Protective effect of endogenous PPARγ against acute gastric mucosal lesions associated with ischemia-reperfusion

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Wada, Koichiro, Atsushi Nakajima, Hirokazu Takahashi, Masato Yoneda, Nobutaka Fujisawa, Emi Ohsawa, Takashi Kadokawa, Naoto Kubota, Yasuo Terauchi, Nobuyuki Matsushahi, Lawrence J. Saubermann, Noriko Nakajima, and Richard S. Blumberg. Protective effect of endogenous PPARγ against acute gastric mucosal lesions associated with ischemia-reperfusion. Am J Physiol Gastrointest Liver Physiol 287: G452–G458, 2004; 10.1152/ajpgi.00523.2003.—Acute gastric mucosal lesions (AGMLs) are an important cause of gastrointestinal bleeding. Herein, we demonstrate that peroxisome proliferator-activated receptor-γ (PPARγ), a member of a nuclear receptor family, functions as an endogenous anti-inflammatory pathway in a murine model of AGML induced by ischemia-reperfusion (I/R). Treatment with specific PPARγ ligands such as BRL-49653, pioglitazone, or troglitazone was examined for their response to I/R injury. Specific PPARγ ligands exhibited dramatic and rapid protection against AGML formation associated with I/R in mice in a dose-dependent manner. In contrast, the AGML induced by I/R in PPARγ-deficient mice was more severe than that observed in wild-type mice. Administration of the PPARγ ligand significantly inhibited the upregulation of TNF-α, ICAM-1, inducible nitric oxide synthase, apoptosis, and nitrotyrosine formation induced by I/R in the stomach. These data indicate that an endogenous pathway associated with PPARγ plays an important role in the pathogenesis of I/R-associated injury in the stomach.

peroxisome proliferator-activated receptor-γ nitrotyrosine; ICAM-1

ALTHOUGH GREAT PROGRESS has been made in the prevention and therapy of acute gastric mucosal lesions (AGML), AGML is still recognized as an important cause of gastrointestinal bleeding (21). Moreover, the pathogenesis of AGML has yet to be fully elucidated. AGML is frequently observed in clinical situations, such as stress-induced gastric ischemia-reperfusion (I/R) injury, a major cause of AGML. Low blood flow with subsequent reflow induces the generation of reactive oxygen species (ROS), such as superoxide radicals, hydroxyl radicals, and peroxynitrite. These ROS attack and damage the gastric mucosa leading to the formation of AGMLs (19, 22, 23). I/R injuries continue to be important clinical problems in addition to their contribution to the formation of AGMLs (7). In the gastrointestinal tract, I/R injuries are associated with significant morbidity and mortality during the course of hemorrhagic shock, abdominal aortic aneurysm repair, ischemic bowel disease, and necrotizing enterocolitis (7, 8, 25). It also remains unclear whether the pathophysiological mechanisms associated with I/R-induced AGML are also involved in the generation of stress-related gastritis (7, 19, 21).

Peroxisome proliferator-activated receptor-γ (PPARγ) is one of the nuclear receptors involved in adipocyte differentiation and insulin sensitivity (9, 12). PPARγ is activated by several ligands including 15-deoxy-Δ12,14-prostaglandin J2 and thiazolidinediones (long-term thiazolidinediones, such as troglitazone, pioglitazone, and rosiglitazone (BRL-49653) that are used as oral anti-hyperglycemic agents in the therapy of diabetes mellitus (5, 9). Recently, our group and others (3, 14, 20, 26) reported that PPARγ plays an anti-inflammatory role in intestinal inflammation associated with administration of either dextran sodium sulfate or trinitrobenzene sulfonic acid and exposure to I/R injury.

On the basis of these observations, we investigated whether PPARγ had a similar protective effect in the AGML induced by I/R. Specifically, we assessed PPARγ ligands such as BRL-49653, pioglitazone, or troglitazone in an I/R-induced AGML model involving clamping the celiac artery that is commonly used by many investigators for assessing such responses (15, 16). Furthermore, to clarify the role of endogenous PPARγ in the stomach, we also examined the I/R-induced AGML model in heterozygous PPARγ-deficient (+/−) mice (13). Results from these investigations provide strong evidence supporting an important role for endogenous PPARγ activity in the pathogenesis of I/R injury in the stomach.

MATERIALS AND METHODS

Animals. BALB/c mice were purchased from Charles River Japan (Yokohama, Japan). PPARγ-deficient mice were generated by our group and are previously described (13). Because homozygous PPARγ-deficient embryos (−/−) die due to placental dysfunction, we used heterozygous PPARγ-deficient mice (+/−) in this study as previously described (14).

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Reagents. The PPARγ-specific agonist BRL-49653 was a kind gift of SmithKlineBeecham (Tokyo, Japan). Pioglitazone was kindly provided by Takeda (Osaka, Japan). Troglitazone was kindly provided by Sankyo (Tokyo, Japan). All other chemicals were of reagent grade.

I/R-induced AGML. All animal experiments were performed in accordance with The Guidelines for Animal Experimentation of the Yokohama City University School of Medicine. Adult male BALB/c mice, heterozygous PPARγ-deficient mice (+/−), or wild-type control mice (+/+ ) (weighing 20–25 g) were fasted for 12–14 h before all experiments, except for free access to water. I/R of the stomach was produced by the occlusion of the celiac artery as previously described (15, 16, 22, 23). Briefly, under pentobarbital sodium (50 mg/kg ip) anesthesia, the celiac artery was occluded with a small clamp (Sugita standard aneurysm clip, holding force: 140–150 g; Mizuho Ikakogyo, Tokyo, Japan) for 30 min. Reperfusion was achieved by removing the clamp for 60 min. Sham-operated animals underwent the same surgical procedure without clamping. BRL-49653 (1–10 mg/kg ip), pioglitazone (10 mg/kg ip), and troglitazone (30 mg/kg ip) were administered 15 min before ischemia. Mice were killed, and tissue samples were collected.

Evaluation of injury. A picture of the whole stomach was obtained and analyzed with a digital imaging analyzer (model FAS3; Toyobo, Osaka, Japan). Areas of macroscopic damage associated with the AGML (recognized as reddish bleeding areas by the computer system) were measured and summed together as the total injured areas (mm²) using the National Institutes of Health Imaging system. Evaluation of the macroscopic stomach damage was performed according to a method described previously (23).

Histological analysis. Tissue samples were fixed in 3.7% formaldehyde-PBS overnight at 4°C. Samples were dehydrated and embedded in paraffin. Three-micrometer sections were stained with hematoxylin and eosin.

Immunohistochemical staining of PPARγ, ICAM-1, inducible nitric oxide synthase, and nitrotyrosine. Expression and localization of PPARγ in tissues was detected by standard immunohistochemical techniques using the anti-PPARγ polyclonal antibody (Cayman Chemical, Ann Arbor, MI) on paraffin sections. The vectastain ABC kit (Vector Laboratories, Burlingame, CA) was used with the 3,3’-diaminobenzidine substrate kit (Vector Laboratories) according to the manufacturer’s instructions for detection as previously described (11, 14).

Alterations of ICAM-1, inducible nitric oxide synthase (iNOS) expression, and nitrotyrosine deposition in tissues were detected by standard immunohistochemical techniques using either anti-ICAM-1, anti-iNOS polyclonal antibody (Santa Cruz Biotech, Santa Cruz, CA), or anti-nitrotyrosine antibody (mouse anti-nitrotyrosine, clone HM11; Zymed Laboratories) on paraffin sections. The vectastain ABC kit (Vector Laboratories) was used with the 3,3’-diaminobenzidine substrate kit (Vector Laboratories) according to the manufacturer’s instructions for detection.

Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling staining. Alterations of apoptotic cells in the tissues was detected by standard immunohistochemical techniques using the DeadEnd fluorometric terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) System (Promega, Madison, WI)

RNA protection assay for TNF-α expression. RNA protection assays for quantification of TNF-α expression were performed according to a method described previously (6). Briefly, RNA was extracted from tissues, and 10 μg of total sample RNA was hybridized with a 32P-labeled anti-sense RNA probe. The RibonQuant Multi-Probe RNase Protection Assay System (Pharmingen, San Diego, CA) was used according to the manufacturer’s instructions. Quantification was performed by using a Phosphorimager (Bio-Rad, Hercules, CA) and was normalized to expression of a housekeeping gene (GAPDH).

Statistical analysis. All results are expressed as means ± SE. Statistical comparisons were made by using either Student’s t-test or Scheffe’s method after ANOVA. Results were considered significantly different at P < 0.05.

RESULTS

Effect of PPARγ ligands on I/R-induced AGML. Previous studies have established that PPARγ is present in the stomach, albeit at levels that are much less than in the small and large intestines (14). Therefore, to investigate whether PPARγ ligand was protective in gastric I/R, the effect of a specific PPARγ ligand examined. BALB/c mice were subjected to I/R injury of the stomach in either the presence or absence of the PPARγ ligand BRL-49653. Macroscopic damage was observed in the stomachs of the group subjected to I/R. The total area of damage was significantly diminished by treatment with BRL-49653 in a dose-dependent manner (Fig. 1). Compared with sham-operated animals not subjected to I/R, hemorrhage and a loss of villi were observed in the I/R-injured stomach as defined microscopically (Fig. 2, I/R). Treatment with BRL-49653 dramatically diminished the microscopic alterations observed in the I/R-stomach (Fig. 2, I/R + BRL). Furthermore, apoptotic cells, a characteristic feature of the AGML lesion, were observed in the I/R-injured stomach as defined by the levels of green fluorescence observed by using a fluorometric TUNEL system method (Fig. 3B). Treatment with BRL-49653 (10 mg/kg) dramatically diminished the proportion of apoptotic cells in the I/R-stomach (Fig. 3C). These data demonstrate that the PPARγ agonist BRL-49653 can inhibit AGML formation induced by I/R.

To confirm the effect of BRL-49653 is dependent on PPARγ pathway, we used other PPARγ agonists (pioglitazone and troglitazone) on the AGML model. Treatment with pioglitazone (10 mg/kg) and troglitazone (30 mg/kg) showed the significant inhibition of tissue damages in stomach induced by I/R. The efficacy of troglitazone was much weaker than those
of BRL-49653 and pioglitazone (Fig. 4). These results clearly indicate that the inhibition of the formation of AGML by PPARγ agonists is dependent on the PPARγ pathway.

Expression of ICAM-1 and iNOS in stomach tissue. Adhesion molecules play an important role in the transendothelial migration of leukocytes into inflammatory tissues (14, 19, 22, 25). Therefore, the expression of ICAM-1 in stomach tissues was investigated by immunohistochemistry after the I/R procedure. When BALB/c mice were exposed to I/R, strong positive staining for ICAM-1 expression was detected in the stomach (Fig. 5A). This positive staining was markedly diminished by treatment with BRL-49653 (Fig. 5B) at a dose of 10 mg/kg, suggesting that activation of PPARγ prevented the upregulation of this adhesion molecule.

It is well known that increased expression of iNOS is directly associated with the degree of inflammation (10). Therefore, we also investigated the expression of iNOS in the stomach in response to I/R. Increased expression of iNOS was detected in I/R stomach (Fig. 5C), which was diminished by treatment with BRL-49653 at a dose of 10 mg/kg (Fig. 5D).
These studies show that activation of PPARγ leads to downregulation of several pathways involved in amplifying the inflammatory response associated with I/R injury in the stomach.

**Nitrotyrosine formation in stomach tissue.** It is well known that nitric oxide and superoxide radicals react with each other to form peroxynitrite, which attacks tyrosine residues in proteins to form nitrotyrosine (18, 24). Therefore, nitrotyrosine formation is indicative of the presence of ROS. Formation of nitrotyrosine was observed in the I/R-injured stomach as defined microscopically (Fig. 6, I/R). Treatment with BRL-49653 dramatically diminished the levels of detectable nitrotyrosine in the I/R-stomach (Fig. 6, I/R + BRL). These results indicate that the PPARγ ligand BRL-49653 can reduce the injury associated with the formation of free radicals that occurs during acute injury by I/R.

**PPARγ ligand inhibits the increase in mRNA for TNF-α in stomach.** It has been reported that I/R-induced injury is associated with the production of various proinflammatory cytokines including TNF-α (14, 17). Therefore, we measured the levels of mRNA for TNF-α in stomach tissues in response to I/R in either the presence or absence of the PPARγ ligand. Increased mRNA levels for TNF-α could be detected in I/R-stomach in control mice, and these were significantly diminished by treatment with BRL-49653 (Fig. 7). These results show that activation of PPARγ leads to downregulation of a proinflammatory cytokine associated with amplification of the inflammatory response during I/R injury in stomach. In the presence of diminished levels of PPARγ as observed in heterozygous PPARγ-deficient mice, the AGML associated with I/R was exacerbated. Despite this diminution in PPARγ levels and increased susceptibility to I/R injury, BRL-49653 (10 mg/kg) was able to inhibit tissue damage in the heterozygous PPARγ-deficient mice (14). As can be seen in Fig. 8A, a greater degree of injury was observed in PPARγ-deficient mice (+/-), compared with wild-type mice (+/+), after the I/R procedure as defined by both macroscopic and microscopic analyses (data not shown). To confirm and quantify these results, tissue damage was directly measured by using an image analysis system. A significant increase in the total area of injury was observed in I/R stomach from PPARγ-deficient mice compared with the wild-type mice (Fig. 8B). These results strongly indicate that endogenous PPARγ activity plays a direct role in the protection from injury related to I/R.

Effects of PPARγ deficiency on AGML induced by I/R. To directly confirm a role for endogenous PPARγ activity in the protection against gastric I/R injury, the response of heterozygous PPARγ-deficient mice in the formation of AGML induced by I/R was compared with wild-type mice (+/+) of the same strain (13, 14). Notably, messenger RNA and protein levels of PPARγ in PPARγ-deficient mice (+/-) within the gastrointestinal tract were significantly lower than those observed in wild-type mice (+/+ ) (14). As can be seen in Fig. 8A, a greater degree of injury was observed in PPARγ-deficient mice (+/-), compared with wild-type mice (+/+), after the I/R procedure as defined by both macroscopic and microscopic analyses (data not shown). To confirm and quantify these results, tissue damage was directly measured by using an image analysis system. A significant increase in the total area of injury was observed in I/R stomach from PPARγ-deficient mice compared with the wild-type mice (Fig. 8B). These results strongly indicate that endogenous PPARγ activity plays a direct role in the protection from injury related to I/R.

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gous PPARγ-deficient mice as defined by macroscopic examination of the stomach (Fig. 8C) and quantitative assessment of the damaged area (Fig. 8D). These results indicate that activation of even reduced levels of PPARγ in tissues is sufficient to provide some protection against I/R injury of the stomach.

**Localization of PPARγ in stomach.** To clarify the localization of PPARγ within stomach, we performed immunohistochemical analysis of PPARγ protein. As shown in Fig. 9, the expression of PPARγ was observed in mucosal epithelial cells and parietal cells. PPARγ observed in both epithelial and parietal cells in stomach might be potential targets for PPARγ agonists.

**DISCUSSION**

In this study, we demonstrated the dramatic protective effects of PPARγ agonists, BRL-49653, pioglitazone, and troglitazone on the AGML associated with an I/R injury. We observed that BRL-49653 significantly diminished the total areas of mucosal damage in a dose-dependent fashion after I/R. Other PPARγ agonists, pioglitazone and troglitazone, also showed the same protective effects against the AGML. These results suggest a specific role for PPARγ in protecting against this type of tissue injury. We further defined the protective effect of endogenous PPARγ activity in AGML induced by I/R through an examination of PPARγ-deficient mice. Results from these investigations provide strong evidence in support of an important role for endogenous PPARγ activity in protecting from I/R injury in the stomach. In particular, these results strongly suggest that PPARγ plays an intrinsic protective role in the stomach against I/R-induced injury as we have observed with other forms of severe, acute mucosal inflammation (14). These findings support the existence of an endogenous anti-inflammatory pathway in the stomach that is mediated by PPARγ. Actually, expression of PPARγ protein is observed both in mucosal epithelial cells and parietal cells. PPARγ in both epithelial and parietal cells in stomach might be potential targets for PPARγ agonists.

It should be noted that the PPARγ levels within the gastrointestinal tract are higher than that observed in other organs, although the level of PPARγ in stomach is much lower than those of small intestine and colon (2, 14). The reason for these differences is not apparent. However, the low levels observed in the stomach may predispose this organ to AGML. Nonetheless, despite its low levels, PPARγ activation in the stomach does provide endogenous protection against I/R. As shown here, and possibly other injurious events and is of high enough content that it could possibly be engaged by pharmacological activation as predicted by our result with the mice heterozygous for PPARγ expression. The relatively high levels of expression of PPARγ in the small intestine and colon may assist in suppressing inflammation in these organs associated with I/R and other forms of tissue injury (3, 14, 20, 26). Whether the relatively high levels of PPARγ in the small and large intestine is due, in part, to the increased bacterial burden.
present in these organs, at least in the colon, is unknown. However, these results suggest that this indeed may be the case, because PPARγ/H9253 might be regulated by the intestinal microflora (4). If so, this may be particularly important for the pathogenesis of inflammatory bowel disease.

Through the use of the PPARγ agonist BRL-49653, we have also established that one possible mechanism by which PPARγ activation leads to protection against I/R-related injury is through inhibition of expression of proinflammatory cytokines (e.g., TNF-α), inflammatory mediators (e.g., iNOS), adhesion molecules (e.g., ICAM-1), and apoptosis. In our previous study (14), we also reported that a PPARγ ligand inhibited the expression of ICAM-1 protein and TNF-α mRNA in intestinal I/R injury via inhibition of NF-κB activation. As TNF-α and other cytokines and adhesion molecules are downstream targets of NF-κB (1), this implies a similar role for PPARγ-mediated inhibition of NF-κB activation in gastric tissues. Further investigation of this hypothesis is required to clarify whether this and/or other molecular targets of PPARγ are involved in this protection.

Furthermore, activation of PPARγ might inhibit the formation of ROS. We observed strong staining for nitrotyrosine products in the tissue sections of mice exposed to I/R injury.
compared with those of normal, nonexposed mice. However, inhibition of nitrotyrosine formation was observed in the tissue sections of mice treated with BRL-49653. These results indicate that formation of ROS is induced by the I/R procedure and, in turn, inhibited by treatment with BRL-49653. Thus inhibition of the formation of injurious ROS products may be a major mechanism by which PPARγ agonists provide protective from I/R.

In this study, we have also demonstrated that the AGML induced by I/R in PPARγ-deficient mice was more severe than that observed in wild-type mice. Our results clearly indicate that an endogenous PPARγ activation pathway plays a direct role in protection from injury related to I/R. Decreased areas of tissue injury that observed in wild-type mice. Our results clearly indicate that an endogenous PPARγ is present in the stomach such that activation provides potent protection against the injury associated with gastric I/R injury. Activation of PPARγ in the stomach diminishes the increase in proinflammatory cytokine expression associated with I/R, likely via an NF-κB-mediated pathway. Findings of this study open up the new possibility for PPARγ agonist therapy in AGML and potentially, other forms of tissue injury in the stomach that involve similar pathophysiological mechanisms.

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