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Chronic ethanol consumption increases reactive oxygen species generation and the synthesis of pro-inflammatory proteins in the heart through TNFR1-dependent mechanisms

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ABSTRACT

We evaluated the role of tumor necrosis factor (TNF)-α receptor 1 (TNFR1) on ethanol-induced cardiac dysfunction. Male C57BL/6J wild-type (WT) or TNFR1-deficient mice (TNFR1$^{-/-}$) were treated with ethanol (20% v/v) for 10 weeks. Increased protein expression of TNFR1 and NFκB p65 was detected in the left ventricle (LV) of WT mice chronically treated with ethanol. Echocardiographic analysis showed that ethanol consumption increased left ventricular posterior wall end-diastolic diameter and left ventricular posterior wall end-systolic diameter in WT, but not TNFR1$^{-/-}$ mice. Increased levels of TNF-α, interleukin (IL)-6, superoxide anion ($O_2^{\cdot -}$), thiobarbituric acid reactive substances (TBARS) as well as increased nitrotyrosine immunostaining were detected in the LV from WT, but not TNFR1$^{-/-}$ mice. Conversely, treatment with ethanol decreased nitrate/nitrite (NOx) concentration in the LV. Histopathological analysis showed that ethanol did not induce inflammatory infiltrates, necrosis or edema in the LV. No differences in the ventricular expression of iNOS, Nox2 or COX-2 as well as in the activity of superoxide dismutase (SOD), myeloperoxidase (MPO) and N-acetyl-beta-D-glucosaminidase (NAG) were found after treatment with ethanol. Our study provided novel evidence that ethanol consumption augmented the production of reactive oxygen species (ROS) and the synthesis of pro-inflammatory proteins in the LV through TNFR1-dependent mechanisms. These findings provided novel mechanistic insights about the contribution of TNFR1 in the initial steps of the cardiac damage induced by ethanol.

1. Introduction

Chronic ethanol consumption is related to alcoholic cardiomyopathy. The latter is a specific cardiac disease that occurs in individuals with a history of chronic consumption of higher doses of ethanol [1]. The structural and functional changes found in individuals diagnosed with alcoholic cardiomyopathy include fibrosis, increased left ventricle (LV) mass, dilated LV, reduced LV wall thickness, and reduced LV ejection fraction [2]. However, the primary mechanism(s) underlying the initial effects of ethanol in the cardiac tissue is unknown [1]. Much of the research that investigates the toxic effects of ethanol in cardiomyocytes has focused in oxidative stress. In this sense, long-term ethanol consumption has been described to increase the generation of reactive oxygen species (ROS) within the myocardium [3,4]. Ethanol-induced ROS generation in cardiomyocytes is mediated by the enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and this response is associated with lipoperoxidation and nitrative damage [4–6].

Tumor necrosis factor-α (TNF-α), a pro-inflammatory cytokine, is described as a mediator of ethanol-induced toxicity in distinctive tissues and chronic ethanol consumption is associated with increased circulating levels of TNF-α [7–10]. TNF-α induces a wide range of biological effects trough interaction with two distinctive receptors: TNF-α receptor 1 (TNFR1; CD120a; p55/60) and TNF-α receptor 2 (TNFR2; CD120b; p75/80) [11]. Both receptors are expressed in distinctive cell types, including cardiomyocytes, which are also capable of producing

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TNF-α [12]. In cardiomyocytes, TNFR1 is responsible for most of the biological actions of TNF-α such as expression of interleukin (IL)-6 and superoxide anion ($O_2^{\cdot -}$) production by NADPH oxidase [12,13]. The latter is an important source of $O_2^{\cdot -}$ in cardiomyocytes where it mediates the generation of ROS induced by ethanol [4]. Reduction of nitric oxide (NO) bioavailability due to decrease expression of eNOS is another response mediated by TNFR1 [14]. In cardiomyocytes, the enzyme eNOS is an important source of NO, which influences myocardial inotropic and chronotropic responses [15]. On the other hand, TNFR1 activation is described to induce iNOS expression in cardiomyocytes [16]. Excessive NO production mediated by iNOS may contribute to cardiac dysfunction due to an increased production of peroxynitrite ($ONOO^-$), an oxidant molecule that is formed by the reaction of $O_2^{\cdot -}$ with NO [17]. Importantly, chronic ethanol consumption is described to induce iNOS expression and to increase the production of $O_2^{\cdot -}$ and $ONOO^-$ in cardiomyocytes [4].

Increased protein expression of TNFR1 was detected in cultured cardiomyocytes exposed to increasing concentrations of ethanol [18]. Moreover, augmented levels of circulating TNF-α were found in individuals with alcoholic cardiomyopathy [19]. Altogether, these studies suggested a potential link between TNFR1 and ethanol-induced cardiac damage. We hypothesized that TNFR1 could play a role in the cardiac dysfunction induced by ethanol consumption by mediating the generation of ROS and the synthesis of pro-inflammatory proteins. Here, we evaluated the contribution of TNFR1 in chronic ethanol consumption-induced cardiac dysfunction and assessed whether this receptor modulates the initial molecular events trigged by ethanol in the heart.

2. Materials and methods

2.1. Animals and grouping

Treatment of male C57BL/6J wild-type (WT) mice (six to eight weeks old, 20–25 g) was carried out as previously described [20,21]. In brief, mice were distributed into control and ethanol groups. Mice from control group had free access to filtered water. Animals from the ethanol group were submitted to a two-week period of adaptation. In the 1st week, mice had free access to ethanol 5% (vol./vol.), while in the 2nd week animals were treated with a solution of ethanol 10%. In the following weeks (3rd to 12th) mice had free access to ethanol 20%. In order to investigate the role of TNFR1 on the cardiac effects induced by ethanol consumption, TNFR1-deficient mice ($TNFR1^{-/-}$), six to eight weeks old (20–25 g) were treated with ethanol as described for WT mice. TNFR1-deficient mice (B6.129-$Tnfrsf1a^{tm1Mak}$/J; stock number 002818) were originally purchased from the Jackson Laboratories (Bar Harbor, ME, USA). Mice, previously anaesthetized with urethane at 1.25 g/kg (solution of 25%, 5 ml/kg, i.p.) were killed by aortic exsanguination. The left ventricle (LV) was collected for biochemical experiments. The Ethics Committee on Animal Use from the University of São Paulo, campus of Ribeirão Preto (#12.1.1654.53.9) approved the protocols. All protocols were conducted following the guidelines of National Committee for Animal Experimentation Control ("Conselho Nacional de Controle de Experimentação Animal," CONCEA, Brazil). All experiments conform with the ARRIVE guidelines. The protocols were conducted as described by the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

2.2. Echocardiogram

Echocardiograms were performed at baseline and twelve weeks after treatment with ethanol using a Vevo 2100 High-Resolution Imaging System (VisualSonics, Toronto, ON, Canada) as previously described [22].

2.3. Determination of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β) and IL-10 levels in the LV

The LV was collected in tubes containing phosphate buffered saline (PBS, pH 7.4). A protease inhibitor cocktail (#11697498001, Roche, Basel, Switzerland) was used do prevent protein degradation. The LV was homogenized and centrifuged (10,000g, 15 min, 4 °C). The concentration of TNF-α, IL-6, IL-1β and IL-10 was measured by enzyme-linked immunosorbent assay (ELISA) according to the kit instruction (#430904, #431304, #432604 and #431414 respectively - ELISA MAX™ Deluxe Sets, Biolegend, San Diego, CA, USA). Results are expressed as pg/mg protein. The Lowry protein assay method (Bio-Rad Laboratories, Hercules, CA, USA) was used to determinate protein concentration in the samples.

2.4. Detection of $O_2^{\cdot -}$ generation and determination of H_2O_2, nitrate/nitrite (NOx) and thiobarbituric acid reactive substances (TBARS) concentration in the LV

The lucigenin-derived chemiluminescence assay was used to measure the concentration of $O_2^{\cdot -}$ in the samples as described previously [23]. The levels of $O_2^{\cdot -}$ are shown as relative light units (RLU)/mg protein. The levels of H_2O_2 (nmol/mg protein) were determined in a fluorometer (SpectraMax Gemini, Molecular Devices, Sunnyvale, CA, USA) using the fluorogenic substrate Amplex® Red (#A22188, Invitrogen, Carlsbad, CA, USA) as previously described [24].

For NO*x* determination, the LV was homogenized in PBS (pH 7.4) and centrifuged (10,000g, 20 min, 4 °C). Then the samples were ultrafiltered at room temperature (14,000g, 30 min) and the concentration of NO*x* (nmol/mg protein) was determined colorimetrically at 540 nm according to the instructions of a commercially available kit (#780001, Cayman Chemical, Ann Arbor, MI, USA).

A commercially available kit (#10009055, Cayman Chemical, Ann Arbor, MI, USA) was used to determine the concentration of TBARS. The LV was homogenized in radioimmunoprecipitation assay buffer (RIPA). After centrifugation (1600g, 10 min, 4 °C) samples were used to determine the concentration of TBARS (nmol/g protein) colorimetrically at 540 nm.

2.5. Determination of the concentration of reduced glutathione (GSH) and of the activities of catalase or superoxide dismutase (SOD) in the LV

The concentration of GSH (μg/mg protein) was measured in the LV using as previously described [25]. For determination of catalase activity, the LV was homogenized in 200 μl of PBS (pH 7.4). A volume of 100 μl of phosphate buffer (K_2HPO_4, 0.1 mol/l, KH_2PO_4, 0.1 mol/l [pH 6.5]) was added to 100 μl of the homogenate. The samples were then centrifuged at 7800g for 20 min at 4 °C. Twenty microliters of the homogenate were transferred to quartz cuvettes containing 980 μl of the reaction buffer and the absorbance was read (1 min at 240 nm) in a spectrophotometer [25]. Catalase activity is shown as U/min/mg protein.

For determination of SOD activity, the LV was homogenized in 300 μl of PBS (pH 7.4). The homogenate was centrifuged (10,000g, 12 min, 4 °C). SOD activity was measured in supernatants according to instructions of a commercially kit (#19160, Sigma-Aldrich, St. Louis, MO, USA). The results are shown as inhibition rate %/mg protein.

2.6. Determination of myeloperoxidase (MPO) and N-acetyl-beta-D-glucosaminidase (NAG) activities

MPO activity was determined as an estimative of neutrophils accumulation in the LV. In brief, samples of the LV (100 mg of tissue per 1 ml of buffer) were homogenized in ice-cold phosphate buffer (NaCl 0.1 mol/l, $NaPO_4$ 0.02 mol/l, NaEDTA 0.015 mol/l, pH 4.7) and then centrifuged (9600g, 10 min, 4 °C). A volume of 750 μl of NaCl 0.2% was

added to the pellet to induce hypotonic lysis. This procedure was followed by the addition of an equal volume of a solution containing NaCl 1.6% and glucose 5%. The sample was centrifuged and the pellet resuspended in $NaPO_4$ buffer (0.05 mol/l, pH 5.4) containing hexadecyltrimethylammonium bromide 0.5% (HTAB, Sigma-Aldrich, St. Louis, MO, USA). The sample was rehomogenized and centrifuged (9600g, 15 min, 4 °C) after three freeze–thaw cycles using liquid nitrogen. The activity of MPO was determined at 450 nm by measuring the change in optical density (O.D.) of the supernatant in the presence of both 3,3′-5,5′-tetramethylbenzidine (1.6 mmol/l, Sigma-Aldrich) and H_2O_2 (0.002%). The reaction was stopped by the addition of H_2SO_4 (4 mol/l). The results are shown as MPO (relative units O.D.)/100 mg of tissue.

NAG activity was determined as an estimative of macrophages accumulation in the LV. Samples of the LV (100 mg of tissue per 1 ml of buffer) were homogenized in ice-cold phosphate buffer, centrifuged and subjected to hypotonic lysis as described before. After a second centrifugation, the pellet was resuspended in ice-cold saline (Triton X-100 0.1%) and rehomogenized. Homogenates were centrifuged (900g, 15 min, 4 °C). A mixture of p-nitrophenyl-N-acetyl-β-D-glucosaminide (2.2 mmol/l, Sigma-Aldrich) and citrate buffer (0.1 mol/l, pH 4.5) was incubated for 10 min at 37 °C in a microplate. The reaction was stopped by the addition of 100 μl of glycine buffer (0.2 mol/l, pH 10.6). Absorbance was determined at 405 nm and used to calculate NAG activity, which is shown as NAG (relative units O.D.)/100 mg of tissue.

2.7. Histopathological analysis

The LV was fixed using buffered formalin (10%) and paraffin was used to embed the samples. Hematoxylin/eosin or picrosirius red were used to stain the sections (5 μm), which were then analyzed in a microscope (Zeiss, Axioskop 2 plus, Jena, Germany). The presence of the following morphological changes was evaluated: inflammatory infiltrates (neutrophils), necrotic cardiomyocytes, interstitial edema and hemorrhagic foci. The images were captured at ×1000 using a digital camera AxioCam Hrc® (Zeiss).

2.8. Immunohistochemistry

The LV was embedded in paraffin and cut (5-μm-thick slices) using a Reichert Jung 2040 microtome (Leica, Wetzlar, Germany). The assay was carried out using the primary antibody for nitrotyrosine (1:300, #06-284, Millipore Billerica, MA, USA) as previously described [26]. Images were captured at ×400 and quantification of nitrotyrosine staining was performed as previously described [26].

2.9. Immunofluorescence determination of TNFR1 expression

Expression of TNFR1 was measured using an antibody conjugated to fluorescein (1:250, sc-8436, Santa Cruz Biotechnology, Dallas, Texas, USA). The LV was embedded in Tissue-tek® and sectioned in slices of 5 μm. The slides were incubated for 30 min with an antibody anti-TNFR1. Then, cold PBS (pH 7.4) was used to wash the slides. Sections were analyzed at 480/510 nm (λexcitation/λemission) in a microscope (Leica Model SPE, Leica Imaging Systems Ltd., Wetzlar, Germany). The images were captured at x400. The fluorescence was measured using the ImageJ Program (National Institutes of Health, USA). An area of 20–30% of the LV was analyzed (10 fields). For each slide, the arithmetic mean of the fluorescence from these fields was calculated.

2.10. Western immunoblotting

Proteins were separated by electrophoresis as previously described [27]. Membranes of nitrocellulose were incubated with one of the following primary antibodies: anti-TNFR1 (1:1000, sc-8436, Santa Cruz Biotechnology, Dallas, Texas, USA), anti-NF-κB p65 (1:500, sc-398442, Santa Cruz Biotechnology), anti-gp91^{phox} (1:500, sc-5827, Santa Cruz Biotechnology), iNOS (1:500, sc-650, Santa Cruz Biotechnology), anti-COX-2 (1:500, sc-1746, Santa Cruz Biotechnology) or anti-eNOS (1:2000, BD610296, Biosciences, San Francisco, CA, USA). The membranes were then incubated with secondary antibodies. A ChemiDoc™ XRS + system (Bio-Rad, USA) was used to visualize the bands, which were detected by chemiluminescence. The bands were quantified by densitometry. GAPDH (1:1000, sc-25778, Santa Cruz Biotechnology) was used as an internal control.

2.11. Statistical analysis

Results are shown as the means ± standard error of the mean (S.E.M.) and were analyzed by Student's *t* test or two-way analysis of variance (ANOVA) followed by Bonferroni's comparison test (GraphPad® Prism 5.01, GraphPad Software Inc., San Diego, CA, USA). $P < 0.05$ was considered as significant.

3. Results

3.1. Effects of chronic ethanol consumption on TNFR1 expression

Increased fluorescence for TNFR1 was detected in the LV of WT mice treated with ethanol (Fig. 1A and B). Similarly, Western immunoblotting analyzes showed that chronic ethanol consumption increased protein expression of TNFR1 in the LV (Fig. 1C). Increased expression of NFκB p65 was detected in the LV from WT mice treated with ethanol when compared to control mice (Fig. 1D). TNFR1 was not detected in the LV of TNFR1-deficient mice (Fig. S1, supplementary material online).

3.2. Effects of chronic ethanol consumption on heart function

Representative images of the LV of mice from WT, ethanol, TNFR1$^{-/-}$ and TNFR1$^{-/-}$ethanol groups (Fig. 2A). Treatment with ethanol increased both left ventricular posterior wall end-diastolic diameter and left ventricular posterior wall end-systolic diameter (LVPWd and LVPWs, respectively) in WT, but not TNFR1$^{-/-}$ mice (Fig. 2B and C). The ejection fraction and fractional shortening were not affected by ethanol consumption (Table S1, supplementary material online). On the other hand, ethanol consumption did not affect heart rate (HR), cardiac output (CO), stroke volume (SV), end systolic/diastolic left ventricular internal diameter (LVIDs/d) or interventricular septal end systole/diastole (IVSs/d) (Table S1, supplementary material online).

3.3. Effects of chronic ethanol consumption on the levels of cytokines and on the activities of MPO and NAG in the LV

Treatment with ethanol increased TNF-α and IL-6 levels in the LV from WT, but this response was not found in the LV from TNFR1-deficient mice (Fig. 3A and B). Conversely, ethanol consumption decreased IL-10 levels in the LV from both WT and TNFR1-deficient mice (Fig. 3C). Treatment with ethanol did not affect the levels of IL-1β in the LV of WT or TNFR1-deficient mice (Fig. 3D). Similarly, ethanol consumption did not affect MPO or NAG activity in the LV (Fig. 3E and F).

3.4. Effects of chronic ethanol consumption on the levels of ROS, TBARS, NOx, GSH and on the activities of SOD and catalase in the LV

Ethanol consumption increased lucigenin-derived luminescence in the LV of WT, but not TNFR1-deficient mice (Fig. 4A). No differences in the concentration of H_2O_2 were found after treatment with ethanol (Fig. 4B). Increased levels of TBARS were found in the LV of WT mice treated with ethanol, but this response was not observed in TNFR1-

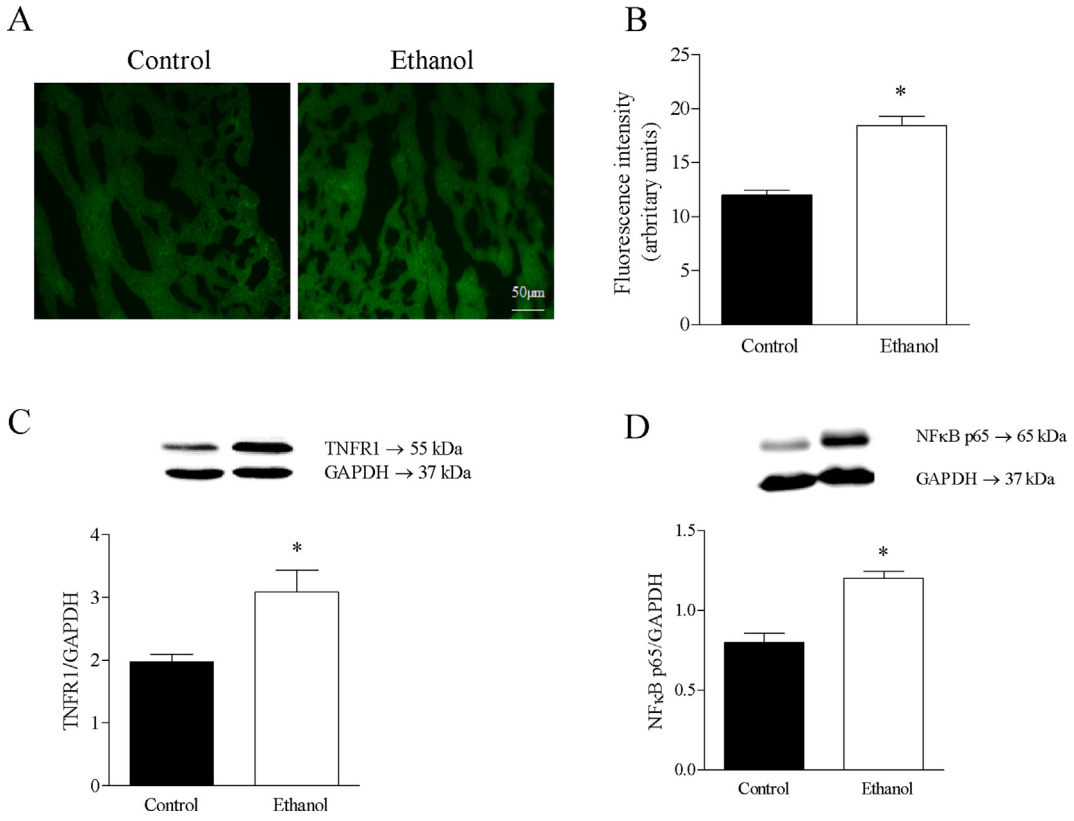

Fig. 1. Effects of chronic ethanol consumption on TNFR1 expression in the left ventricle (LV) of WT mice. (A) Representative immunofluorescence photomicrographs showing TNFR1 expression. (B) Relative fluorescence intensity was calculated for TNFR1 expression in transverse sections of the LV. (C and D) Top panels: representative immunoblots for TNFR1 or NFκB p65. Bottom panel: corresponding bar graphs show densitometric data for protein expression of TNFR1 or NFκB p65. Values given as means ± S.E.M. from 4 to 5 LV for each group. *Compared to control (WT) group ($p < 0.05$, Student's *t* test).

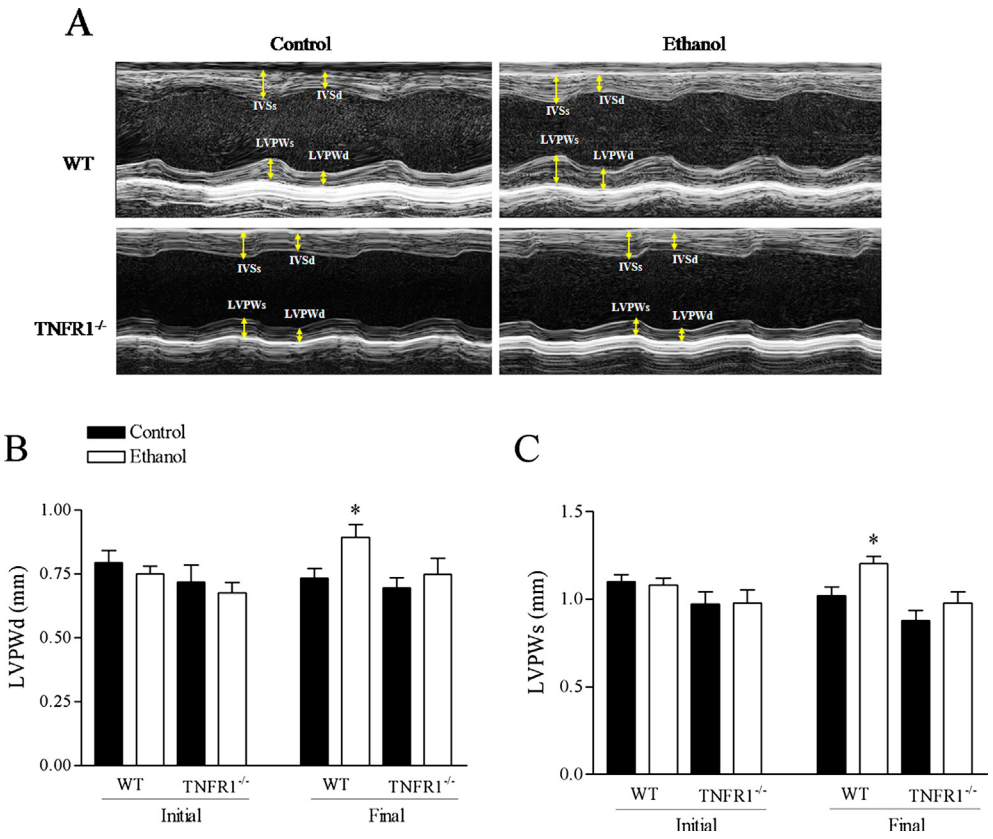

Fig. 2. Echocardiographic images of mice left ventricle (LV) and diameter of the left ventricular posterior wall (end-diastole and end-systole). (A) Representative short-axis of LV of mice from WT, ethanol, TNFR1$^{-/-}$ and TNFR1$^{-/-}$ ethanol groups. (B and C) Left ventricular posterior wall end-diastolic diameter and left ventricular posterior wall end-sistolic diameter (LVPWd and LVPWs, respectively) in WT and TNFR1$^{-/-}$ mice. Values are shown as the means ± S.E.M of 10 mice for each group. *Compared to control (WT), TNFR1$^{-/-}$ and TNFR1$^{-/-}$ ethanol groups ($p < 0.05$, two-way ANOVA followed by Bonferroni's comparison test). IVSs: interventricular septal end systole; IVSd: Interventricular septal end-diastole; LVPWs: left ventricular posterior wall end-systolic diameter; LVPWd: left ventricular posterior wall end-diastolic diameter.

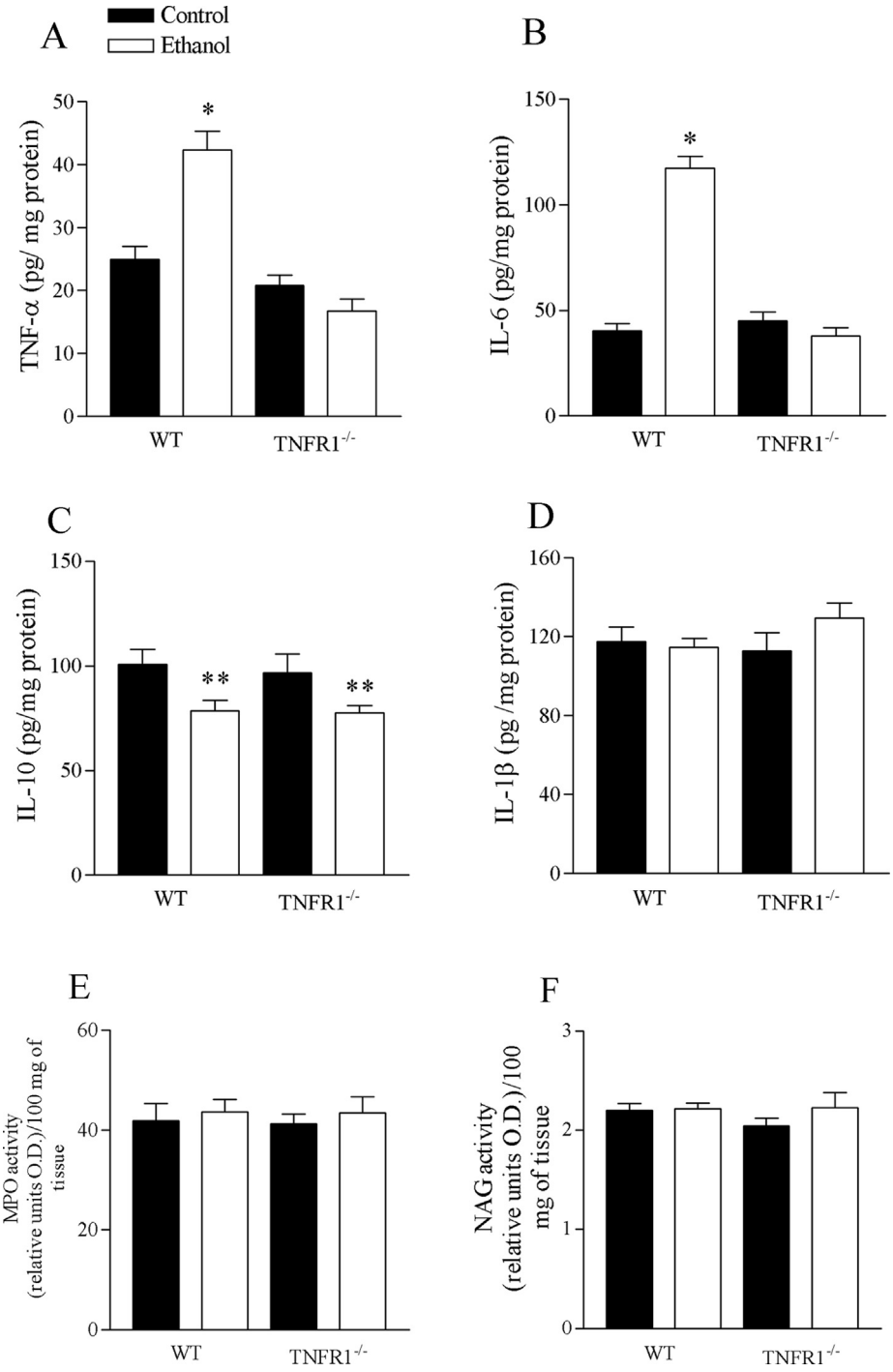

Fig. 3. Effects of chronic ethanol consumption on the levels of cytokines and the activity of MPO and NAG in the left ventricle (LV). Bar graphs represent the concentrations of (A) TNF-α, (B) IL-6, (C) IL-10 and (D) IL-1β or the activities of (E) MPO and (F) NAG in the LV. Values given as means ± S.E.M. from 7 to 10 LV for each group. *Compared to control (WT), TNFR1$^{-/-}$ and TNFR1$^{-/-}$ ethanol groups;**Compared to control (WT) and TNFR1$^{-/-}$ groups ($p < 0.05$, two-way ANOVA followed by Bonferroni's comparison test).

deficient mice (Fig. 4C). NO*x* concentration was decreased in the LV from WT mice treated with ethanol and this response was not found in the LV of TNFR1-deficient mice (Fig. 4D). SOD activity was not affected by chronic ethanol consumption (Fig. 4E). However, increased activity of catalase was found in the LV of WT mice (Fig. 4F). Ethanol-induced increase in catalase activity was attenuated in the LV of TNFR1-deficient mice. Ethanol consumption did not alter the concentration of GSH (μg/mg protein) in the LV (13.9 ± 0.7, n = 10), as compared to WT (14.6 ± 1.1, n = 9), TNFR1$^{-/-}$ (12.3 ± 1.2, n = 9) and TNFR1$^{-/-}$ ethanol (11.8 ± 0.9, n = 8) groups.

3.5. Histopathological and immunohistochemical analysis

Photomicrographs on Fig. 5 show that chronic ethanol consumption did not induce inflammatory infiltrates, necrosis or edema. Increased staining for nitrotyrosine was found in the LV of WT, but not TNFR1-deficient mice (Fig. 6A and B).

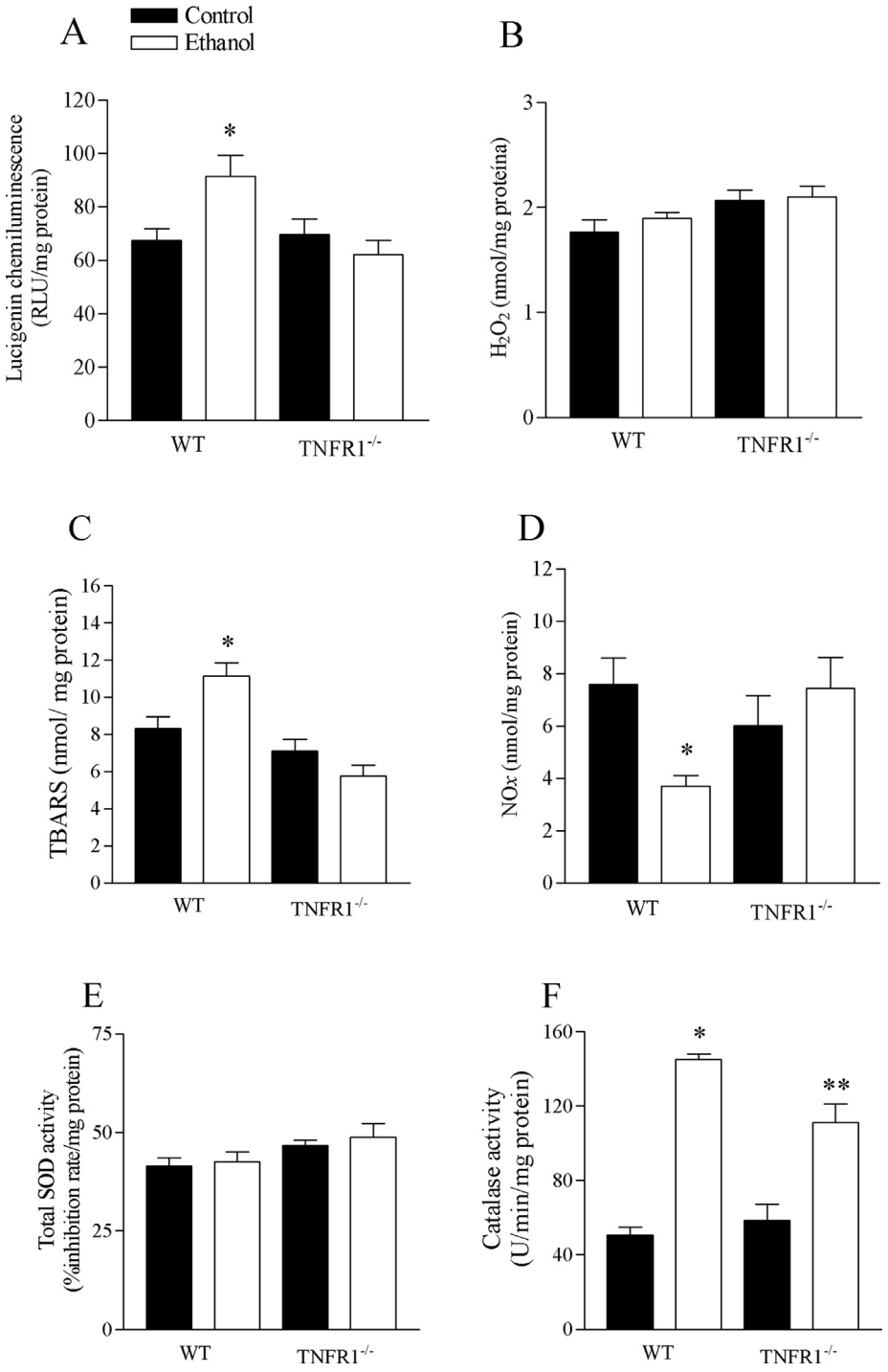

Fig. 4. Effects of chronic ethanol consumption on oxidative stress in the left ventricle (LV). Bar graphs represent the levels of (A) $O_2^{\cdot -}$, (B) H_2O_2, (C) TBARS, (D) NO*x* or the activities of (E) SOD and (D) catalase in the LV. Values given as means ± S.E.M. from 7 to 10 LV for each group. *Compared to control (WT), TNFR1$^{-/-}$ and TNFR1$^{-/-}$ ethanol groups;**Compared to control (WT) and TNFR1$^{-/-}$ groups ($p < 0.05$, two-way ANOVA followed by Bonferroni's comparison test).

3.6. Effects of chronic ethanol consumption on protein expression of eNOS, iNOS, Nox2 and COX-2

Chronic ethanol consumption did not alter the expression of eNOS in the LV. However, up-regulation of eNOS was found in the LV of TNFR1-deficient mice (Fig. 7A). Protein expression of iNOS, Nox2 and COX-2 was not affected by chronic ethanol consumption (Fig. 7B, C and D).

4. Discussion

TNFR1 is associated with myocardial damage and dysfunction [12,13,16], and increased expression of this receptor was previously detected in cultured cardiomyocytes exposed to ethanol [18]. Here we first show that ethanol consumption increased the expression of TNFR1 in the LV of WT mice. This response was accompanied by up-regulation of NF-κB p65, which is a central downstream protein that mediates intracellular TNFR1 signaling.

TNFR1 is expressed in cardiomyocytes, where it acts as a regulator

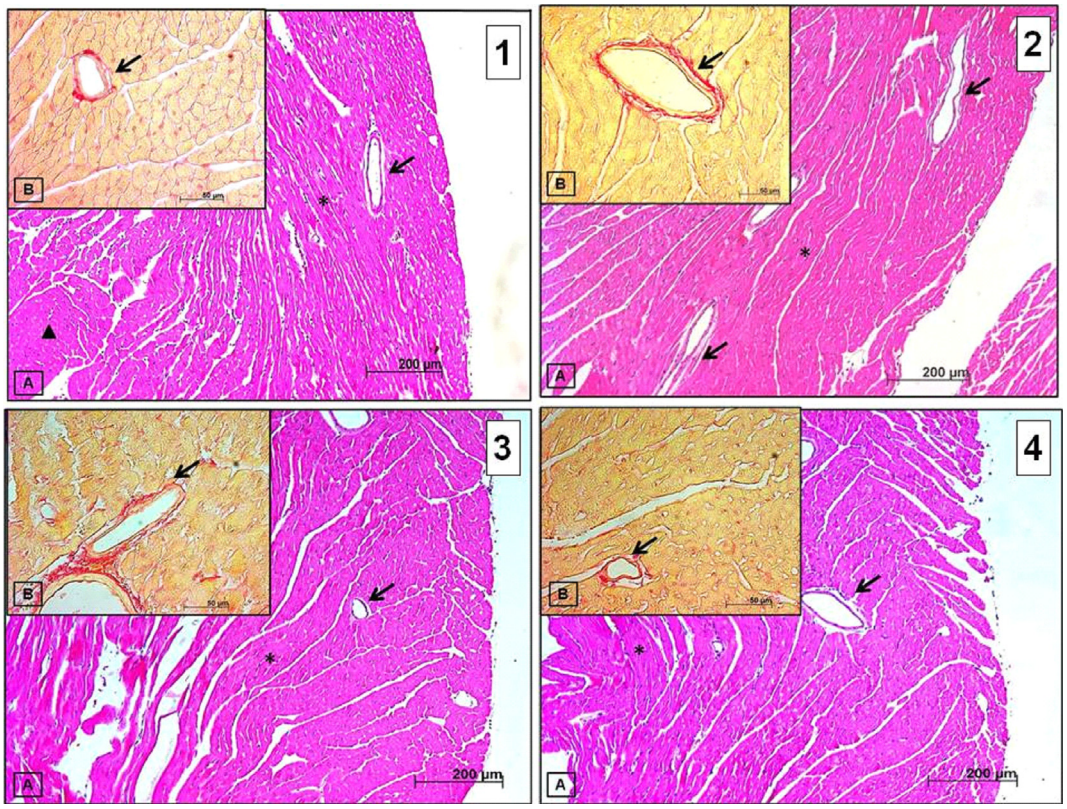

Fig. 5. Photomicrographs of the left ventricle (LV) stained with hematoxylin and eosin or Sirius-red. Sections of the LV of control WT (panel 1), ethanol (panel 2), TNFR1$^{-/-}$ (panel 3) or TNFR1$^{-/-}$ mice were stained with hematoxylin and eosin (A) or Sirius-red (B). (A) Images show a general view of normal cardiomyocytes with central nuclei in longitudinal (*) sections, without the presence of inflammatory infiltrates (neutrophils), necrotic cardiomyocytes, interstitial edema and hemorrhagic foci. The images were captured with a magnification of 100. (B) Images show in detail the absence of fibrosis in the cardiac tissue. The images were captured with a magnification of 400. Black arrows indicate blood vessels.

of inflammation [28]. However, TNFR1 also regulates cardiac structure and function under physiological and non-physiological conditions [11]. The functional and structural changes induced by chronic ethanol consumption are dependent on the amount and duration of ethanol consumption. These changes include dilatation of the LV, increase in LV mass, and, in advanced stages reduction of LV ejection fraction [1]. We found that ethanol consumption did not change the ejection fraction or the fractional shortening. On the other hand, ethanol consumption increased both left ventricular posterior wall end-diastolic diameter and left ventricular posterior wall end-systolic diameter in WT, but not TNFR1$^{-/-}$ mice. These findings suggested that TNFR1 contributes to the cardiac changes induced by chronic ethanol consumption. The lack of effect of ethanol consumption in the functional parameters (ejection fraction) is possibly related to the duration of treatment with ethanol. In fact, clinical and experimental studies have shown that ethanol-induced functional abnormalities in the heart are dependent of the period of ethanol consumption [1,29–31]. It is important to note that using this same model of ethanol feeding [20], we observed that plasma ethanol levels in mice were in the range detected in individuals who chronically consume ethanol [32].

Ethanol consumption was described to decrease the synthesis of the anti-inflammatory cytokine IL-10 in blood and the central nervous system [33,34]. Our findings are in accordance with these results since decreased IL-10 levels were found in the LV of ethanol-treated mice. The mechanism whereby ethanol reduced IL-10 production is unclear but seemed not to involve TNFR1 activation. TNFR1 is involved in the initiation and maintenance of inflammatory process in the heart. In the cardiac tissue, TNFR1 displays its inflammatory actions by increasing

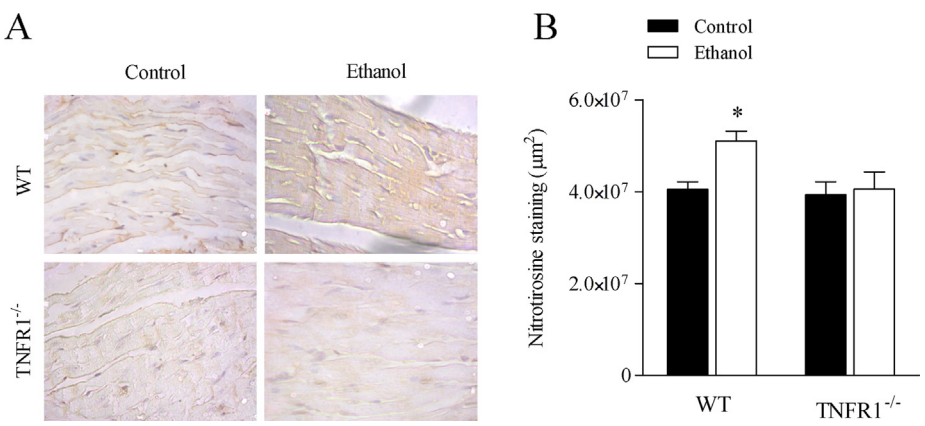

Fig. 6. Effects of chronic ethanol consumption on protein nitration in the left ventricle (LV). (A) Representative photomicrographs of nitrotyrosine immunostaining in the LV. (B) Bar graphs represent the quantification of brown staining of nitrotyrosine. Values given as means ± S.E.M. from 5 to 6 LV for each group. The images were captured with a magnification of 400. *Compared to control (WT), TNFR1$^{-/-}$ and TNFR1$^{-/-}$ ethanol groups ($p < 0.05$, two-way ANOVA followed by Bonferroni's comparison test).

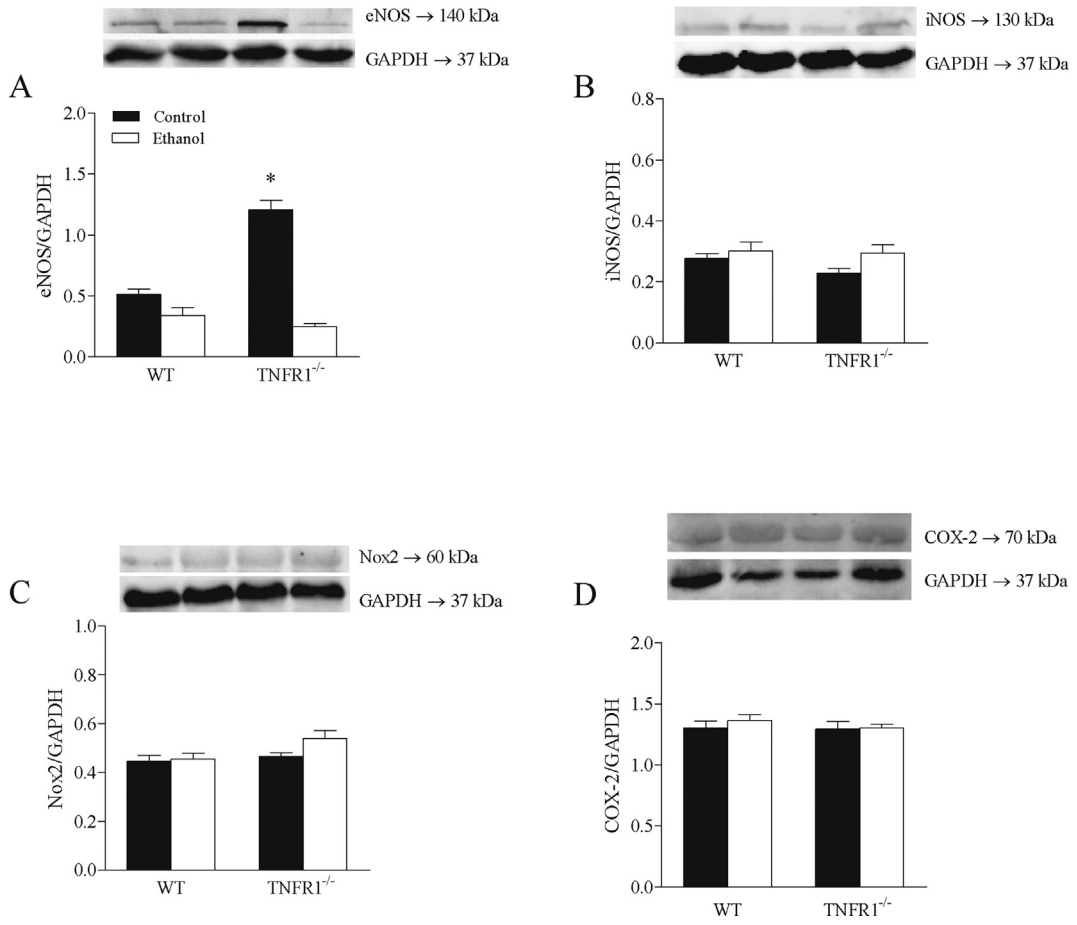

Fig. 7. Effects of chronic ethanol consumption on protein expression of eNOS, iNOS, Nox2 and COX-2 in the left ventricle (LV). Top panels: representative immunoblots for eNOS, iNOS, Nox2 and COX-2. Bottom panels: corresponding bar graphs show densitometric data for protein expression of eNOS (A), iNOS (B), Nox2 (C) or COX-2 (D). Values given as means ± S.E.M. from 4 to 5 LV for each group. *Compared to control (WT), ethanol and TNFR1$^{-/-}$ ethanol groups ($p < 0.05$, two-way ANOVA followed by Bonferroni's comparison test).

the production of pro-inflammatory cytokines including IL-6 and TNF-α [12,13]. Our present findings showed that both TNF-α and IL-6 levels were higher in the LV from WT mice treated with ethanol. This response was not found in TNFR1-deficient mice, indicating that TNFR1 mediated the up-regulation of TNF-α and IL-6 induced by ethanol consumption. This finding is in accordance with previous reports describing that the expression of TNF-α and IL-6 is modulated by TNFR1 by a mechanism that involves NF-κB activation [35–37]. Activation of TNFR1 in the cardiomyocytes could be being mediated by systemic TNF-α or TNF-α derived from cardiomyocytes or inflammatory cells. Recently we described that ethanol consumption did not change plasma TNF-α levels [20]. The histopathological analysis of the LV showed no inflammatory infiltrates, necrosis or edema. Moreover, we did not detected alteration in MPO or NAG activities in the LV after treatment with ethanol, suggesting that ethanol did not induce recruitment/activation of neutrophils or macrophages. Thus, it is possible that TNF-α produced within cardiomyocytes is responsible for TNFR1 activation in our model. Thus, increased expression of TNFR1 combined to an augmented production of TNF-α within cardiomyocytes could be an important mechanisms by which ethanol initiates its deleterious effects in the heart.

TNFR1 also modulates the expression of IL-1β, COX-2 and iNOS, which are pro-inflammatory proteins that play a role in cardiac dysfunction and remodeling [11]. Previous studies have described that ethanol consumption increased the expression of these pro-inflammatory proteins [20,21,38]. Our results showed that ethanol consumption did not affect IL-1β, COX-2 or iNOS expression in the LV. A possible explanation for such response is that the effect of ethanol on the expression of these proteins is tissue-specific and dependent of the period of treatment with ethanol [21,27,38].

In cardiomyocytes TNFR1 modulates the activation of NADPH oxidase with further increase in $O_2^{\cdot -}$ generation [12,13]. This response seems to be crucial to the deleterious effects mediated by TNF-α/TNFR1 in the heart under distinctive circumstances [39]. Measurement of lucigenin luminescence revealed increased levels of $O_2^{\cdot -}$ in the LV from WT mice chronically treated with ethanol. Additionally, our results suggested that TNFR1 plays a role in ethanol-induced ROS generation. Moreover, our findings also implicated TNFR1 receptors in ethanol-induced lipoperoxidation in the LV. Superoxide anion is produced by Nox2 and for this reason this catalytic subunit of NADPH has been associated with cardiac dysfunction [40]. However, up-regulation of Nox2 seems not to be a mechanism whereby ethanol increases $O_2^{\cdot -}$ generation in our model. Thus, our results suggested that TNFR1 mediates $O_2^{\cdot -}$ generation and lipoperoxidation in the LV in response to ethanol.

Myocardial inotropic and chronotropic responses may be influenced by NO [15], and TNFR1 is described to reduce the bioavailability of NO in cardiomyocytes [14]. Our results showed that TNFR1 mediated the decrease of NO levels in the LV of mice chronically treated with ethanol. Decreased levels of NO are usually the result of: (1) inactivation by $O_2^{\cdot -}$; (2) reduction on the activity/expression of the enzyme eNOS. Ethanol consumption did not affect eNOS expression in WT mice, but up-regulation of eNOS was observed in the LV of TNFR1-deficient mice, which is in accordance with the observation that TNF-α decreases

eNOS expression [11]. Interestingly, this response was not found in the LV of TNFR1-deficient mice treated with ethanol, suggesting that ethanol consumption counteracted the inhibitory action of TNF-α on eNOS expression. Moreover, NO inactivation by $O_2^{\cdot -}$ could be also contributing to the reduced bioavailability of NO since augmented levels of $O_2^{\cdot -}$ were found in the LV of WT mice treated with ethanol. This hypothesis is supported by the observation that the augmented generation of $O_2^{\cdot -}$ as well as the reduction of NO levels was not detected in TNFR1-deficient mice. The reaction of $O_2^{\cdot -}$ with NO leads to the generation of $ONOO^-$, which will ultimately induce protein nitration [17]. Nitration of tyrosine by reactive nitrogen species (e.g. $ONOO^-$) leads to the formation of nitrotyrosine, which has been used as a biomarker for endogenous $ONOO^-$ production [41]. Our results showed increased nitrotyrosine immunostaining in the LV from WT mice treated with ethanol, suggesting oxidative damage mediated by $ONOO^-$.

The imbalance between the generation of ROS and their elimination by antioxidant enzymes could be responsible for the alteration of the cardiac oxidative state induced by ethanol consumption. Oxidoreductases known as SODs (SOD1-3) are the primary defense against ROS-induced tissue damage [42]. SODs reduce $O_2^{\cdot -}$ leading to the formation of H_2O_2 that can be then converted into H_2O and O_2 by the enzyme catalase [42]. Here we showed that ethanol consumption did not change the levels of H_2O_2 as well as SOD activity in the LV. However, ethanol augmented the activity of catalase in the LV. Similar results were found in the mice aorta and rat renal cortex [20,43]. This response may be part of a compensatory mechanism that decreases ROS levels and protects the tissue from the oxidative damage caused by ethanol. The increase in catalase activity induced by ethanol was attenuated in TNFR1-deficient mice, which is in accordance with the fact that TNF-α can modulate catalase activity in cardiomyocytes [44]. Finally, no differences in the concentration of GSH were detected among groups, suggesting that ethanol did not affect non-enzymatic antioxidant capacity.

Ethanol consumption also increases the circulating levels of pro-inflammatory cytokines [20,45]. There is a positive relationship between circulating levels of inflammatory biomarkers and the cardiovascular damage induced by chronic ethanol consumption [46]. Recently, we found that mice treated with ethanol (20% v/v) for 12 weeks showed increased levels of circulating IL-6. This response was accompanied by systemic lipoperoxidation and decreased levels of GSH in plasma [10,21]. Thus, ethanol-induced increase in circulating cytokines is accompanied by increased systemic oxidative stress. Moreover, it is important to note that pro-inflammatory cytokines play a role in ethanol-induced systemic lipoperoxidation [10].

In conclusion, chronic ethanol consumption increased ROS generation and the synthesis of pro-inflammatory proteins in the LV through TNFR1-dependent mechanisms. These findings provided novel mechanistic insights about the contribution of TNFR1 in the initial steps of the cardiac injury induced by ethanol consumption.

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Declaration of Competing Interest

The authors have no disclosures or conflicts of interest.

Author contribution

M.A.N. contributed to data curation and formal analysis; N.A.G. contributed to conceptualization, data curation and formal analysis; C.B.P.S. contributed to conceptualization, data curation and formal analysis; J.A.S. contributed to data curation and formal analysis; A.C.M.O contributed to data curation and formal analysis; L.F.T contributed to data curation and formal analysis; J.E.T.S contributed to writing - review & editing; C.R.T. contributed to supervision, roles/writing - original draft; writing - review & editing.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2019.154734.

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