Ethanol increases tumor necrosis factor-alpha receptor-1 (TNF-R1) levels in hepatic, intestinal, and cardiac cells

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Abstract

Chronic ethanol consumption leads to cell injury in virtually every tissue. Tumor necrosis factor-alpha (TNF-α) constitutes a major factor in the development of alcohol-induced liver injury. In alcohol-dependent subjects, elevated levels of plasma TNF-α are strongly predictive of mortality. Binding of TNF-α to TNF-α receptor-1 (TNF-R1) activates death domain pathways, leading to necrosis and apoptosis in most tissues, and it also increases the expression of intercellular adhesion molecules (i.e., ICAM-1), which promote inflammation. We determined whether ethanol exposure leads to increases in cellular TNF-R1. We incubated HepG2 human hepatoma cells and H4-II-E-C3 rat hepatoma cells with 25, 50, and 100 mM ethanol for various intervals of time up to 48 h. Human colonic adenocarcinoma cells (Caco-2 cells) and neonatal rat primary cardiomyocytes were also incubated with different concentrations of ethanol. Levels of TNF-R1 were measured either by a sandwich enzyme-linked immunosorbent assay (ELISA) method or by determining the extracellular transmembrane domain of TNF-R1 by an intact-cell ELISA method. Ethanol exposure for 48 h increased TNF-R1 levels in human hepatoma cells in a dose-dependent manner. Levels increased significantly by 164% at 50 mM and by 240% at 100 mM ethanol. Effects were time dependent and did not reach a plateau at 48 h. Similar increases in TNF-R1 were also observed in rat hepatoma cells (90% at 50 mM and 230% at 100 mM ethanol). Under similar conditions, Caco-2 cells showed a significant 80% increase in TNF-R1 levels at 200 mM ethanol, a concentration found in intestine. Neonatal rat primary cardiomyocytes showed TNF-R1 increases of 36% at 50 mM and 44% at 100 mM ethanol. These results indicate that exposure of different cell types to pharmacologic concentrations of ethanol increases TNF-R1 levels and may augment TNF-α-mediated cell injury in different tissues.

Keywords: Tumor necrosis factor-alpha (TNF-α); Ethanol; Hepatoma; Tumor necrosis factor receptor-1 (TNF-R1); Liver injury

1. Introduction

Tumor necrosis factor-alpha (TNF-α) mediates liver damage and inflammation in animal models of alcoholic liver damage (Iimuro et al., 1997; Yin et al., 1999). Administration of antibodies against TNF-α markedly reduces the injury (Iimuro et al., 1997), whereas knockout mice for the TNF-α receptor-1 (TNF-R1) fail to show ethanol-induced damage (Yin et al., 1999). In patients with alcoholic hepatitis, elevated plasma TNF-α levels are strongly predictive of mortality (Felver et al., 1990). Chronic ethanol consumption is also toxic to a variety of other cell types (Rubin & Farber, 2001). Alcohol abuse accounts for more than half of the cases of cardiomyopathy (Piano, 2002; Rubin & Farber, 2001), and TNF-α is well known to mediate heart injury (Edmunds et al., 1999; Levine et al., 1990). In animals, overexpression of TNF-α leads to cardiomyopathy (Kubota et al., 1997). The increased release of TNF-α from Kupffer cells induced by ethanol is mediated partly by the higher plasma endotoxin levels that occur after chronic ethanol consumption (Enomoto et al., 2001; Nanji et al., 2001; Thurman, 1998). The latter increases likely result from the injury to intestinal cells induced by ethanol (Beck & Dinda, 1981; Lambert et al., 2003).

Activation of TNF-R1 engages death domains and apoptotic pathways (Aggarwal, 2000; Chen & Goeddel, 2002)

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and mediates increases in the synthesis of intercellular adhesion molecule-1 (ICAM-1), which, in turn, promote inflammation (Fan et al., 2002; Sakamoto et al., 1997). Apoptosis has been demonstrated in hepatocytes of rats fed ethanol (Baroni et al., 1994; Deaciuc et al., 2001; Mi et al., 2000), as well as in patients with alcoholic hepatitis (Natori et al., 2001). The degree of apoptosis correlates well with neutrophil infiltration (Ziol et al., 2001). Increases in ICAM-1 levels have also been reported in the liver of rats fed ethanol chronically (Kono et al., 2001b; Nanji et al., 1995; Sakamoto et al., 1997). These findings might be explained by elevated TNF-α levels, but also, conceivably, by increased TNF-R1 levels or by a combination of both.

The aim of the current study was to assess whether exposure to ethanol increases TNF-R1 levels. To evaluate such a possibility, we have determined TNF-R1 levels in a variety of cells, including human hepatoma cells, human colonic adenocarcinoma cells, rat hepatoma cells, and neonatal rat primary cardiomyocytes, after incubation with ethanol.

2. Materials and methods

2.1. Antibodies

All polyclonal antibodies and the recombinant TNF-R1 peptide used were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell culture and ethanol treatment

H4-II-E-C3 (ATCC CRL-1600) rat hepatoma cells, obtained from American Type Culture Collection [(ATCC), Rockville, MD, USA], were cultured in Dulbecco's modified Eagle's medium [(DMEM); Gibco BRL, Bethesda, MD, USA] containing penicillin (100 U/ml) and streptomycin (0.1 mg/ml) and supplemented with 5% fetal bovine serum (FBS) and 10% equine serum (Hyclone, Logan, UT, USA). Human colonic adenocarcinoma cells [(Caco-2) ATCC HTB-37] were cultured to confluence in DMEM supplemented with 10% FBS (approximately 1.5×10^6 cells per 15-mm dish), as previously described (Tapia et al., 1996). Differentiated human colonic adenocarcinoma cells (Caco-2 cells) are able to express features characteristic of mature intestinal cells such as enterocytes. HepG2 (ATCC HB-8065) human hepatoma cells were cultured in minimum essential medium (MEM), supplemented with sodium pyruvate (0.11 g/l), penicillin (100 U/ml), and streptomycin (0.1 mg/ml) (Gibco BRL) and complemented with 10% FBS (Hyclone). Cell culture flasks and plates were obtained from Sarstedt (Newton, NC, USA). Cells were subcultured at 1:10 ratio once a week. Studies with hepatoma cells were conducted with cells at 80%–90% confluence (approximately 5×10^6 cells per 35-mm dish) incubated under an atmosphere of 95% air and 5% CO_2 at 37ºC. To prevent evaporation of ethanol, 35-mm plates containing the cells were placed inside a 100-mm dish containing twice the ethanol concentration used in experiments (in 35-mm plates). Such a procedure helps to maintain the concentration of ethanol in the cell culture medium at 95%–100% of the desired concentration. Rat and human hepatoma cells were exposed to various concentrations of ethanol for 48 h, with medium replaced every 8 h. At the end of these treatments, TNF-R1 levels were determined both in cell lysates and in intact cells (vide infra) by enzyme-linked immunosorbent assay (ELISA).

Neonatal rat primary cardiomyocytes were prepared from 1- to 3-day-old rats delivered by Harlan Sprague Dawley rats bred at the University of Chile, as previously described (Foncea et al., 1997). In essence, ventricles of at least 50 neonates (routinely prepared in S. Lavandero's laboratory) are trisected and pooled and myocytes are subsequently dissociated with a solution of collagenase and pancreatin. Yield is approximately $2–5 \times 10^6$ cardiomyocytes per rat. After the enzymatic dissociation, cardiomyocytes in the suspensions were enriched by centrifugation. Cardiomyocytes (5×10^5 cells) were plated on 35-mm dishes (Sarstedt) precoated with gelatin. Cardiomyocytes were allowed to stabilize for 24 h in DMEM containing penicillin (100 U/ml) and streptomycin (0.1 mg/ml) and supplemented with 5% FBS and 10% equine serum (Hyclone). Thereafter, cells were exposed for 48 h to 0, 50, and 100 mM ethanol in the same culture medium.

2.3. Enzyme-linked immunosorbent assay for tumor necrosis factor-alpha receptor-1 determination

2.3.1. Sandwich enzyme-linked immunosorbent assay on membranes of H4-II-E-C3 rat hepatoma cells

The TNF-R1 was initially determined by a sandwich ELISA in cell membrane fragments. After ethanol preexposure, the cells were washed twice with phosphate-buffered saline [(PBS); pH 7.4], harvested on ice (0ºC–4ºC) by using a scraper, and suspended in 200 µl of buffer containing 75 mM sodium chloride (NaCl), 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 2.5 mM magnesium chloride ($MgCl_2$), 0.5 mM dithiothreitol (DTT), and protease inhibitor cocktail [2 mM 4-(2-aminoethyl)-benezensulfonyl fluoride (AEBSF); 1 mM ethylenediaminetetraacetic acid (EDTA); 130 µM bestatin; 1.4 µM E-64; 1 µM leupeptin; and 0.3 µM aprotinin) (Sigma, St. Louis, MO, USA)]. Cells were subjected to three cycles of freezing and thawing. The cell lysate was centrifuged for 15 min (4ºC) at 7,500*g*, and the supernatant containing the cell membrane fragments was stored at –80ºC until used. The sandwich ELISA was initiated by adsorbing the primary antibody (2 µg/ml; 1:100 goat anti TNF-R1 Sc-1070, E-20) onto the plates (BD Falcon; BDBioscience, San Diego, CA, USA) overnight at 4ºC. The antibody recognizes the carboxyl-terminal region of TNF-R1. Unbound sites on the plate were blocked by incubation with bovine serum albumin [(BSA) 1 mg/ml] for 2 h at 25ºC. The plates were washed with 0.05% Tween-20 in PBS (Tween-20/PBS), and serial dilutions of

the cell-membrane fraction in PBS were added and allowed to bind to the primary antibody for 4 h at 25ºC. Plates were washed (with 0.05% Tween-20/PBS), followed by the addition of 0.4 μg/ml (1:500) of a secondary polyclonal antibody (rabbit anti TNF-R1 Sc-7895, H-271) that recognizes the amino-terminal region of the receptor. After completion of the wash with 0.05% Tween-20/PBS, a tertiary goat anti rabbit-immunoglobulin (Ig)G bound to peroxidase (0.2 μg/ml; 1:2,000) in BSA (1 mg/ml) that recognizes the secondary polyclonal antibody was added in the same buffer and incubated for 1 h at 25ºC. At such time, 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase substrate (100 μl) was added and allowed to generate color (about 15 min). The reaction was stopped by the addition of 3 N H_3PO_4 (25 μl). The absorbance of the color developed was measured at 450 nm in an ELISA reader (Labsystems Multiskan MS, Washington, DC, USA).

Control studies of antibody reactivity were initially conducted to confirm that the antibodies used did recognize the TNF-R1. In these studies, recombinant TNF-R1 was plated directly on the wells, and goat anti-TNF-R1 antibody was used to bind to the recombinant receptor, followed by a secondary rabbit anti-goat IgG-peroxidase antibody, which led to high optical density readings. Further studies were conducted in which the rabbit anti-TNF-R1 antibody was preincubated with increasing amounts of the recombinant receptor before adding it to the ELISA plates, which fully blocked the color developed.

2.3.2. Enzyme-linked immunosorbent assay on intact hepatoma cells and cardiomyocytes

The TNF-R1 also was measured in intact cells by ELISA. Primary polyclonal antibodies anti-TNF-R1 (H-271 for rat cells; N-20 for human cells) bind to the extracellular domain. Antibodies (H-271 or N-20) at 0.4 μg/ml (1:500) in BSA (1 mg/ml) were added to the respective undetached cells and incubated for 20 min. Excess antibody was removed, and plated cells were washed in ice-cold 0.001% Tween-20/PBS. The primary antibody bound to the extracellular domain of the receptor was detected by the addition of a secondary antibody linked to peroxidase (goat anti-rabbit for rat hepatoma cells and rabbit anti-goat for human hepatoma cells): IgG-horseradish peroxidate (HRP). The secondary antibodies (0.2 μg/ml, 1:2,000) in BSA (1 mg/ml) were added to the same medium and incubated at 37ºC for 20 min. The cells were subsequently washed twice with ice-cold 0.001% Tween-20/PBS, and the enzymatic peroxidase reaction was initiated after addition of the TMB substrate (500 μl) and kept at room temperature. After the incubation medium was quantitatively transferred from the plates to a new set of tubes, the reaction was stopped by the addition of 3 N H_3PO_4 (500 μl). The absorbance of the developed color was measured spectrophotometrically at 450 nm. The ELISA readings (optical density) obtained were expressed per milligram of total protein in each well, determined with the use of the Micro BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

2.3.3. Enzyme-linked immunosorbent assay on Caco-2 cell lysates

Because Caco-2 cells in confluence are polarized, TNF-R1 was determined by ELISA on cell lysates. After ethanol preexposure (0, 100, and 200 mM), the cells were washed twice with PBS (pH 7.4), harvested on ice (0ºC–4ºC), and suspended in 200 μl of lysis buffer containing 75 mM NaCl, 10 mM HEPES, 2.5 mM $MgCl_2$, 0.5 mM DTT, 0.1% Triton X-100, and protease inhibitor cocktail (2 mM AEBSF; 1 mM EDTA; 130 μM bestatin; 1.4 μM E-64; 1 μM leupeptin; and 0.3 μM aprotinin) (Sigma). Lysates were cleared by centrifugation for 15 min (4ºC) at 7,500*g*, and protein concentration was measured by using the Micro BCA Protein Assay Kit (Pierce). The ELISA was initiated by adsorbing the cell lysate in PBS overnight at 4ºC. Unbound sites on the plate were blocked by incubation with 350 μl of BSA (1 mg/ml) for 2 h at 25ºC. The plates were washed with 0.05% Tween-20 in PBS. The primary antibody (0.6 μg/ml), a 1:350 goat anti TNF-R1 Sc-1067, was added onto the ELISA plates (Falcon BD) for 2 h at 25ºC. This antibody recognizes the amino-terminal region of TNF-R1. Plates were washed with 0.05% Tween-20/PBS, followed by the addition of a secondary polyclonal antibody (0.2 μg/ml), a 1:2,000 dilution of rabbit anti goat-IgG bound to peroxidase, Sc-2768, and incubated for 1 h at 25ºC. After completion of the wash with 0.05% Tween-20/PBS, TMB peroxidase substrate (50 μl) was added and allowed to develop color (about 15 min). The reaction was stopped by the addition of 3 N H_3PO_4 (25 μl). The absorbance of the color developed was measured at 450 nm in an ELISA reader (Labsystems) and expressed per milligram of protein.

2.4. Statistical analyses

All data were subjected to an analysis of variance, with means separated by a multiple comparison test. All data are reported as means ± standard error of the mean (S.E.M.). Differences were considered significant at a P value of $< .05$.

3. Results

In initial studies, we investigated the effect of ethanol (0, 50, and 100 mM) on TNF-R1 levels in H4-II-E-C3 rat hepatoma cells by the sandwich ELISA method. Fig. 1 shows that ethanol at 50 and 100 mM significantly increased TNF-R1 levels, ranging from 50% to 200% or higher. However, the relative effect depended on the membrane dilution used, supporting the suggestion that, at higher membrane concentrations, nonspecific binding of the membrane fragments to the primary antibody may occur. In subsequent experiments, TNF-R1 was determined directly on the surface of intact hepatoma cells with the use of an antibody that recognizes

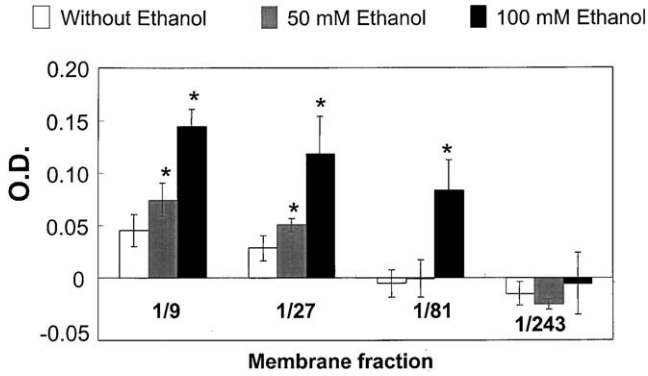

Fig. 1. Effect of ethanol exposure on tumor necrosis factor-alpha receptor-1 (TNF-R1) levels in the membranes of rat hepatoma cells. H4-II-E-C3 rat hepatoma cells were incubated in the presence or absence of 50 or 100 mM ethanol for 48 h. At the end of the incubation period, cells were lysed by freezing and thawing, and membrane fractions were prepared as described in the Materials and Methods section. The TNF-R1 levels in the membrane fractions (serial dilutions of the membrane: 1/9,1/27, 1/81, and 1/243, as shown) were detected by a sandwich enzyme-linked immunosorbent assay method that binds both the intracellular and the extracellular domains of TNF-R1 (see Materials and Methods section). Results shown are a representative experiment conducted in quadruplicate. Bars show means ± standard error of the mean of replicates. *$P < .05$ versus control without ethanol by the Fisher least significant difference test. O.D. = Optical density.

the extracellular domain of the receptor, coupled to a secondary antibody linked to horseradish peroxidase. Fig. 2A shows marked increases in TNF-R1 levels determined by this method after a 48-h incubation of H4-II-E-C3 rat hepatoma cells with ethanol (90% at 50 mM ethanol; 230% at 100 mM ethanol; $P < .05$). To determine whether the changes observed were actually the result of increases in TNF-R1 levels, the anti-TNF-R1 antibody was preincubated with soluble recombinant TNF-R1. As can be seen in Fig. 3, the effect induced by ethanol was fully blocked by preincubation of the antibody with the recombinant TNF-R1. In the absence of ethanol, ELISA readings were marginally reduced by recombinant TNF-R1, again indicative of a degree of nonspecific baseline binding to the cell membrane. These findings seem to indicate that the percentage increases in TNF-R1 indicated above are likely underestimated, and this is also consistent with the studies with ELISA sandwich at high membrane dilutions (see dilution 1/81, Fig. 1). To err on the safe side, we have presented the results without subtracting the baseline values.

As shown in Fig. 2B, ethanol also markedly increased TNF-R1 levels in HepG2 human hepatoma cells (50% at 25 mM ethanol, $P = .06$; 164% at 50 mM ethanol, $P < .05$; and 240% at 100 mM ethanol, $P < .05$; at 48 h). Additional studies on HepG2 cells showed that the increases in TNF-R1 levels at 50 mM ethanol were essentially linear from 8 to 48 h of incubation with ethanol, without reaching a plateau at 48 h (increases were 30% at 8 h, 50% at 24 h, 100% at 32 h, and 164% at 48 h).

To determine whether other cell types known to be susceptible to injury by ethanol are also affected in a similar manner, we studied the effect of ethanol on the levels of

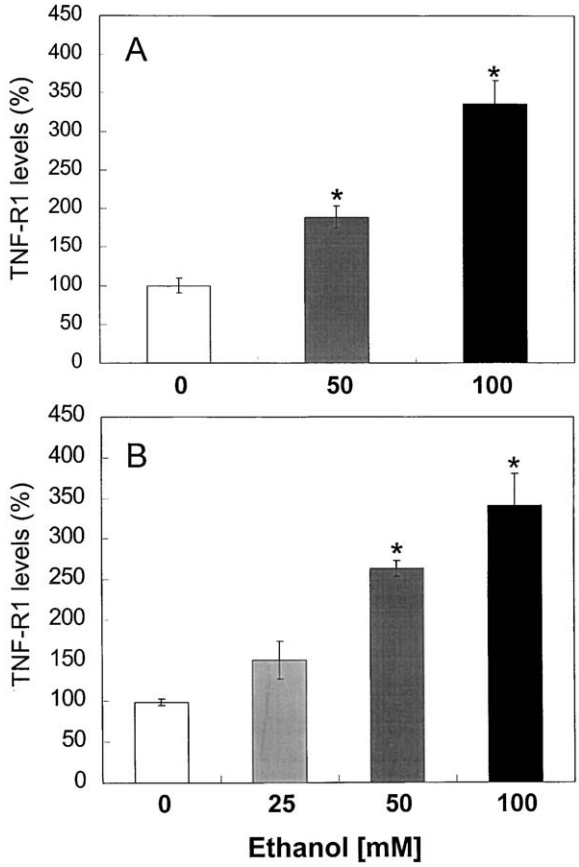

Fig. 2. Effect of ethanol on tumor necrosis factor-alpha receptor-1 (TNF-R1) levels in intact H4-II-E-C3 rat hepatoma cells and HepG2 human hepatoma cells. A. H4-II-E-C3 rat hepatoma cells were incubated in the presence or absence of 50 or 100 mM ethanol for 48 h. At the end of the incubation period, the extracellular transmembrane domain of TNF-R1 was determined by enzyme-linked immunosorbent assay (ELISA) on intact cells. Ethanol increased the levels of TNF-R1 by 90% at 50 mM and by 230% at 100 mM ethanol. Data represent the means ± standard error of the mean (S.E.M.) from three independent experiments with H4-II-E-C3 plated in duplicates or triplicates. Values were calculated as optical density per milligram of protein for each plate and subsequently converted to percentages relative to the mean of the plates without ethanol. *$P < .05$ versus control without ethanol by the Fisher least significant difference test. B. HepG2 human hepatoma cells were incubated for 48 h with various concentrations of ethanol ranging from 0 to 100 mM. At the end of the incubation period, the extracellular transmembrane domain of TNF-R1 was determined by ELISA on intact cells. Ethanol exposure increased the levels of TNF-R1 by 50% at 25 mM ($P = .06$), by 164% at 50 mM, and by 240% at 100 mM ethanol. Data presented are the means of three independent experiments with triplicates at zero and at each of the concentrations of ethanol, and two additional experiments with triplicates at zero and 50 mM ethanol for HepG2 cells plated in each experiment ± S.E.M. Values were calculated as optical density per milligram of protein for each plate and subsequently converted to percentages relative to the mean of the plates without ethanol. *$P < .05$ versus control without ethanol by the Fisher least significant difference test.

TNF-R1 in intact Caco-2 cells and neonatal rat primary cardiomyocytes (Fig. 4). Fig. 4A shows that, after a 48-h incubation with ethanol, levels of TNF-R1 in Caco-2 cells were increased by 80% ± 20% at 200 mM ($P < .05$) ethanol; increases at 100 mM ethanol were not significant. It should

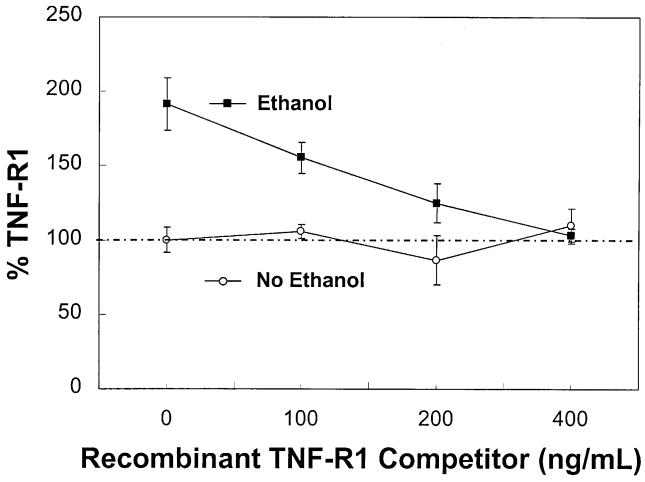

Fig. 3. Preabsorption of anti TNF-R1 polyclonal antibodies with soluble recombinant tumor necrosis factor-alpha receptor-1 (TNF-R1) reduces enzyme-linked immunosorbent assay signal induced by ethanol treatment. Various concentrations of soluble recombinant TNF-R1 [in bovine serum albumin (1 mg/ml)] were incubated with primary anti-TNF-R1 antibody (0.4 μg/ml), and this mixture was added to intact H4-II-E-C3 rat hepatoma cells previously incubated for 48 h with or without 100 mM ethanol. Results shown are for a representative experiment conducted in triplicates for each well and at each concentration of recombinant TNF-R1 competitor means ± standard error of the mean.

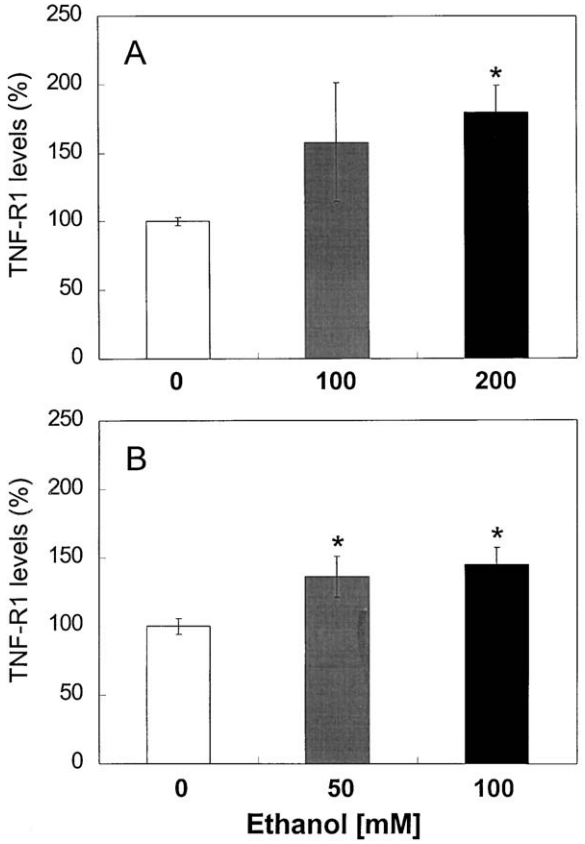

Fig. 4. Effect of ethanol on recombinant tumor necrosis factor-alpha receptor-1 (TNF-R1) levels in human colonic adenocarcinoma cells (Caco-2 cells) and intact neonatal rat primary cardiomyocytes. A. Caco-2 cells were incubated in the presence or absence of 100 or 200 mM ethanol for 48 h. At the end of the incubation period, TNF-R1 was determined by enzyme-linked immunosorbent assay (ELISA) on complete fraction of membranes and cytosol (see Material and Methods section). Ethanol increased the levels of TNF-R1 by 57% at 100 mM and by 80% at 200 mM ethanol. Values were calculated as optical density per milligram of protein for each plate and subsequently converted to percentages relative to the mean of the plates without ethanol. Data presented are the means ± standard error of the mean (S.E.M.) of two independent experiments with triplicates at zero and at each of the concentrations of ethanol, and one additional experiment ($n = 17$) at zero and 200 mM ethanol for Caco-2 cells plated in each experiment $^*P < .05$ versus control without ethanol by the Fisher least significant difference test. B. Neonatal rat primary cardiomyocytes were prepared and cultivated, as previously described (see Materials and Methods section). Finally, the cardiomyocytes were incubated for 48 h with various concentrations of ethanol ranging from 0 to 100 mM. At the end of the incubation period, the extracellular transmembrane domain of TNF-R1 was determined by ELISA on intact cells. Ethanol exposure increased the levels of TNF-R1 by 36% at 50 mM and by 44% at 100 mM ethanol. Values were calculated as optical density per milligram of protein for each plate and subsequently expressed in the same manner as for Caco-2 cells. Results shown represent means ± S.E.M. from three independent experiments with triplicates of neonatal rat primary cardiomyocytes plated in each case. $^*P < .05$ versus control without ethanol by the Fisher least significant difference test.

be noted that convergent Caco-2 cells in culture become polarized. The ELISA data obtained in cell lysate assays do not allow the differentiation between the apical and basolateral aspects. Neonatal rat primary cardiomyocytes (Fig. 4B) showed increases in TNF-R1 levels of 36% ± 14% at 50 mM ($P < .05$) and 44% ± 13% at 100 mM ethanol ($P < .05$).

4. Discussion

Alcohol-related liver injury results from a cascade of events, leading to cell death. Concerted factors (equivalent to a "several-hit" effect) in this cascade include increases in oxidative stress and elevations in serum endotoxin level resulting from increased gut permeability due to intestinal injury. The combination of the above factors activates Kupffer cells, leading to marked elevations of plasma TNF-α. The latter, acting through TNF-R1s, activates death domain pathways. Supporting data for the above cascade come from results of animal studies, showing that increases in mitochondrial glutathione levels can reduce alcohol-induced liver injury (García-Ruiz et al., 1995; Wu & Cederbaum, 2001) and that increases in oxygen radical species result from alcohol-induced elevations in cytochrome P450 2E1 (CYP2E1) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity (Kono et al., 2001a; Marí et al., 2001). Moreover, gut sterilization by oral antibiotics reduces serum endotoxin levels (Nishiyama et al., 2002) and liver damage (Enomoto et al., 1999), whereas destruction of Kupffer cells (Koop et al., 1997) or neutralization of TNF-α by antibodies (Iimuro et al., 1997) reduces alcohol-induced liver injury. Alcohol-induced liver injury fails to develop in knockout animals for the TNF-R1 (Yin et al., 1999).

It has been well established that, in human beings, increased levels of TNF-α are associated with the development of cardiomyopathy (Edmunds et al., 1999; Levine et al., 1990; Sharma et al., 2001). Intracellular signaling mediated by TNF-R1 not only activates death domain pathways but also results in increases in ICAM-1 levels (Oudar et al., 1998; Satoh et al., 1994), which anchor neutrophils and monocytes and result in inflammation.

Findings presented from the current study support the suggestion that an added factor in the "several-hit" effect may be an ethanol-induced increase in TNF-R1 levels, adding to the processes that lead to cell death and inflammation. The mechanism by which ethanol increases TNF-R1 is not known. However, results from preliminary studies indicate that the effect of ethanol is not altered by pyrazole, *N*-acetylcysteine, or cyanamide (data not shown), supporting the suggestion that neither ethanol metabolism nor oxidative stress plays a major role in the increases in TNF-R1 levels induced by ethanol. What seems clear is that different cell types have different sensitivities to the effects of ethanol, with hepatoma cells being most sensitive, followed by cardiomyocytes and intestinal cells, which is consistent with the damage seen in different organs of alcohol-dependent individuals. The effect of ethanol on epithelial intestinal cells is of interest because Caco-2 cells exposed to Gram-negative bacteria present injury, by mechanisms that are nuclear factor-kappa B (NF-κB) and ICAM-1 mediated (Elewaut et al., 1999), that would be potentiated by activation of TNF-R1. An apoptotic effect by this route may result in increases in permeability to endotoxin, known to result from the single administration or the long-term intake of ethanol (Beck & Dinda, 1981; Lambert et al., 2003).

Results from the current study are in line with observations by Deaciuc et al. (1995), who determined the binding of ^{125}I–TNF-α to liver parenchymal and nonparenchymal cells of rats fed ethanol acutely (hours) and chronically (weeks). Findings from their studies showed marked increases in high-capacity low-affinity TNF-α receptors in primary endothelial and Kupffer cells, binding which most likely represents the TNF-R1 (Vandenabeele et al., 1995). However, the Scatchard method used in these studies did not allow detection of TNF-R1s in hepatocytes. In fact, in our early studies the intact TNF-R1 could not be detected by the Western blot technique (with the use of monoclonal antibody Sc 8436, which recognizes the extracellular domain of the receptor) in freshly isolated hepatocytes prepared by protease perfusion, a procedure also used by Deauciuc et al. (1995). It is conceivable that this procedure with nonrecombinant collagenase, which generally contains other proteases, may promote to cleavage of the external side of the receptor from the cells. Observations in patients with severe alcoholic cirrhosis display increases in soluble plasma TNF-R1 (sTNF-R1) levels (Naveau et al., 1998), likely released from cell membranes by proteolytic cleavage of TNF-R1 (Jurewicz et al., 1999).

Two groups of investigators have shown that, in comparison with control cells, primary hepatocytes of rats fed ethanol chronically (Deaciuc et al, 2001) and HepG2 human hepatoma cells preincubated with ethanol (Pastorino & Hoek, 2000) are more susceptible to the apoptotic effects of TNF-α. We have confirmed their studies in rat hepatoma cells preincubated for 48 h with 50 mM ethanol. Ethanol-treated hepatoma cells were significantly more susceptible to the cytotoxic effects of TNF-α (data not shown). Although the exact mechanism is unknown, as indicated earlier, alcohol-induced liver injury fails to develop in TNF-R1 knockout mice (Yin et al., 1999). Conversely, alcohol-induced liver injury does develop in TNF-R2 knockout mice as well as in wild-type mice (Yin et al., 1999).

Overall, findings presented in this article show that ethanol exposure leads to marked increases in TNF-R1 levels both in human cells and in rat cells. Increases in TNF-R1 levels by ethanol could conceivably play a role in the TNF-α-mediated events that result in alcohol-induced cell injury.

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