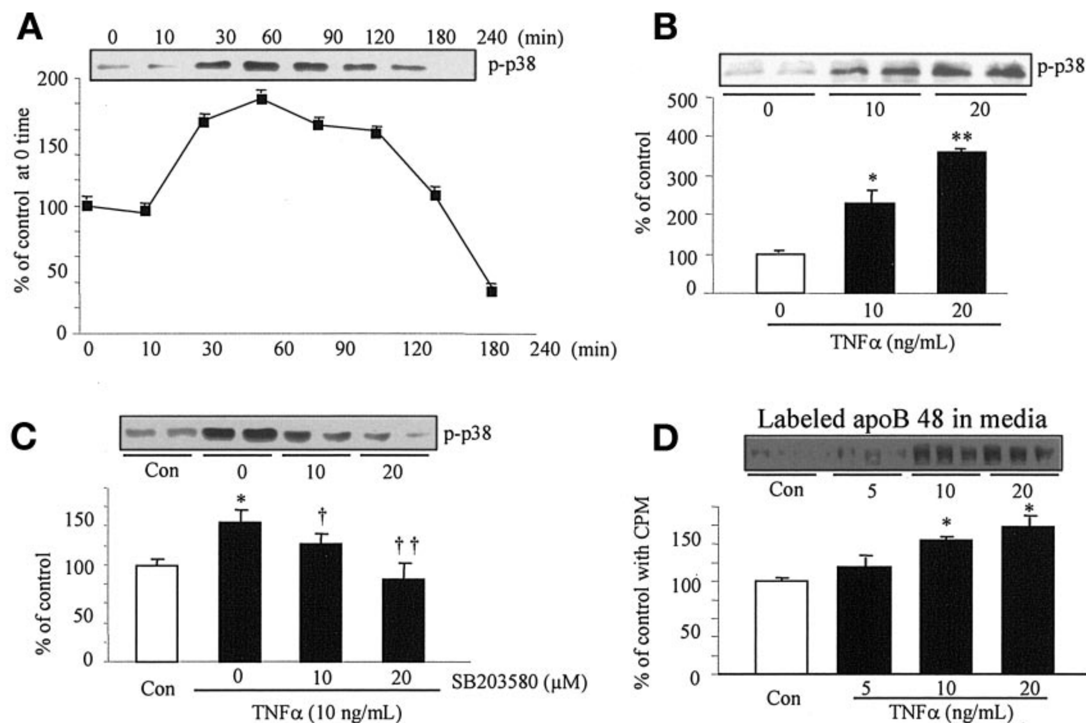

FIG. 7. Time course and dose-dependent effects of TNF-α on the phosphorylation of p38 MAPK. *A*: Enterocytes were stimulated with 10 ng/ml TNF-α for 0–240 min in Dulbecco's modified Eagle's medium at 37°C, and the protein samples were immunoblotted with an anti-phosphorylation p38 MAPK antibody. Immunoblots shown are representative of three experiments with different cell preparations. *B*: SB203580 dose-dependent inhibition of TNF-α–induced p38 MAPK activation. The enterocytes were incubated with the p38 inhibitor SB203580 (10 and 20 μmol/l) for 30 min before adding TNF-α for 30 min, and p38 phosphorylation was measured. Data analysis of two different experiments are shown. *C*: The enterocytes were stimulated with increasing concentrations of TNF-α (10–20 ng/ml) for 30 min. Equal amounts of protein were measured as described above. Results of densitometric analysis of two different experiments are shown. *D*: TNF-α stimulates enterocyte apoB48 secretion in a dose-dependent manner. The enterocytes were pretreated with increasing concentrations of TNF-α (5–20 ng/ml) for 45 min, followed by a pulse-labeling experiment. Media samples were analyzed as described in the RESEARCH DESIGN AND METHODS section. **P* < 0.05 and ***P* < 0.01 vs. saline; †*P* < 0.05 and ††*P* < 0.01 vs. TNF-α. Con, control; CPM, counts per minute.

TNF-α stimulates p38 MAPK phosphorylation and apoB48 production in enterocytes through p55 and p75 TNF-α receptors. To identify the receptor that mediates TNF-α–induced MAPK activation, primary hamster enterocyte lysates were first probed for the presence of TNF-α using specific antibodies. As shown in Fig. 8*A*, both p55 TNF-α receptor-1 and p75 TNF-α receptor-2 were readily detectable in freshly isolated enterocytes. To assess whether TNF-α receptors mediated the effects of TNF-α on p38 phosphorylation and apoB48 levels, cells were pretreated with antibodies against p55 TNF-α receptor-1 and p75 TNF-α receptor-2 and then stimulated with TNF-α (10 ng/ml, 30 min). TNF-α–induced p38 phosphorylation was inhibited by pretreatment with anti–TNF-α receptor-1 ($P < 0.05$). Likewise, anti–TNF-α receptor-2 antibody also impaired TNF-α induced p38 phosphorylation (Fig. 8*B*). These data suggest that TNF-α specifically activates p38 MAPK in enterocytes through both p55 and p75 TNF-α receptors.

Finally, we assessed the effects of antibodies against p55 TNF-α receptor-1 and p75 TNF-α receptor-2 on apoB48 secretion by isolated enterocytes in ex vivo pulse labeling experiments. As shown in Fig. 8*C*, antibodies against both TNF-α receptor-1 and -2 significantly blocked TNF-α–induced enhancement in apoB48 secretion ($P < 0.05$, respectively).

DISCUSSION

In the current study, we examined the effects of TNF-α on intestinal insulin signaling and intestinally derived lipoprotein production. Systemic TNF-α infusion during hyperinsulinemic-euglycemic clamp has been previously shown to induce insulin resistance in rats (43). Moreover, exogenous TNF-α and overexpression of TNF-α in adipose tissue and muscle are known to induce an insulin-resistant state in both animal models and humans (11,31,44). Here, we found that 4-h TNF-α infusion significantly inhibited the glucose infusion rate, as determined by euglycemic clamp studies. TNF-α infusion was also found to alter phosphorylation and/or mass of a number of insulin signaling molecules in the hamster small intestine. TNF-α–induced perturbations in insulin signaling have been well documented in insulin-responsive tissues such as liver, muscle, and adipose tissue (8,45,46). Hotamisligil et al. (11) were the first to demonstrate that TNF-α lowers tissue insulin sensitivity by promoting serine phosphorylation of IRS-1, which in turn causes the serine phosphorylation of insulin receptor. This prevents the normal tyrosine phosphorylation of insulin receptor in adipocytes and thus interferes with phosphorylation of Shc and the downstream activation in vascular smooth muscle cells (47). Other studies have suggested that TNF-α activates MAPKs (p38, ERK-1/2, and JNK), induces insulin resistance, and inhibits insulin signaling by diverse mechanisms in adipocytes (13,48). To date, the majority of investigations have focused on the roles of TNF-α on insulin resistance in liver, adipose, and muscle tissues (8,45,46). However, the effects of TNF-α on intestinal insulin sensitivity and the molecular mechanism(s) by which TNF-α may perturb insulin signaling and lipid metabolism in the intestinal

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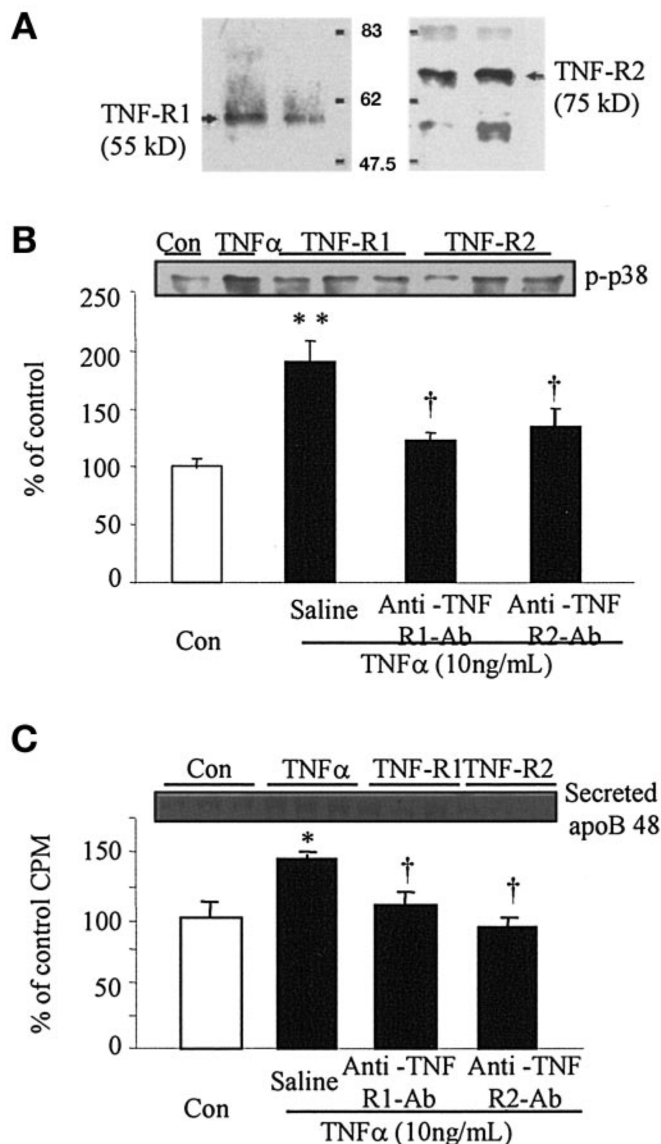

FIG. 8. TNF-α receptors p55 and p75 are present in intestinal enterocytes and mediate the effects of TNF-α on p38 activation and apoB48 production. *A*: Enterocyte extracts were separated on 8% SDS-PAGE gels, followed by immunoblot analysis with antibodies against p55 or p75 TNF-α receptor-1 (TNF-R1) and -2 (TNF-R2). *B*: Enterocytes were pretreated with antibodies against TNF-α receptor-1 and -2 (1:1,000) for 30 min and then stimulated with TNF-α for 45 min in a pulse-labeling experiment. *C*: TNF-α activates MAPK in enterocytes through both TNF-α receptors. The enterocytes were pretreated for 30 min with anti–TNF-α receptor-1 and -2 antibody (1:1,000, respectively) followed by addition of TNF-α. After coincubation with TNF-α and receptor antibodies for 30 min, cells were harvested and immunoblotted with p38 phosphorylation antibody. *P < 0.05 and **P < 0.01 vs. control; †P < 0.05 vs. TNF-α alone. Ab, antibody; Con, control.

tissue are far from clear. This prompted us to investigate the signaling pathways elicited by TNF-α in the intestine. Our results suggest that TNF-α inhibits intestinal insulin receptor-β and IRS-1 tyrosine phosphorylation. This observation is consistent with a number of similar observations in other insulin-sensitive tissues (11,13,48,47).

Interestingly, we also found TNF-α–induced activation of p38 MAPK, ERK-1/2, and JNK. MAPKs are activated by inflammation and have been implicated in the induction of insulin resistance (11,13,48). TNF-α is known to activate the MAPK pathway in endothelial cells and inflammatory cells. Basal phosphorylation of p38 MAPK has also been shown to be increased in skeletal muscle from type 2 diabetic patients (36). However, there have been no reports on intestinal MAPKs in insulin-resistant states and the potential role of TNF-α in the induction of intestinal MAPKs. In our hamster model, TNF-α caused strong activation of both p38 and ERK phosphorylation in postprandial states. In contrast to the postprandial state, TNF-α infusion significantly reduced ERK and p38 phosphorylation in the fasting state. JNK phosphorylation also showed a similar trend (although this did not attain statistical significance). p38 and JNK MAPKs are inflammatory and stress-sensitive kinases. Cross talk is known to exist among MAPK cascades, whereby the activity of one MAPK can be influenced by another. Thus, these proteins collectively integrate the pro-and anti-inflammatory stimuli acting on the cell to produce appropriate downstream effects (49). Taken together, our data suggest that under different conditions (fasting, postprandial, and insulin clamp), TNF-α has various effects on the activity of MAPKs. This raises the possibility that under certain conditions, the release of TNF-α in intestinal tissue may actually exert protective effects by regulating MAPK activation.

Recent studies have shown evidence of intestinal lipoprotein overproduction in both insulin-resistant animals (27) and insulin-resistant humans (50). Here, we postulated that inflammatory cytokines such as TNF-α may play an important role in the induction of intestinal insulin insensitivity and overproduction of apoB48-containing lipoproteins. Both in vivo TNF-α infusion in the hamster as well as ex vivo treatment of isolated hamster enterocytes with TNF-α led to significant increases in apoB48 secretion. TNF-α–induced overproduction of apoB48 lipoproteins was demonstrated not only in response to fat feeding but also in the fasting state. These findings suggest that TNF-α may be an important causative factor in the induction of intestinal overproduction of apoB48-containing lipoproteins in insulin-resistant states, leading to elevation of circulating triglyceride and triglyceride-rich lipoproteins in both fasting and postprandial states. This is the first direct evidence to support the concept that acute inflammatory cytokine infusion may underlie the pathogenesis of intestinal insulin resistance and abnormalities in intestinal lipoprotein production.

It is important to note that TNF-α has been reported to inhibit lipid and lipoprotein transport by Caco-2 cells (51), a human colon carcinoma cell line commonly used for studies of intestinal lipoprotein metabolism. However, the very high dose used in this in vitro study (500 ng/ml) together with a long incubation time of 20 h was likely toxic to the cultured cells. Under these conditions, the authors found decreases in not only apoB100 and apoB48 but also apoAI, suggesting global inhibition of protein secretion. Our in vitro studies used much lower concentrations of TNF-α (10–50 ng/ml) because we have found higher concentrations reduce cell viability. In addition, although Caco-2 has been widely used as a model to study intestinal lipid metabolism, it differs from the adult intestinal absorptive cells in that it secretes predominantly apoB100. ApoB48 is the predominant species of apoB secreted by the human intestine. Although the human intestine synthesizes and secretes small amounts of apoB100, the majority of adult intestinal apoB mRNA is edited (81–97%), resulting in the synthesis and secretion of predominantly apoB48 (52–54). Unlike normal enterocytes, Caco-2 cells produce and secrete both apoB100 and

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apoB48 (51,55). The ratio of secreted B100 to B48 varies somewhat from passage to passage, averaging ~4:1 (55). In the current study, we used an in vivo whole-animal model as well as freshly isolated primary hamster enterocytes. The tissue-specific expression of apoB100 (only in the liver) and apoB48 (only in the intestine) is a distinct advantage of the hamster models in permitting the study of intestinal versus hepatically derived lipoproteins in vivo (56,57). Our data showing TNF-α–induced stimulation of intestinal lipoproteins is in line with several other studies showing cytokine-induced hypertriglyceridemia (58–60). In addition, TNF-α directly stimulates the synthesis of triglycerides in cultured HepG2 cells in vitro (61). Feingold et al. studied in vivo effects of TNF-α and showed considerable elevation in plasma triglycerides after TNF-α administration (17). TNF-α acutely raised serum triglyceride levels in vivo by stimulating VLDL production (17,18). Although the effects were postulated to be caused by hepatic overproduction, their experimental design using Triton WR-1339 could not rule out an intestinal contribution as well.

We also observed that the secretion of apoB48 in TNF-α–treated enterocytes was markedly higher compared with controls, similar to the higher rate of apoB48 production in the fasting state observed in our in vivo Triton-WR1339 experiments. TNF-α infusion not only increased the release of apoB48 into the plasma apoB48, but also increased the total apoB48 protein mass in the intestinal tissue of TNF-α–treated hamsters. This stimulatory effect may be related to increased intracellular stability of apoB48 because there appeared to be a higher recovery of radiolabeled apoB48 in cells treated with TNF-α. Surprisingly, the total labeled apoB recovered in TNF-α–treated conditions was >100% (almost 130%). This may have potentially resulted from some incorporation of radiolabel into newly synthesized apoB during the chase period. TNF-α treatment may have somehow induced continued radiolabel incorporation during the chase period. ApoB recovery was almost 100% under TNF-α plus inhibitor conditions, whereas it was slightly lower under saline-treated conditions (~10% lower with saline alone).

p38 MAPK is well known as a major signaling molecule involved in inflammation, and it is a key regulatory pathway for many genes, including genes regulating TNF-α, interleukin-1β, and TGF-β in a wide variety of cell types (62). Importantly, p38 plays a major role in proinflammatory cytokine production by activating transcription factors binding to the promoter regions of many proinflammatory cytokines, including TNF-α. Moreover, TNF-α is one of the best-characterized agonists of the p38 pathway and is itself regulated by p38 (63,64). Here, we report that TNF-α stimulates p38 phosphorylation and apoB48 secretion in a dose-dependent manner in enterocytes. Interestingly, the pharmacological p38 inhibitor SB203580 was found to block TNF-α–induced p38 phosphorylation and intestinal overproduction of apoB48 in the fasting and postprandial state. These observations were made in both in vivo as well as ex vivo experiments. At higher concentrations, SB203580 was able to completely abolish TNF-α–induced p38 activity, leading to partial inhibition of TNF-α–induced apoB48 oversecretion by enterocytes. Our data clearly suggest that SB203580 inhibits apoB48 overproduction induced by TNF-α through a reduction in the activation of the p38 cascade.

The mechanisms and intracellular signaling pathways leading to apoB48 overproduction by TNF-α are not completely understood. It was also initially unclear whether the signaling and lipoprotein changes observed in the intestine are mediated via TNF-α receptors in intestinal enterocytes. Although many cell types express both TNF-α receptors, TNF receptor-1 and -2 (65,66), the majority of TNF-α–induced signaling events are mediated by TNF-α receptor-1 (67). In the current study, we confirmed the expression of both receptors in the hamster enterocytes and found that neutralizing antibodies to TNF-α receptor-1 or -2 could block TNF-α–induced activation of p38 MAPK as well as apoB48 production. Our data suggest that both TNF-α receptor-1 and -2 mediate TNF-α activation of p38 MAPK as well as TNF-α–induced apoB48 oversecretion by intestinal enterocytes. Previous studies have shown that blocking the activity of TNF-α in vivo in insulin-resistant animal models by infusion of either a TNF-α receptor IgG fusion protein, a soluble TNF-α–binding protein, or a polyclonal anti–TNF-α improves insulin action (10,64,65).

In summary, this study has demonstrated for the first time that an important inflammation factor, TNF-α, is capable of inducing intestinal insulin resistance in the hamster model, accompanied by intestinal overproduction of apoB48-containing lipoproteins in both fasting and postprandial states. TNF-α appears to exert these effects via TNF-α receptors, p55 and p75, and the induction of the p38 MAPK pathway. Based on these observations and previous findings, we postulate that TNF-α may play an important role in the development of intestinal insulin resistance and its associated lipoprotein abnormalities (27,28). Increased TNF-α levels and the induction of an inflammatory state may be at least one mechanism by which high-fructose diets induce insulin resistance at the level of the intestine and lead to overproduction of apoB48-containing lipoproteins in both the fasting and postprandial states. Further direct studies of fructose-fed animal models will be necessary to test this hypothesis.

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