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Glycogen synthase kinase-3 β inhibition attenuates the development of ischaemia/reperfusion injury of the gut

Received: 11 October 2006
Accepted: 21 February 2007
Published online: 24 March 2007
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Abstract *Objective:* This study investigated the effects of TDZD-8, a potent and selective GSK-3 β inhibitor, on tissue injury caused by ischaemia/reperfusion (I/R) of the gut. *Design and setting:* Animal study in the Department of Clinical and Experimental Medicine and Pharmacology, School of Medicine, University of Messina, Italy. *Subjects:* Splanchnic artery occlusion (SAO) shocked rats. *Interventions:* I/R injury of the intestine was caused by clamping both the superior mesenteric artery and the coeliac trunk for 45 min followed by release of the clamp allowing reperfusion for 1 or 6 h. This procedure results in SAO shock. *Measurements and results:* Only 10% of the SAO animals survived the entire 6 h reperfusion period. In a separate set of experiments after 60 min of reperfusion animals were killed for histological examination and biochemical studies. Administration of TDZD-8 (1 mg/kg i.v.) 5 min prior to the reperfusion significantly reduced the (a) fall in mean arterial

blood pressure, (b) mortality rate, (c) infiltration of the reperfused intestine with polymorphonuclear neutrophils (MPO activity), (d) production of pro-inflammatory cytokines (TNF- α and IL-1 β) and (e) histological evidence of gut injury. Administration of TDZD-8 also markedly reduced the immunoreactivity of nitrotyrosine formation and the expression of ICAM-1 and P-selectin during reperfusion. *Conclusions:* Based on these findings we propose that TDZD-8 would be useful in the treatment of various ischaemia and reperfusion diseases.

Keywords 4-Benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione · Ischaemia/reperfusion · Myeloperoxidase · Cytokines · Nitrotyrosine · Adhesion molecules

Introduction

Ischaemia leads to hypoxia, which initiates a series of events primarily related to activation of platelets and release of their vasoconstrictor mediators, which further restrict blood flow to the ischaemic area. It is important to realise that reperfusion of an ischaemic organ is as-

sociated not only with local changes, and that in some situations reperfusion is also associated with systemic changes. For example, in a model of ischaemia/reperfusion (I/R) of the intestine local functional alterations include a progressive fall in the mean arterial blood pressure (MABP), release of pro-inflammatory mediators from the reperfused tissues into the systemic circulation and

ultimately reduced survival [1]. One of the earliest phenomena occurring in I/R is the endothelial dysfunction, which is considered the "trigger" of reperfusion injury [2]. Leukocyte-endothelial interaction involves a complex system of adhesion molecules [3] and begins with polymorphonuclear cell (PMN) rolling, followed by adherence and trans-endothelial migration. The firm adherence involves the interaction between β_2 integrands on the PMN surface and intercellular adhesion molecule (ICAM) 1 on the endothelial cell surface [3]. ICAM-1 is normally expressed at a low basal level, but its expression can be enhanced by various inflammatory mediators [4]. Glycogen synthase kinase (GSK) 3 was first identified in 1980 as a ubiquitous serine-threonine protein kinase involved in glycogen metabolism [5]. Two isoforms have been isolated in mammals, GSK-3 α and GSK-3 β . GSK-3 is constitutively active in cells, although phosphorylation of a specific serine residue (Ser21 in GSK-3 α and Ser9 in GSK-3 β) located in its N-terminal domain inhibits GSK-3 activity and hence reduces its activity to alter cell function [6]. A wide variety of extracellular stimuli exert their effects by inhibiting GSK-3 activity [7]. Unique to GSK-3 β is its reported ability to influence the activity of the transcription factor nuclear factor (NF) κ B [8]. The regulatory influence of GSK-3 β on the activity of NF- κ B, which has since been confirmed in a range of systems [9, 10], is the basis for the hypothesis that GSK-3 β may play a key role in the regulation of the inflammatory response.

Recent *in vivo* studies have demonstrated that 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8) and SB 415286, potent selective inhibitors of GSK-3 β , reduces the renal and liver dysfunction caused by both endotoxaemia and administration of endotoxin and peptidoglycan [11, 12]. In addition, there is also evidence that TDZD-8 reduces the development of colon injury associated with experimental colitis [13], as well as the development of acute and chronic inflammation in mice [14], and modulates secondary damage following spinal cord injury [14].

The aim of this study was to investigate the effects of TDZD-8, a potent and selective GSK-3 β inhibitor, on the tissue injury caused by ischaemia/reperfusion (I/R) of the gut.

Methods

Animals

Male Sprague-Dawley rats (250–300 g; Harlan Nossan, Milan, Italy) were housed in a controlled environment and allowed access to food and water *ad libitum*. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purpose (D.M. 116192) and with EEC regulations (O.J. of E.C.L 358/1, 18 December 1986).

Surgical procedures

Rats were anaesthetised with sodium pentobarbital (45 mg/kg, intraperitoneally). Following anaesthesia catheters were placed in the carotid artery and jugular vein as described previously [15]. Blood pressure was monitored continuously by a Maclab A/D converter (Ugo Basile, Varese, Italy) and stored and displayed on a Macintosh personal computer. After midline laparotomy the coeliac and superior mesenteric arteries were isolated near their aortic origins. Rats were observed for a 30 min stabilisation period before either splanchnic ischaemia or sham ischaemia. SAO shock was induced by clamping both the superior mesenteric artery and the coeliac trunk, resulting in a total occlusion of these arteries for 45 min. After this period of occlusion the clamps were removed and the splanchnic circulation was allowed to reperfuse for 2 h at which time the experiment was terminated. Sixty minutes after reperfusion animals were killed and an 8 to 10 cm length of ileum, 30 cm distal to the stomach, was isolated, washed in saline and used for histological examination and for biochemical studies as previously described by Hayward and Lefer [16].

Experimental groups

Rats were randomly allocated into the following groups ($n = 10$ each):

- Sham +vehicle group: rats were treated with 10% dimethyl sulfoxide (DMSO, 1 ml/kg, intravenously) and subjected to the surgical procedure alone except that the blood vessels were not occluded and the rats were maintained under anaesthesia for the duration of the experiment.
- Sham +TDZD-8 group: identical to sham +vehicle group except for the administration of TDZD-8 (1 mg/kg intravenous bolus) 5 min prior to surgical procedures.
- I/R +vehicle group: rats were subjected to SAO shock and were administered DMSO.
- TDZD-8 group: identical to the I/R +vehicle group but were administered TDZD-8 (1 mg/kg intravenous bolus) 5 min prior to reperfusion.

In the experiments investigating the survival rates and survival times the rats ($n = 15$ from each group) were monitored for 6 h after reperfusion. The doses of TDZD-8 used here to reduce ischaemia-reperfusion injury in the gut were based on previous *in vivo* studies [17–19].

Light microscopy

Ileum biopsy samples were taken after 60 min of reperfusion. The specimens were fixed for 1 week in buffered

formaldehyde solution (10% in phosphate-buffered saline) at room temperature, dehydrated by graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, N.J., USA). Tissue sections (thickness 7 μ m) were deparaffinised with xylene, stained with haematoxylin/eosin and studied using light microscopy (Dialux 22 Leitz). In order to have a quantitative estimation, section ($n=6$ for each animals) was scored by an independent observer blinded to the experimental protocol. The morphological criteria considered were previously described [20].

Myeloperoxidase activity

Myeloperoxidase (MPO) activity, an indicator of PMN accumulation, was determined as previously described in intestinal tissues collected after 60 min of reperfusion [21]. MPO activity was defined as the quantity of enzyme degrading 1 μ mol peroxide/min at 37 °C and was expressed as MPO units per milligram of protein.

Malondialdehyde measurement

Thiobarbituric acid reactant malondialdehyde (MDA) measurement, which is considered a good indicator of lipid peroxidation, was determined, as previously described in intestinal tissues collected at after 60 min of reperfusion [22].

Measurement of cytokines

TNF- α and IL-1 β levels were evaluated in the plasma and ileum tissues collected after 60 min of reperfusion as previously described [23], using a colorimetric commercial enzyme-linked immunosorbent assay kit (Calbiochem-Novabiochem, Milan, Italy).

Immunohistochemical localisation of ICAM-1, P-selectin, nitrotyrosine, Bax and Bcl-2

After 60 min of reperfusion ileum tissues were fixed in 10% (w/v) PBS-buffered formaldehyde and 7- μ m sections were prepared from paraffin embedded tissues, as previously described [1]. Sections were incubated overnight with (a) purified goat polyclonal antibody directed towards P-selectin which reacts with rats, (b) with purified hamster anti-mouse ICAM-1 (CD54; 1:500 in PBS, w/v), (c) with anti-nitrotyrosine rabbit polyclonal antibody (Upstate Biotechnology, 1:500 in PBS, v/v), (d) with anti-Bax antibody (Santa Cruz Biotechnology, Calif., USA, 1:500 in PBS, v/v) or (e) with anti-Bcl-2 antibody (Santa Cruz Biotechnology, 1:500 in PBS, v/v). Sections were

incubated with secondary antibody. Specific labelling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (DBA, Milan, Italy).

Western blot analysis for Bax and Bcl-2

Ileum tissues were homogenated in a buffer containing: 1% nonidet P-40 (NP-40), 150 mM NaCl, 10 mM NaF, 20 mM Tris pH 7.5, phenylmethylsulphonyl fluoride (PMSF) 1 mM, trypsin inhibitor 10 μ g/ml, leupeptin 10 μ g/ml, sodium orthovanadate (1 mM). The filters were blocked with 1 \times PBS, 5% (w/v) non-fat dried milk (PM) for 40 min at room temperature and subsequently probed with specific monoclonal antibodies against Bax (Santa Cruz Biotechnology, 1:100) or Bcl-2 (Santa Cruz Biotechnology, 1:100) in 1 \times PBS, 5% w/v non-fat dried milk, 0.1% Tween-20 (PMT) 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, West Grove, Pa., USA) for 1 h at room temperature. The immune complexes were visualised using the SuperSignal West Pico chemiluminescence Substrate (Pierce, Milan, Italy).

Subsequently the relative expression of the protein bands of Bax (approx. 23 kDa), Bcl-2 (approx. 29 kDa) was quantified by densitometric scanning of the radiographic films with GS-700 Imaging Densitometer (GS-700, Bio-Rad, Milan, Italy). Sub-cellular fractionation and western blot analysis for I κ B- α , phospho-NF- κ B p65 (serine 536), and phospho-GSK-3 β Ser 9.

Cytosolic and nuclear extracts were prepared as previously described [24] with slight modifications. Briefly, ileum tissues from each rat were suspended in extraction Buffer A containing 0.2 mM PMSF, 0.15 μ M pepstatin A, 20 μ M leupeptin, 1 mM sodium orthovanadate, homogenised at the highest setting for 2 min and centrifuged at 1,000 g for 10 min at 4 °C. Supernatants represented the cytosolic fraction. The pellets, containing enriched nuclei, were re-suspended 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 μ M, 0.2 mM sodium orthovanadate. After centrifugation, 30 min at 15,000 g at 4 °C, the supernatants containing the nuclear protein were stored at -80 °C for further analysis. The levels of I κ B- α , phospho-NF- κ B p65 (serine 536), were quantified in cytosolic fraction from ileum tissue collected after 60 min of reperfusion. The filters were blocked with PM for 40 min at room temperature and subsequently probed with specific monoclonal antibodies I κ B- α (Santa Cruz Biotechnology, 1:1000), or phospho-NF- κ B p65 (serine 536; Cell Signaling, 1:1000) or phospho-specific GSK-3 β Ser 9 (Stressgene, 1:1000) in PMT 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) for 1 h at room temperature.

To ascertain whether blots were loaded with equal amounts of proteic lysates, they were also incubated in the presence of the antibody against β -actin protein (1:10,000 Sigma-Aldrich). The relative expression of the protein bands of $I\kappa B$ - α (approx. 37 kDa), phospho NF- κB (75 kDa) and GSK-3 β Ser 9 (110 kDa) was quantified by densitometric scanning of the radiographic films with GS-700 imaging densitometer (Bio-Rad) and a computer program (Molecular Analyst, IBM) were quantified by scanning densitometry (GS-700).

Terminal deoxynucleotidyl transferase mediated UTP nick end labelling (TUNEL) assay

We carried out terminal deoxynucleotidyl transferase (TdT) mediated UTP nick end labeling (TUNEL) using a detection kit according to the manufacturer's instructions (Apotag, HRP kit DBA, Milan, Italy), as previously described [19]. 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-horseradish peroxidase-conjugated antibody, and the signals were visualised with diaminobenzidine.

Materials

Unless otherwise stated, all compounds were obtained from Sigma-Aldrich (Poole, Dorset, UK). TDZD-8 was obtained from Calbiochem (Merck Biosciences, Beeston, Nottingham, UK) All other chemicals were of the highest commercial grade available. All stock solutions were prepared in non-pyrogenic saline (0.9% NaCl; Baxter, Italy, UK). We obtained 1,1,3,3-tetramethoxypropan, 99%, and

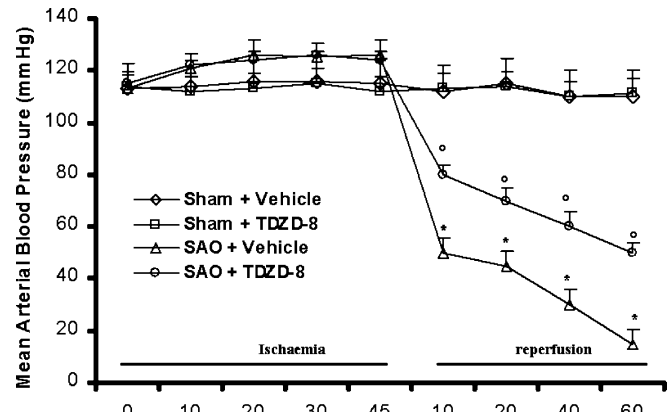


Fig. 1 No significant alteration in MABP was observed in sham-operated rats. Fall in MABP in SAO rats ($n = 10$) is blocked by treatment with TDZD-8. Data are means \pm SD of 10 rats for each group. * $p < 0.01$ vs. sham, $^{\circ} p < 0.01$ vs. I/R

Fig. 2 Reperfusion of the ischaemic splanchnic circulation leads to profound increase in plasma and ileum TNF- α (a, b) and IL-1 β (c, d) production. This enhance were significantly inhibited by the treatment with TDZD-8 (1 mg/kg). Data are means \pm SD of 10 rats for each group. * $p < 0.01$ vs. sham, $^{\circ} p < 0.01$ vs. I/R

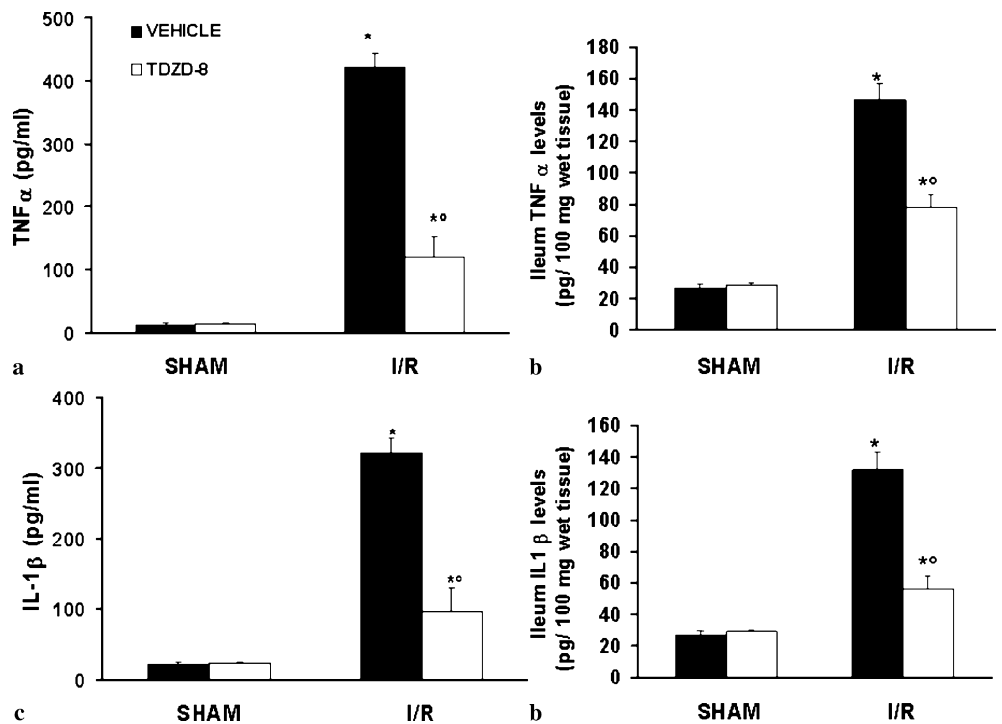
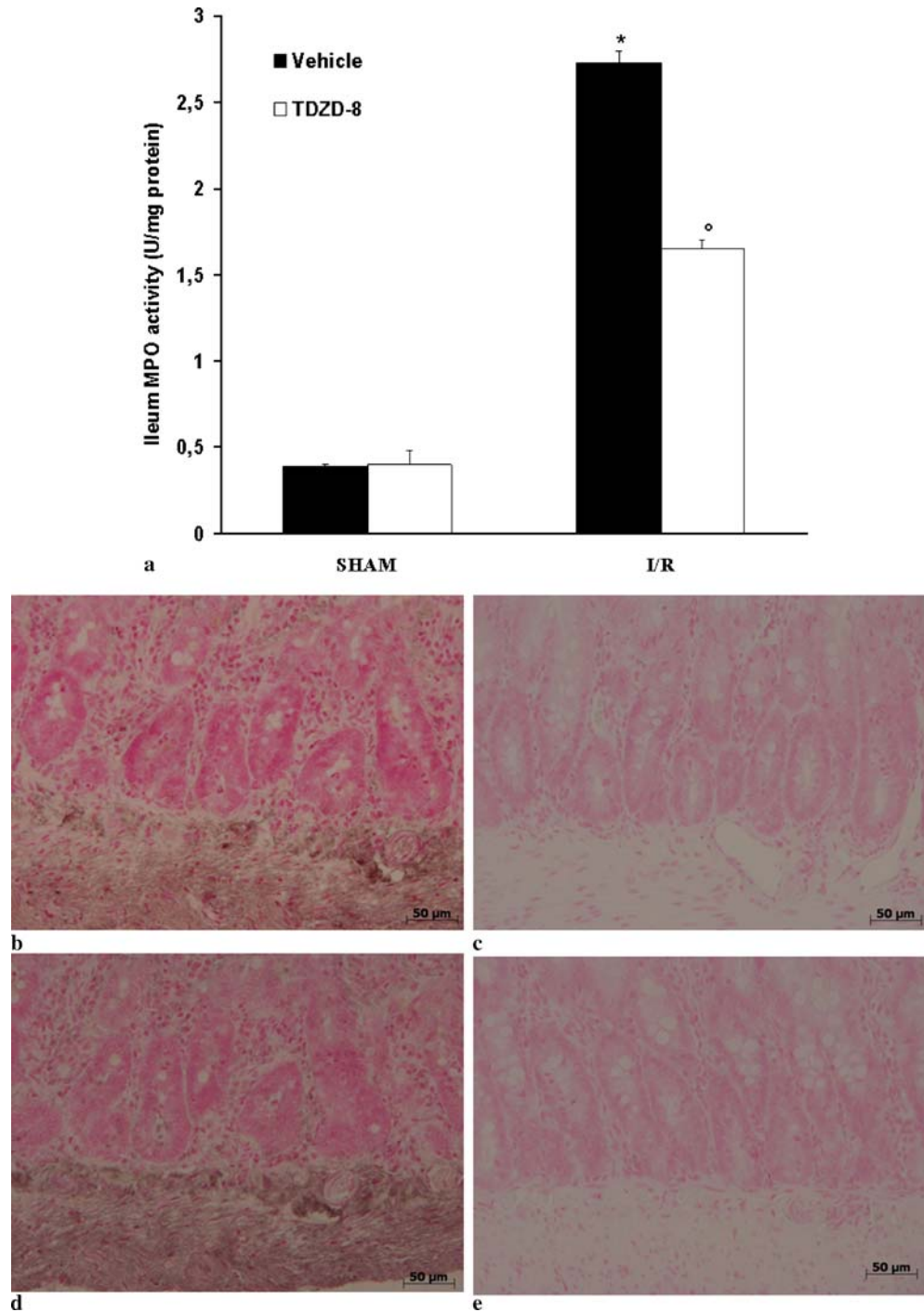


Fig. 3 a Reperfusion of the ischaemic splanchnic circulation leads to increase in MPO activity, which are inhibited by the treatment with TDZD-8. **b, d** In addition, immunohistochemical analysis of intestinal sections obtained from rats subjected to splanchnic ischaemia/reperfusion revealed a positive staining for ICAM-1 (**b**) and for P-selectin (**d**) in the injured tissues, which was localised primarily around the vessel. **c, e** There was no detectable immunostaining for ICAM-1 (**c**) or for P-selectin (**e**) in the ileum from TDZD-8 treated rats. The figure is representative of at least three experiments performed on different experimental days. Data are means \pm SD of 10 rats for each group. * $p < 0.01$ vs. sham, ° $p < 0.01$ vs. I/R



MDA bis(dymethyl acetal), 99%, from Sigma-Aldrich (Milan, Italy).

Statistical evaluation

All values are expressed as mean \pm standard error (SEM) of n observations; in the in vivo studies n represents

the number of animals studied. In the experiments involving histology or immunohistochemistry figures are representative of at least three experiments (histological or immunohistochemistry coloration) performed on different experimental days on the tissue sections collected from all the animals in each group. The results were analysed by one-way analysis of variance followed by Bonferroni's post-hoc test for multiple comparisons.

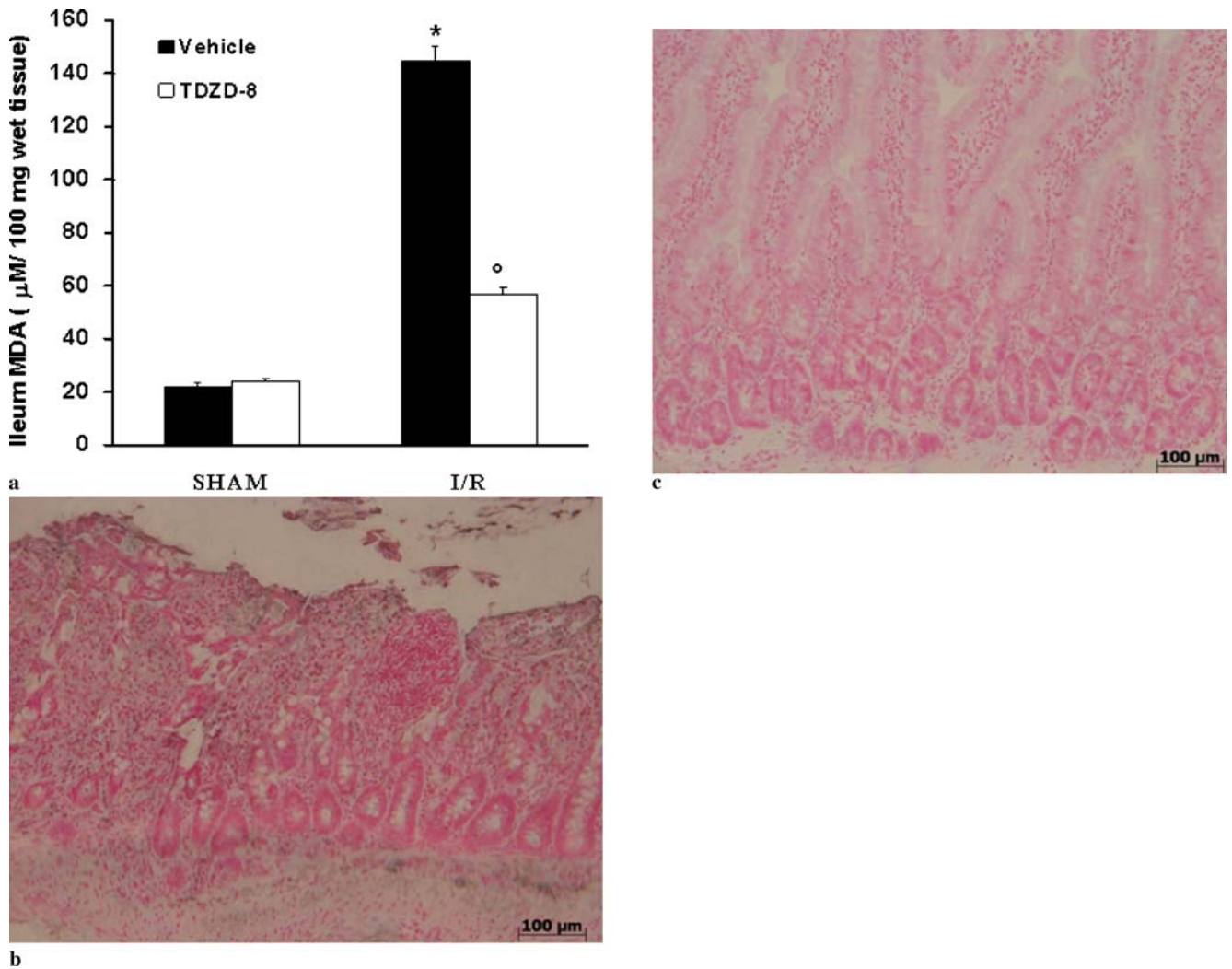


Fig. 4 **a** Reperfusion of the ischaemic splanchnic circulation leads to profound increase in MDA levels in ileum tissues which is inhibited by treatment with TDZD-8. **b** Immunohistochemical analysis of intestinal sections obtained from rats subjected to splanchnic ischaemia/reperfusion revealed a positive staining for nitrotyrosine in

the injured tissues. **c** There was no detectable immunostaining in the ileum from TDZD-8 treated rats. Data are means \pm SD of 10 rats for each group. * $p < 0.01$ vs. sham, ° $p < 0.01$ vs. I/R. The figure is representative of at least three experiments performed on different experimental days

Differences with a p value less than 0.05 were considered statistically significant, and individual group means were then compared using Student's unpaired t test. A p value less than 0.05 was considered statistically significant.

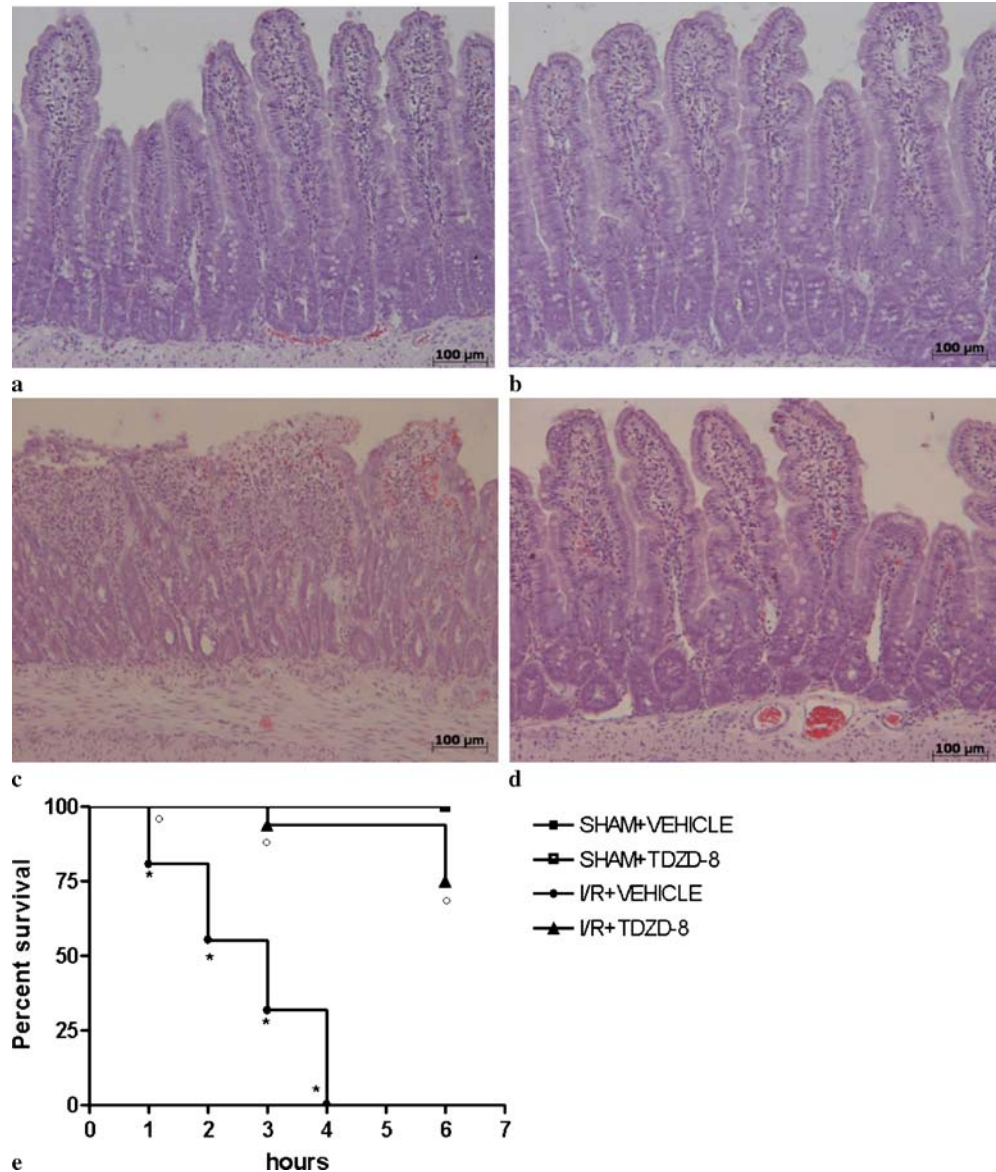
Results

Effects of TDZD-8 in SAO shock

Occlusion of the splanchnic arteries produced an increase in MABP which then decreased until death (Fig. 1). The TDZD-8 treatment significantly prevented the fall in blood

pressure seen after reperfusion (Fig. 1). Animals were killed either after the period of ischaemia or after 60 min of reperfusion to collect blood and tissues for biochemical analysis. Reperfusion of the ischaemic splanchnic circulation led to the following events: substantial increase in intestinal TNF- α and IL-1 β levels (Fig. 2) and a profound infiltration of neutrophils into the intestine (Fig. 3a). These inflammatory events were triggered by the reperfusion phase since no changes were observed when blood or tissues were removed after the period of ischaemia alone (data not shown). TDZD-8 (1 mg/kg) 5 min prior to reperfusion significantly reduced TNF- α and IL-1 β plasma and ileum levels (Fig. 2) as well as the neutrophil infiltration into the ileum (Fig. 3a).

Fig. 5 **a, b** Histological features of normal gut tissue were observed in gut tissues prepared from sham-operated rats treated with vehicle (**a**) or with TDZD-8 (**b**). **c** Distal ileum section from SAO-shocked rats showed inflammatory infiltration by PMNs and lymphocytes extending through the wall and concentrated below the epithelial layer and demonstrating oedema of the distal portion of the villi. **d** Distal ileum from TDZD-8 (1 mg/kg) treated rats shows reduced SAO-induced organ injury. **e** In addition, survival was monitored for 6 h after SAO shock. Data are means \pm SD of 20 rats for each group. * $p < 0.01$ vs. sham, ° $p < 0.01$ vs. I/R. The figure is representative of at least three experiments performed on different experimental days



Immunohistochemical localisation of ICAM-1 and P-selectin in the reperfused intestine

To further elucidate the effect of TDZD-8 on neutrophil accumulation in reperfused ileum we evaluated the intestinal expression of P-selectin and ICAM-1. Staining of ileum tissue sections obtained from sham-operated rats with anti-ICAM-1 antibody showed no positive for ICAM-1 (data not shown). After 1 h of reperfusion significant positive staining for ICAM-1 localised mainly around the vessels was observed (Fig. 3b). Sections from TDZD-8 treated rats did not reveal positive staining for ICAM-1 (Fig. 3c). Ileum tissue sections obtained from at SAO-shocked rats after 60 min of reperfusion showed positive staining for P-selectin localised in the vessels

(Fig. 3d). No positive staining for P-selectin was found in the ileum tissue obtained after 60 min of reperfusion from TDZD-8 treated rats (Fig. 3e). No staining was observed in sham-operated animals (data not shown).

TDZD-8 reduced lipid peroxydation and nitrotyrosine formation

After 60 min of reperfusion small intestine was investigated for MDA levels, indicative of lipid peroxydation. As shown in Fig. 4a, MDA levels were significantly increased in the ileum from SAO-shocked rats ($p < 0.01$). The TDZD-8 (1 mg/kg) when given intravenously 5 min prior to reperfusion significantly inhibited the increased ileum

Fig. 6 Representative western blots showing the effects of TDZD-8 on I κ B- α degradation (**a, a1**) and phosphorylation of Ser536 on NF- κ B subunit p65 (**b, b1**) after SAO-shock.

A representative blot of lysates (**a, b**) obtained from 5 animals per group is shown and densitometry analysis of all animals is reported. The results in **a1** and **b1** are expressed as mean \pm SEM from 5 or 6 ileum tissues for each group. * $p < 0.01$ vs. sham, $^{\circ}p < 0.01$ vs. I/R

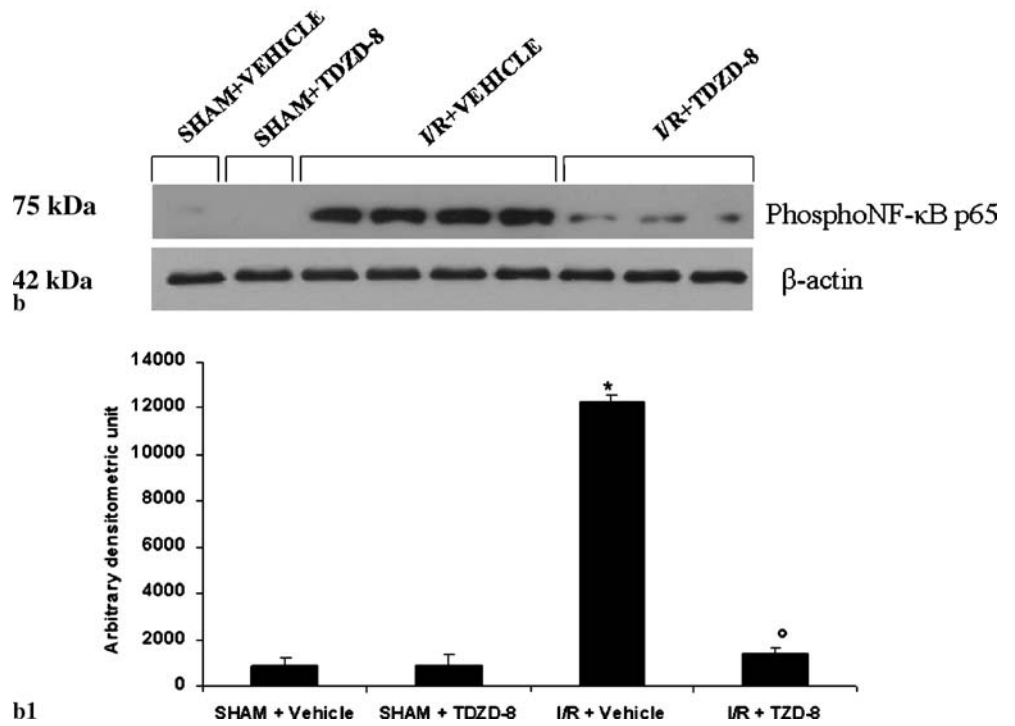
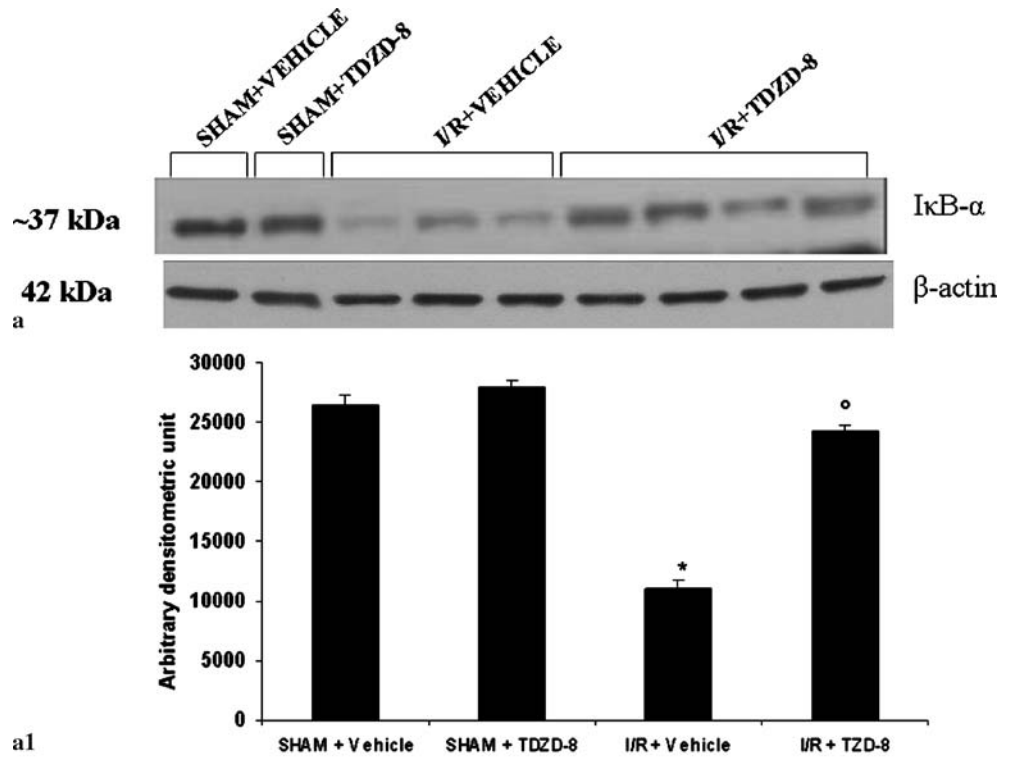
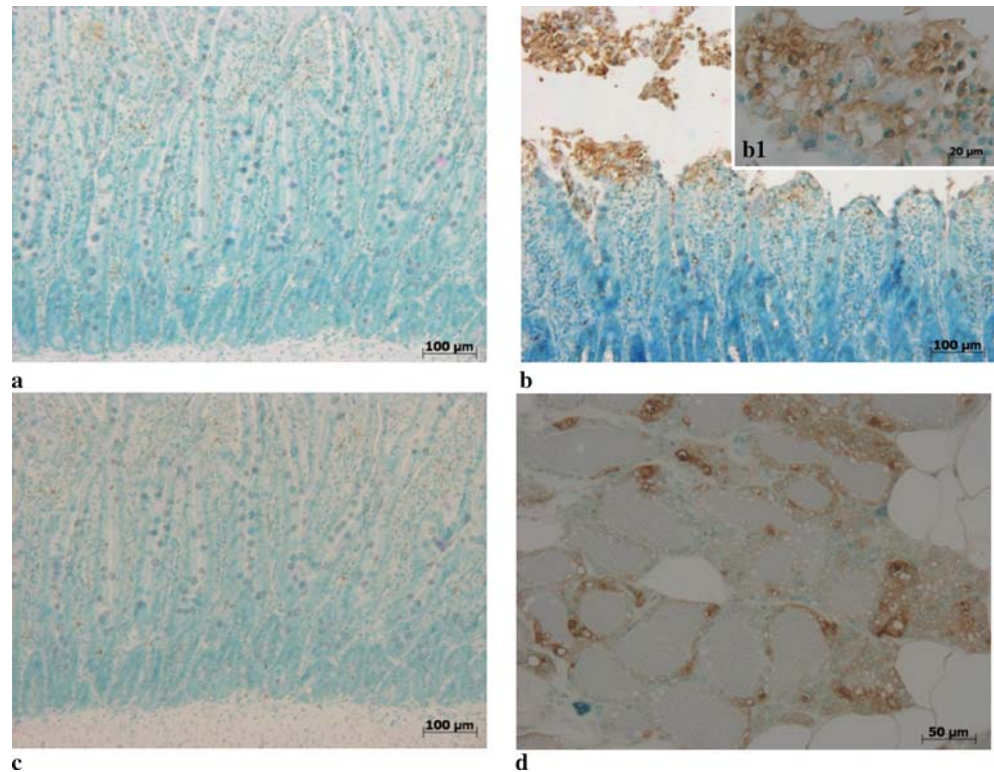


Fig. 7 Effect of TDZD-8 on SAO-shocked induced apoptosis as measured by TUNEL-like staining. (a) No apoptotic cells were observed in ileum from sham-operated rats. (b) Positive staining was observed in ileum sections taken from SAO-shock rats treated with vehicle (see particles **b1**). (c) In contrast, tissues obtained from SAO-shock treated rats treated with 1 mg/kg TDZD-8 demonstrated no apoptotic cells or fragments. (d) A positive control is also included. The figure is representative of at least three experiments performed on different experimental days



levels of MDA (Fig. 4a). The increase in lipid peroxidation activity was triggered by the reperfusion phase since no changes were observed when tissues were removed after the period of ischaemia alone (data not shown). In addition, ileum tissue sections obtained from SAO-shocked rats undergoing 45 min of ischaemia followed by 1 h of reperfusion showed positive staining for nitrotyrosine (Fig. 4b). Administration of TDZD-8 (1 mg/kg) significantly reduced the degree of immunostaining for nitrotyrosine in the reperfused intestine (Fig. 4c). No staining for nitrotyrosine in intestine was obtained from sham-operated rats (data not shown).

Effects of TDZD-8 on histological changes caused by gut I/R

Histological examination of the small intestine after 60 min of reperfusion revealed expected and characteristic pathological changes (see representative sections in Fig. 5). Histological features of normal gut tissue were observed in gut tissues prepared from sham-operated rats. 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. 5c). The degree of the tissue injury was 3.75 ± 0.11 . TDZD-8 (1 mg/kg) treatment (Fig. 5d) significantly prevented ileum damage induced by I/R, with a significant reduced damage score (1.1 ± 0.05).

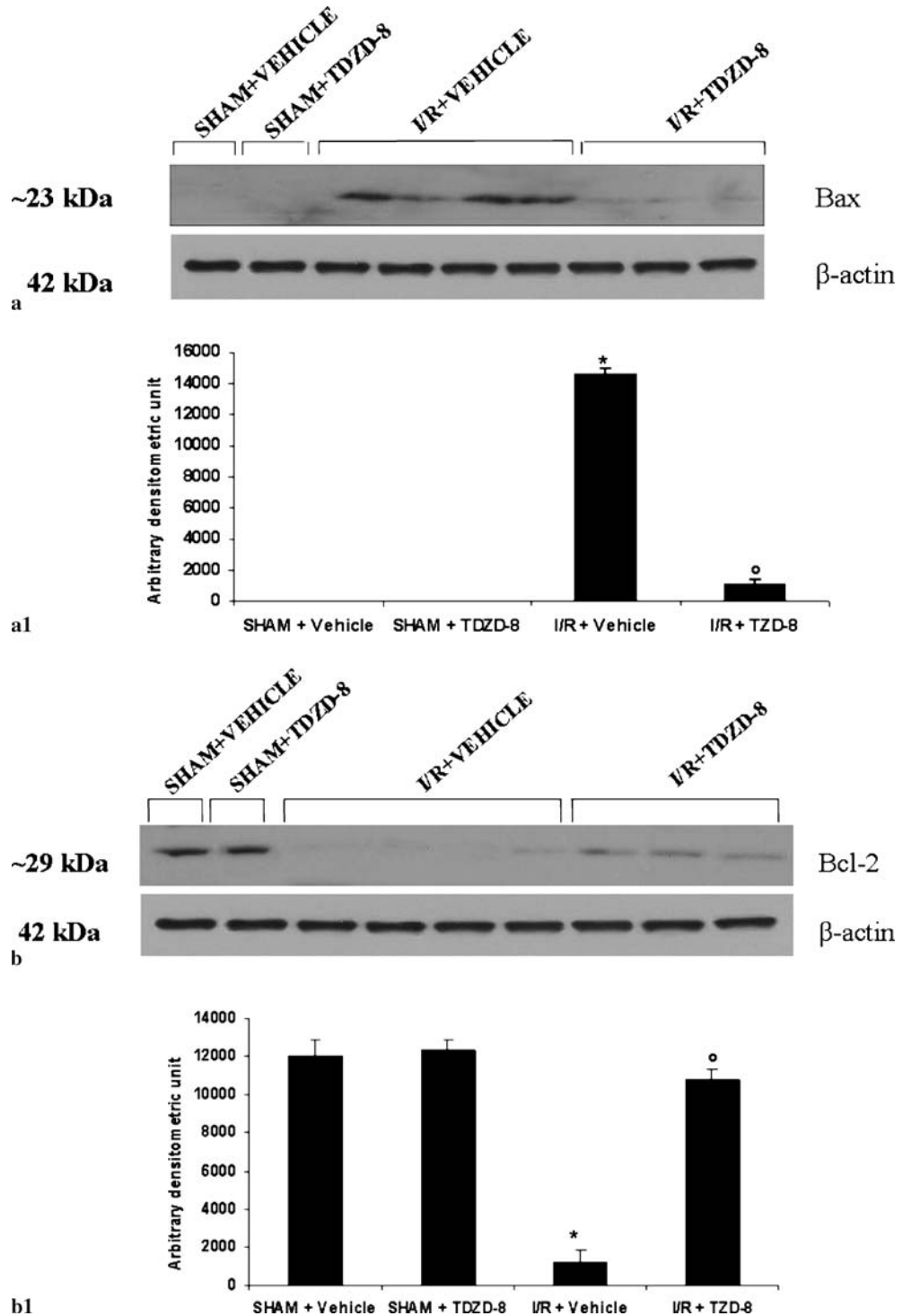
Effects of TDZD-8 on survival rate

All sham-operated rats survived the entire 6 h reperfusion-period (Fig. 5e). In contrast, splanchnic artery occlusion produced a profound shock state characterised by a 100% lethality at the end of the 6-h reperfusion period (Fig. 5). The mean survival time was found to be 65 ± 1.5 min whereas control sham animals survived for the entire period of reperfusion period (6 h; Fig. 5e). TDZD-8 (1 mg/kg) treatment significantly prevented the mortality induced by I/R (Fig. 5e).

Effect of TDZD-8 on I κ B- α degradation and phosphorylation of Ser536 on p65

To investigate the cellular mechanisms by which treatment with TDZD-8 attenuates the development of intestinal injury associated with ischaemia and reperfusion we evaluated both I κ B- α degradation and phosphorylation of Ser536 on the NF- κ B subunit p65. After 60 min of reperfusion a basal level of I κ B- α was detected in the ileum tissues from sham-animals (Fig. 6a; see densitometry analysis, b) whereas in ileum from SAO-shocked rats I κ B- α expression was substantially reduced (Fig. 6a; see densitometry analysis, a1). TDZD-8 (1 mg/kg) prevented SAO-shock induced I κ B- α degradation. The I κ B- α levels observed in these animals were similar to those of the sham group (Fig. 6a; see densitometry analysis,

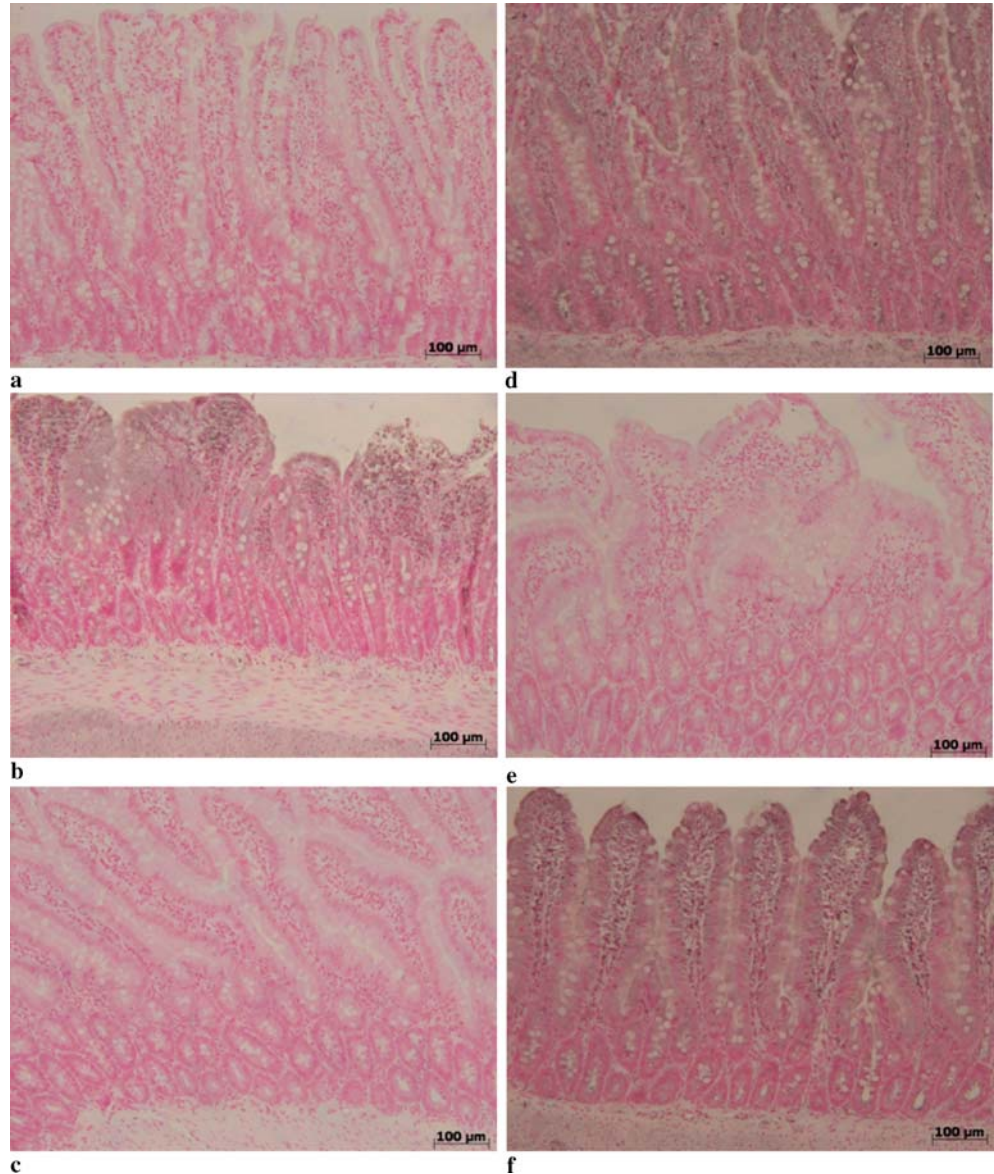
Fig. 8 Representative western blots showing the effects of TDZD-8 on Bax (**a, a1**) and Bcl-2 (**b, b1**) expression after SAO-shock. A representative blot (**a, b**) is shown and densitometry analysis of all animals is reported. The results in **a1** and **b1** are expressed as mean \pm SEM from 5 or 6 ileum tissues for each group. * $p < 0.01$ vs. sham, $^{\circ}p < 0.01$ vs. I/R



a1). We also evaluated the phosphorylation of Ser536 on the NF- κ B subunit p65 by western blot analysis in ileum collected after 60 min of reperfusion. A significant increase in the phosphorylation of Ser536 was observed

in ileum tissues from SAO-shocked rats (Fig. 6b; see densitometry analysis, b1). Treatment with the GSK-3 β inhibitor significantly reduced the phosphorylation of p65 on Ser536 (Fig. 6b; see densitometry analysis, b1).

Fig. 9 Immunohistochemical expression of Bax and Bcl-2. (a) No positive staining for Bax was observed in the tissue section from sham-operated rats. (b) SAO-shocked after 60 min of reperfusion, an increase in the release of Bax expression. (c) Treatment with TDZD-8 significantly inhibited the SAO-induced increase in Bax expression. (d) On the other hand, positive staining for Bcl-2 was observed in the ileum tissues of sham-operated rats. (e) After 60 min of reperfusion significantly less staining for Bcl-2 was observed. (f) TDZD-8 treatment significantly prevented the loss of Bcl-2 expression induced by SAO-shock. The figure is representative of at least three experiments performed on different experimental days



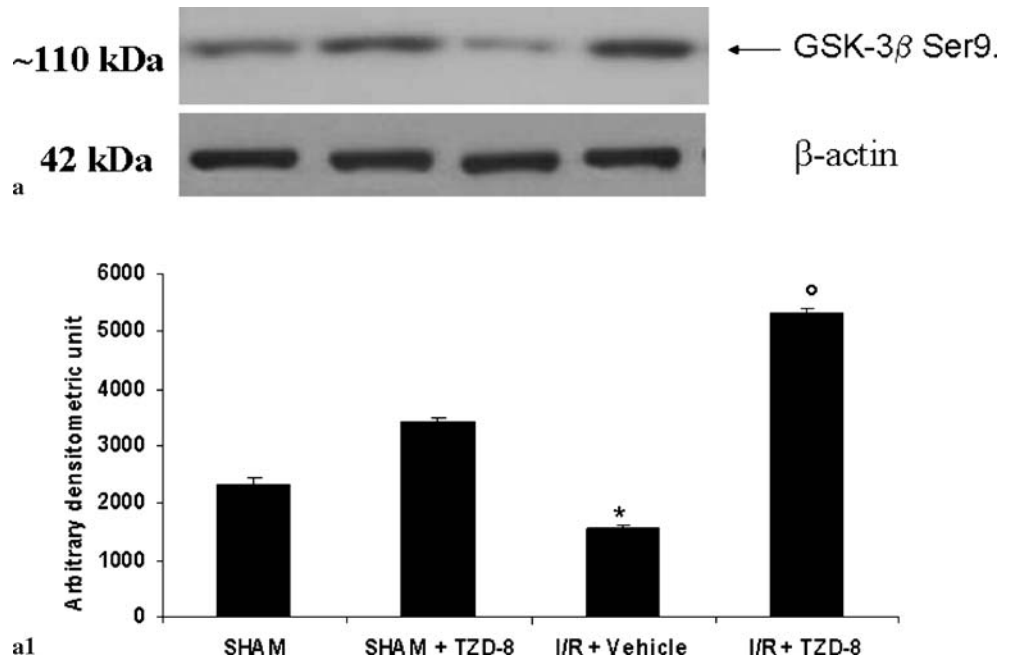
Effects of TDZD-8 on apoptosis in ileum tissues after gut I/R

To investigate whether gut injury is associated with apoptotic cell death we measured TUNEL-like staining in ileum tissues. After 60 min of reperfusion ileum tissues demonstrated a marked appearance of dark brown apoptotic cells and intercellular apoptotic fragments (Fig. 7b; see particles, b1). In contrast, no apoptotic cells or fragments were observed in the tissues obtained from SAO-shocked rats treated with TDZD-8 (1 mg/kg; Fig. 7c). Similarly, no apoptotic cells were observed in ileum of sham-treated rat (Fig. 7a). A positive control is also included (Fig. 7d).

Western blot analysis and immunohistochemistry for Bax and Bcl-2

No Bax level was detected in ileum tissues obtained from sham-treated animals by western blot analysis (Fig. 8a; see densitometry analysis, a1). Bax expression were significantly increased in the ileum tissues obtained from SAO-shocked rats (Fig. 8a; see densitometry analysis, a1). On the other hand, TDZD-8 (1 mg/kg) treatment prevented shock-induced Bax expression (Fig. 8a; see densitometry analysis, a1). To detect Bcl-2 expression whole extracts from ileum tissues were also analysed by western blot analysis. A basal level of Bcl-2 expression was detected in ileum tissues from sham-treated rats (Fig. 8b; see

Fig. 10 Effects of TDZD-8 on the phosphorylation of GSK-3 β Ser9. (a) Western blot analysis of the phosphorylation of GSK-3 β on Ser9 was carried out in the intestinal tissues collected after 60 min of reperfusion. TDZD-8, a GSK-3 β inhibitor, induced a significant increase in phosphorylation of GSK-3 β at Ser9, consistent with inactivation of the kinase. The results in (a1) are expressed as mean \pm SEM from 5 or 6 ileum tissues for each group. * p < 0.01 vs. sham, $^{\circ}$ p < 0.01 vs. I/R



densitometry analysis, b1). Sixty minutes after reperfusion Bcl-2 expression was significantly reduced (Fig. 8b; see densitometry analysis, b1). Treatment with TDZD-8 significantly blunted SAO-induced reduction in Bcl-2 expression (Fig. 8b; see densitometry analysis, b1).

Ileum samples were also collected after 60 min of reperfusion to determine the immunohistological staining for Bax and Bcl-2. Ileum tissues taken from sham-treated rats did not stain for Bax (Fig. 9a) whereas ileum sections obtained from SAO-shocked rats exhibited positive staining for Bax (Fig. 9b). TDZD-8 treatment reduced the degree of positive staining for Bax in the ileum from rats subjected to SAO shock (Fig. 9c). In addition, ileum sections from sham-treated rats demonstrated positive staining for Bcl-2 (Fig. 9d) whereas in SAO-shocked rats Bcl-2 staining was significantly reduced (Fig. 9e). TDZD-8 treatment significantly attenuated the loss of positive staining for Bcl-2 in rats subjected to SAO shock (Fig. 9f).

Effects of TDZD-8 on the phosphorylation of GSK-3 β Ser9

To obtain a mechanistic insight into the effects of the GSK-3 β inhibitor used here a western blot analysis of the phosphorylation of GSK-3 β on Ser9 was carried out. The GSK-3 β inhibitor TDZD-8 induced a significant increase in the phosphorylation of GSK-3 β at Ser9, consistent with inactivation of the kinase (Fig. 10a, a1).

Discussion and conclusions

Several animal models have been studied to understand the pathophysiological mechanism. Among these SAO is a severe form of circulatory shock produced by ischaemia and reperfusion of the splanchnic organs. This type of shock is characterised by a decrease in systemic blood pressure upon release of the splanchnic arteries, which leads to a fatal outcome [25].

Our data demonstrate that TDZD-8 treatment exerts an important protective effect against SAO shock. The present study provides evidence that TDZD-8 attenuates: (a) the development of SAO-induced shock, (b) neutrophil infiltration, (c) lipid peroxidation, (d) the upregulation of P-selectin and ICAM-1 in the ileum, (e) the nitration of tyrosine residues, (f) NF- κ B expression, (g) apoptosis, (h) Bax and Bcl-2 expression and (i) the degree of ileum injury. All of these findings support the view that TDZD-8 attenuates the degree of gut ischaemia and reperfusion in the rats. What then is the mechanism by which TDZD-8 reduces SAO-shock?

The confirmation of a role of GSK-3 β in the regulation of gut ischaemia and reperfusion injury is of special interest because several transcription factors important to the regulation of acute inflammation serve as substrates for GSK-3 β . Among these is the transcription factor NF- κ B, whose function is strikingly altered by GSK-3 β [9]. NF- κ B plays a central role in the regulation of many genes, for example, TNF- α , IL-1 β , iNOS and COX-2 [26]. In 2000 Hoefflich and colleagues [9] first demonstrated that

deletion of GSK-3 β has no effect on the TNF- α induced I κ B- α degradation or on the nuclear translocation of the subunit p65 but does prevent the activation of NF- κ B by an unknown mechanism. On the other hand, other studies have provided evidence of an inverse association between the activity of GSK-3 β and NF- κ B signalling. We report here that SAO-shock causes a significant increase in the phosphorylation of Ser536 on p65 in the ileum tissues whereas treatment with TDZD-8 significantly reduces this phosphorylation. Moreover, we also demonstrate that TDZD-8 inhibits I κ B- α degradation. Taken together, the balance between pro-inflammatory and pro-survival roles of NF- κ B may depend on the phosphorylation status of p65, and GSK-3 β may play a central role in this process. However, the reasons for the apparent discrepancies in the modulatory effects of GSK-3 β on NF- κ B activity remain to be fully elucidated. There is good evidence that TNF- α and IL-1 β help to propagate the extension of ischaemia and reperfusion of the gut [27]. This study demonstrates that TDZD-8 attenuates the TNF- α and IL-1 β production in the plasma and ileum from SAO-shocked rats. Therefore the inhibition of the production of TNF- α and IL-1 β by TDZD-8 described in the present study is most likely due to the inhibitory effect the activation of NF- κ B. Furthermore, we observed that SAO shock induced the appearance of P-selectin on the endothelial vascular wall and the expression of ICAM-1 on endothelial cells. Treatment with TDZD-8 abolished the expression of P-selectin and ICAM-1. These results demonstrate that inhibition of the GSK-3 β pathway may interrupt the interaction between neutrophils and endothelial cells both 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 rats treated with TDZD-8 is correlated with the reduction in leukocyte infiltration and with the attenuation in the ileum tissue damage. Activation and accumulation of leukocytes is one of the initial events of tissue injury due to release of oxygen free radicals [28]. Inhibition of reactive oxygen species prevents the infiltration of neutrophils at inflamed sites, as shown by the use of different therapeutic interventions including melatonin [29], a vitamin E-like antioxidant [30], a superoxide dismutase-mimetic [31] and a peroxynitrite decomposition catalyst [32].

In the present study the levels of MDA were significantly increased by ischaemia-reperfusion. This observation is in agreement with previous studies which detected elevated levels of lipid peroxidation products [32]. TDZD-8 treatment reduced the increase in lipid peroxidation products, probably in part dependent on the observed reduction in neutrophil infiltration into the ileum. Reduction in lipid peroxidation was also paralleled with the inhibition of nitrotyrosine immunoreactivity as index of nitrosative stress. Generation of free radicals has also been implicated in causing oligodendrocyte apoptosis. We have demonstrated that treatment with TDZD-8 attenuates the degree of apoptosis measured by TUNEL in the ileum. There is evidence that direct over-expression of GSK-3 β is known to induce apoptosis in different cells line, and specific inhibitors of GSK-3 β ameliorate this apoptotic response [33]. It is known that pathways which inhibit GSK-3 β activity often lead to the induction of the NF- κ B cell survival pathway [34]. GSK-3 β is a major target of Akt/PKB, which is activated by the phosphatidylinositol 3-kinase mediated signalling pathway [35]. Inhibition of this signalling cascade has been shown to lead to cell death in several paradigms [36]. We identified pro-apoptotic transcriptional changes, including upregulation of pro-apoptotic Bax and down-regulation of anti-apoptotic Bcl-2 using western blot analysis and by immunohistochemical staining. We report in the present study for the first time that the treatment with TDZD-8 in gut ischaemia and reperfusion documents features of apoptotic cell death after SAO shock, suggesting that protection from apoptosis is a prerequisite for anti-inflammatory approaches. In particular, we demonstrated that the treatment with TDZD-8 lowers the signal for Bax in treated group when compared with ileum sections obtained from SAO-shocked rats, while, on the other hand, the signal is much more express for Bcl-2 in TDZD-8 treated rats than in SAO-shocked rats. Taken together, the results of the present study enhance our understanding of the role of GSK-3 β in the pathophysiology of ischaemia and reperfusion. Our results imply that inhibitors of the activity of GSK-3 β may be useful in the therapy of ischaemia and reperfusion.

Acknowledgements. This study was supported by grant from a University Minister grant. The authors thank Giovanni Pergolizzi and Carmelo La Spada for their excellent technical assistance during this study, Mrs. Caterina Cutrona for secretarial assistance and Miss Valentina Malvagni for editorial assistance with the manuscript.

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