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TNF-α induces apoptosis of parietal cells

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Abstract

Helicobacter pylori infection can be associated with chronic gastric inflammation and hypochlorhydria with increased levels of the proinflammatory cytokines. The current study investigated the effects of TNF-α on programmed death of gastric parietal cells. TNF-α induced apoptosis of parietal cells in isolated perfused rat stomachs at 10 ng/mL. In isolated and highly enriched rat parietal cells, 10 ng/mL TNF-α induced a 2.6-fold increase in the apoptotic rate. The 55 kDa protein of TNFR-1 but not the 75 kDa of TNFR-2 was detected by Western blot analysis. TNF-α-induced apoptosis of isolated parietal cells was inhibited by pretreatment with different NF-κB-inhibitors, nitric oxide synthase inhibitors and with antisense-oligodeoxynucleotides against the p65 subunit of NF-κB. Investigation of downstream signaling pathways of apoptosis revealed that TNF-α induced the expression of iNOS, but failed to stimulate the activity of caspase 3. The TNF-α effect on gastric parietal cells may contribute to the atrophy and hypochlorhydria of the gastric mucosa observed during chronic *H. pylori* infection.

Keywords: Atrophy; Cytokine; Oxyntic mucosa; TNFR

1. Introduction

Colonisation of the gastric mucosa with *Helicobacter pylori* causes infiltration by polymorphonuclear cells, macrophages, and T and B lymphocytes [1]. In the gastric mucosa acutely infected by *H. pylori*, epithelial cell replication and rates of apoptotic cell death are both increased [2], whereas after long-term infection, apoptosis of epithelial cells predominates [3]. A characteristic feature of atrophic corpus gastritis is hypochlorhydria caused by the loss of parietal cells. Several mechanisms of parietal cell death might be involved: apoptosis induced by inflammatory cytokines or cell death by activated T-cells. However, the precise mechanisms are unknown.

Infection of the gastric mucosa with *H. pylori* results in elevated tissue levels of proinflammatory cytokines such as IL-1β, IL-6, IL-8, and TNF-α [4,5]. TNF-α is synthesized and secreted by mononuclear phagocytes [6] and mucous pit cells [7] and exerts various effects on different cell types, e.g. activation of genes involved in inflammatory responses, cell proliferation, growth inhibition, and cell death. Most of the known biological activities of TNF-α are believed to be mediated *via* TNFR-1. Ligand binding to the TNFR-1 receptor is generally thought to activate two main signaling pathways, one leading to cell death and the other leading to gene transcription [8–10].

In this study, we show that stimulation of parietal cells with TNF-α results in an increased apoptotic rate of parietal cells. We present evidence that TNF-α-induced

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Abbreviations: ALLN, *N*-acetyl-Leu-Leu-norleucinal; AS-PS-ODN, antisense-phosphorothioate oligodeoxynucleotides; CASPASE, cytosolic aspartate-specific protease; FAM-AS-PS-ODN, 5′-carboxyfluorescein-phosphoramidite antisense oligodeoxynucleotides; *H. pylori*, *Helicobacter pylori*; iNOS, inducible nitric oxide synthase; L-NMMA, N^G-monomethyl-L-arginine acetate; PSI, proteasome inhibitor I; TNF-α, tumor necrosis factor alpha; TNFR, tumor necrosis factor receptor; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling.

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apoptosis is associated with the activation of NF-κB as well as stimulation of inducible nitric oxide synthase, whereas caspase 3 activity was not stimulated by TNF-α. These data underline the special importance of this cytokine for the development of gastric acid hyposecretion observed during long term *H. pylori* infection.

2. Materials and methods

We studied the effect of TNF-α in the intact gastric mucosa: isolated rat stomachs were vascularly perfused *via* the celiac artery in a single-pass perfusion system for 90 min at 1.5 mL/min with a modified Krebs–Ringer buffer solution supplemented with vehicle or 10 ng/mL TNF-α (Sigma) [11]. TUNEL staining was performed on paraformaldehyde-fixed and paraffin-embedded sections of corpus mucosa (*in situ* cell death detection kit, Boehringer). The animal studies were approved by the responsible authorities of the County of Upper Bavaria (approval 211-2351.3-45/92).

For isolation of parietal cells, rat stomachs were prepared by the everted sac method as previously described [12]. Enzymatically dispersed cells (1.3 mg/mL pronase E, Boehringer M.) were subjected to counterflow elutriation followed by a density gradient (60% Percoll). Almost pure parietal cells ($97 \pm 5\%$) were collected at the interface between Percoll and buffer.

For electron microscopy studies, parietal cells were fixed with 4% paraformaldehyde/0.5% glutaraldehyde and postfixed with 4% OsO_4/potassium hexacyanoferrate (II). After embedding in Epon, thin sections were cut, contrasted with uranylacetate (2%)/lead citrate (2.7%) and examined with an EM10 electron microscope (Carl Zeiss) [13].

For quantitative measurement of parietal cell apoptosis we stained semi-thin sections of the same preparations, which were used for electron microscopic studies, with azur-II/methylenblue (1:1). A characteristic feature of apoptotic parietal cells both at light microscopical and electron microscopical levels were the shrunken, condensed nuclei. Therefore, the number of cells with condensed nuclei was determined. Furthermore, for quantitative determination of parietal cell apoptosis single strand breaks in high molecular weight DNA were detected by labeling free 3′-OH termini with modified nucleotides in an enzymatic reaction [14]. This was performed using the *in situ* cell death detection kit. Cells with dark brown nuclei or apoptotic bodies were considered positive. The apoptotic index was calculated after counting 500 cells in randomly selected fields using an 40× objective and 10× ocular.

Transfection studies were performed as described by Reuning *et al.* [15]. Oligodeoxynucleotides complementary to RelA of the NF-κB protein p65 were synthesized in a phosphorothioate-modified form and purified by HPLC (MWG Biotech). The sequence for spanning the translation initiation codon was 5′-GGGGAACAGTTCGTCCATGGC-3′. Missense PS-ODN with an identical nucleotide content but in random order (5′-GTACGCGGTGAAGCTGCGATC-3′) or lipofectin alone served as control. For the use in internalization studies, AS-PS-ODN were 5′-end-labelled using FAM-(5′-carboxyfluorescein)-phosphoramidite. Parietal cells (3×10^5 cells/mL) were incubated with 10 μM FAM-AS-PS-ODN, AS-PS-ODN, or missense PS-ODN in presence of 10 μg/mL lipofectin at 37° and 100% O_2 for 8 hr. Transfection was examined with FAM-AS-PS-ODN by confocal microscopy using a Confocal Laser Scanning Microscope (CLSM) LSM 510 (Carl Zeiss Jena GmbH).

The caspase 3 activity was determined by a fluorogenic substrate assay [16]. We incubated 20 μg of parietal cell extract and 50 μM of DEVD-AMC for 30 min at 37° and quantified liberated fluorescence by fluorospectrophotometry (Perkin-Elmer LS-3).

Primer pairs for PCR yielding iNOS (5′-gaaagaactcgggcatacct-3′, 5′-ggcgaagaacaatccacaac-3′) and GAPDH (5′-tgaaggtcggtgtcaacggatttggc-3′, 5′-catgtaggccatgaggtccaccac-3′) were synthesized by MWG Biotech. The primers were designed in a way that the amplified sequence spanned at least two different exons to avoid amplification of genomic DNA. The housekeeping gene GAPDH was chosen to determine integrity of the synthesized cDNA.

3. Results and discussion

Increased apoptosis of gastric epithelial cells is a consequence of *H. pylori*-induced gastritis [2]. Apoptosis gradually overcomes cell proliferation with increasing duration and severity of the inflammatory response [3]. Death of gastric epithelial cells may result from direct contact with *H. pylori*, from secreted bacterial products and, indirectly, from the release of inflammatory cytokines [17]. Several studies have shown that tissue levels of TNF-α are elevated in the gastric mucosa infected with *H. pylori* [4,5].

We studied the *in vivo* relevance of TNF-α on epithelial death in the gastric mucosa. Isolated rat stomachs were perfused with 10 ng/mL TNF-α or vehicle for 90 min. The nuclei of many cells located in the gastric crypts of the TNF-α perfused stomachs showed the dark brown pattern of TUNEL staining (Fig. 1a). Whereas, perfusion with buffer without TNF-α did not result in apoptosis of gastric epithelial cells (Fig. 1b). To investigate the apoptotic effect of TNF-α on the acid secreting cells of the gastric mucosa, we isolated rat parietal cells and enriched them to a purity of over 97%. By using electron microscopy we showed, that parietal cells were killed by apoptosis (Fig. 1c and d). The cells showed the characteristic features of apoptosis described by Wyllie *et al.* [18]. Shrunken, condensed nuclei of apoptotic parietal cells were also noticed under light microscopy (Fig. 1e). Using this feature for quantitative determination, we noticed a 2.4-fold increase in

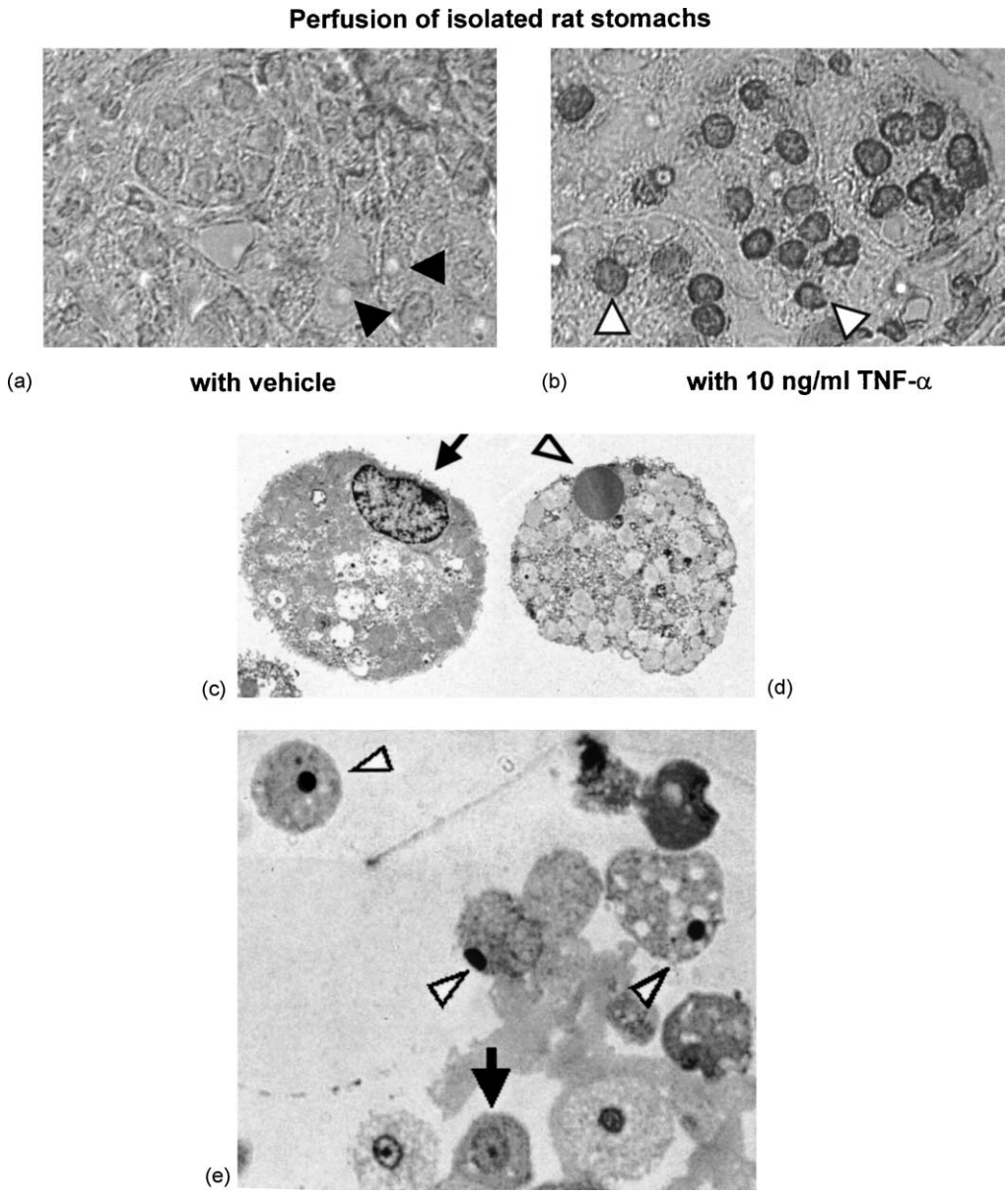

Fig. 1. Isolated rat stomachs were perfused *via* the celiac artery with buffer supplemented with vehicle (a) or with 10 ng/mL TNF-α (b) for 90 min. Paraformaldehyde-fixed and paraffin-embedded sections of corpus mucosa were stained for apoptotic cells by TUNEL. The nuclei of epithelial cells in the gastric crypts were negative in the vehicle perfused stomachs (black arrows), whereas perfusion with TNF-α resulted in a positive, dark brown staining of most of the cell nuclei (open arrows). TNF-α-induced apoptosis of parietal cells was confirmed by electron microscopy. (c) A parietal cell incubated for 4 hr with vehicle only. The normal cell is characterized by a large nucleus with heterochromatin (arrow), many mitochondria, and microvilli, which are found on the surface of the cell and inside the canaliculi. (d) After incubation with TNF-α for 4 hr a great number of parietal cells shows a small nucleus with condensed chromatin (arrowhead); 6400×. This typical change was also visible at the light microscope level. For quantification of apoptosis thin sections of the same preparations of parietal cells, which were used for electron microscopy, were stained with azur-II/methylenblue (e). The cells with dark, shrunken nuclei were considered as apoptotic (arrowheads), whereas cells with normal configured nuclei were evaluated as non-apoptotic (arrow); Zeiss Axioplan, objective 100×.

parietal cell apoptosis induced by 10 ng/mL TNF-α for 4 hr (basal apoptosis: 18.4 ± 1.3%, TNF-α-induced apoptosis: 44.2 ± 1.3%). We confirmed these results by TUNEL staining of TNF-α-exposed parietal cells (under the same conditions 2.6-fold increase in the apoptotic rate, Fig. 2a, bars 1 and 2). This underlines that the effect of TNF-α did not differ in isolated cells or in gastric epithelial cells remaining in their natural setting.

We further investigated parietal cell expression of the specific TNF-α receptor subtypes TNFR-1 and TNFR-2 by Western blot analysis. In general, TNF-α exerts its apoptotic effects by binding to specific receptors. Two high affinity TNF receptors of 55 kDa (TNFR-1, CD120a) and 75 kDa (TNFR-2, CD120b) have been described, both of which are expressed ubiquitously [8]. The transduction of the death signal is believed to be mainly mediated by

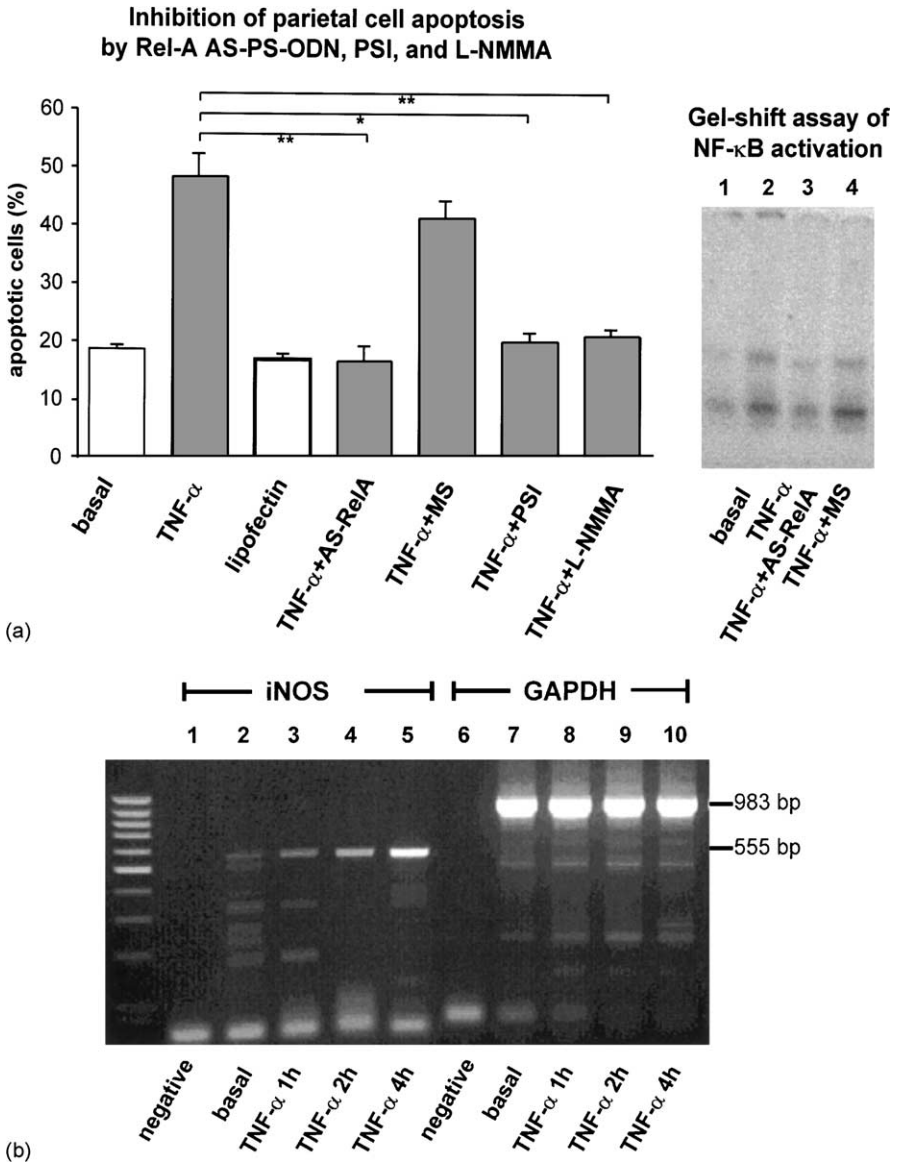

Fig. 2. (a) Left side: inhibition of NF-κB activation, p65 expression, and nitric oxide synthase abolished TNF-α-stimulated parietal cell apoptosis. 10 ng/mL TNF-α for 4 hr induced a 2.6-fold increase in the apoptotic rate of parietal cells as determined by TUNEL staining. Preincubation of the cells with 60 μM PSI inhibited TNF-α-stimulated apoptosis ($^{*}P = 0.02$ vs. TNF-α). Transfection with AS-PS-ODN directed to RelA inhibited TNF-α-stimulated parietal cell apoptosis ($^{**}P = 0.005$ vs. TNF-α). Exposure of the cells to lipofectin alone did not alter the basal apoptotic rate. Missense ODN showed no significant effect on TNF-α-induced apoptosis. Incubation with L-NMMA (10^{-4} M) inhibited TNF-α-stimulated parietal cell apoptosis ($^{**}P = 0.005$ vs. TNF-α). Right side: EMSA of nuclear extracts of PC stimulated with vehicle (lane 1) or TNF-α (lanes 2–4). TNF-α-activated (10 ng/mL) NF-κB DNA-complexes (lane 2) were reduced by previous transfection of the cells with antisense-oligodeoxynucleotides to RelA (lane 3). Transfection with missense ODN (lane 4) showed no distinct effects on TNF-α-induced NF-κB activation. (b) Detection of iNOS and GAPDH expression by PCR. cDNA of isolated rat parietal cells, which were stimulated with TNF-α for 1 hr (lane 3), for 2 hr (lane 4), and for 4 hr (lane 5), showed increasing expression of iNOS from 1 up to 4 hr. GAPDH expression remained constant in unstimulated and TNF-α-stimulated parietal cells of the same preparations (lanes 7–10). Negative controls were run without cDNA (lanes 1 and 6). The expected size of the PCR products of iNOS was 555 bp, of GAPDH 983 bp. The molecular weight standard represents steps of 100 bp where the bright band equals 500 bp.

TNFR-1. However, there is controversy about the apoptotic role of TNFR-2, which contains no intracellular death domains [19]. Expression of the 55 kDa protein of TNFR-1 was found, whereas a signal of the 75 kDa protein of TNFR-2 was not detected (not shown). In line with these findings, immunocytochemical stainings for both receptors confirmed the presence of only the TNFR-1 receptor on parietal cells (not shown). Therefore, we suggest that TNF-α-induced apoptosis of parietal cells is mediated by TNFR-1.

To examine the involvement of the transcription factor NF-κB in the regulation of TNF-α-induced apoptosis, we preincubated parietal cells with peptidyl aldehyde inhibitors 60 min before exposition to 10 ng/mL TNF-α. The NF-κB inhibitors were used at the same concentrations, which were shown in EMSAs to be sufficient to inhibit NF-κB

activation in parietal cells: 60 μM PSI, 50 μM MG115, 100 μM ALLN, 50 μM MG132 (not shown). Each of the tested peptidyl aldehydes abolished TNF-α-induced apoptosis fully (only shown for PSI, Fig. 2a, bar 6). Thus, blocking IκBα degradation and NF-κB activation inhibits TNF-α-induced apoptosis in parietal cells.

To investigate the functional role of NF-κB's p65 subunit in the mediation of TNF-α-induced apoptosis, we transfected parietal cells with antisense oligodeoxynucleotides directed against RelA. TNF-α-stimulated apoptosis was abolished by preincubation of cells with AS-PS-ODN directed to RelA for 8 hr (Fig. 2a, bar 4). In contrast, pretreatment with the transfection medium lipofectin or with missense PS-ODN for 8 hr showed no significant effect on basal or TNF-α-stimulated apoptosis (Fig. 2a, bars 3 and 5). Thus, inhibition of translation of RelA mRNA and expression of NF-κB's protein p65 resulted specifically in abrogation of TNF-α-induced apoptosis. Our finding of a proapoptotic effect of NF-κB in TNF-α-stimulated parietal cells is in line with results from various cell types, e.g. glutamate-stimulated neurons [20], serum-deprived transformed human embryonic kidney cells [21], Fas-stimulated T cell hybridomas [22], and α-CD3-activated thymocytes [23]. Furthermore, it has been shown that TNF-α-induced apoptosis is mediated by NF-κB activation in myeloid leukemic progenitor cells [24], in fibrosarcoma cells [25], and in clonal osteoblasts [26]. In contrast to these and our present results, NF-κB has been found to counteract the proapoptotic effects of TNF-α in several other cell types [27–31]. The different pro- or anti-apoptotic roles of NF-κB observed in previous studies may depend to a great extent on different kinetics of cell exposure to the death stimulus or by a pre-exposure to stress stimuli. Finally, it has to be mentioned that induction of apoptosis may not only be triggered by NF-κB alone; factors such as AP1, p53 and others may further be involved in the apoptotic signaling pathway.

The generation of nitric oxide is known to induce apoptosis by DNA breakdown. To examine the apoptotic effect of NO, we preincubated parietal cells with the nitric oxide synthase inhibitor L-NMMA (10^{-4} M). As indicated in Fig. 2a (bar 7), L-NMMA prevented significantly parietal cell apoptosis induced by TNF-α. We also studied with PCR the stimulation of inducible nitric oxide synthase by TNF-α. As indicated in Fig. 2b, mRNA expression of iNOS is stimulated increasingly from 1 to 4 hr, whereas GAPDH mRNA remained constant over this period. Therefore, we showed that a NF-κB target gene with pro-apoptotic function, the inducible nitric oxide synthase, is stimulated by TNF-α. The promoter region of the rat iNOS gene contains the NF-κB consensus sequence at several positions (bp 71–79, 134–142, 888–896 and 930–938). Activation of iNOS results in generation of NO-radical from L-arginine. Although iNOS and NO-radical have been shown to exert pro-apoptotic function, they have also been implicated with an anti-apoptotic action [32]. It seems that the effect of NO-radical is concentration-dependent and may be influenced by the presence of other reactive oxygen species. In our cell model by using the nitric oxide synthase inhibitor L-NMMA, we demonstrated that TNF-α-stimulated apoptosis is triggered by iNOS. Similar observations were made in pancreatic β-cells following IL-1β stimulation. In these cells, NF-κB activation is linked to apoptosis, and this effect appeared to be mediated by induction of iNOS and generation of NO [33].

To investigate other possible execution pathways of apoptosis, which were stimulated by TNF-α in parietal cells, we measured the activity of the key effector caspase, caspase 3. Using a fluorogenic substrate assay we determined the liberated fluorescence in lysated parietal cells and detected only a small stimulation of caspase 3 activity by TNF-α (background was set to 1.0; unstimulated parietal cells: 1.68; parietal cells stimulated with 10 ng/mL TNF-α for 30 min: 1.65, for 1 hr: 1.72, for 2 hr: 1.72, for 4 hr: 1.78). Whereas, the positive control, rat gastric epithelial cells, which were kept in suspension for 3 hr to induce anoikis, revealed a strong stimulation (7.30).

In summary, our present data show that non-transformed parietal cells differ from several other cell systems with respect to the apoptosis-regulating function of NF-κB. By ligation of TNFR-1, TNF-α activates NF-κB and stimulates expression of iNOS leading to apoptosis. We speculate that this mechanism may contribute to atrophy of the gastric mucosa infected by *H. pylori*.

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