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Monochloramine Impairs Caspase-3 Through Thiol Oxidation and Zn^{2+} Release

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Abstract

Background—Caspase-3, a pro-apoptotic enzyme, represents a class of proteins in which the active site contains reduced thiol (S-H) groups and is modulated by heavy metal cations such as Zn^{2+}. We explored the effects of the thiol oxidant monochloramine (NH_2Cl) on caspase-3 activity within cells of isolated rabbit gastric glands. In addition, we tested the hypothesis that NH_2Cl-induced alterations of caspase-3 activity are modulated by oxidant-induced accumulation of Zn^{2+} within the cytoplasm.

Materials and Methods—Isolated gastric glands were prepared from rabbit mucosa by collagenase digestion. Caspase-3 activity was measured colorimetrically in suspensions of healthy rabbit gastric glands, following exposure to various concentrations of NH_2Cl with or without the zinc chelator TPEN for 1 hour, and re-equilibration in Ringer's solution for 5 hours. Conversion of procaspase 3 to active caspase-3 was monitored by Western blot.

Results—Monochloramine inhibited caspase-3 activity in a dose dependent fashion. At concentrations of NH_2Cl up to 100μM, these effects were prevented if TPEN was given concurrently and were partly reversed if TPEN was given one hour later. Caspase-3 activity was preserved by concurrent treatment with a thiol-reducing agent, dithiothreitol (DTT).

Conclusions—At pathologically relevant concentrations, NH_2Cl impairs caspase-3 activity through oxidation of its thiol groups. Independently from its thiol oxidant effects on the enzyme, NH_2Cl-induced accumulation of Zn^{2+} in the cytoplasm is sufficient to restrain endogenous caspase-3 activity. Our studies suggest that some bacterially generated oxidants such as NH_2Cl impair host pathways of apoptosis through release of Zn^{2+} from endogenous pools.

Keywords

monochloramine; caspase-3; zinc; Helicobacter pylori; gastritis; oxidants; oxidative stress

Introduction

Infestation of the gastric mucosa by *Helicobacter pylori* leads to persistent mucosal injury. In Western countries, *H. pylori* infestation mucosal injury is often muted, and is associated with chronic inflammatory infiltrates, chronic peptic ulcer disease, and malignancy (1). In acute *H.*

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pylori gastritis, the intense inflammatory response is marked by neutrophil-enriched infiltrates that lead to severe erosions and bleeding (1,2).

The acute inflammatory respose is characterized by the uncontrolled accumulation of a diverse array of oxidants, including monochloramine (2-4). Monochloramine (NH_2Cl) is produced from the spontaneous reaction of hypochlorous acid (HOCl), generated by the activated neutrophil, and ammonia (NH_3), produced through the action of *H. pylori* ureases (2,5). It has been estimated that NH_2Cl concentrations of up to 200μM may accumulate in acutely inflamed regions of gastrointestinal mucosa (5). Among the principal intracellular targets of monochloramine are cysteine-rich proteins containing reduced sulfhydryl (thiol) residues (6, 7).

Apoptosis, or programmed cell death, is activated in response to different forms of stress, including stimuli that are exogenous to the cell and signals that are generated within the cell (1,8,9). A key enzyme in the initiation of apoptosis is caspase-3. Activation of caspase-3 occurs through proteolytic cleavage of the Ile-Glu-Thr-Asp motif in the inactive procaspase-3, which after acidification dissipates the tri-aspartate residue “safety catch” that resists autocatalysis or premature cleavage by initiating caspases (10).

The active site of the caspase-3 molecule is composed of reduced cysteine residues that are potentially susceptible to oxidation and inactivation by thiol directed oxidants (10,11). In addition, reversible modulation of caspase-3 activity by variation of intracellular zinc ($[Zn^{2+}]_i$) has been demonstrated in cell-free experimental systems (12-15). Recent studies in our laboratory indicate that exposure of gastric parietal cells to NH_2Cl at pathologically relevant concentrations releases Zn^{2+} from thiol-rich intracellular pools in alimentary tract epithelial cells, including the gastric parietal cell (16,17).

These considerations led us to hypothesize that caspase-3 activity is susceptible to oxidation by NH_2Cl and modulated by the concurrent accumulation of Zn^{2+}. The goals of this study were: first, to determine whether loading of gastric epithelial cells with oxidant-relevant concentrations of Zn^{2+} (nanomolar range (16)) alters endogenous caspase-3 activity; second, to determine whether exposure to NH_2Cl leads to decreases in endogenous caspase-3 activity that are modulated by the concurrent accumulation of Zn^{2+}; and, third, to determine whether these effects are attributable to oxidation of intracellular thiol groups.

Materials and Methods

Gland isolation

Anesthesia and euthanasia for New Zealand White rabbits were approved according to policies of Harvard Medical School. Gastric glands were harvested as described previously (17,18). Rabbit gastric glands prepared by this method yield a homogenous gland preparation consisting of approximately 50% parietal cells and 40% chief cells (19-22).

Preparation of Monochloramine

Monochloramine (NH_2Cl) was prepared as described previously (16,17). Briefly, a 200μl solution containing 500mM NaOCl in water was added dropwise to 10ml of 20mM NH_4Cl and 5mM Na_2HPO_4 in water at 0°C. This procedure resulted in a 5mM NH_2Cl solution. Use of concentrated NH_2Cl solutions was completed within 6 hours of preparation. Over this time course we observed that NH_2Cl remained stable in Ringer solution at concentrations ranging from 50 to 200μM, with <10% loss of absorbance at 242nm. The concentrated solution was kept on ice and then diluted to the final concentration just before each experiment. Taurine monochloramine (TaurNHCl) was generated under similar conditions by including taurine instead of NH_4Cl in the reaction mixture (5,16,23). Concentrations were verified by measuring

absorbance in a UV spectrophotometer at 242, 292, and 252nm for NH_2Cl, HOCl, and TaurNHCl, respectively. [NH_2Cl], [HOCl], and [TaurNHCl] were then quantified with molar extinction coefficients reported previously (23).

Caspase-3 activity

Caspase-3 activity was measured using assay systems obtained from Sigma-Aldrich, which are based on the hydrolysis of the peptide substrate Ac-DEVD-pNA (acetyl-Asp-Glu-Val-Asp p-nitroanilide). This leads to release of p-nitroaniline, which can be measured by absorbance at 405nm. Suspensions of healthy glands (~800μl, which ultimately provides 3μg/ml to 5μg/ml protein by Bradford assay) were exposed to control and experimental Ringer's solutions for a total of 6 hours at 37°C, then lysed and subjected to brief centrifugation (16,100 × g for 5 minutes). Cytosol supernatants were assayed on 96 well plates per protocol (CASP3C, Sigma-Aldrich, St. Louis), with readout (Ex 485nm; Em 528nm) after 18 to 20 hours of incubation at 37°C with Ac-DEVD-pNA. In preliminary studies, we confirmed the stability of both Ac-DEVD-pNA alone and p-nitroaniline (after hydrolysis using purified caspase-3) in the presence of 200μM NH_2Cl (data not shown). As NH_2Cl appeared to interfere with the Bradford assay dose-dependently, results were normalized to control glands from the same aliquot as experimental groups.

Additional Reagents and Methods

TPEN (tetrakis-(2-pyridylmethyl)ethylene diamine) and BAPTA (tetramethyl1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra acetate) in its acetoxymethyl ester form were obtained from Molecular Probes (Eugene, OR). TPEN was dissolved directly in Ringer's, BAPTA was first dissolved in DMSO and then diluted 1:1000 in Ringer's solution. Dithiothreitol (DTT) and ascorbic acid (both from Sigma) were dissolved in Ringer's. All solutions were checked for changes in pH and adjusted if necessary to baseline pH. For Ringer's and intracellular buffer (ICB), calculations of free and bound concentrations of Ca^{2+}, Zn^{2+}, TPEN, BAPTA and EGTA were performed using the internet-based WEBMAXSTANDARD program (http://www.stanford.edu/~cpatton/webmaxc/webmaxcS.htm).

Data Summary and Statistical Analysis

Luminescence intensities were monitored at discrete time intervals and measurements were summarized as means ± SE. Unless stated otherwise, comparisons were performed using analysis of variance for sequential or multiple measurements using standard statistical software (Sigma Stat, Version 2.0, Jandel Corp.).

Results

Modulation of endogenous caspase-3 activity though exogenous Zn^{2+} loading

Caspase-3 activity was monitored using a colorimetric method that monitors release of p-nitroaniline from hydrolysis of Ac-DEVD-pNA, a specific substrate of caspase-3. In preliminary studies, we confirmed the stability of both Ac-DEVD-pNA alone and p-nitroaniline (after hydrolysis using purified caspase-3) in the presence of 200μM NH_2Cl (data not shown).

We then performed studies to determine whether endogenous caspase-3 activity in the gastric gland is susceptible to short-term increases in $[Zn^{2+}]_i$ at concentrations (2.5nM, 25nM, 250nM). These concentrationsspan the range (24,25) that is relevant to pathological release by oxidants such as NH_2Cl (16). Glands were incubated at 37°C with Ringer's solutions containing EGTA (0.5mM) and the Zn^{2+} ionophore Na-pyrithione (50μM). Ringer's solutions containing EGTA (0.3mM free) and [Ca^{2+}] (1.0mM free) were prepared with either no added Zn^{2+} or

sufficient Zn^{2+} to achieve free concentrations of 2.5nM, 25nM or 250nM. The highest dose of Zn^{2+} administered represents an approximately 10-fold excess of the highest level of Zn^{2+} we would anticipate to have been released to the cytoplasm of a gastric gland. After 3 hours, glands were processed for caspase-3 activity. As shown in Figure 1, increases in $[Zn^{2+}]_i$ led to dose-dependent decreases in activity, while exposure to TPEN (free concentration 10μM) elicited a modest, statistically insignificant activity increase in this experiment.

Inactivation of caspase-3 by NH2Cl

To evaluate effects of NH_2Cl on endogenous caspase-3 activity in glands, two protocols were used to determine responses to different doses and different intervals of exposure. In these studies, the goals were: first, to determine responses to short-term, but continuous exposures to NH_2Cl; and, second, to determine whether responses were influenced by an equilibration in NH_2Cl-free solution after exposure to NH_2Cl. The first set of conditions might be considered as an *in vitro* test of acute toxicity of the oxidant, while the second set of conditions was intended to simulate disease in which oxidants reach a steady state.

In the first protocol, glands were exposed to NH_2Cl at different concentrations (50μM, 100μM, or 200μM) for 3 hours and were then processed immediately for caspase-3 activity. In the second protocol, we measured generation of caspase-3 activity after isolated glands were incubated at different concentrations for varying intervals (1hr, 2hr, 3hr) and then allowed to equilibrate in Ringer's (5hr, 4hr, 3hr for each group respectively) to achieve a total experiment time of 6 hours. A maximum exposure to NH_2Cl of 3 hours was chosen because exposure of glands to NH2Cl for longer time periods leads to complete destruction of the cells (data not shown.) In both protocols, concentration-dependent decreases in caspase-3 activity were observed, with severe inactivation during exposure to 200μM NH_2Cl. (Figure 2) The findings of the first protocol (Figure 2A) indicate that the effects of NH_2Cl are immediate. Those of the second protocol (Figure 2B) indicate that these effects are not reversed simply with removal of the oxidant, and that prolonged exposure beyond 1 hour increases inactivation at low and intermediate concentrations of NH_2Cl.

Modulation of NH_2Cl-induced inactivation of caspase-3 by the concurrent release of Zn^{2+} into the cytoplasm

We next sought to determine whether NH_2Cl-induced alterations in caspase-3 activity are influenced by the concurrent accumulation of Zn^{2+} in the cytoplasm. In these studies, the strategy was to expose glands to NH_2Cl, either alone or in the presence of a heavy metal chelator, TPEN (20μM). First, we evaluated the effects of TPEN on acute administration of NH_2Cl. In these studies, TPEN was present from the beginning of the 1 hour exposure to NH_2Cl that was followed by immediate processing of glands for caspase-3 activity. As shown in Figure 3A, in the absence of NH_2Cl, exposure to TPEN alone did not alter baseline caspase-3 activity. However, TPEN enhanced caspase-3 activity during exposure to NH_2Cl at all concentrations tested, suggesting that endogenous release of free Zn^{2+} contributes to the inactivation of caspase-3.

We then evaluated the effects of TPEN on the steady state model of NH_2Cl exposure. In these studies, glands were exposed to escalating concentrations of NH_2Cl for 1 hour and subsequently equilibrated for 5 hours in Ringer's, with or without 20μM TPEN, which was present from the start of the experiment. As shown in Figure 3B, caspase-3 activity was preserved when glands were exposed to NH_2Cl and TPEN concurrently, at low (50μM) and intermediate (100μM) concentrations of NH_2Cl. In control studies (not shown), we added 20μM TPEN directly to purified caspase-3, at levels observed in our experiments (dilution of pure enzyme 1:500 to 1:1000). There was no change in activity (data not shown), confirming

that the observed effect is not attributable simply to chemical (i.e., non-biological) enhancement of caspase-3 activity by TPEN.

Finally we tested whether a reversal of caspase-3 inactivation is observed if TPEN is administered following the exposure to NH_2Cl (100μM) rather than concurrently. In these experiments, one group of glands was exposed to Ringer's for 1 hour while another group was exposed to Ringer's plus 100μM NH_2Cl for 1 hour; both groups were then equilibrated with Ringer's for 5 hours. We then compared caspase-3 activity generated in wells that had TPEN present from the beginning of the experiment and in wells in which TPEN was added 1 hour later. As summarized in Figure 4, delayed administration of TPEN achieved a response similar to that when TPEN was present concurrently. Additional experiments showed that the effects of monocloramine are almost immediate, with cell viability decreasing as early as 30 minutes after monochloramine exposure (data not shown.) These findings indicate that Zn^{2+} released to the cytoplasm by NH_2Cl continues to elicit its inhibitory effect on caspase-3 activity, well past the period of exposure to the oxidant.

DTT preserves caspase-3 activity during exposure to thiol oxidant NH_2Cl

We next performed studies to confirm that the effects of NH_2Cl are due to its targeting of thiol groups, by determining whether a thiol-specific reducing agent, dithiothreitol (DTT) would preserve endogenous caspase-3 activity during exposure of glands. Glands were exposed for 2 hours to different concentrations of NH_2Cl, in the presence or absence of 1mM DTT. A comparison group of glands was incubated with NH_2Cl in the presence of 20μM TPEN. As shown in Figure 5, dose-dependent inactivation of caspase-3 activity were observed in response to NH_2Cl. In the presence of the metal chelator, TPEN, this activity was partially restored. However, in the presence of DTT, caspase-3 activity was fully preserved, indicating that thiol oxidation is a critical determinant of the effects of NH_2Cl.

Discussion

Our studies indicate that exposure of the gastric gland to pathologically relevant concentrations of NH_2Cl leads to inactivation of caspase-3, a cysteine-rich intracellular protein (10,11) that play a key role in cell death. This inactivation is greatly dependent on the concentration of the oxidant and, to a much lesser extent, on the duration of exposure. These effects are completely prevented by concurrent exposure to the thiol-reducing agent DTT. This observation supports the hypothesis that the effects of NH_2Cl are due to its targeting of thiol residues in cysteine-rich structures (6,7,26).

In addition, we find that controlled increases in $[Zn^{2+}]_I$, within the limits of known physiologic conentration, dose-dependently inhibit activation and activity of caspase-3, a key step in programmed cell death (13-15). Moreover, our studies indicate that application of the heavy metal chelator TPEN unmasks a component of endogenous caspase-3 activity that is otherwise be suppressed by endogenous Zn^{2+}. Thus TPEN maintains or even elevates Caspase-3 activity, even in the presence of NH_2Cl-induced Zn^{2+} release. For caspase-3, the apparent binding constant for Zn^{2+} has been reported in the range of 0.1μM to 10μM, although in ultra-pure systems 50% inhibition at concentrations as low as 1.7nM has been reported (12). In addition, it has been proposed that the activation of caspase-3 from procaspase-3 occurs in response to $[Zn^{2+}]_i$ in the range of 1 to 10nM (14). Thus, it is plausible that NH_2Cl-induced increases in $[Zn^{2+}]_i$ above this level are sufficient to inhibit endogenous activity of caspase-3 and to modulate other intracellular proteins that depend on Zn^{2+} for proper function or structure. The continued activity of Caspase-3 even at very high doses of Zn^{2+}, however, suggests that Zn is not the sole modulator of Caspase-3 activity. Our studies provide novel evidence for this kind of modulation, *in situ*, in a primary epithelial structure such as the gastric gland.

These findings raise three additional questions for discussion. The first is technical and involves the quantitation of caspase-3 activity and whether it simply is proportional to viability of glands. In this study, valid comparison of caspase-3 activity between groups depends on the equivalence of the mass of viable glands allotted to each well. We controlled for equivalence of loading by monitoring cell protein levels (Bradford assay). In addition, the comparison between groups exposed to different concentrations of NH_2Cl might depend on overall viability of the glands. Since NH_2Cl is a known oxidant and can cause cell death at concentrations >200μM (4,5,27), it is possible that NH_2Cl-induced decreases in caspase-3 activity, particularly at our highest concentration, are due to cell death as well as the postulated inactivation of the enzyme.

The major goal of this study was to evaluate the hypothesis that oxidant-induced accumulation of Zn^{2+} modulates the activity of caspase-3 within a cell exposed to pathologically relevant concentrations of NH_2Cl for time periods sufficient to cause cell injury without immediate cellular destruction (Figure 2). Our studies show that, for each level of exposure to NH_2Cl (50μM, 100μM, 200μM) an unmeasured component of caspase-3 inhibition was unmasked by chelation of Zn^{2+} by the high affinity chelator TPEN (Figure 3). Moreover, we observed this unmasking of caspase-3 inhibition even when glands were exposed to the chelator after the oxidant had been removed from the system (Figure 4). These findings suggest that, within each concentration of NH_2Cl, the effects of TPEN cannot be attributed solely to effects on gland viability.

The second question is also technical and involves whether the effects of TPEN might be due not to the Zn^{2+} released by NH_2Cl, but to chelation of heavy metal cations already bound to caspase-3 by the higher-affinity chelator. As shown in Figures 3 and 5, significant effects of TPEN were not observed in groups of glands that were not exposed to NH_2Cl, making it unlikely that the endogenous pool of caspase-3 was already “loaded” with Zn^{2+}.

The third question is the relationship between Zn^{2+}-induced suppression of caspase-3 activity and viability of the gastric gland. In other cell systems, it has been suggested that release of Zn^{2+} by reactive nitrogen species, such as nitric oxide, may be anti-apoptotic and therefore protective (13-15,28). In our experimental system, the reactive nitrogen species, NH_2Cl, is likely to be toxic to the cell. In addition, Zn^{2+} accumulation in response to oxidant stress may be protective if brief and controlled or toxic if high and sustained. Thus, low-level, oxidant-induced increases in intracellular Zn^{2+} may have an overall protective effect when necrotic cell death is rare and apoptosis is the dominant pathway for cell demise. When oxidant stress is high, however, the concurrent Zn^{2+}-induced suppression of caspase-3 activity may accelerate chaotic cell death (necrosis) by suppressing pathways that lead the gland to a more orderly and controlled demise (apoptosis). Further studies are underway to clarify the distinction between these two types of cell death in a complex epithelial unit such as the gastric gland, and to determine how oxidant-induced release of Zn^{2+} may influence the balance between apoptosis and necrosis in the setting of acute mucosal inflammation.

In summary, our results suggest that caspase-3, a zinc-depdendent protein present in the cytoplasm of rabbit gastric glands, is inhibited by the rise in intracellular Zn^{2+} associated with thiol oxidative stress. This effect can be prevented by treatment with DTT, a thiol oxidant, and can be both arrested and reversed using the selective zinc chelator TPEN. Though the role of Zn^{2+} as a pro-apoptotic signal has not been established, these experiments serve as a reminder of the many essential physiologic roles played by this cation.

Acknowledgments

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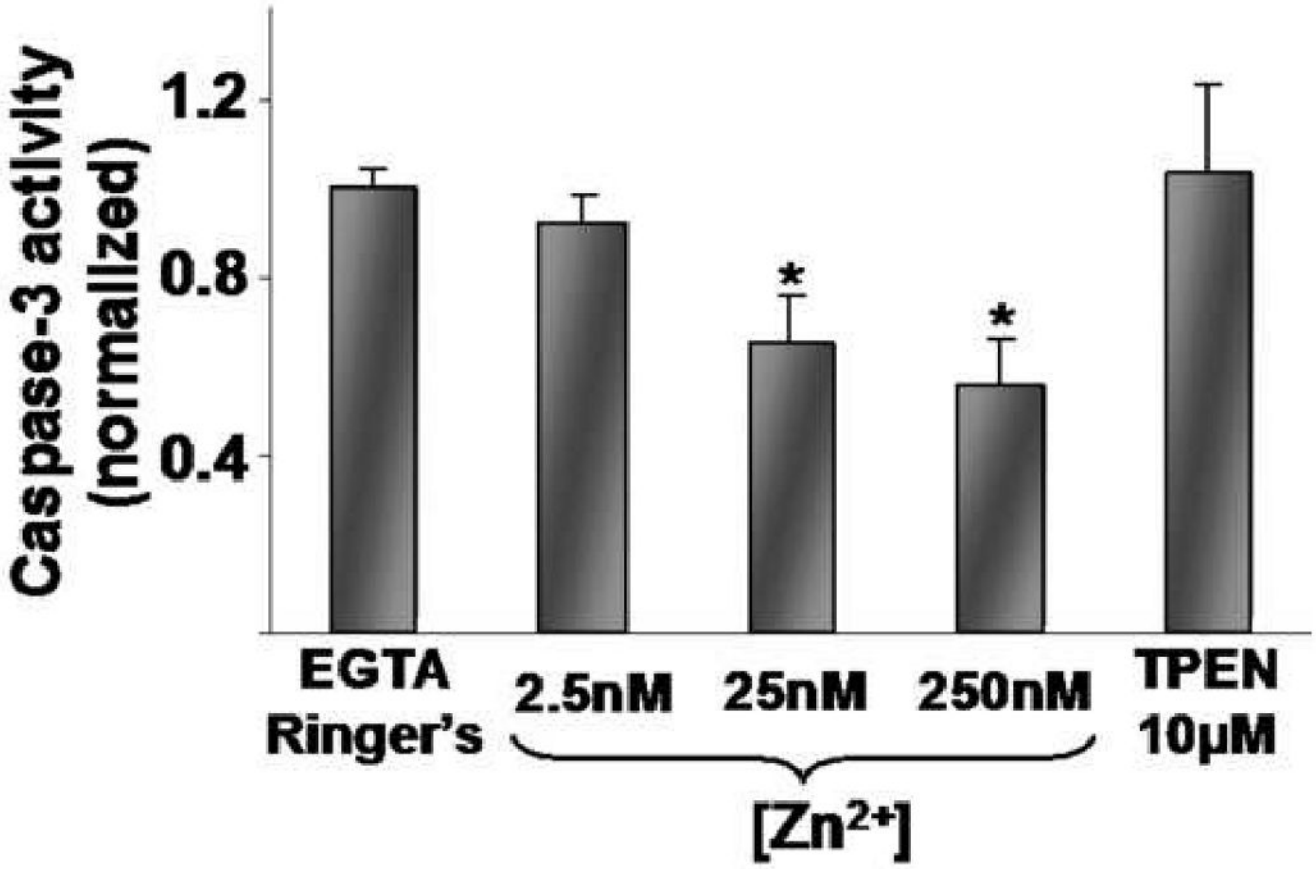

Figure 1.

Caspase-3 activity in isolated gastric glands in response to $[Zn^{2+}]$ in the presence of pyrithione (50μM) at increasing doses (2.5nM, 25nM, 250nM) for 3 hrs. Results are normalized to Ca^{2+} and Zn^{2+} depleted Ringer's controls (EGTA 0.5mM, pyrithione 50μM; average value 1.0) run concurrently and expressed as means ± SE, n=5 wells, *$p<0.05$ compared to Ringer's alone (ANOVA).

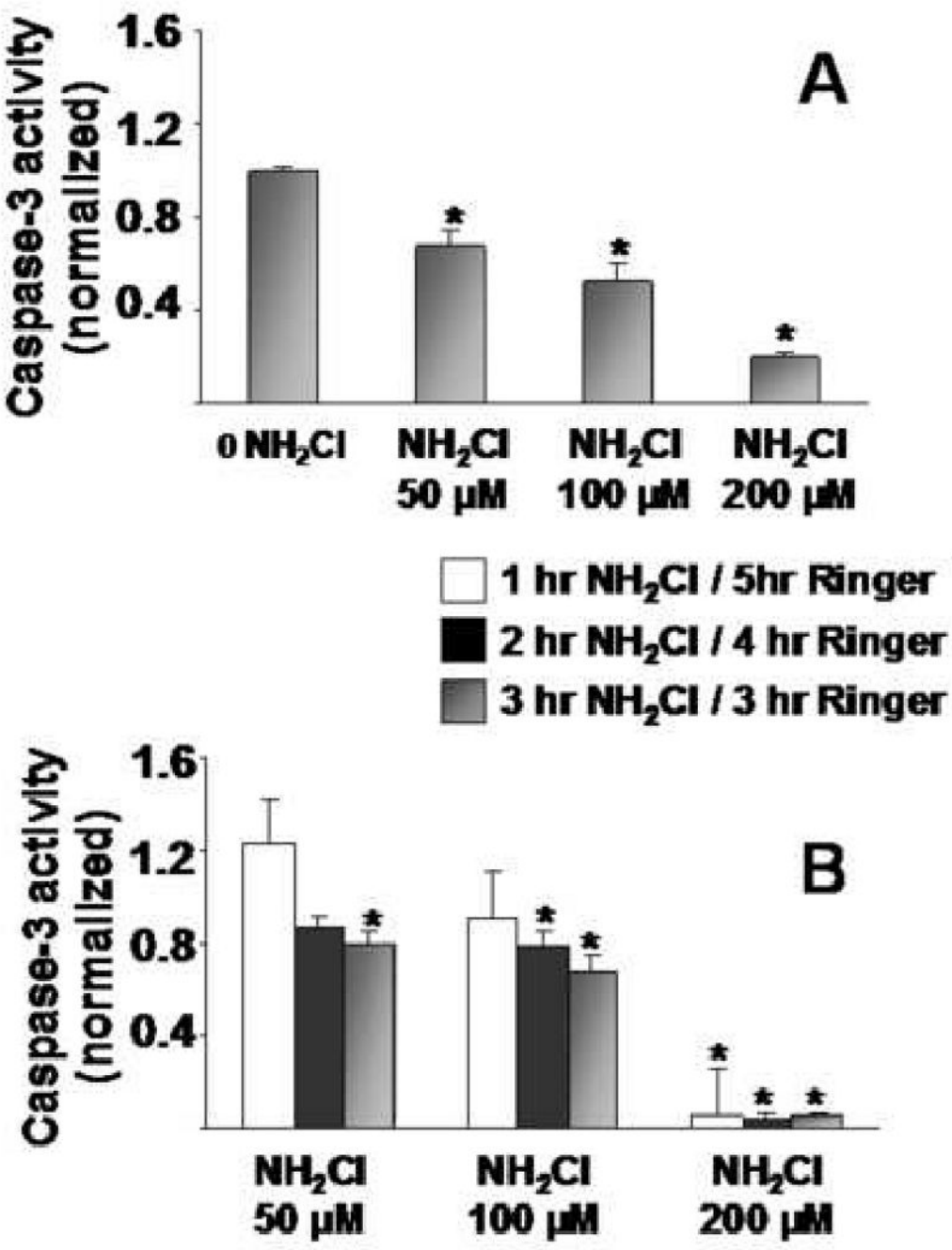

Figure 2.

Caspase-3 activity in Isolated gastric glands in response to monochloramine at increasing doses (50 μM, 100 μM, 200 μM). ***Panel 2A:*** Exposure of glands to different concentrations of NH_2Cl for 2 hr, with immediate processing for caspase-3 activity. ***Panel 2B:*** Exposure of glands to NH_2CI at different doses for different periods of time (1hr, 2hr, 3 hr), after which glands were equilibrated with Ringer's for 5hr, 4hr, or 3hr, respectively, before processing. Each column represents 10 to 12 wells of glands from 3 to 4 separate preparations. Results are normalized to protein content (Bradford assay) and then to Ringer's controls (first white column in each group = 1.00) and expressed as means ± SE, *$p<0.05$ compared to Ringer's alone (ANOVA).

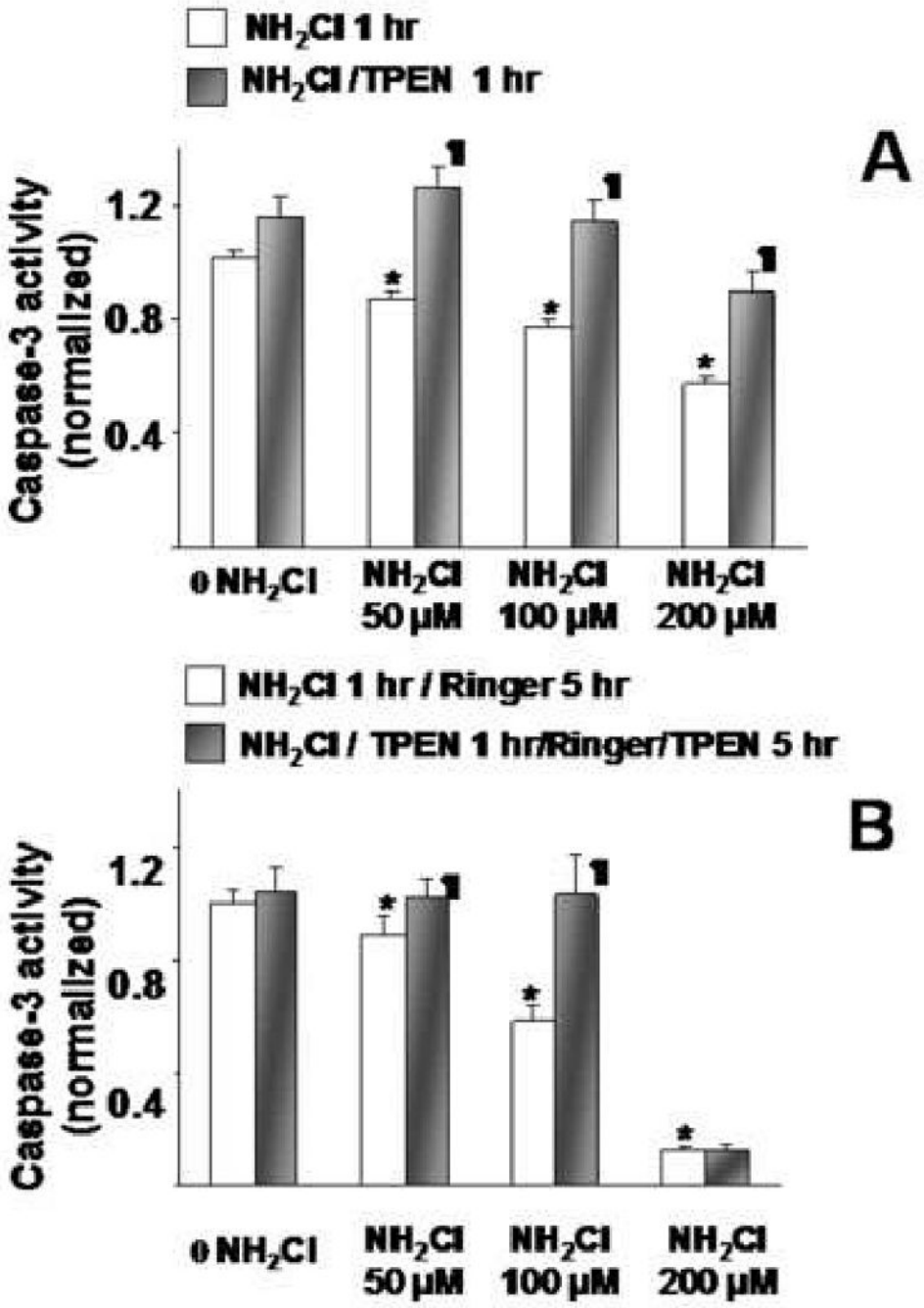

Figure 3.

Caspase-3 activities in glands exposed to NH_2Cl at different concentrations, in the presence or absence of TPEN 20µM. ***Panel 3A:*** Glands were exposed to NH_2Cl alone (white bars) or TPEN (shaded bars) for 1 hr. ***Panel 3B:*** Glands were exposed to NH_2Cl for 1 hr, followed by equilibration in Ringer's, alone (white bars) or in the presence of TPEN (shaded bars). Each column represents 5-6 wells of glands from 3 separate preparations. Results are normalized to protein content (Bradford assay) and then to Ringer's controls (first white column in each group = 1.00) and expressed as means ± SE, *$p<0.05$ compared to Ringer's alone (ANOVA), ¶ indicates $p<0.05$ compared to effects without TPEN at the corresponding concentration.

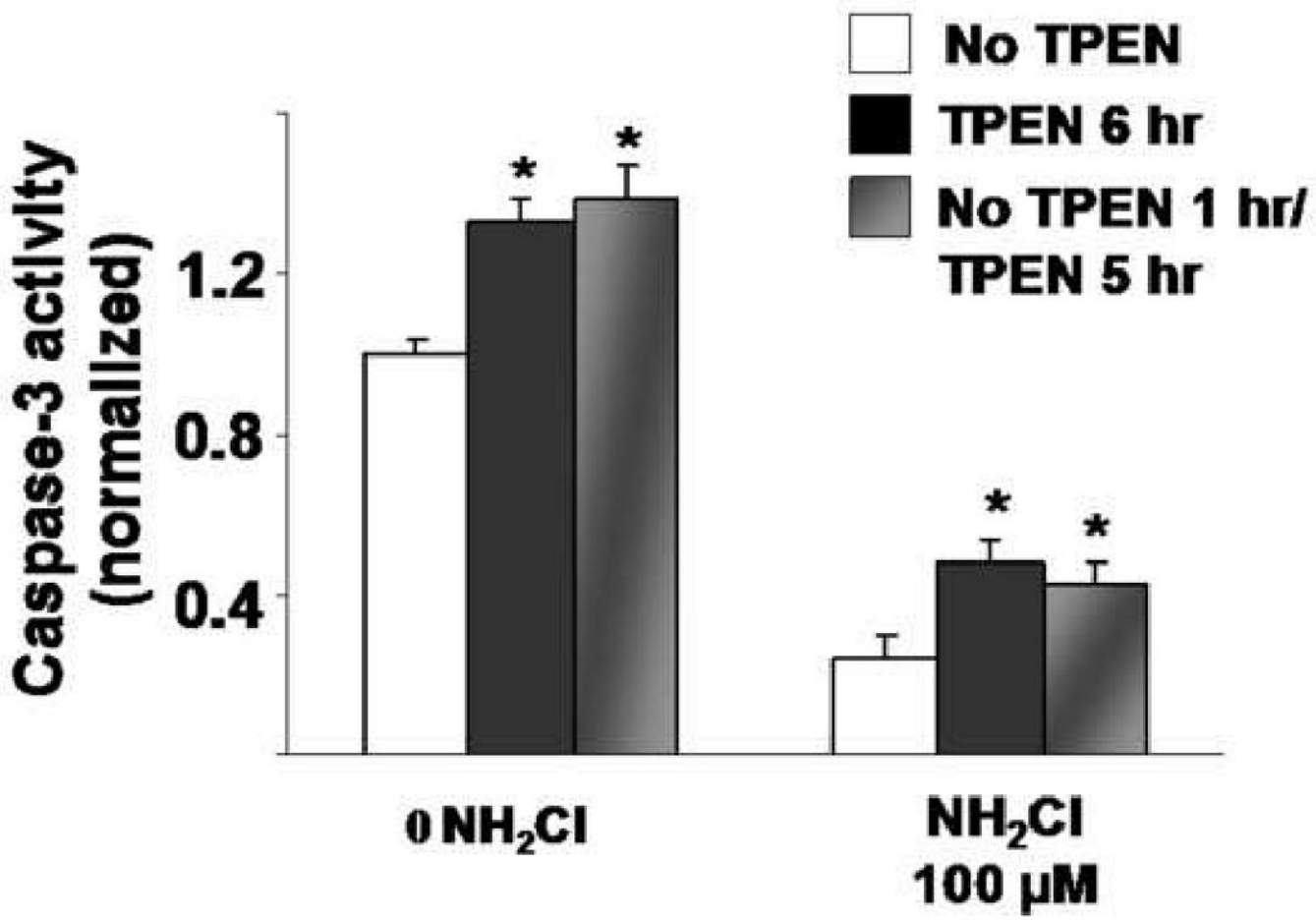

Figure 4.

Effects of delayed TPEN treatment after exposure to NH_2CI. Glands were perfused with Ringer's alone or Ringer's plus NH_2CI (100μM) for 1 1hr. Glands were perfused under similar conditions with TPEN present for the entire 6 hr incubation, or only for the 5 hr following exposure to NH_2CI. Each column represents 15 wells of glands from 3 separate preparations. Results are normalized to protein content (Bradford assay) and then to Ringer's controls (first white column in each group = 1.00) and expressed as means ± SE, *$p<0.05$ compared to Ringer's alone (ANOVA).

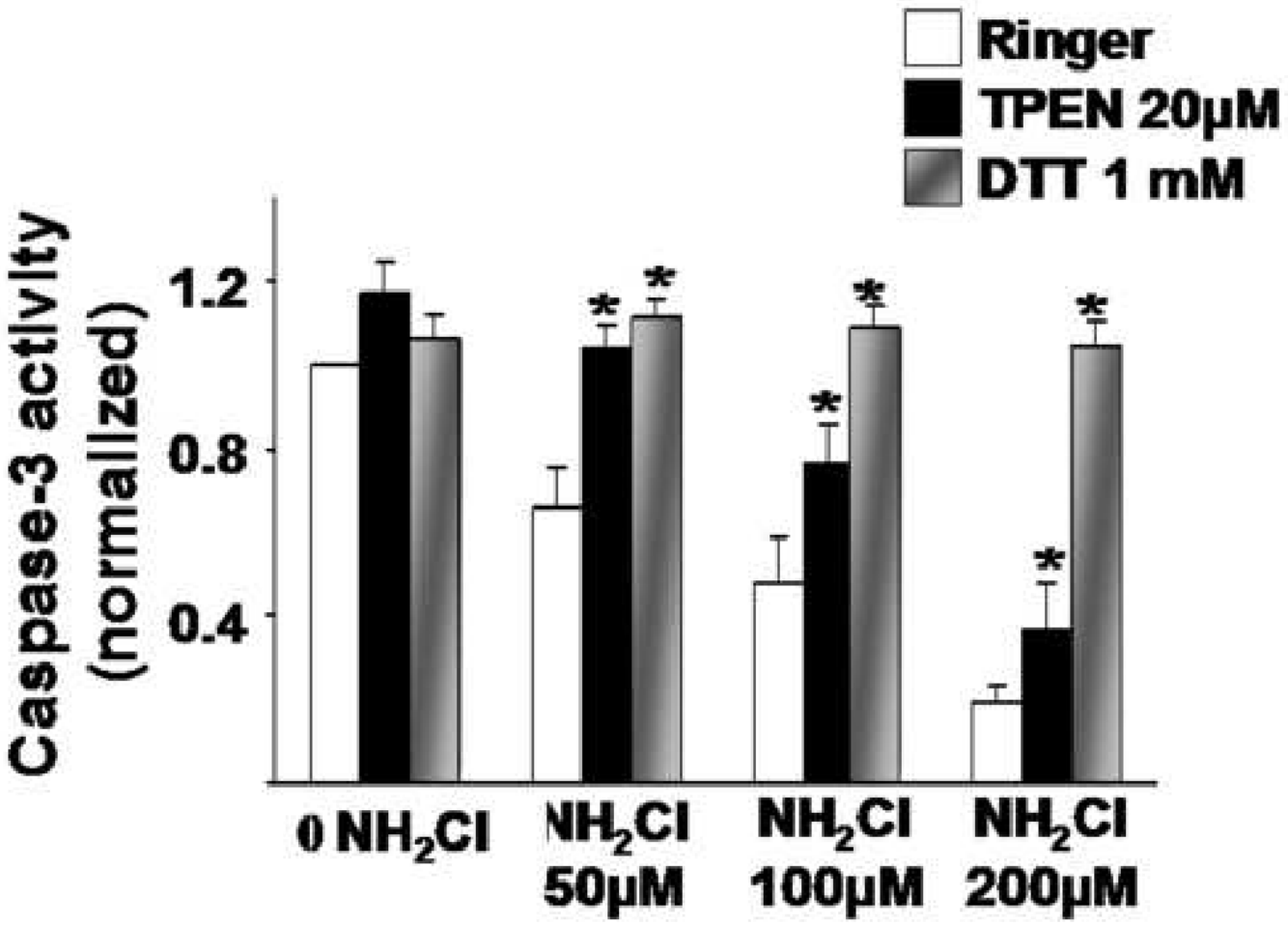

Figure 5.

Comparison of effects of TPEN and DTT on inactivation of caspase-3 activity by NH_2CI. Glands were incubated for 2 hours at different concentrations of NH_2CI, alone or in the presence of the metal chelator TPEN or thiol reducing agent diothiothreitol (DTT). Results are normalized to protein content (Bradford assay) and then to Ringer's controls (first white column in each group = 1.00) and expressed as means ± SE, *$p<0.05$ compared to Ringer's or Ringer's plus NH_2Cl (ANOVA).