Role of TNF-α in ileum tight junction alteration in mouse model of restraint stress

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Mazzon E, Cuzzocrea S. Role of TNF-α in ileum tight junction alteration in mouse model of restraint stress. Am J Physiol Gastrointest Liver Physiol 294: G1268–G1280, 2008.—Restraint stress induces permeability changes in the small intestine, but little is known about the role of tumor necrosis factor (TNF)-α in the defects of the TJ function. In the present study, we used tumor necrosis factor-R1 knockout mice (TNF-α-R1KO) to understand the roles of TNF-α on ileum altered permeability function in models of immobilization stress. The genetic TNF-α inhibition significantly reduced the degree of 1) TNF-α production in ileum tissues; 2) the alteration of zonula occludens-1 (ZO-1), claudin-2, claudin-4, claudin-5, and β-catenin (immunohistochemistry); and 3) apoptosis (TUNEL staining, Bax, Bcl-2 expression). Taken together, our results demonstrate that inhibition of TNF-α reduces the tight junction permeability in the ileum tissues associated with immobilization stress, suggesting a possible role of TNF-α on ileum barrier dysfunction.

apoptosis; TNF-α-deficient mice; zonula occludens-1; claudin-2; β-catenin; tight junction

STRESS IS DEFINED AS A STATE of disharmony or threatened homeostasis and results in various physiological and behavioral changes (3). It has been suggested that stress influences brain activity and promotes long-term changes in various neural systems. Stress therefore elicits a cluster of neuronal disorders that is implicated in cognitive, endocrinial, and psychiatric problems (39, 40, 56). In addition, a series of studies has demonstrated that stress generally decreases the nociception referred to as a stress-induced analgesia that is considered to be implicated with endogenous opioid systems (30, 54, 56). Moreover, the same authors also demonstrated enhanced intercellular uptake of macromolecules (29), hypothesizing that this mechanism may contribute to the clinically described flare-ups in patients with inflammatory bowel disease after stressful events. In a recent study Mazzon and colleagues (38) have demonstrated that immobilization stress induced an increase in tight junction (TJ) permeability in the rat terminal ileum. These changes were mainly due to modifications and redistribution of the TJ transmembrane protein occludin and of the plaque protein zonula occludens (ZO-1) whereas protein synthesis, at least that of occludin, was not affected by stress (38).

Epithelial and endothelial cell sheets form semipermeable barriers to the passage of cells, molecules, and ions across two compartments of the extracellular space. To achieve this barrier function, cells develop a circumferential seal around the apical pole of the cell, which is part of the junctional complex (15) and is called the TJ or ZO. TJ can finely regulate the passage of molecules through the paracellular pathway (gate function) and are also located precisely at the boundary between distinct apical and basolateral domains of the plasma membranes of polarized epithelial and endothelial cells (fence function).

Thus, TJ have a key role in the function of all epithelia involved in polarized secretion or absorption and in the formation of barriers between different tissue and organ compartments. Furthermore, TJ can be targets of toxins, and TJ modulation may be important in the pathogenesis of diseases and in the therapeutic delivery of drugs across physiological barriers (31, 44, 45, 60, 61). As a result, TJ have been extensively studied at the morphological, functional, and molecular levels. Several studies have contributed to elucidate the molecular structure of TJ (36, 37, 57). The transmembrane portion of TJ contains at least two distinct sets of integral membrane proteins, occludin (molecular mass 88–82 kDa) and claudins (molecular mass 22 kDa) (19, 32, 34, 47, 51).

Activation of epithelial proinflammatory signaling cascades is mediated by tumor necrosis factor (TNF)-α, a prototypic member of a cytokine family that regulates essential biological functions (e.g., cell differentiation, proliferation, survival, apoptosis) and a broad spectrum of responses to stress and injury (2). It is primarily produced by immune cells such as monocytes and macrophages, but it can also be released by many other cell types, including acinar cells. Membrane-bound or soluble TNF-α interacts with two different surface receptors, TNF-α receptor 1 (TNFR1), or p55, and TNF-α receptor 2 (TNFR2), or p75 (1). Although the extracellular domains of TNFR1 and TNFR2 are homologous and manifest similar affinity for TNF-α, the cytoplasmic regions of the two receptors are distinct and mediate different downstream events. Although most cell lines and primary tissues express both isoforms (25), most of the biological activities of TNF-α are mediated through TNF-R1 (20). TNF-R2 is a poor inducer of apoptosis (33), and binding affinities of soluble TNF-α are significantly higher to TNF-R1 (20).

After exposure to TNF-α, target cells may downregulate their responsiveness to the cytokine by shedding the receptors into the circulation. A natural mechanism that has been hypothesized to counteract excessive concentrations of circulating TNF-α (and the subsequent enhanced surface receptor

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activation) is the release of soluble receptors. The two soluble receptor forms (sTNFR1 and sTNFR2) have longer half-lives than TNF-α, and their concentration may reflect TNF-α activity (27).

A primary role for TNF-α in inflammatory process (e.g., sepsis, endotoxic shock, and acute pancreatitis) is suggested by several studies conducted on cell lines, animal models, and human beings (11, 43, 59). In inflammation, overproduction of TNF-α is pivotal in the induction of inflammatory genes, cell death, and endothelial upregulation and in the recruitment and activation of immune cells (52, 62). It has been also regarded as one of the major mediators of systemic progression and tissue damage in severe disease. In addition, it has been demonstrated that TNF-α plays a role in the control of epithelial permeability (5, 22, 24, 35). Moreover, TNF-α at higher concentrations leads to downregulation of ZO-1 protein expression and disturbance in junction localization of ZO-1 protein and functional opening of TJ barrier (9, 35, 53). Moreover, an important increase of plasma TNF-α production was observed at 2 h after immobilization period in mice (42). On the basis of this evidence, we have hypothesized that increased production of TNF-α might lead to structural and functional alterations in restraint stress-induced TJ alteration in mice. Herein, we confirm that restraint stress is associated with decreased expression and function of several TJ proteins in the ileum. Moreover, we also demonstrate that TNF-α plays an important role in the TJ alteration associated with restraint stress.

**MATERIALS AND METHODS**

**Animals.** Mice (4–5 wk old, 20–22 g) with a targeted disruption of the TNF-α-R1 (TNF-α-R1KO) and C57BL/6J wild-type (WT) controls (TNF-α-WT) were purchased from Jackson Laboratories (Charles River, Messina, Italy). The study was approved by the University of Messina Review Board for the care of animals. The animals were housed in a controlled environment and provided with standard rodent chow and water ad libitum. Animal care was in compliance with regulations in Italy (Ministerial Declaration 116192), Europe (O.J. of European Commission L 358/1 12/18/1986), and the United States (Animal Welfare Assurance no. A5594-01, Department of Health and Human Services).

**Experimental protocol.** Immobilization stress was induced in non-fasted TNF-α-R1KO (n = 16) and in TNF-α-WT (n = 16) mice by fixation for 2 h of the four extremities with an adhesive tape under brief ether anesthesia. Because fecal pellet output is known to increase under stress conditions, the feces were collected to document the stress effect during the 2-h stress period. In addition, we also used TNF-α-R1KO (n = 16) and in TNF-α-WT (n = 16) mice as “ether controls,” and the mice were only briefly anesthetized with ether and thereafter allowed to move freely in their cages over the following 2 h. Mice were euthanized after a 2-h observation period that included only a brief initial handling of the animals. All experiments were carried out between 8:00 and 10:00 AM to minimize any effect of the
cycadical rhythm. The stomach of these animals was opened along the greater curvature and examined for macroscopic lesions. For sample preparation see below.

**Histological examination.** After fixation for 1 wk at room temperature in 10% (wt/vol) PBS-buffered formaldehyde, samples were dehydrated in graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ). Thereafter, 70-μm sections were deparaffinized with xylene, stained with Alcian blue-periodic acid-Schiff, and observed in a Dialux 22 microscope (Leitz, Wetzlar, Germany).

**Measurement of cytokines.** TNF-α production was evaluated in the ileum tissues after 2 h of the restraint period as previously described (10). The assay was carried out by use of a colorimetric commercial ELISA kit (Calbiochem-Novabiochem, Milan, Italy) with a lower detection limit of 10 pg/ml.

**Immunohistochemical localization of TNF-α, Bax, Bcl-2, occludin, ZO-1, and β-catenin.** Tissues were fixed in 10% (wt/vol) PBS-buffered formaldehyde, and 50-μm sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% (vol/vol) hydrogen peroxide in 60% (vol/vol) methanol for 30 min. Nonspecific adsorption was minimized by incubating the section in 2% (vol/vol) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with biotin and avidin (DBA, Milan, Italy), respectively. Sections were incubated overnight with anti-TNF-α antibody (Santa Cruz; 1:100 in PBS wt/vol), 2) anti-Bax antibody (Santa Cruz; 1:100 in PBS vol/vol), or 3) anti-Bcl-2 antibody (Santa Cruz; 1:100 in PBS vol/vol). After deparaffinization, for occludin, ZO-1, and β-catenin detection, slices were treated with protease (type XIV, Sigma; 2 mg/ml) for 10 min at 37°C. Detection of BCL-2 and Bax was carried out after boiling in citrate buffer, 0.01 M pH 6 for 4 min. Sections were incubated overnight with polyclonal rabbit anti-occludin, ZO-1, and β-catenin antibody (1:100 in PBS, wt/vol). Sections were washed with PBS and incubated with secondary antibody. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (DBA). The counterstain was developed with diaminobenzidine (brown color) and nuclear fast red (red background). To verify the binding specificity, some sections were also incubated with only the primary antibody (no secondary) or with only the secondary antibody (no primary). In these situations no positive staining was found in the sections, indicating that the immunoreaction was positive in all the experiments carried out.

**SEM.** The morphological alteration of gastric mucosa were followed by scanning electron microscopy (SEM). Tissues were fixed (at +4°C in modified Karnovsky fixative (1.5% glutaraldehyde and 1.5% paraformaldehyde in 0.1 M cacodylate buffer), transferred to ice-cold 0.1 M phosphate buffer, and postfixed in 1% OsO4 in 0.1 M cacodylate buffer for 1 h. After thorough rinsing in 0.1 M phosphate buffer, samples were dehydrated in a graded series of ethanol and transferred into liquid CO2 in a critical point dryer. The dried specimens were mounted, sputtercoated with gold, and examined in a SEM at 20 W.

**TUNEL assay.** Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was conducted by using a TUNEL detection kit according to the manufacturer’s instructions (Apopag, HRP kit, DBA). Briefly, sections were incubated with 15 μg/ml proteinase K for 15 min at room temperature and then washed with PBS. Endogenous peroxidase was inactivated by 3% H2O2 for 5 min at room temperature and then washed with PBS. Sections were immersed in terminal deoxynucleotidyl transferase (TdT) buffer containing deoxynucleotidyl transferase and biotinylated dUTP in TdT buffer, incubated in a humid atmosphere at 37°C for 90 min, and then washed with PBS. The sections were incubated at room temperature for 30 min with anti-horseradish peroxidase-conjugated antibody, and the signals were visualized with diaminobenzidine.

**Statistical evaluation.** All values in the figures and text are expressed as means ± SE from 10 mice for each group. For the in vivo studies n represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative at least three experiments (histological or immunohistochemistry coloration) performed on different experimental days on the tissues section collected from all the animals in each group. The results were analyzed by one-way ANOVA followed by a Bonferroni’s post hoc test for multiple comparisons. A P value of less than 0.05 was considered significant.

Fig. 2. Effects of TNF-α gene deletion on restraint stress-induced colon alteration. The histological alteration of colon tissues collected from sham TNF-α-R WT (A) and sham TNF-α-R KO mice (B) was typical of a normal architecture. On the contrary, using an Alcian blue-periodic acid-Schiff stain an important presence of alterations of colon mucosa characterized by the acid mucin secretion was observed in the tissue section from TNF-α-R1WT at 2 h after restraint (C). Colon section from stressed mice lacking TNF-α receptor 1 gene (TNF-α-R1KO) (D) showed significantly less histological alterations. The figure is representative of at least 3 experiments performed on different experimental days.
Fig. 3. Effects of TNF-α gene deletion on restraint stress induced ileum alteration. Distal ileum section from sham TNF-α-R WT (A) and sham TNF-α-R KO mice (B) demonstrating the normal architecture of the intestinal epithelium and wall. Distal ileum section from stressed TNF-αWT mice showed an important presence of acid mucin secretion assessed with an Alcian blue-periodic acid-Schiff stain (C). On the contrary, ileum section from mice lacking TNF-α receptor 1 gene (TNF-α-R1KO) (D) showed significantly less histological alteration. The figure is representative of at least 3 experiments performed on different experimental days.

Fig. 4. Effects of TNF-α gene deletion on restraint stress-induced TNF-α production in the ileum (A) and plasma (B). TNF-α production was evaluated in the serum by use of a colorimetric commercial ELISA kit. Two hours of restraint stress leads to profound TNF-α increase in ileum (A) and plasma (B) in TNF-αWT mice. TNF-α was not detected in the ileum and plasma from stressed TNF-α-R1KO. ND, not detectable. Data are means ± SE of 16 mice for each group. *P < 0.01 vs. Sham. °P < 0.01 vs. TNF-αWT mice.
RESULTS

Effects of TNF-α-R1 gene deletion on fecal pellet output induced by restraint stress. A significant increase in fecal pellet output was observed in TNF-α-R1WT during the 2-h immobilization period compared with the corresponding sham-operated mice (Table 1). The fecal pellet output were significantly reduced in the absence of a functional TNF-α-R1 gene in TNF-α-R1KO mice at 2 h after immobilization (Table 1). Please note that in sham TNF-α-R WT and TNF-α-R KO mice no difference in the in fecal pellet output was observed (Table 1).

Effects of TNF-α-R1 gene deletion on stomach alteration induced by restraint stress. The stomach alteration was studied by SEM observation at 2 h after restraint. Marked ultrastructural changes characterized by ulcer microlesion in the appearance of surface epithelial cells of the stomach (see arrow Fig. 1B) were observed in the tissues from TNF-α-R1WT after 2 h of restraint. On the contrary, in immobilized TNF-α-R1KO mice, no stomach ultrastructural changes (Fig. 1C) were observed. Please note that in sham TNF-α-R WT (data not shown) and TNF-α-R KO (Fig. 1A) mice the ultrastructural of the stomach were typical of a normal architecture.

Effects of TNF-α-R1 gene deletion on colon and ileum alteration induced by restraint stress. Light microscopy with Alcian blue-periodic acid-Schiff stain revealed alterations of colon (Fig. 2C) and ileum (Fig. 3C) mucosa characterized by the acid mucin secretion in TNF-α-R1WT at 2 h after restraint. On the contrary, in immobilized TNF-α-R1KO mice, no colon (Fig. 2D) and ileum (Fig. 3D) mucosa alteration were observed. Please note that in sham TNF-α-R WT and TNF-α-R KO mice, the histological structure of the colon (Fig. 2, A and B, respectively) and ileum (Fig. 3, A and B, respectively) were typical of a normal architecture.

Fig. 5. Immunohistochemical localization of TNF-α in the ileum. No positive staining for TNF-α was observed in the ileum tissues collected from sham WT mice (A) and from sham TNF-α-R1KO mice (B). On the contrary, tissue sections obtained from WT animals after a 2-h stress period (C) demonstrate positive staining for TNF-α mainly localized in the infiltrated inflammatory cells. In immobilized TNF-α-R1KO mice, no positive staining for TNF-α was observed in the ileum tissues (D). The figure is representative of at least 3 experiments performed on different experimental days.
**Effects of TNF-α gene deletion on ileum TNF-α production induced by restraint stress.** To test whether TNF-α gene may modulate the structural and functional alterations in ileum TJ function in vivo, we analyzed the levels of this proinflammatory cytokine in TNF-α-R1KO and WT mice. A substantial increase of TNF-α production was found in ileum tissues (Fig. 4A) as well as in the plasma (Fig. 4B) collected from WT mice at 2 h after immobilization. No detectable TNF-α production was found in the ileum tissues (Fig. 4A) as well as in the plasma (Fig. 4B) from immobilized TNF-α-R1KO. Therefore, we also evaluate the TNF-α expression in the ileum tissues by immunohistochemical detection. No positive staining for TNF-α was observed in the ileum tissues collected from sham WT mice (Fig. 5A) and from sham TNF-α-R1KO mice (Fig. 5B). On the contrary, tissue sections obtained from WT animals at 2 h after immobilization (Fig. 5C) demonstrate positive staining for TNF-α mainly localized in the inflammatory cells. In immobilized TNF-α-R1KO mice, no positive staining for TNF-α was observed in the ileum tissues collected at 2 h after immobilization (Fig. 5D).

**Effects of TNF-α gene deletion on apoptosis in the ileum induced by restraint stress.** To test whether functional TNF-α gene plays a role on apoptosis in ileum tissues during restraint stress, we measured TUNEL-like staining in the ileum tissues at 2 h after restraint stress. Almost no apoptotic cells were detected in the ileum from sham WT mice (Fig. 6A) and from sham TNF-α-R1KO mice (Fig. 6B). At 2 h after immobilization (Fig. 6C), ileum tissues from immobilized WT mice demonstrated a marked appearance of dark brown apoptotic cells and intercellular apoptotic fragments. The presence of apoptotic cells or fragments was significantly reduced in the absence of a functional TNF-α-R1 gene in TNF-α-R1KO mice at 2 h after immobilization (Fig. 6D).
Moreover, samples of ileum tissue were taken at 2 h after immobilization to determine the immunohistological staining for Bax and Bcl-2.

No positive staining for Bax was observed in the ileum tissues collected from sham WT mice (Fig. 7A) and from sham TNF-α-R1KO mice (Fig. 7B). On the contrary, tissue sections obtained from immobilized WT mice at 2 h after restraint stress (Fig. 7D) demonstrate positive staining for Bax. In immobilized TNF-α-R1KO mice, no positive staining for Bax were observed in the ileum tissues collected at 2 h after restraint stress (Fig. 7D).

In addition, ileum tissues collected from sham WT mice (Fig. 8A) and from sham TNF-α-R1KO mice (Fig. 8B) demonstrated Bcl-2 positive staining whereas in the ileum from immobilized WT mice the staining was significantly reduced at 2 h after restraint stress (Fig. 8C). In immobilized TNF-α-R1KO mice, a regular presence of Bcl-2 distribution was observed in the ileum tissues collected at 2 h after restraint stress (Fig. 8D).

Effects of TNF-α gene deletion on epithelial/endothelial function during restraint stress. During the inflammatory process, tissue permeability is modified in part by changes in TJs (16, 50). Since ZO-1 is implicated in TJ regulation and was used as a marker of cellular barrier integrity (28), we performed immunohistochemistry experiments to evaluate ZO-1 distribution in ileum sections. Results indicate that, in the ileum tissues collected from sham WT mice (Fig. 9A) and from sham TNF-α-R1KO mice (Fig. 9B), ZO-1 was uniformly and continuously distributed along the epithelium and the vascular endothelium. In contrast, a significant disruption of immunosignal for ZO-1 was observed along the epithelium and the vascular endothelium in the ileum sections of immobilized WT mice.
mice at 2 h after restraint stress (Fig. 9C). In the ileum of from immobilized TNF-α-R1KO mice, a more regular distribution pattern of ZO-1 along the epithelium and the vascular endothelium was found at 2 h after restraint stress (Fig. 9D), thus indicating that in immobilized TNF-α-R1KO mice restraint stress provokes a lower degree of disorganization of ZO-1.

In addition, results indicate that in the ileum tissues collected from sham WT mice and from TNF-α-R1KO mice, occludin (Fig. 10, A and B, respectively) and β-catenin (Fig. 11, A and B) were uniformly and continuously distributed along the epithelium and the vascular endothelium. On the contrary, a significant disruption of immunohistochemical localization signal for occludin (Fig. 10C) and β-catenin (Fig. 11C) was observed in the ileum tissue sections of immobilized WT mice at 2 h after restraint stress. In ileum from immobilized TNF-α-R1KO mice we found a less irregular distribution pattern of occludin (Fig. 10D) and β-catenin (Fig. 11D) at 2 h after restraint stress.

DISCUSSION

The normal functioning of the lungs, liver, kidneys, and intestine, among other organs, depends on the establishment and maintenance of compositionally distinct compartments that are lined by sheets of epithelial cells (4). An essential element in this process is the formation of TJs between adjacent cells making up the epithelial sheet (21–23, 63). The TJ serves as a fence that differentiates the cytosolic membrane into apical and basolateral domains. This fence function is essential for establishing and maintaining cellular polarity (21–23, 63). Transcellular vectorial transport processes, which depend on proper cellular polarization, generate distinct internal environments in the opposing compartments defined by the epithelial sheet (21–23, 63). In addition, the TJ acts as a regulated semipermeable barrier that limits the passive diffusion of solutes across the paracellular pathway.
Fig. 9. Immunohistochemical localization of zonula occludens (ZO)-1 in the ileum. In tissue sections collected from sham WT mice (A) and from sham TNF-α-R1KO mice (B), ZO-1 was uniformly and continuously distributed along the intestinal epithelium and the vascular endothelium. In contrast, a significant disruption of immunosignal for ZO-1 was observed along the intestinal epithelium and the vascular endothelium in the ileum sections of immobilized WT mice at 2 h after restraint stress (C). In the ileum of immobilized TNF-α-R1KO mice, a more regular distribution pattern of ZO-1 along the epithelium and the vascular endothelium was found at 2 h after restraint stress (D). The figure is representative of at least 3 experiments performed on different experimental days.

between adjacent cells (21–23, 63). Thus the barrier function of the TJ is necessary to prevent dissipation of the concentration gradients that exist between the two compartments defined by the epithelium (21–23, 63). In some organs, notably the gut and the lung, this barrier function is also important to prevent systemic contamination by microbes and toxins that are present in the external environment.

The TJ is a complex of several integral membrane proteins and peripheral membrane proteins that interact strongly with the cytoskeleton (19). Integral membrane proteins involved in TJ formation include occludin and members of a large class of proteins called claudins (19). Cosedimentation assays of TJ proteins suggest that there is a strong interaction between occludin and another protein associated with TJ formation, ZO-1 (14, 41). ZO-1 has been shown to interact with the cytoplasmic tails of occludin and the claudins. In addition, ZO-1 interacts with two additional members of the membrane-associated guanylate kinase family of proteins, ZO-2 and ZO-3 (18). The TJ serves as a fence that differentiates the plasma membrane into apical and basolateral domains (64). TJs also serve as a regulated semipermeable barrier that limits the passive diffusion of solutes across the paracellular pathway between adjacent cells (4). These properties of TJs, in combination with transcellular vectorial transport processes, generate distinct internal environments in the opposing compartments.

Recently, we have demonstrated, using an experimental model of colitis, that the structure and function of TJs in the intestinal epithelial is dependent of the production of proinflammatory cytokines (e.g., TNF-α) (36). In particular in one study, we have showed that the structure and function of TJs in the ileum were altered in mice at 4 days after the induction of experimental colitis (36). These changes were ameliorated when mice were treated with thalidomide.
Moreover, Mazzon and colleagues (38) have demonstrated that a 2-h period of immobilization of the four extremities in rats induced an increase in fecal pellet output and a reduction in goblet cell count in the upper compartments of the colonic mucosa together with vacuolization of crypt bottom goblet cells, indicating an effective stressful event (38). In addition, in this study it has been also demonstrated that these changes were associated with an alteration in the immunofluorescence signals of both junctional proteins investigated (occludin and ZO-1).

In the present study, we showed that the structure and function of TJs in the ileum were altered in mice subjected to 2-h period of immobilization of the four extremities. Moreover, these changes were ameliorated in mice with a targeted deletion of the TNF-α-R1 gene. As a result of these efforts, we point out that restraint stress-induced alterations in the function of ileum are due, at least in part, to massive changes in the expression and localization of key TJ proteins. Furthermore, we propose that excessive TNF-α production is an important component of this process. What is then the mechanism by which inhibition of TNF-α induced the structure and function of TJs alteration in restraint stress?

There is good evidence that TNF-α help to propagate the extension of a local or systemic inflammatory process (17, 58). Moreover, Nukina and colleagues have also clearly demonstrated that restraint stress induced a significant increase of plasma levels of TNF-α at 2 h after the immobilization (4). Similarly, we confirm here a that 2-h period of immobilization of the four extremities induced a substantial increase of TNF-α in the plasma from TNF-α R1WT that likely contribute in different capacities to the evolution of ileum functional alteration. As expected, the TNF-α levels were abolished in TNF-α-R1KO mice. Moreover various study have clearly point out that the immune system plays an important role in modulating

![Image](AJP-Gastrointest Liver Physiol • VOL 294 • MAY 2008 • www.ajpgi.org)
epithelial permeability in different organs such as gut and lung (6, 7). The regulation of epithelial barrier function by cytokines has been studied most extensively in the gut by using different epithelial cell lines. For examples some studies have demonstrated that two cytokines, interferon-γ (IFN-γ) and TNF-α, decreased barrier function of cultured intestinal epithelial monolayers (55, 65). Moreover, incubation of intestinal epithelial cell monolayers with both IFN-γ and TNF-α leads to reorganization of many TJ proteins, including ZO-1, junction adhesion molecule 1, occludin, claudin-1, and claudin-4 (5). In the present study, we also demonstrate that restraint stress in TNF-α R1WT mice induced an alteration of immunohistochemical localization signal for ZO-1 and occludin at 2 h after immobilization. The genetic inhibition of TNF-α abolished the alteration of TJ permeability. Thus a key step in the pathogenesis of restraint-induced ileum functional alteration may be myosin light chain kinase activation by TNF-α, leading to epithelial barrier dysfunction. There is extensive literature examining the consequences of specific proinflammatory cytokines, particularly TNF-α, on the induction of apoptosis. An important biological effect of TNF-γ is the induction of apoptosis through its interaction with its receptor, TNFR. Recently we have demonstrated that a significant expression of FAS ligand was observed in the ileum from TNF-α R1WT mice after ischemia and reperfusion (12). Similarly, we have demonstrated that restraint stress induced a significant expression of FAS-ligand and that the genetic inhibition of TNF-α-R1 attenuates the degree of FAS-ligand expression apoptosis. Thus the activation of proinflammatory signaling pathways can elicit apoptotic responses as well as the expression of cytokines. The response of the normal epithelial cell to inflammatory stimuli may consist of activation of both apoptotic and proinflammatory cascades (26). In the present study, using the TUNEL, coloration we have clearly confirmed that TNF-α plays an important role in the induction of apoptosis during restraint stress-induced ileum functional alteration and that the genetic inhibition of TNF-α-R1 attenuates the degree of apoptosis. Moreover, it is well known that Bax, a proapoptotic gene, plays an important role in developmental cell death (13). Similarly, it has been shown that the overexpression of Bcl-2, a known antiapoptotic factor, significantly reduced the ileum tissues as well as intestinal epithelial apoptosis (8, 13, 46). On the basis of these evidences, we have identified proapoptotic transcriptional changes, including upregulation of proapoptotic Bax and downregulation of antiapoptotic Bcl-2 by immunohistochemical staining in the ileum tissues collected from immobilized TNF-α R1WT mice. We report in the present study for the first time that the genetic inhibition of TNF-α-R1 prevents in restraint stress the loss of the antiapoptotic way activation and reduced the proapoptotic pathway activation. Thus we propose the following positive feedback cycle: restraint stress → TNF-α production → apoptosis activation → epithelial injury → TJ alteration → ileum permeability. Inhibition of TNF-α would intercept this cycle at the early time at the level of apoptosis activation. Further studies are needed to address this point. In conclusion, we have demonstrated in vivo that the inhibition of TNF-α attenuates the development of TJ alteration in the ileum in a model of restraint stress in mice.

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