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Brief Conclusive Report

Effects of palmitoylethanolamide on intestinal injury and inflammation caused by ischemia-reperfusion in mice

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ABSTRACT

Our primary aim in this study was to test the hypothesis that PEA, a member of the fatty acid ethanolamide family and an endogenous PPAR-α ligand, exerts anti-inflammatory effects on SAO shock, causing a severe form of circulatory shock and enhanced formation of ROS. SAO shock was induced by clamping the superior mesenteric artery and the celiac trunk, resulting in a total occlusion of these arteries for 30 min. After this period of occlusion, the clamps were removed. In this study, we demonstrated that the administration of PEA, 5 min before reperfusion, significantly reduced all of the parameters involved during inflammation, such as pro-inflammatory cytokine production (TNF-α, IL-1β), adhesion molecules (ICAM-1, P-selectin) expression, NF-κB expression, and apoptosis (Bax, Bcl-2, TUNEL assay) activation. In addition, to study whether the protective action of PEA on SAO shock is also related to the activation of PPAR-α, we have investigated the effect of PEA in PPAR-α KO mice subjected to SAO shock. Our study clearly demonstrates that PEA significantly attenuated the degree of intestinal injury and inflammation caused by I/R injury. Moreover, the positive effects of PEA were at least in part dependent on the PPAR-α pathway. The results clearly indicate that PEA exerts an anti-inflammatory effect, also in a SAO shock model, which could imply a future use of PEA in the treatment of I/R shock. *J. Leukoc. Biol.* 91: 911–920; 2012.

Introduction

In humans, intestinal ischemia usually results from impaired perfusion of blood to the bowel as a result of a variety of causes, including cardiac insufficiency, sepsis, vaso- and cardiodepressant drugs, and complications of long-lasting surgery [1]. Ischemia leads to hypoxia, which initiates a series of events primarily related to activation of platelets and release of their vasoconstrictor mediators (e.g., thromboxane A2 and 5-hydroxytryptamine), which further restrict blood flow to the ischemic area. The pathophysiology of intestinal ischemia and its sequelae has been widely investigated using animal models of SAO, followed by reperfusion, which is characterized by a marked decrease in systemic blood pressure and leucopenia [2], as well as by disturbances in reticuloendothelial system activity and elevated plasma levels of platelet-activating factor. Ischemia progressively damages the cell structures, and following the restoration of blood flow, lesions produced are exacerbated further [3]. Moreover, it is believed that several mediators, such as ROS [4], proinflammatory cytokines [5], chemokines, and excess of NO, contribute significantly to the degree of the injury.

PPARs are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors that are related to retinoid, steroid, and thyroid hormone receptors [1, 6]. PPARs are expressed in the intestine at various levels [7]; recently, it has been demonstrated that PPAR-α is also expressed in the digestive tract, mainly localized in the intestinal mucosa, in the small intestine, and in the colon [7]. PPAR-α binds to a diverse set of ligands, namely, arachidonic acid metabolites (PGs and leukotrienes) and synthetic fibrate drugs, including clofibrate, fenofibrate, and bezafibrate [8]. Many PPAR-α ligands, including fibrate, show only modest selectivity over the

Abbreviations: Bax=B cell lymphoma 2-associated X protein, Bcl-2=B cell lymphoma 2, DBA=Dolichos biflorus agglutinin, eCB=endocannabinoid, I/R=ischemia/reperfusion, Isc=short-circuit current, KO=knockout, MC=mast cell, MDA=malondialdehyde, PAR=prostate-apoptosis response, PEA=palmitoylethanolamide, PPAR=peroxisome proliferator-activated receptor, Rt=transepithelial resistance, SAO=splanchnic artery occlusion, Vt=transepithelial potential differences

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other PPAR subtypes. However, a potent thioisobutyric acid, GW7647, has been identified, which shows excellent selectivity for murine and human PPAR-α [9]. Moreover, PPAR-α is a receptor for a diverse set of fatty acid derivatives, including oleoylethanolamide, which binds to the purified ligand-binding domain of PPAR-α with a K_D of 40 nM and activates it with a median effective concentration (EC50) of 120 nM [10], and PEA, an endogenous fatty acid amide belonging to the family of the *N*-acylethanolamines. Recently, several studies demonstrated that PEA is an important analgesic, anti-inflammatory, and neuroprotective mediator, acting at several molecular targets in the CNS and sensory nervous systems, as well as immune cells [11]. Different mechanisms have been proposed to explain the anti-inflammatory and antihyperalgesic effects of PEA, including: the activation of a cell surface receptor (i.e., the cannabinoid receptors CB2-like or alternatively, the orphan GPR55 receptor) or otherwise, a nuclear receptor of the PPAR family [11, 12]; the down-modulation of MC hyperactivity (Autacoid Local Inflammation Antagonism mechanism) [13]; an action as an "entourage" compound, i.e., the augmentation of eCB activities at their receptors and/or the inhibition of eCB degradation [14].

Based on this evidence, in the present study, we investigate whether PEA confers any protection against the effect of the intestinal injury resulting from SAO shock.

MATERIALS AND METHODS

Animals

Male adult CD1 mice (25–30 g, Harlan, Milan, Italy) and mice with a targeted disruption of the PPAR-α gene (PPAR-αKO, 4–5 weeks old, 20–22 g) and littermate WT controls (WT mice) purchased from The Jackson Laboratory (Bar Harbor, ME, USA; Charles River, Calco, Italy) were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (D.M. 116,192), as well as with the European Economic Community regulations (O.J. of E.C. L 358/1 12/18/1986).

Surgical procedures

Male mice were allowed access to food and water ad libitum. The mice were anesthetized with chloral hydrate (400 mg/kg i.p.). After midline laparotomy, the celiac and superior mesenteric arteries were isolated near their aortic origins. During this procedure, the intestinal tract was maintained at 37°C by placing it between gauze pads soaked with warmed, 0.9% NaCl solution. Mice (n=10 for each group) were observed for a 30-min stabilization period before splanchnic ischemia or sham operation. SAO shock was induced by clamping the superior mesenteric artery and the celiac trunk, resulting in a total occlusion of these arteries for 30 min. After this period of occlusion, the clamps were removed. In one study, the various groups of mice were sacrificed at 60 min for histological examination of the ileum and for biochemical studies, as described below. In another set of studies, following reperfusion, the various groups (n=10 for each group) of mice were observed for 24 h to determine survival differences.

Experimental groups

Mice were randomly allocated to the following groups:

●*Sham + vehicle group.* Mice were subjected to the surgical procedure alone, except that the blood vessels were not occluded, and mice were maintained under anesthesia for the duration of the experiment and treated with vehicle (n=10).

●*Sham + PEA group.* Mice were subjected to the surgical procedure alone, except that the blood vessels were not occluded, and the mice were maintained under anesthesia for the duration of the experiment and treated with PEA (10 mg/kg i.p., 10% ethanol; n=10).

●*I/R + vehicle group.* Mice were subjected to SAO shock and were administered with vehicle (n=10).

●*PEA group.* Mice were subjected to I/R, described as above, and PEA (10 mg/kg i.p. in 10% ethanol) was administered 5 min before reperfusion (n=10).

●*Sham + PEA group.* Identical to sham-operated r mice except for the administration of PEA (10 mg/kg i.p. in 10% ethanol), 5 min before reperfusion (n=10).

In a separate set of experiments, SAO shock was induced in PPAR-α KO mice to evaluate the involvement of the PPAR-α receptor on the effects of PEA. For this purpose, the following groups were used:

●*I/R PPAR-α KO + vehicle group.* PPAR-α KO mice underwent SAO shock for 30 min, followed by reperfusion for 1 h (n=10).

●*I/R PPAR-α KO + PEA group.* Identical to I/R PPAR-α KO + vehicle group except that PEA (10 mg/kg i.p.) was administered 5 min before reperfusion (n=10).

●*I/R WT mice + vehicle group.* WT mice, which underwent SAO shock for 30 min, followed by reperfusion for 1 h (n=10).

●*I/R WT mice + PEA group.* Identical to I/R WT + vehicle group except that PEA (10 mg/kg i.p.) was administered 5 min before reperfusion (n=10).

●*Sham PPAR-α KO + vehicle group.* Sham-operated WT mice were subjected to the surgical procedures described but were not subjected to SAO shock (n=10).

●*Sham PPAR-α KO + PEA group.* Identical to Sham + vehicle group except that PEA (10 mg/kg i.p.) was administered 5 min before surgical procedures (n=8).

In the experiments, investigating the survival mice (n=15 from each group) was monitored for 24 h after reperfusion. We have used three doses of PEA, respectively: 1 mg/kg, 3 mg/kg, and 10 mg/kg. PEA doses were chosen on the basis of previous experiments [15, 16].

Evaluation of survival

The various groups of mice were monitored for 24 h after SAO and reperfusion, and survival rates and survival times were evaluated.

Light microscopy

For histopathological examination, biopsies of small intestine were taken 60 min after reperfusion. Tissues were fixed in Dietric solution (14.25% ethanol, 1.85% formaldehyde, 1% acetic acid) for 1 week at room temperature, dehydrated by graded ethanol, and embedded in Paraplast (Sherwood Medical, Mahwah, NJ, USA). From each biopsy, 7-μm sections were obtained with H&E to evaluate intestine morphology. For quantitative estimation of damage caused by I/R, sections were scored by two independent observers blinded to the experimental protocol. The following morphological criteria were used for scoring, as described previously [17]: 0, no visible damage; 1, slight injury with focal epithelial edema and necrosis; 2, moderate injury with diffuse swelling and necrosis of the villi; 3, severe injury with necrosis and evidence of neutrophil infiltration in the submucosa; 4, severe injury with widespread necrosis and massive neutrophil infiltration plus evidence of hemorrhage.

Staining of MCs

Ileum sections were cut 5-μm-thick and stained with 0.25% toluidine blue, pH 2.5, for 45 min at room temperature [18]. The sections were then dehydrated and mounted in xylene-based medium for viewing. Three nonsequential sections were chosen from one random block from each ileum for examination. All sections were evaluated at ×200, whereas some sections were photographed at ×400 using a Nikon inverted microscope.

Measurement of cytokines

TNF-α and IL-1β levels were evaluated in plasma samples at 60 min after reperfusion. The assay was carried out by using a colorimetric commercial kit (Calbiochem-Novabiochem, San Diego, CA, USA).

MPO activity

MPO activity, an indicator of PMN accumulation, was determined as described previously [19] in intestinal tissues collected after 60 min of reperfusion. MPO activity was defined as the quantity of enzyme degrading 1 μmol peroxide/min at 37°C and was expressed as MPO units/mg protein.

MDA measurement

MDA levels, as an indicator of lipid peroxidation, were determined in the intestinal tissue, which was weighed and homogenized in 1.15% (w/v) KCl solution. An aliquot (100 μl) of the homogenate was added to a reaction mixture containing 200 μl 8.1% (w/v) SDS, 1500 μl 20% (w/v) acetic acid (pH 3.5), 1500 μl 0.8% (w/v) thiobarbituric acid, and 700 μl distilled water. Samples were then boiled for 1 h at 95°C and centrifuged at 3000 *g* for 10 min. The absorbance of the supernatant was measured by spectrophotometry at 515–553 nm.

Immunohistochemical localization of chymase, ICAM-1, P-selectin, nitrotyrosine, PAR, Bax, and Bcl-2

Immunohistochemical localization of ICAM-1, P-selectin, nitrotyrosine, PAR, Bax, and Bcl-2 was determined following a protocol described previously [20]. The ileum tissues were fixed in 10% buffered formaldehyde, and 8 μm sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% H_2O_2 in 60% methanol for 30 min. The sections were permeabilized with 0.1% Triton X-100 in PBS for 20 min. Nonspecific adsorption was minimized by incubating the section in 2% normal goat serum in PBS for 20 min. Endogenous biotin or avidin-binding sites were blocked by sequential incubation for 15 min with avidin and biotin. The sections were then incubated overnight with primary antichymase (purified human skin chymase) polyclonal antibody (1:1000), anti-P-selectin antibody (BD PharMingen, San Diego, CA, USA; CD62P 1:500), anti-ICAM-1 antibody (BD PharMingen; CD54, 1:500), and anti-Bax polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:500 in PBS, v/v), with anti-Bcl-2 polyclonal antibody (Santa Cruz Biotechnology; 1:500 in PBS, v/v), antinitrotyrosine polyclonal antibody (Santa Cruz Biotechnology; 1:500 in PBS, v/v), anti-PAR (Santa Cruz Biotechnology; 1:500 in PBS, v/v), or control solutions. Controls included buffer alone or nonspecific, purified rabbit IgG. 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 (Vector Laboratories, Burlingame, CA, USA; DBA). To verify the binding specificity for ICAM-1, P-selectin, nitrotyrosine, PAR, Bax, and Bcl-2, 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 immunoreactions were positive in all of the experiments carried out.

Western blot analysis for IκB-α, NF-κB p65, ICAM, P-selectin, Bax, and Bcl-2

In brief, ileum tissues from each mouse were suspended in extraction buffer A containing 0.2 mM PMSF, 0.15 μM pepstatin A, 20 μM leupeptin, and 1 μM sodium orthovanadate; homogenized at the highest setting for 2 min; and centrifuged at 1000 *g* for 10 min at 4°C. Supernatants represented the cytosolic fraction. The pellets, containing enriched nuclei, were resuspended in buffer B containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM PMSF, 20 M leupeptin, and 0.2 mM sodium orthovanadate. After centrifugation at 15,000 *g* for 30 min at 4°C, the supernatants containing the nuclear protein were stored at −80°C for further analysis. The levels of IκB-α, ICAM, P-selectin, Bax, and Bcl-2 were quantified in cytosolic fraction from ileum tissue collected after I/R, whereas NF-κB p65 levels were quantified in nuclear fraction. The filters were blocked with 1× PBS, 5% (w/v) nonfat dried milk, for 40 min at room temperature, and they were subsequently probed with specific antibodies, anti-IκB-α (1:1000; Santa Cruz Biotechnology), anti-ICAM (1:500; Santa Cruz Biotechnology), anti-P-selectin (1:500; Santa Cruz Biotechnology), anti-NF-κB p65 (1:1000; Santa Cruz Biotechnology), anti-Bax (1:500; Santa Cruz Biotechnology), and anti-Bcl-2 (1:500; Santa Cruz Biotechnology) in 1× PBS, 5% (w/v) nonfat dried milk, and 0.1% Tween 20 at 4°C overnight. Membranes were incubated with peroxidase-conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h at room temperature. To ascertain that blots were loaded with equal amounts of protein lysates, they were also incubated in the presence of the antibody against β-actin protein (1:2000; Sigma-Aldrich, Milan, Italy). The relative expression of the protein bands of IκB-α (~37 kDa), ICAM (~110 kDa), P-selectin (~140 kDa), NF-κB p65 (~65 kDa), Bax (~23 kDa), and Bcl-2(~29 kDa) was quantified by densitometry scanning of the X-ray films with a GS-700 imaging densitometer (GS-700; Bio-Rad, Milan, Italy) and a computer program (Molecular Analyst, IBM, Armonk, NY, USA).

TUNEL assay

TUNEL assay was conducted by using a TUNEL detection kit, according to the manufacturer's instructions (ApopTag, HRP kit DBA, Milan, Italy). 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% H_2O_2 for 5 min at room temperature and then washed with PBS. Sections were immersed in 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-HRP-conjugated antibody, and the signals were visualized with diaminobenzidine. The number of TUNEL-positive cells/high-power field was counted in five to 10 fields for each coded slide.

Ussing chamber experiments

The small intestine of the mouse was removed, stripped of muscle layers using two pairs of fine forceps, and mounted vertically in a modified Ussing chamber (CHM4, World Precision Instruments, Berlin, Germany; membrane area: 0.13 cm^2), where it was perfused on both sides by isotonic saline (Ringer solutions), and compositions were: NaCl 107 mM, KCl 4.5 mM, $NaHCO_3$ 25 mM, $MgSO_4$ 1.0 mM, $CaCl_2$ 1.25 mM, NaH_2PO_4 0.2 mM, Na_2HPO_4 1.8 mM, and glucose 12 mM; osmolarity = 290 mOsm kg^{-1}. Solution was bubbled with a mixture of 95% O_2 and 5% CO_2 to yield pH = 7.4 ± 0.1. The temperature of the perfusing solution was kept constant at 37°C. Tissues were connected to an automatic Isc device (DVC-1000, World Precision Instruments) by four Ag/AgCl electrodes (two voltage electrodes and two current electrodes), which made contact with the bathing solutions via agar-Ringer-filled cartridges. Isc was measured by passage of sufficient current through Ag/AgCl electrodes to reduce the spontaneous Vt to zero (resistance of the chamber fluid was subtracted automatically). The preparations were kept open, circuited throughout the experiments, except for a few seconds every 10 min for recording the Isc. Vt was measured with respect to the mucosal side. The Isc is referred to as positive when current flows across the tissue from the apical side to the serosal side. Rt was measured from spontaneous Vt and Isc, according to Ohm's law (Rt=V/I). All data are expressed as mean ± SE. Statistical analyses were performed using the one-way ANOVA test, followed by a Dunnett's multiple comparison test and the Student's *t*-test. Differences were considered significant at $P < 0.05$.

Reagents

Unless otherwise stated, all compounds were obtained from Sigma-Aldrich. Primary monoclonal P-selectin (CD62P) or ICAM-1 (CD54) for immunohis-

tochemistry was purchased by BD PharMingen. Reagents and secondary and nonspecific IgG antibody for immunohistochemical analysis were from Vector Laboratories. DMSO was obtained by EuroClone (Italy). All other chemicals were of the highest commercial grade available. All stock solutions were prepared in nonpyrogenic saline (0.9% NaCl, Baxter Healthcare, Thetford, Norfolk, UK).

Statistical analysis

All values in the figures and text are expressed as mean ± SEM of n animals or ileal segments. In the experiments involving histology or immunohistochemistry, the figures are representative of at least three experiments, each performed on different days. Data sets were examined by one- and two-way ANOVA, and individual group means were then compared with Student's unpaired t-test. Nonparametric data were analyzed with the Fisher's exact test. $P < 0.05$ was considered significant. Calculations were performed using the GraphPad Prism 2.0 statistical program (GraphPad Software, San Diego, CA, USA). *$P < 0.01$ versus Sham; °$P < 0.01$ versus SAO.

RESULTS AND DISCUSSION

Gut injury as a result of I/R is a life-threatening, clinical emergency. Interruption of blood supply causes ischemia, which rapidly damages metabolically active tissues. Restoration of blood flow after a period of intestinal ischemia is necessary to maintain cell function and viability. However, restoration of blood flow to the ischemic tissues can initiate a cascade of pathophysiologic responses that lead to additional cell or tissue injury.

We demonstrate here that the treatment with PEA significantly reduced: the degree of ileum injury, the degree of mortality, the infiltration of the ileum PMNs, the proinflammatory cytokine production, as well as the increased expression of ICAM-1 and P-selectin caused by SAO shock in the ileum. All of these findings support the view that PEA exerts

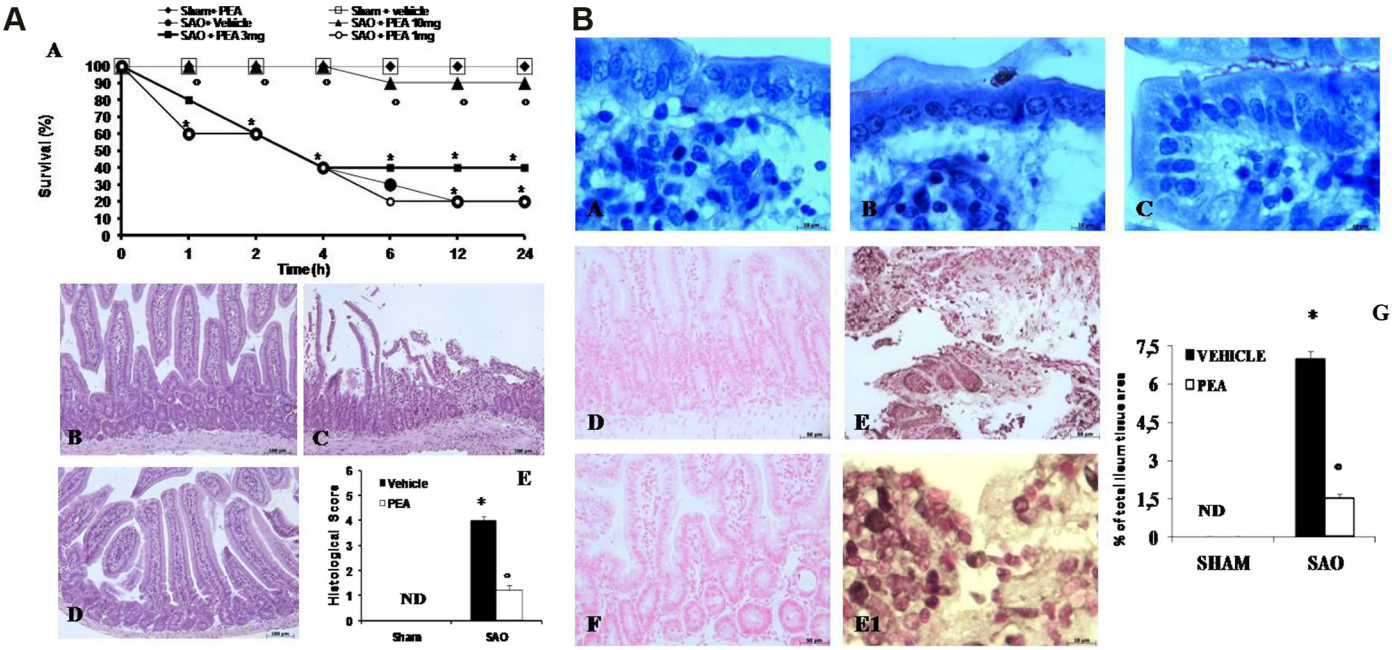

Figure 1. Effect of PEA on survival rate, histological alteration, and chymase expression. Survival was monitored for 24 h after SAO shock. SAO produced a profound shock state characterized by a 100% lethality at the end of the 24-h reperfusion period (A,A). Control sham animals survived for the entire 24-h period of reperfusion. PEA (10 mg/kg) treatment significantly prevented the mortality induced by I/R (A,A). The survival rate of mice treated with PEA (1 mg/kg and 3 mg/kg) showed the same trend of animals affected only by SAO shock (A,A). Data are mean ± SD of 10 mice for each group. *$P < 0.01$ versus sham; °$P < 0.01$ versus I/R (A,A). Histological features of gut were observed in gut tissues prepared from sham-operated mice (A,B; see A,E, Histological Score). Distal ileum section from SAO-shocked mice showed inflammatory infiltration by PMNs and lymphocytes extending through the wall, concentrated below the epithelial layer, demonstrating edema of the distal portion of the villi (A,C; see A,E, Histological Score). Distal ileum from PEA-treated mice shows reduced SAO-induced organ injury (A,D; see A,E, Histological Score). Data are mean ± SD of 10 mice for each group. *$P < 0.01$ versus sham; °$P < 0.01$ versus I/R (A,E). The figure is representative of at least three experiments performed on different experimental days. The slides stained with acidified Toluidine blue were also shown to have dark lilac-blue granules, which identifies the cells as MCs. Many of the MCs are arranged separately in concentric rings around small blood vessels. Different gradients of staining intensity were evident in the granulated and degranulated MCs. In the ileum tissues collected from SAO-shocked mice, there is the presence of MCs (B,B), localized mainly in the perivascular area. On the contrary, PEA reduced MC infiltration in the ileum tissues from SAO-shocked mice (B,C). No granules were found in the ileum tissues from sham-operated mice (B,A). There was no staining for chymase in ileum tissues obtained from the sham-operated mice (B,D). A substantial increase in serine peptidase chymase expression was found mainly localized in MCs in the ileum tissues collected from SAO-shocked mice (B,E and E1). Chymase expression was attenuated in mice treated with PEA (B,F). This figure is representative of at least three experiments performed on different experimental days. Values shown are mean ± SE; mean of 10 mice for each group. *$P < 0.05$ versus sham; °$P < 0.05$ versus I/R (B,G).

potent anti-inflammatory effects. What, then, is the mechanism by which PEA inhibit the gut injury caused by I/R?

We report here that I/R caused a significant infiltration (identified morphologically by Toluidine blue staining) and activation (evaluated as chymase and tryptase expression) of MCs in ileum tissues after 1 h. Different gradients of staining intensity were evident in the granulated and degranulated MCs. In the ileum tissues collected from mice, subjected to SAO shock, there is the presence of MCs (**Fig. 1B,B**) mainly localized in the perivascular area. On the contrary, PEA treatment reduced MC infiltration in ileum tissues (Fig. 1B,C). No granules were found in the ileum tissues from sham-operated mice (Fig. 1B,A). There was no staining for chymase in ileum tissues obtained from the sham-operated mice (Fig. 1B). Instead, a substantial increase in serine peptidases chymase expression was found mainly localized in MCs in the ileum tissues collected from mice subjected to SAO shock (Fig. 1B,E and E1). Chymase levels were attenuated in mice treated with PEA (Fig. 1B,F), whereas treatment with PEA reduced the infiltration and the activation of MCs.

Moreover, histological features of gut tissue from sham-operated mice were observed (Fig. 1A,B; see A,E, Histological Score). Ileum sections revealed PMN infiltration through the gut wall and concentrated below the epithelial layer, as well as an alteration of the villi tips (Fig. 1A,C; see A,E, Histological Score). PEA treatment reduces the degree of tissue injury (Fig. 1A,D; see A,E, Histological Score). MCs possess several biological mediators that are released from cytoplasmic granules primarily as a result of stimulus-induced degranulation, including vasoactive amines, such as histamine, proteoglycans (mainly heparin and chondroitin sulfate), cytokines, and growth factors, such as VEGF, bFGF, NGF, TGF-β, and TNF-α [21]. We report here that the levels of proinflammatory cytokines, such as IL-1β and TNF-α, were increased in SAO mice tissue (**Fig. 2C** and **D**, respectively), whereas the treatment with PEA significantly attenuated the expression of IL-1β and TNF-α (Fig. 2C and D, respectively). Moreover, MPO activity in ileum homogenates from SAO-shocked mice was elevated significantly (Fig. 2E). A decrease in MPO activity was observed in the ileum from mice treated with PEA after 60 min of reperfusion (Fig. 2E).

The synthesis and/or release of inflammatory cytokines, chemokines, growth factors, and ECM proteins in the I/R process are a result of NF-κB activation.

NF-κB belongs to a family of dimeric transcription factor complexes and is normally found in the cytoplasm of unstimulated cells in their inactive forms; stimulation of cells with cytokines, oxidants, or inflammatory mediators results in phosphorylation and ubiquitination of the IκB. This results in translocation of the subunit to the nucleus to activate transcription of its target genes.

The mechanisms by which PEA suppress NF-κB activation in inflammation are not known. We report here that SAO shock caused a significant increase in nuclear p65 expression, an index of NF-κB translocation, in the nuclear extract of ileum tissues at 1 h of reperfusion (Fig. 2B and B1), whereas PEA treatment significantly reduced the NF-κB translocation (Fig. 2B and B1). Moreover, we also demonstrate that the PEA inhibited IκB-α degradation (Fig. 2A and A1).

NF-κB activation mediates the expression of a number of genes involved in the inflammatory response to injury, some of which are potential key targets for intervention in treating inflammatory conditions. It has been demonstrated that NF-κB activation increases the expression of the adhesion molecules E-selectin, VCAM-1, and ICAM-1, whereas NF-κB inhibition reduces leukocyte adhesion and transmigration [22]. We con-

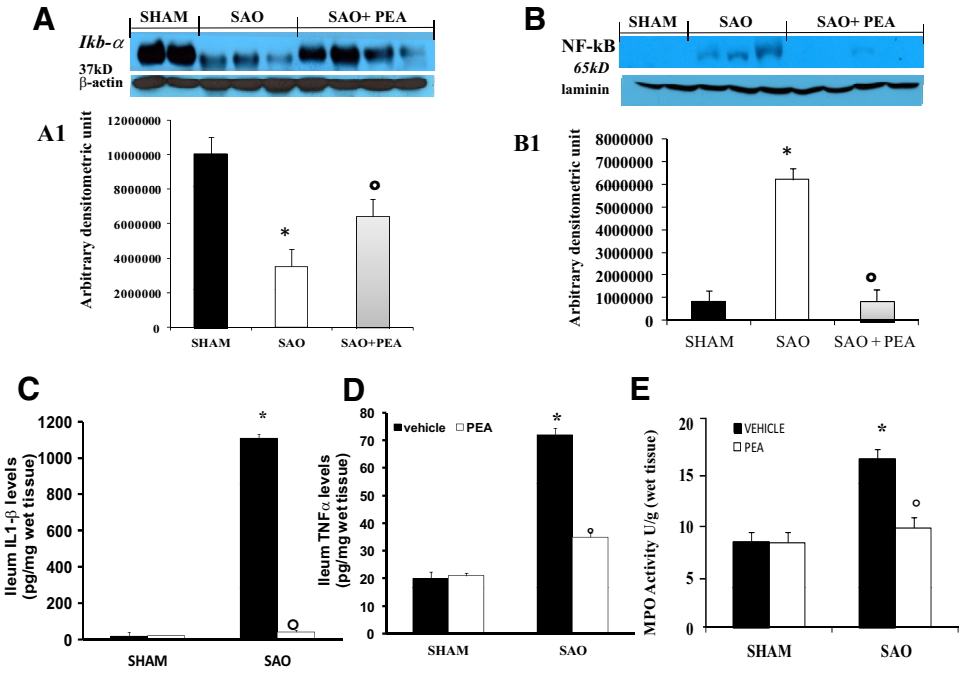

Figure 2. Effect of PEA on IκB-α degradation, NF-κB p65 translocation, and cytokines levels. Representative Western blots showing the effects of PEA on IκB-α degradation (A and A1) and phosphorylation of Ser536 on NF-κB subunit p65 (B and B1) after SAO shock. A representative blot of lysates (A and B) obtained from five animals/group is shown, and densitometry analysis of all animals is reported. The results in A1 and B1 are expressed as mean ± SEM from five or six ileum tissues for each group. *$P < 0.01$ versus sham; °$P < 0.01$ versus SAO (A1 and B1). Moreover, tissue levels of cytokines TNF-α (D) and IL-1β (C) increased in samples obtained from SAO mice and when compared with sham-operated mice. PEA treatment reduced the tissue levels of TNF-α (D) and IL-1β (C). Moreover, MPO activity (index of PMN infiltration) was elevated significantly at 60 min after SAO shock (E). PEA significantly reduced MPO activity in the ileum (E). The results are expressed as mean ± SEM from $n = 10$ mice for each group. *$P < 0.01$ versus sham group; °$P < 0.01$ versus SAO shock plus vehicle (C–E).

firmed here that SAO shock induced the appearance of P-selectin on the endothelial vascular wall (**Fig. 3E**) and the expression of ICAM-1 on endothelial cells (Fig. 3B). Treatment with PEA abolished the expression of P-selectin and ICAM-1 (Fig. 3C and F, respectively). These results demonstrate that PEA treatment may interrupt the interaction between neutrophils and endothelial cells at the early rolling phase mediated by P-selectin and at the late firm-adhesion phase mediated by ICAM. The absence of an increased expression of the adhesion molecule in the ileum tissue from SAO-shocked mice treated with PEA is correlated with the reduction in leukocyte infiltration and with the attenuation in the ileum tissue damage.

Moreover, PEA also prevents lipid peroxidation and nitrosative stress. In fact, in the present study, we clearly demonstrated that PEA treatment fully inhibited the appearance of nitrotyrosine (**Fig. 4A,C**), as an indication of "increased nitrosative stress", staining in the inflamed tissue.

Recent evidence has shown that ischemia and/or I/R induce apoptosis in several tissues, such as brain [23], myocardium [24], intestine [25], and liver [26]. Moreover, the Bcl-2 family of apoptotic regulator is in addition to caspase, another one of the functional components of the apoptosis pathway. Moreover, in this study, we have identified proapoptotic transcriptional changes, including up-regulation of proapoptotic Bax and down-regulation of antiapoptotic Bcl-2. We report in

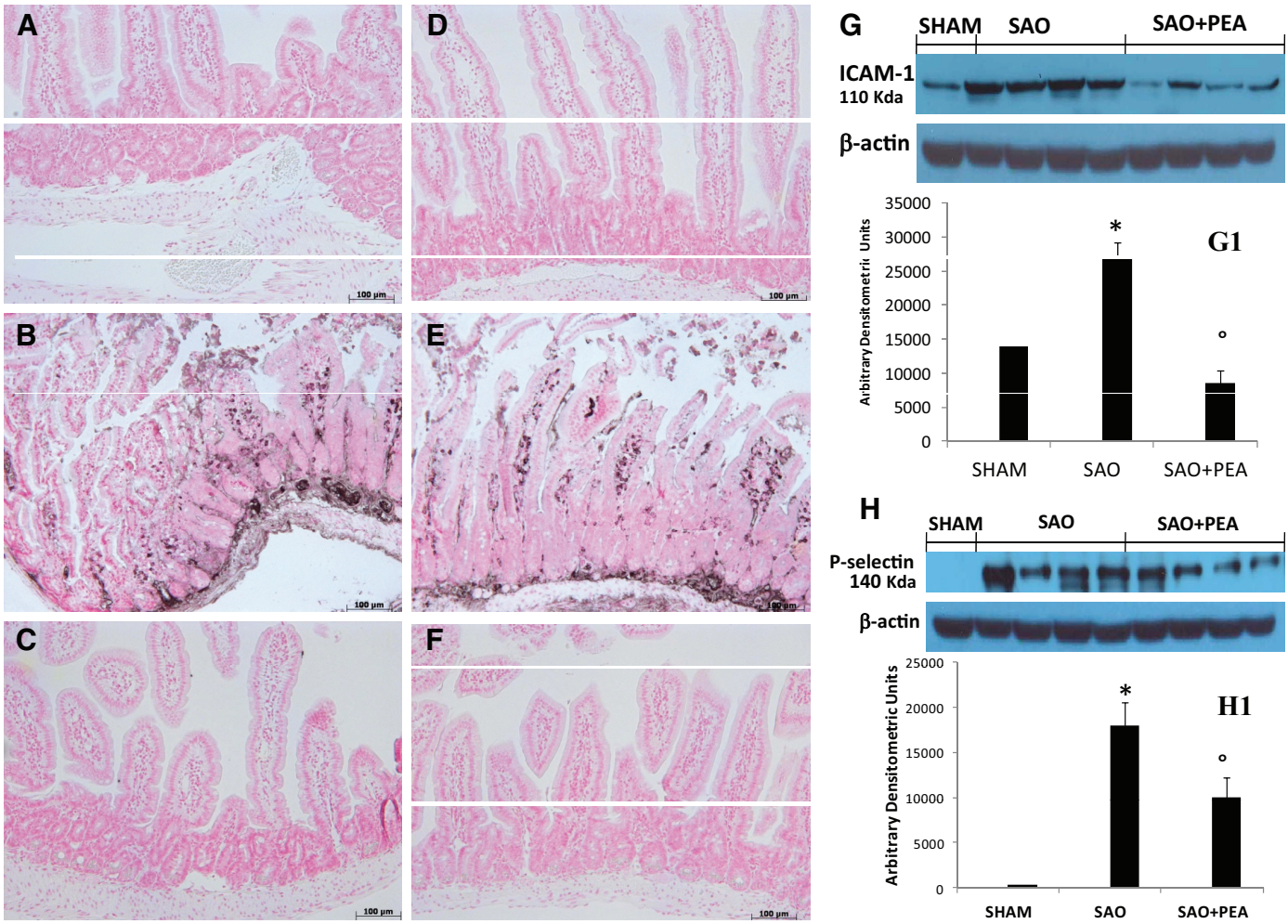

Figure 3. Effect of PEA on adhesion molecule expression. Ileum sections taken from SAO shock-treated mice showed positive staining for ICAM-1 (B) and for P-selectin (E) compared with sham-operated mice (A and D, respectively). The degree of positive staining for adhesion molecules was reduced markedly in tissue sections obtained from mice treated with PEA (C and F, respectively). Moreover adhesion molecule expression was evaluated by Western blot analysis. A basal level of ICAM-1 was detected in ileum tissues obtained from sham-treated animals by Western blot analysis (G and G1). ICAM-1 expression was increased significantly in the ileum tissues obtained from SAO-shocked mice (G and G1). PEA treatment prevented shock-induced ICAM-1 expression (G and G1). No level of P-selectin expression was detected in ileum tissues from sham-operated mice (H and H1). P-selectin expression was increased significantly in SAO-shocked mice (H and H1). PEA treatment prevented shock-induced P-selectin expression. The figures are representative of at least three experiments performed on different experimental days. A representative blot of lysates obtained from five animals/group is shown, and densitometry analysis of all animals is reported. The results in G1 and H1 are expressed as mean ± SEM from $n = 5/6$ ileum tissues for each group. *$P < 0.01$ versus sham group; °$P < 0.01$ versus SAO shock (G1 and H1).

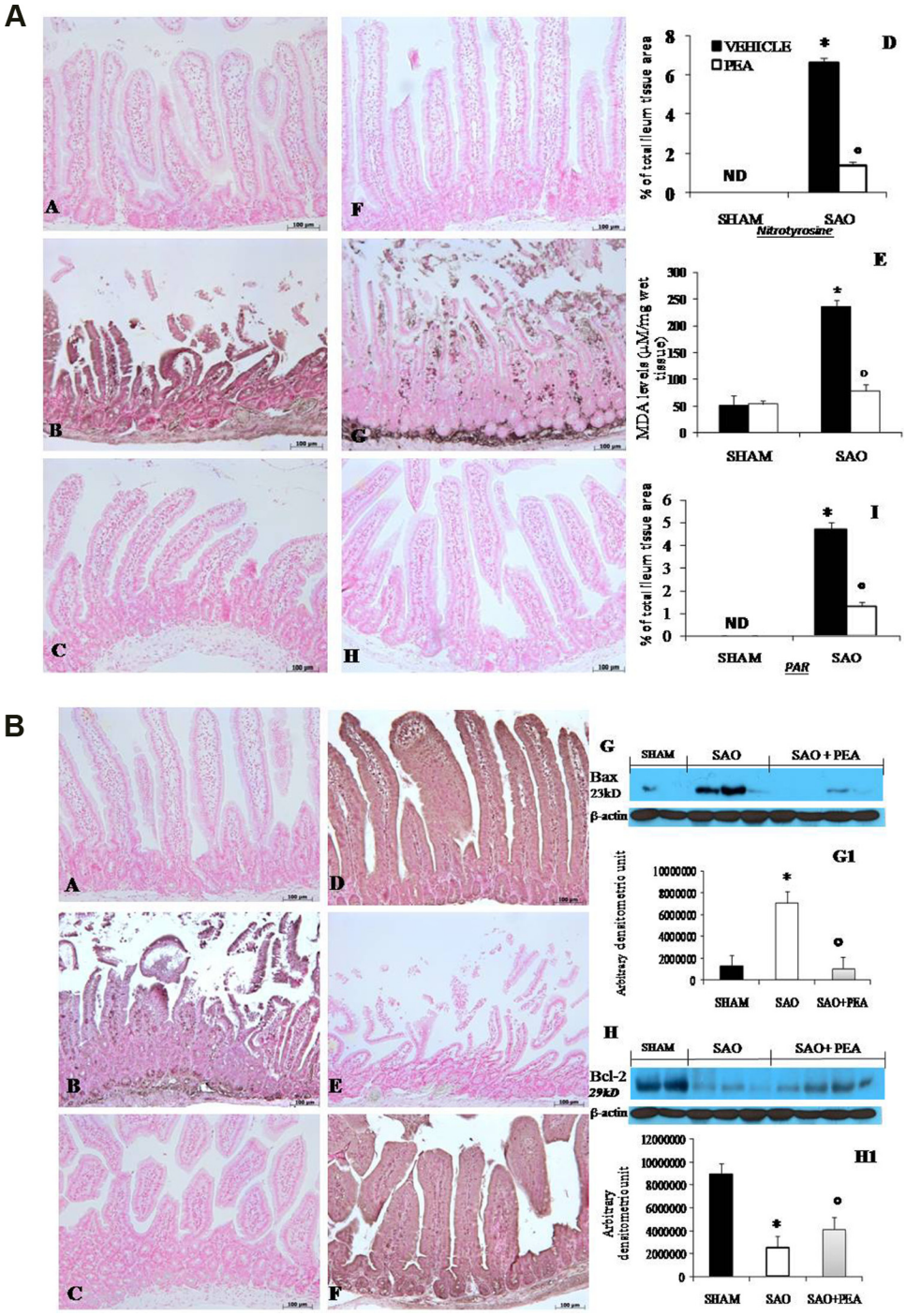

Figure 4. Effect of PEA on nitrotyrosine, PAR formation, and on apoptotic pathways. Immunohistochemical analysis of intestinal sections obtained from mice subjected to splanchnic I/R revealed a positive staining for nitrotyrosine in the injured tissues (A,B) compared with sham-operated mice (A,A). There was no detectable immunostaining in the ileum from PEA-treated mice (A,C). Reperfusion of the ischemic splanchnic circulation leads to a profound increase in MDA levels in ileum tissues, which is inhibited by treatment with PEA (A,E). Immunohistochemistry for PAR revealed positive staining for PAR localized in the inflammatory cells in the ileum tissues from mice subjected to SAO shock (A,G). PEA treatment reduced the degree of positive staining for PAR (A,H) in the ileum. Densitometry analysis of immunocytochemistry photographs (n=5 photos from each sample collected from all mice in each experimental group) for nitrotyrosine (A,D) and PAR (A,I) from ileum tissues was assessed. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3/266). Data are expressed as percent of total tissue area. The figure is representative of at least three experiments performed on different experimental days. Data are mean ± SD of 10 mice for each group. *$P < 0.01$ versus sham; °$P < 0.01$ versus I/R (A,D, E, and I). Moreover, ileum sections taken from SAO shock mice showed positive staining for Bax (B,B), localized mainly in the inflammatory cells. The degree of positive staining for Bax was reduced markedly in ileum sections obtained from mice treated with PEA (B,C). No staining for Bax was observed in ileum tissues obtained from sham-operated animals (B,A). Positive staining for Bcl-2 was observed in ileum sections taken from sham mice (B,D). The degree of positive staining for Bcl-2 was reduced markedly in ileum sections obtained from SAO shock mice (B,E). PEA treatment significantly attenuated the reduction in Bcl-2 expression caused by SAO shock (B,F). Moreover, representative Western blots show no Bax expression in ileum tissues obtained from sham-operated animals (B,G and G1). Bax levels were increased in the ileum tissues from SAO shock-treated mice (B,G and G1). PEA treatment prevented the SAO shock-induced Bax expression (B,G and G1). A basal level of Bcl-2 expression was detected in ileum tissues from the sham group (B,H and H1). At 60 min after reperfusion, Bcl-2 expression was reduced significantly (B,H and H1). Treatment of mice with PEA significantly attenuated SAO shock-induced inhibition of Bcl-2 expression (B,H and H1). The figures are representative of at least three experiments performed on different experimental days. A representative blot of lysates obtained from five animals/group is shown, and densitometry analysis of all animals is reported. The results in G1 and H1 are expressed as mean ± SEM from n = 5/6 ileum tissues for each group. *$P < 0.01$ versus sham group; °$P < 0.01$ versus SAO shock (B,G1 and H1).

the present study for the first time that the treatment with PEA significantly reduced the apoptotic cell death after SAO shock, suggesting that protection from apoptosis may be a prerequisite for anti-inflammatory approaches.

In particular, we demonstrated that the treatment with PEA lowers the signal for Bax in the shocked group (Fig. 4B,C) when compared with ileum sections obtained from SAO-shocked mice (Fig. 4B,B), whereas on the contrary, the signal

for Bcl-2 is expressed much more in PEA-treated mice (Fig. 4B,F) than in SAO-shocked mice (Fig. 4B,F).

Furthermore, intestinal permeability reflects the integrity of the intestinal mucosal barrier, which restricts the passive permeation of luminal substances [27]. It is well known that altered intestinal permeability has been reported in various intestinal conditions associated with diarrhea, such as celiac disease [28], infectious gastroenteritis [29], and food intolerance or allergy.

It is possible to assess intestinal permeability in vivo by Ussing chamber experiments. In particular, in our study using the Ussing chamber, we have suggested that PEA stimulates the recovery of the barrier function of injured mouse ileum. In fact, ischemic tissues of untreated animals showed an evident reduction of Rt with respect to control (nonischemic tissues; **Fig. 5F**); this reduction was not observed in ischemic tissues from PEA-injected animals (Fig. 5F). It is known that the barrier properties of the intestinal epithelium and hence, Rt are regulated by the permeability of the tight junctions [30–33] and that barrier function is re-established predominantly by restoration of tight junction [34, 35]. The observation that the serosa-positive Isc of ischemic tissues, isolated by PEA-injected animals, was higher (Fig. 5E) than Isc measured in the control tissues (Fig. 5E) suggests that the drug could stimulate Cl secretion and that this stimulation could be responsible of the restoration of the barrier function of the epithelium. This hypothesis is consistent with the observation that the reparative role of PGs occurs via the stimulation of the Cl channel located in the tight junction of the villus, a ClC-2 Cl channel. The role of this channel in the repair of the mucosal barrier was strengthened by the effect of the selective agonist, lubiprostone, a bicyclic fatty acid of the prostone group [36]. The authors showed that the luminal addition of the drug in ischemic-injured porcine ileum and colon, isolated and mounted in an Ussing chamber, produced an increase of Isc and a restoration of Rt.

It has been shown previously that some anti-inflammatory actions of PEA are mediated by PPAR-α activation and are accompanied by a decrease in NO production [37] and expression of proinflammatory proteins [38]. For these reasons, to

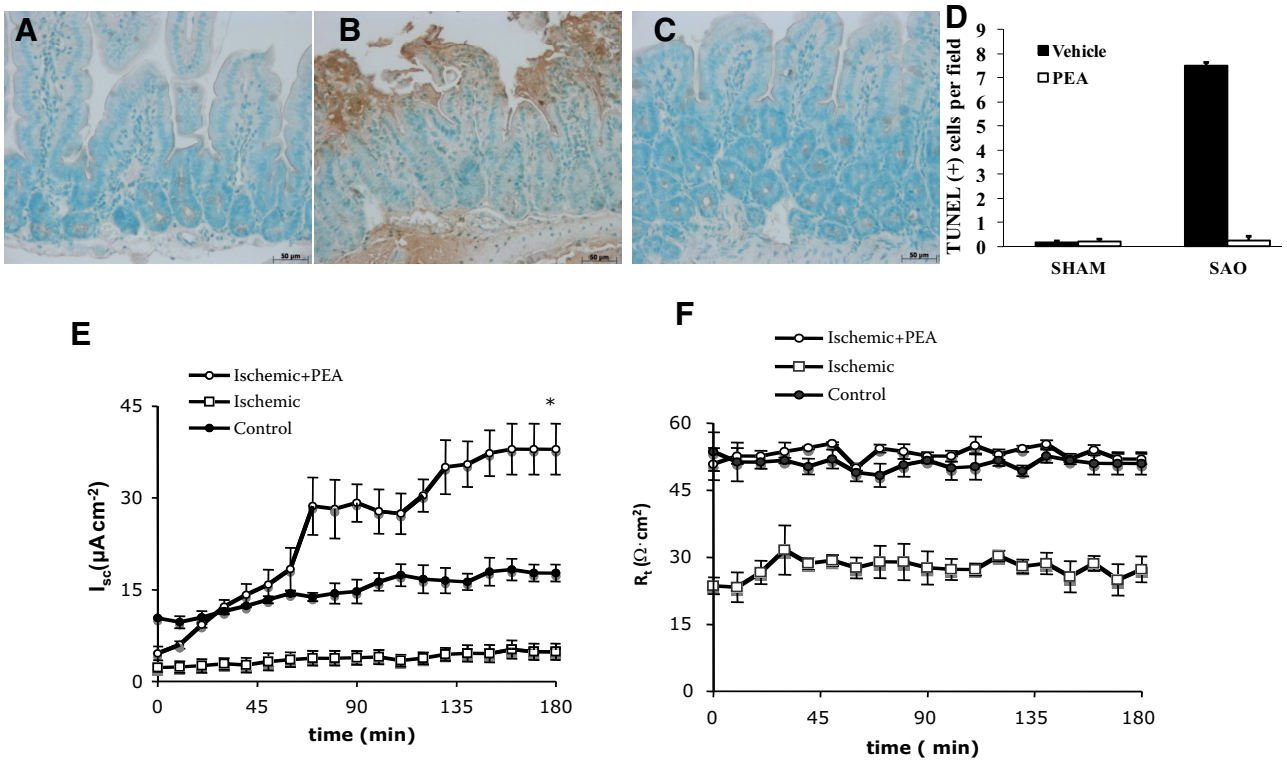

Figure 5. Effect of PEA on apoptosis, as measured by TUNEL-like staining and on intestinal permeability. Positive TUNEL staining was observed in ileum sections taken from SAO shock-treated mice treated with vehicle (B). In contrast, tissue obtained from SAO shock-treated mice treated with PEA demonstrated no apoptotic cells or fragments (C). Almost no apoptotic cells were observed in ileum from sham mice (A). Moreover, we evaluated a number of dark-brown apoptotic cells for field, and we counted a high number of apoptotic cells in ileum sections obtained from SAO mice (D). On the contrary, the number of dark-brown cells was reduced significantly in PEA-treated mice (D). The figure is representative of at least three experiments performed on different experimental days. The results in D are expressed as mean ± SEM from five or six ileum tissues for each group. Moreover, SAO shock-treated mice mounted in an Ussing chamber and perfused with identical saline from both sides—mucosal and serosal—generated a serosa-positive (I_{sc}), which did not exhibited a significant increase during the entire observation period (3 h; E). The value of animals treated with PEA showed a continuous increase, reaching a significantly higher value after 3 h (E). The value of R_t of the ischemic tissues was significantly lower than R_t of the controls (F). R_t of ischemic tissues from PEA-treated animals was not different from the nonischemic (control; F). The results are expressed as mean ± SEM from $n = 10$ mice for each group.

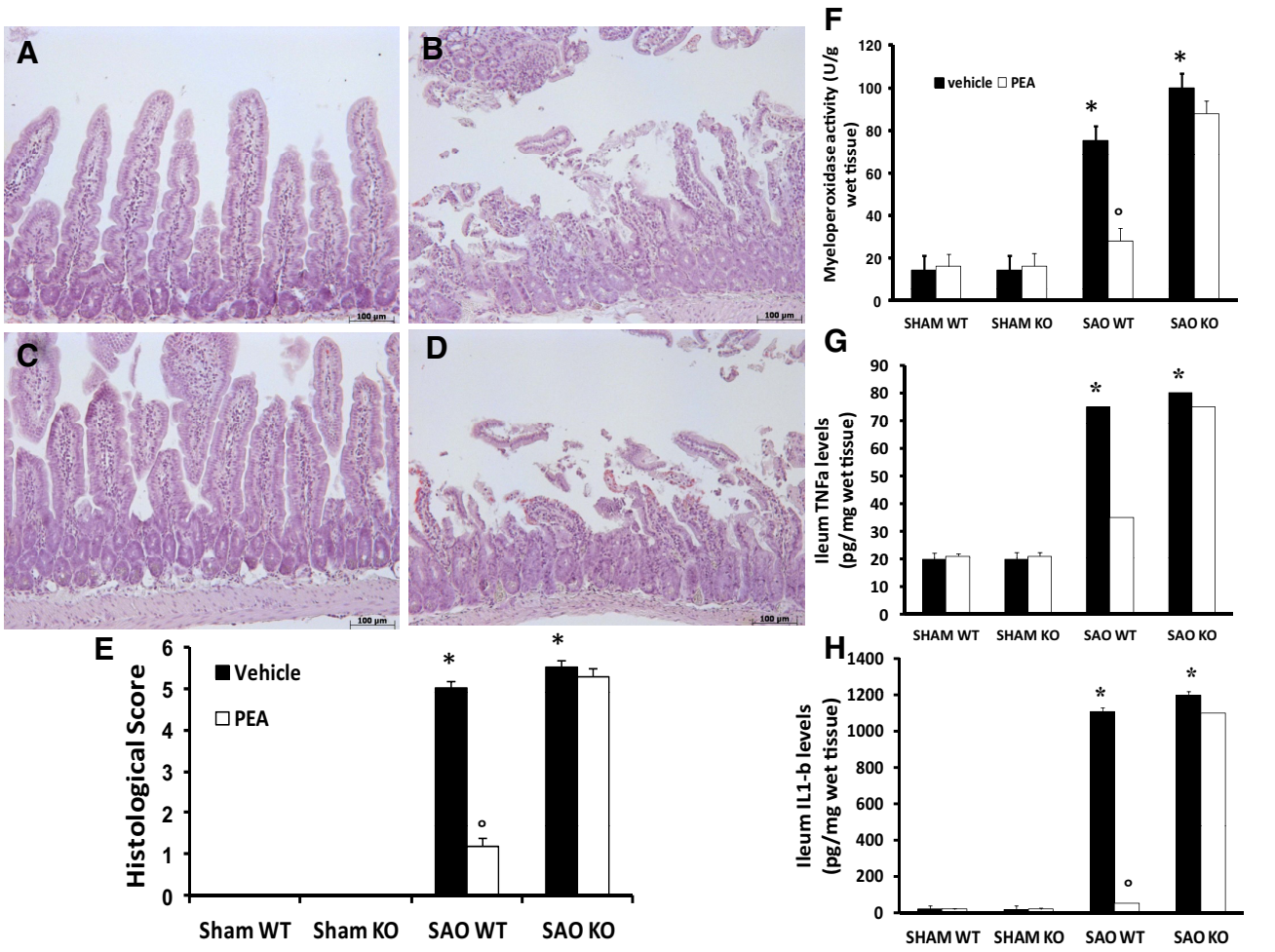

Figure 6. Effect of PPAR-α gene deletion and PEA administration. Histological examination of the small intestine after 60 min of reperfusion revealed expected and characteristic pathological changes (B). Histological features of normal gut tissue were observed in gut from sham-operated mice (A). PEA treatment reduces the degree of tissue injury in ileum tissue from WT mice (C). On the contrary, PEA treatment did not reduce the ileum injury in tissue sections from PPAR-α KO mice (D). The histological score (E) was made by an independent observer. Moreover, the increase in neutrophil infiltration seen in PPAR-α KO mice subjected to I/R was significantly higher than that seen in WT mice (F). However, the degree of protection from neutrophil infiltration observed with PEA treatment in WT mice was not seen in PPAR-α KO mice treated with PEA (F). Furthermore, when compared with sham animals, PPAR-α KO and WT SAO mice resulted in an increase in the levels of proinflammatory cytokines, such as TNF-α (G) and IL-1β (H), in the tissue homogenates. The release of TNF-α (G) and IL-1β (H) was attenuated significantly by treatment with PEA in PPAR-α WT mice, but it was not seen in PPAR-α KO mice treated with PEA. This figure is representative of at least three experiments performed on different experimental days. The results are expressed as mean ± SEM from $n = 10$ mice for each group. *$P < 0.01$ versus sham group; °$P < 0.01$ versus SAO shock plus vehicle (E–H).

evaluate the possible involvement of PPAR-α in the protective effect of PEA in this experimental model, SAO shock was also induced in PPAR-α KO mice (**Fig. 6**). Our results, in agreement with a precedent study [11], clearly demonstrated that exogenous administration of PEA attenuates inflammation, but PEA was protective in PPAR-α KO animals, although the protection from intestinal dysfunction observed in PPAR-α KO mice was lower than that observed in WT mice. Thus, the effect of PEA treatment on NF-κB activation may be related to PPAR-α activation, resulting in an up-regulation of PPAR-α expression.

In conclusion, taken together, our data suggested that PEA may be useful in the treatment of intestinal I/R injury. The mechanism of action of PEA, however, remains unclear. Future studies will provide useful information concerning this elusive mechanism of action.

AUTHORSHIP

R.D.P. performed experiments and prepared the manuscript. D.I. performed the biochemical analysis. A.T. performed the permeability study. E.M. performed the histological and immunohistochemistry analysis. A.C. analyzed the results. C.F. performed the permeability study and analyzed the results., E.E. performed the Western blot analysis and analyzed the results. F.T. analyzed the results. S.C. planned the study, analyzed the results, and prepared the manuscript.

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DISCLOSURES

None of the other authors has financial or other conflicts of interests to disclose.

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KEY WORDS:
circulatory shock · fatty acid ethanolamide family · cytokines · adhesion molecules · apoptosis