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Antioxidant pre-treatment prevents omeprazole-induced toxicity in an *in vitro* model of infectious gastritis

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Abstract

Omeprazole is a mainstay of therapy for gastroesophageal reflux disease (GERD) and gastritis, and is increasingly used as an over-the-counter remedy for dyspepsia. Omeprazole acts by selectively oxidizing thiol targets in the gastric proton pump, but it also appears to be toxic to the gastric mucosa. We hypothesized that omeprazole toxicity is due to non-specific oxidation of cell structures other than the proton pump, and tested the efficacy of antioxidants to prevent omeprazole-induced toxicity in isolated rabbit gastric glands. Toxicity was measured by uptake and conversion of calcein-AM, following three hours of exposure to omeprazole and a non-selective thiol-oxidant, monochloramine. Intracellular concentration of Zn^{2+} and the capacity to maintain luminal acidity were monitored using the fluorescent reporters fluozin-3 and Lysosensor DND-160, respectively. Both omeprazole and monochloramine caused marked reduction in cell viability. The toxicity of omeprazole was independent of monochloramine toxicity. The thiol reducing agent dithiothreitol protected gastric glands from injury. The oxidant scavenger Vitamin C also protected, and did not impair the anti-secretory effects of omeprazole. Thus, omeprazole toxicity appears to be oxidative and preventable with antioxidant therapy, including Vitamin C. Vitamin C may be a safe and efficacious addition to treatments requiring the use of PPIs.

Keywords

omeprazole; gastritis; Vitamin C; *Helicobacter pylori*; gastric mucosa; antioxidants

INTRODUCTION

Omeprazole is a prototypical proton pump inhibitor (PPI), which covalently binds and inhibits the H^+/K^+ ATPase necessary for acidification of the tubulovesicle of the parietal cell. This prevents acid production and raises the pH of the stomach lumen, which has been shown to be an important component of therapy for the treatment of gastritis. Gastritis, defined as inflammation of the stomach mucosa, is a common clinical condition associated with local infection of the gastric mucosa. The predominant organism associated with gastritis is the urealytic bacterium *Helicobacter pylori*. Chronic gastritis has been estimated

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to be present in up to 37% of asymptomatic adults. Incidence increases with age and is almost always associated with *H. pylori* infection [1]. Omeprazole, in combination with antibiotics, effectively eradicates *H. pylori* infection. However, evidence suggests that omeprazole treatment may itself cause acute and chronic injury to the cells of the gastric gland. In experimental studies in rabbits, it has been shown that omeprazole leads to acute depletion of parietal cells, increased parietal cell turnover, and accumulation of chronic inflammatory cells [2]. This effect has been attributed to CCK-2 mediated proinflammatory effects of compensatory hypergastrinemia in response to decreased acid production [3]. In human studies, use of omeprazole alone as a treatment for *H. pylori* gastritis has also been demonstrated to increase mucosal inflammation. Prolonged inflammation, in turn, leads to destruction of the gastric glands and persistent hypergastrinemia, a condition known as atrophic gastritis. Atrophic gastritis resulting from omeprazole monotherapy in the setting of chronic *H. pylori* infection has been associated with increased risk of mucosal dysplasia and gastric cancers [4–6]. To our knowledge, direct toxicity of omeprazole to the cells of the gastric gland has not been previously reported.

In the setting of *H. pylori* gastritis, polymorphonuclear cells (PMNs) and macrophages are recruited to the gastric mucosa, where their antibacterial action includes the production and secretion of hypochlorous acid (HOCl). HOCl in turn can react with ammonia (NH₃) produced by the metabolism of urea in *H. pylori* to form the potent thiol oxidant monochloramine (NH₂Cl). A number of reports have suggested that NH₂Cl can act as a significant agent of injury in gastric mucosa [7–10]. In addition, recent work in our laboratory has demonstrated that thiol oxidation with NH₂Cl results in cell death associated with disruption of Ca²⁺ and Zn²⁺ homeostasis in the rabbit gastric gland [11,12].

The mechanism of action of omeprazole also depends on thiol oxidation. Within the acidic environment of the parietal cell, omeprazole is converted into a thiophilic sulfenamide, which binds to a cysteine residue of the H⁺/K⁺ ATPase and oxidizes a thiol bond to render the pump inoperative. While this effect is generally believed to be specific to thiol groups of the H⁺/K⁺ ATPase, recent studies have suggested targets for oxidation that are apart from the gastric proton pump [13–16]. These considerations suggest that, in the context of oxidative stress induced by gastritis, omeprazole may amplify injury to the gastric mucosa rather than accelerating healing.

In the studies reported here, we use a recently described *in vitro* assay [11] to evaluate the toxicity of a prototypical PPI, omeprazole, on gastric secretory glands. We demonstrate that omeprazole's effects on the viability of the cells of the gastric gland may be through a previously underappreciated role as a thiol oxidant against multiple cellular targets. Our findings suggest that exposure to omeprazole amplifies cellular injury caused by NH₂Cl, but that toxicity of both omeprazole and NH₂Cl can be abrogated using concurrent antioxidant therapy.

METHODS

Reagents and Solutions

Unless otherwise specified, reagents were obtained from Sigma (St. Louis, MO). Ringer's Solution: NaCl 145mM, KH₂PO₄ 2.5mM, MgSO₄ 1.0mM, CaCl₂ 1mM, HEPES 10mM, glucose 10mM, pH 7.4. Zinc and calcium-free Ringer's solution: NaCl 145mM, KH₂PO₄ 2.5mM, MgSO₄ 1.0mM, HEPES 10mM, glucose 10mM, 0.5 mM EGTA, pH 7.4. Omeprazole, TPEN (tetrakis-(2-pyridylmethyl) ethylenediamine), dithiothreitol (DTT) and ascorbic acid (VitC) were dissolved in Ringer's directly. BAPTA-AM and FluoZin-3 AM (Invitrogen) were dissolved in DMSO. All solutions were checked for changes in pH and adjusted if necessary to baseline pH. Calculations of free and bound concentrations of Ca²⁺,

Zn²⁺, TPEN, BAPTA and EGTA were performed using the internet-based WEBMAXSTANDARD program (<http://www.stanford.edu/~cpatton/webmaxc/webmaxcS.htm>).

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 [12,17]. Enrichment for parietal cells in gastric glands was previously confirmed [11] by immunofluorescent imaging using a mouse monoclonal antibody against the H⁺/K⁺-ATPase (ABCAM, no. ab2866) probed using a secondary donkey anti-mouse fluorescent antibody (Invitrogen, no. 10037). Other cell types present in the gastric mucosa are present in low numbers, with parietal cells accounting for a high percentage of the gland volume [18,19]

Effects of omeprazole, monochloramine, intracellular zinc chelators, and antioxidants on cell viability

Glands were suspended in Ringer's solutions with or without omeprazole (100μM) and monochloramine (0, 50, 100, and 200μM). After 2.5 hours of incubation at 37°C in sealed Eppendorf tubes, cell viability was assessed by monitoring uptake and intracellular conversion of non-fluorescent calcein-acetoxymethyl ester (calcein-AM) to fluorescent calcein by intracellular esterases, using a modification of previously reported methods [20,21]. Calcein-AM was added to each tube (final concentration 8μM) for thirty minutes, after which glands were washed twice in Ringer's solution and transferred to a 96-well plate for measurement of calcein fluorescence (Ex 485nm; Em 528nm; Syngery 2, BioTek Inc, Winooski, VT). To determine the effect of intracellular zinc on cell viability in the presence of monochloramine and omeprazole, the above conditions were recapitulated in the presence of the Zn²⁺ chelators BAPTA and TPEN. BAPTA is delivered across cell membranes in an acetoxymethyl ester form as BAPTA-AM, and is converted in the cytoplasm to BAPTA when, like calcein, the acetoxymethyl ester group is cleaved by intracellular esterases. For experiments including BAPTA-AM, glands were allowed to incubate with BAPTA-AM (10μM) for 45 minutes at room temperature before addition of omeprazole or monochloramine. For experiments including TPEN, 20μM was added simultaneously with omeprazole and monochloramine. To study the effects of antioxidants, glands were co-incubated with omeprazole, monochloramine and Vitamin C (1mM) or DTT (1mM).

Determination of intracellular Zn²⁺

Fluozin-3 acetoxymethyl ester (Fluozin-3 AM) was dissolved in DMSO then loaded (30 minutes at room temperature) into gastric glands suspended in DMEM containing 100μM cimetidine, pH 7.4, using dye concentrations between 4μM and 8μM. Subsequently, glands were rinsed several times in dye-free DMEM, mounted on glass coverslips, and transferred to the microscope stage, where they were superfused with Ringer's solution at room temperature. Fluorescence was monitored concurrently in 6 to 10 individual parietal cells in each isolated gland using a real-time fluorescence imaging microscope (Nikon TE-2000U, Nikon, Inc, Melville, NY). Glands were excited at 488nm with emission measurement at 520 nm ± 15 nm. Digital images of glands were captured using a digital CCD camera (Hamamatsu ORCA-ER, Bridgewater, NJ). Images were processed using image processing software (Universal Imaging Corp., Downingtown, PA) to yield quantitative measurements of fluorescence intensity. Images were acquired every 10 seconds, using the minimum exposure time possible, to minimize photobleaching.

Determination of acidity in the tubulovesicle/lumen compartments of isolated gastric glands

Glands were loaded with 4 μ M Lysosensor DND-160 (Invitrogen) in DMEM for 15 minutes at room temperature. Following loading, solutions were exchanged for Ringer's containing combinations of omeprazole (100 μ M), vitamin C (1 mM) and DTT (1mM). In order to ensure that adequate substrate was available for mitochondrial function and optimal acid production, Ringer's was supplemented with succinate (100 μ M) and pyruvate (100 μ M). Excitation ratiometric measurements were performed using excitation wavelengths of 340 and 400nm, with emission filtering at 528nm [17,22]. Results are presented as the ratio of relative fluorescence units (RFU): RFU_(340nm)/RFU_(400nm), with a higher ratio corresponding to a more alkaline pH [17].

Data Summary and Statistical Analysis

Fluorescence measurements were made at a single time point, and summarized as means $\pm$ SEM. For non-parametric comparisons of data normalized to control, Kruskal-Wallis one-way analysis of variance on ranks was performed, with multiple pairwise comparisons using Dunn's method. For raw RFU measurements or comparisons of ratiometric values, the Holm-Sidak test for multiple comparisons was used, after testing for normal distribution of the data. Statistics were performed using standard statistical software (Sigma Stat, Version 3.5, Systat Software Inc., Port Richmond, FL).

RESULTS

Omeprazole is toxic, and enhances NH₂Cl toxicity to gastric glands

We first tested the effect of omeprazole on the viability of rabbit gastric glands, both independently and in the presence of monochloramine (NH₂Cl), which we have previously demonstrated to have toxic effects on gastric glands through a thiol oxidant mechanism [11]. For our assay of cell viability, we used the fluorescent dye calcein-AM. This dye is able to cross the cell membrane while associated with its acetoxymethyl (AM) group, but only becomes fluorescent after the -AM group is cleaved by functional intracellular esterases. In dead or dying cells, esterases are not present to perform this conversion, providing an effective loss-of-function assay for cell viability [23]. As shown in Figure 1, a 3-hour exposure to omeprazole (100 μ M) alone caused a 38% decrease in cell viability compared to control ($\pm$ 6.4%, $P < 0.01$). This dose of omeprazole was chosen to produce a maximal inhibition of the H⁺/K⁺ ATPase in this *in vitro* model [24–26]. When combined with monochloramine, omeprazole augmented its toxic effects at all but the highest dose used (200 μ M).

Omeprazole toxicity does not depend on dysregulation of intracellular Zn²⁺ levels

We have previously demonstrated that exposure to monochloramine leads to mobilization of intracellular Zn²⁺ stores, and that chelation of intracellular zinc improves cell viability when exposed to monochloramine [11]. In order to understand whether omeprazole also dysregulates intracellular Zn²⁺ homeostasis, we exposed gastric glands to omeprazole, using fluozin-3 to measure [Zn²⁺]_i in individual parietal cells of isolated gastric glands [11,22]. As shown in Figure 2, exposure to omeprazole did not itself lead to release of Zn²⁺ to the cytoplasm.

In order to further confirm the zinc-independent nature of omeprazole toxicity, we next pre-treated gastric glands with metal chelators before exposure to omeprazole. Shown in Figure 3 are the effects of the chelator BAPTA, which has affinity for Ca²⁺ (K_d~100nM) and for Zn²⁺ (~4nM) [27] levels released during exposure to NH₂Cl but above expected resting levels of either cation [11,12,22]. By itself, BAPTA is not membrane-permeable; it is taken

up as BAPTA-acetoxymethyl (AM) ester and activated by intracellular esterases. Glands were pretreated with low concentrations of BAPTAAM (10 μ M), to minimize over-loading of chelator in the cell [12,28,29]. When glands were exposed to omeprazole (100 μ M), in the presence or absence of NH₂Cl, gland viability was not improved by pre-incubation with BAPTA-AM.

Our previous work [11] suggests that a significant component of NH₂Cl-induced toxicity is related to the release of Zn²⁺. However, the results of studies summarized in Figure 3 suggest that if Zn²⁺ contributes to omeprazole-induced toxicity, it would be at concentrations below the range chelated by BAPTA. We therefore tested the effects of high-affinity zinc chelation, effectively eliminating free intracellular Zn²⁺, using the zinc chelator TPEN (tetrakis-(2-pyridylmethyl) ethylenediamine), which is membrane permeable and has a K_d for zinc in the femtomolar (10⁻¹⁴M) range [30]. Figure 4 shows that concurrent treatment of glands with 20 μ M TPEN prevented NH₂Cl toxicity, while providing no protection against omeprazole toxicity. Interestingly, the degree of toxicity displayed by omeprazole continued to increase with increasing doses of NH₂Cl, even when the effects of monochloramine alone were abrogated by TPEN pre-treatment. The findings summarized in Figures 3 and 4 confirm that while oxidant-induced accumulations of intracellular Ca²⁺ and Zn²⁺ may influence viability of gastric glands exposed to NH₂Cl, they do not contribute directly to the toxicity of omeprazole.

Effect of thiol reduction on omeprazole toxicity

To determine whether omeprazole exerts its toxic effects through a thiol oxidative pathway, we treated gastric glands with the selective thiol reducing agent dithiothreitol (DTT) concurrent with exposure to NH₂Cl and omeprazole. As shown in Figure 5, preventing thiol oxidation with DTT completely protects glands from both omeprazole and NH₂Cl toxicity. Results of these studies suggest that the cellular toxicity associated with both omeprazole and NH₂Cl depends on thiol oxidation.

Effect of Vitamin C on toxicity and therapeutic function of omeprazole

We next tested the effect of a commonly consumed nutritional supplement and known oxidant scavenger, ascorbic acid (Vitamin C), on omeprazole-induced toxicity. As shown in Figure 6, concurrent treatment with Vitamin C (1 mM) preserves gland viability in the presence of omeprazole, alone and in the presence of all tested concentrations of NH₂Cl. These findings suggest that a non-thiol anti-oxidant may antagonize the toxic effects of omeprazole, both in isolation and in a cellular level model of gastritis-induced oxidative stress.

Because omeprazole depends on the thiol oxidation of the H⁺/K⁺ ATPase to exert its anti-secretory effects, the use of Vitamin C could potentially act as an inhibitor not just of the toxic effects of omeprazole, but of its desirable therapeutic effects. We therefore tested the effect of Vitamin C on the baseline acidity of the tubulovesicle/lumen compartment and its effect on the ability of omeprazole to inhibit the proton pump. Summarized in Figure 7 are fluorescence measurements of ratiometric excitation for the acid-responsive fluorophore Lysosensor DND-160 (340nm/400nm) [22]. Vitamin C alone does not alter pH of the tubulovesicle lumen compartment. As reported previously [17,22], inhibition of the proton pump by omeprazole led to alkalization of the tubulovesicle/lumen compartment. As shown in the figure, concentrations of Vitamin C (1mM) that were protective against the toxic effects of omeprazole did not influence its ability to block acid secretion.

DISCUSSION

Omeprazole is the prototypical proton pump inhibitor, available over-the-counter and in inexpensive generic formulations. It is promoted as a therapy for a range of disease states, from mild heartburn to aggressive *H. pylori* gastritis, where it forms one component of the triple-agent therapy (clarithromycin, amoxicillin, and omeprazole) that is commonly used to eradicate *H. pylori* infection [6]. However, it is increasingly well-recognized that omeprazole may also contribute to gastric gland toxicity. In this report we use a recently described gland viability assay to show that omeprazole is toxic to cells of the rabbit gastric gland at physiologically relevant doses (Figure 1). We also demonstrate that this toxic effect occurs without the same disturbances to Zn²⁺ homeostasis that we have previously observed from a non-specific thiol oxidant, monochloramine (Figure 2) [11].

Like monochloramine, the toxic effects of omeprazole can be reversed using both a thiol reducer (DTT) and a generic antioxidant (Vitamin C) (Figures 5 & 6), suggesting that omeprazole's effect on cell viability is through its action as a thiol oxidant. However, while chelating zinc with TPEN protects the gastric gland from monochloramine, chelation does not prevent injury by omeprazole (Figure 4). These data suggest that the two agents, while both acting as thiol oxidants, operate through different pathways to exert their toxic effects – monochloramine through a mechanism at least in part dependent on zinc-mediated toxicity, and omeprazole through a zinc-independent pathway.

The utility of Vitamin C to abrogate the toxicity of omeprazole may have clinical importance. Clinical studies have demonstrated that *H. pylori* eradication is improved when supplemented with Vitamin C [31] and that Vitamin C may decrease the total dose of antibiotic necessary for eradication [32,33]. In addition, Vitamin C therapy has been shown to reduce histological and serological markers of inflammation in patients taking omeprazole as a monotherapy for *H. pylori* gastritis [34]. Because omeprazole is now available over-the-counter for treatment of dyspepsia, such potentially inappropriate monotherapy is increasingly common [35]. To our knowledge, this is the first report to identify a potential mechanism by which Vitamin C may provide benefit when co-administered with omeprazole in *H. pylori* gastritis, by protecting the cells of the gastric gland against the toxic effects of both monochloramine and omeprazole. Because our studies suggest that pre-treatment with Vitamin C does not significantly impair the antisecretory potency of omeprazole, it may be a valuable adjunct to existing triple therapy for *H. pylori* disease, or to monotherapy with omeprazole for non-infectious conditions.

In addition to potential direct effects on the cells of the gastric gland by Vitamin C, omeprazole users may benefit from Vitamin C supplementation for more indirect reasons. Inhibition of the proton pump has been shown to decrease active secretion of Vitamin C into the lumen of the stomach [36] and to decrease plasma Vitamin C [37]. Decreased availability of Vitamin C may have multiple deleterious effects. Luminal Vitamin C, in its active, ascorbic acid form, scavenges nitrites, converting them to nitric oxide and preventing their transformation into potentially mutagenic N-nitroso compounds [38,39]. Vitamin C has also been demonstrated to increase absorption of non-heme iron [40]. At higher pH, such as in the stomach of patients taking PPIs, ascorbic acid is oxidized to inactive dehydroascorbic acid, meaning that decreased Vitamin C in the gastric lumen of patient using PPIs is further impaired by the elevated pH of the gastric lumen [37,41]. Oxidation of ascorbic acid has also been shown in *H. pylori* infection, particularly in atrophic gastritis [42–45]. This effect may be due to inflammatory mediators and to local pH elevation from ammonia produced by the bacterium [36].

Taken together, these data suggest that omeprazole acts through a previously unappreciated thiol oxidation pathway to cause a decrease in cell viability of the rabbit gastric gland *in vitro*. A large number of potential targets for thiol oxidation exist within the cells of the gastric gland, as a wide range of intracellular proteins are susceptible to thiol oxidation. However, these data suggest that differential thiol oxidation occurs between monochloramine (which appears to act through a zinc-dependent pathway) and omeprazole (which does not depend on zinc to exert its toxic effect). That both monochloramine and omeprazole toxicity can be reversed with Vitamin C provides a mechanistic explanation for previously observed benefits to Vitamin C co-administration in *H. pylori* gastritis treated with omeprazole. Further elucidation of the mechanism of action and the benefits of ascorbic acid in treating and preventing gastritis may improve patient outcomes in the future.

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LIST OF ABBREVIATIONS

BAPTA	1,2-bis(0-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
DTT	dithiothreitol
HOCl	hypochlorous acid
GERD	gastro-esophageal reflux disease
NH ₃	ammonia
NH ₂ Cl	monochloramine
PMN	polymorphonuclear cell
PPI	Proton Pump Inhibitor
TPEN	tetrakis-(2-pyridylmethyl) ethylenediamine
VitC	Vitamin C/Ascorbic Acid

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Figure 1. Toxicity of omeprazole and monochloramine (NH₂Cl) as measured by calcein-AM uptake/conversion to fluorescent calcein

Glands were exposed to omeprazole (100μM) with or without NH₂Cl (0, 50, 100, 200μM) for 2.5 hours at 37°. Gland viability was assessed by uptake of calcein and its conversion of fluorescent calcein. Calcein-AM (8μM) was added for 30 minutes, and fluorescence was measured (Excitation 485, Emission 528). Data are expressed as % of Ringer's control, mean ± SEM, N = 8. * = P < 0.05 vs. Ringer's control. † P < 0.05 vs. 0 μM NH₂Cl.

Figure 2. Representative recording of $[Zn^{2+}]$ in an individual gastric gland, during exposure to omeprazole

Glands were loaded with FluoZin-3 AM ($8\mu M$, 30 minutes), placed on coverslips for real-time imaging, then equilibrated initially by perfusion with Ringer's solution containing no added Ca^{2+} or Zn^{2+} and $0.5mM$ EGTA. Ringer's solution containing omeprazole ($100\mu M$) was added after approximately 5 minutes. Each line represents a separate parietal cell in the gland, fluorescence in each cell being normalized to its baseline at the beginning of the experiment. Similar results were observed in three other glands.

Figure 3. Effect of pretreatment with BAPTA-AM on omeprazole and NH₂Cl toxicity
 Glands were pre-treated with BAPTA-AM for 45 minutes, then exposed to omeprazole (100μM) and NH₂Cl for three hours. Gland viability was assessed by uptake of calcein and its conversion of fluorescent calcein. Data are expressed as % of Ringer's control, mean ± SEM, N = 8. * = P < 0.05 vs. Ringer's control. † P < 0.05 vs. 0 μM NH₂Cl.

Figure 4. Effect of TPEN on toxicities associated with omeprazole and NH₂Cl

Glands were co-incubated with omeprazole (100μM), NH₂Cl, and TPEN (20μM) for three hours. Gland viability was assessed by uptake of calcein and its conversion of fluorescent calcein. Data are expressed as % of Ringer's control, mean ± SEM, N = 8. * = P < 0.05 vs. Ringer's control. † P < 0.05 vs. 0 μM NH₂Cl.

Figure 5. Thiol reducing agent DTT (dithiothreitol) prevents both monochloramine and DTT damage

Glands were co-incubated with omeprazole (100μM), NH₂Cl, and DTT (1mM) for three hours. Gland viability was assessed by uptake of calcein and its conversion of fluorescent calcein. Data are expressed as % of Ringer's control, mean ± SEM, N = 8. * = P < 0.05 vs. Ringer's control. † P < 0.05 vs. 0 μM NH₂Cl.

Figure 6. Oxidant scavenger Vitamin C prevents omeprazole toxicity

Glands were co-incubated with omeprazole (100μM), NH_2Cl , and Vitamin C (1mM) for three hours. Gland viability was assessed by uptake of calcein and its conversion of fluorescent calcein. Data are expressed as % of Ringer's control, mean $\pm$ SEM, N = 8. * = P < 0.05 vs. Ringer's control. † P < 0.05 vs. 0 μM NH_2Cl .

Figure 7. Effects of Vitamin C effect on acidity in the tubulovesicle/lumen compartment of isolated gastric glands

Glands were co-incubated with omeprazole (100 μ M), NH_2Cl , and Vitamin C (1mM) for three hours. Lysosensor DND-160 (8 μ M) was added for 15 minutes, following which fluorescence was measured ratiometrically (Ex. 340nm/ 400nm, Em. 528nm). Data are expressed as the ratio of 340/400 excitations, mean $\pm$ SEM, N = 9. * = P < 0.05 vs. Ringer's control. † P < 0.05 vs. Vitamin C (1mM) in Ringer's.