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# Proton Pump Inhibitors Accelerate Endothelial Senescence

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## Abstract

**Rationale—**Proton pump inhibitors (PPIs) are popular drugs for gastroesophageal reflux, now available for long-term use without medical supervision. Recent reports suggest that PPI use is associated with cardiovascular, renal and neurological morbidity.

**Objective—**To study the long-term effect of PPIs on endothelial dysfunction and senescence and investigate the mechanism involved in PPI induced vascular dysfunction.

**Methods and Results—**Chronic exposure to PPIs impaired endothelial function and accelerated human endothelial senescence by reducing telomere length.

**Conclusion—**Our data may provide a unifying mechanism for the association of PPI use with increased risk of cardiovascular, renal and neurological morbidity and mortality.

### Keywords

Proton pump inhibitors; lysosomal function; proteostasis; senescence and telomere length; cardiovascular disease risk factor; aging

### Subject Terms

Vascular Biology; Vascular Disease; Angiogenesis; Oxidative Stress

## INTRODUCTION

Proton pump inhibitors (PPIs) like Esomeprazole (Nexium) are widely used drugs for the treatment of gastroesophageal reflux disease (GERD). In the US these drugs are sold over-

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**DISCLOSURES**

None.

the-counter and thus medical supervision is not required. Although these agents are effective, they were never approved by regulatory authorities for long-term use. Furthermore, evidence suggests that up to 70% of PPI use may be inappropriate.[1] Recent large and well-controlled epidemiological and retrospective studies have found associations between the use of PPIs, and an increased prevalence of myocardial infarction, renal failure, and dementia.[2–5] However, in the absence of a mechanism and without evidence of causality, global regulatory authorities have not restricted the use of PPIs. In this paper, we provide evidence that chronic exposure to proton pump inhibition accelerates senescence in human endothelial cells, a unifying mechanism which may explain the association of adverse cardiovascular, renal and neurological effects with the use of PPIs.

In the low pH conditions of the gastric parietal cell, PPIs are converted to the active sulfenic acid form.[3, 6] When activated the PPIs form a mixed disulfide with the proton pump of the parietal cell to inhibit its secretion of HCl into the stomach.[7, 8] Physicians have prescribed these drugs with the perception that these agents have specificity for the parietal cells of the stomach. However, similar proton pumps are also found in cell lysosomes.[9] An earlier publication found no evidence that the PPI rabeprazole impaired lysosomal activity in hepatic cells.[10] However, we wondered if PPIs may also affect endothelial lysosomes and disrupt proteostasis. Our rationale for testing this hypothesis is that endothelial dysfunction is known to contribute to the pathogenesis of myocardial infarction, renal failure, and dementia.[11–13]

## METHODS

A detailed materials and methods section is available in the online supplement data

## RESULTS

### The PPI esomeprazole impairs human lysosomal function and proteostasis

We cultured human microvascular endothelial cells (ECs) continuously for 3 passages (passage 4–6) in media containing a clinically relevant concentration of the PPI esomeprazole (ESO; 5 and 10 μmol/L) or vehicle (DMSO). Using a pH sensitive fluorescent dye that is taken up by endocytosis, we observed fluorescence in a perinuclear distribution consistent with lysosomal localization in EC treated with vehicle. In ECs chronically exposed to ESO, fluorescence intensity was significantly reduced, consistent with an increase in lysosomal pH (Figure 1A). We repeated these studies using a second pH sensitive fluorescent dye and obtained qualitatively similar findings (Online Figure I). An impairment in the lysosomal proton pump and an increase in lysosomal pH would be expected to impair lysosomal enzymes which are optimally active at a pH of about 4.80.[14, 15] Indeed the activity of lysosomal cathepsin-B and acid phosphatase were reduced in ECs treated chronically with ESO (Figure 1B, C, E). We did not observe any difference in N-acetyl-β-d-glucosaminidase activity (Online figure II). Using a commercially available protein aggregation detection dye, together with image quantification software to quantify protein aggregates, we observed an increase in protein aggregates in the ESO treated ECs (Figure 1D, F). These studies indicate that PPIs impair endothelial lysosomal acidification, enzyme activity and proteostasis.

### The PPI esomeprazole impairs endothelial function

Disruption of proteostasis is associated with a global deterioration of cell function and accelerated cell aging.[16–18] A hallmark of endothelial dysfunction is an increase in the generation of superoxide anion[19, 20] and a decrease in nitric oxide (NO) levels.[21] Using fluorescent live cell imaging dyes we observed that by comparison to EC treated with vehicle, those treated chronically with ESO produced more superoxide anion as measured by dihydroethidium (DHE), and generated less NO as measured by di-amino fluorescein 2-diacetate (DAF-2DA) staining. This impairment in EC function was confirmed by a decrease in total nitrate levels as detected by Griess colorimetric assay (Figure 2A–E) in the ESO treated group. We also observed a decrease in the expression of DDAH1/2, eNOS and iNOS (Online figure IIIA–D); a reduced expression of these critical enzymes in the NO synthase pathway would explain a decline in EC NO generation. Because NO plays a key role in EC proliferation and angiogenesis[22] we also assessed these EC functions. Chronic exposure to ESO dose- dependently impaired cell proliferation as measured by BrdU assay (Figure 2F), a finding which was confirmed using a real-time cell analyzer which assesses cell growth (Figure 2G). Additional studies revealed that chronic exposure (3 passages) to a concentration of ESO as low as 1μM significantly reduced endothelial cell proliferation as measured by real time cell analyzer (Online figure IV). Consistent with these observations, we observed that chronic ESO treatment increased expression of cell cycle inhibitor p21 gene (Figure 2H). Finally, we noted that ESO impaired the angiogenic capacity of ECs as measured by network formation on growth factor depleted matrigel (Figure 2I–L). These results indicate that esomeprazole impairs multiple endothelial functions.

### The PPI esomeprazole accelerates endothelial aging

Impairment of proteostasis, and reduced cell proliferation, are hallmarks of cellular senescence.[18,23] To determine if cells chronically treated with PPIs exhibited other features of senescence, we assessed the impact of chronic treatment with ESO or with SCH-28080 (another H+K+ ATPase inhibitor with a potency similar to omeprazole, $IC_{50}$ of 2.5 and 4.0μM respectively). We found that senescence associated β-galactosidase (SA-β-gal) positive cells were increased by comparison to vehicle (Figure 3A, B, D, E) as early as P6 in both ESO and SCH-28080 treated groups. Also we observed a decrease in total cell count per microscopic field (figure 3E–F) by SYTO-green staining consistent with a decline in cell proliferation. We also noted a change in the morphology in some of the PPI-treated cells, some of which adopted the “fried egg” morphology characteristic of senescent EC. Interestingly, we did not see any significant difference in SA-β-gal positive cell or total cell count upon treatment with ranitidine (Online figure VA–C; ranitidine is a $H_2$ histamine receptor antagonists which is used as an alternative treatment for GERD). We further investigated expression of 331 genes from five different molecular pathways (Cellular Senescence, Endothelial Cell Biology, Angiogenesis, TGF-β – BMP and Epithelial to Mesenchymal Transition signaling pathways) involved in esomeprazole-induced endothelial dysfunction using PCR array. We observed 52 genes were up-related (>2 fold increase) and 49 genes were down-regulated (>0.5 fold of control value). In general, the changes in gene expression were consistent with those observed in endothelial senescence, e.g., increased expression of genes involved in endothelial-to-mesenchyme transition (EndoMT), inflammation and increased oxidative stress. (Online table 1, 2). We selected several of these

genes for validation. Plasminogen activator inhibitor (PAI-1) is a well-known marker for endothelial dysfunctions, e.g., increased thrombogenicity, immune activation, oxidative stress, and senescence[24]. We found that PAI-1 message and protein expression was upregulated in ESO treated cells (figure 3G–I). We also found that genes associated with EndoMT including TWIST1, COL1A1 and SMAD3 (Online figure VI-A–C) were upregulated, together with a decline in the expression of vWF (Online figure VI–D), a marker for vascular endothelium. In additional studies, after treating ECs with ESO (5 or 10μM) or vehicle for 3 passages, we discontinued treatment and maintained the ECs in endothelial growth medium at the same passage for approximately three months. At the three month time point, the ECs that had been exposed to vehicle remained confluent, with occasional apoptotic and senescent cells. By contrast, there was a qualitative difference in the cells that had been exposed to ESO, with most high power fields showing some cell loss and/or endothelial-to-mesenchyme transition (Online figure VII). To conclude, chronic exposure to a PPI induces endothelial dysfunction consistent with EndoMT and senescence.

### PPIs induce telomere shortening

Endothelial senescence is associated with attrition of telomere length[25], whereas restoration of EC telomere length can reverse senescence-associated endothelial dysfunction.[26,27] As expected of mature somatic cells, ECs in either group did not manifest telomerase expression (dns) or activity (Online Figure VIII). Using monochrome multiplex qPCR (mmqPCR) as we have previously described,[27] we observed a significant decrease in telomere length in ESO treated group compared to vehicle (Figure 4A). To assess the mechanism of telomere shortening, we examined the expression of genes involved in regulating the shelterin complex. The shelterin complex is encoded by six genes (TRF1, TRF2, POT1, RAP1, TIN2, and TPP1) involved in regulation and maintenance of telomere length and function.[28] We observed a global downregulation of all six genes of the shelterin complex (Figure 4C–H), which could explain in part the effect of the PPI to accelerate telomere erosion.

## DISCUSSION

The salient findings of this study are that long-term exposure to proton pump inhibition: 1) impairs lysosomal acidification and enzyme activity, in association with protein aggregate accumulation; 2) increases the generation of reactive oxygen species, and impairs the NO synthase pathway; 3) accelerates telomere erosion in association with reduced expression of the shelterin complex; and 4) speeds endothelial aging as manifested by impaired cell proliferation and angiogenesis, together with histological markers of senescence and endothelial-to-mesenchyme transition. Our results in primary human EC are consistent with the recent finding that PPIs impair the activity of lysosomal enzymes in several immortalized cell lines including A549, Caco2, HEK293, and HepG2.[15] Lysosomes bind to autophagosomes to complete the process of autophagy,[29] which comprises the degradation and elimination of unwanted cellular products including misfolded proteins.[14, 15] An impairment of lysosomal acidification and reduced lysosomal enzyme activity might be expected to result in an accumulation of protein aggregates.

Our studies were conducted in a clinically relevant dose range. In adults, the peak plasma concentration ($C_{max}$) of esomeprazole is 4.7 μmol/L with the 40mg dose[30, 31]. The metabolism of esomeprazole is dependent on the isoenzyme CYP2C19 which exhibits polymorphism. About 3% of Caucasians and 23% of Asians are poor metabolizers and may experience a threefold increase in plasma concentration of esomeprazole[30, 32].

In addition, we find that chronic PPI exposure upregulates genes that are involved in endothelial-to-mesenchyme transition (EndoMT) and is associated with histological changes consistent with EndoMT. EndoMT is a feature of senescent ECs, and may itself play an important role in cardiovascular disease, as well as other disorders characterized by fibrosis and loss of the microvasculature.[33] Furthermore, we show that esomeprazole downregulates the expression of the shelterin complex genes, in association with a reduction in telomere length. There is little known regarding the association of endothelial dysfunction with downregulation of the shelterin complex and this observation merits further investigation.[34] An observation of clinical importance is that ranitidine, an alternative treatment for GERD which acts by a different mechanism than the PPIs, does not have an adverse effect on endothelial aging.

A limitation of this paper is that we have not we have not tested the full range of PPIs that are commercially available. Furthermore we have not determined how the PPIs are altering lysosomal pH, although we believe the effect is related to their binding to the lysosomal proton pump. Finally, we have not determined if the effect of the PPIs to accelerate aging of human endothelial cells occurs in vivo. Whereas our previous work has indicated that short-term use of PPIs does not significantly alter endothelial function[35], chronic use must now be addressed in randomized clinical trials.

To conclude, we find that chronic exposure of human endothelial cells to the PPIs esomeprazole or SCH-28080 accelerates endothelial aging. This adverse effect appears to be due to an inhibition of lysosomal acidification and subsequent impairment of proteostasis. The accumulation of protein aggregates is associated with an increase in oxidative stress, endothelial dysfunction and senescence. Vascular senescence would provide a mechanistic explanation[11–13] for the accumulating evidence that PPIs increase the risk of cardiovascular morbidity and mortality, renal failure, and dementia.[2–5] In the presence of consistent epidemiological evidence of harm, and a unifying mechanism for the disparate disorders linked to PPI use; and with the knowledge that PPIs are being used by millions of people for indications and durations that were never tested or approved; it is time for the pharmaceutical industry and regulatory agencies to re-visit the specificity and the safety of these agents.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Nonstandard Abbreviations and Acronyms

| | |
|---|---|
| **PPIs** | Proton Pump Inhibitors |
| **ECs** | Endothelial Cells |
| **GERD** | Gastroesophageal Reflux Disease |
| **ESO** | Esomeprazole |
| **EndoMT** | Endothelial to Mesenchymal Transition |
| **CVDs** | Cardiovascular Diseases |

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## Novelty and Significance

**What Is Known?**

- Proton pump inhibitors (PPIs) inhibit H+K+ATPase proton pumps in the stomach to reduce acid secretion.
- PPIs are commonly used for gastroesophageal reflux.
- Accumulating observational data indicates an association between the use of PPIs and increased risk of heart attack, dementia and renal failure.

**What New Information Does This Article Contribute?**

- PPIs impair acidification and enzyme activity in endothelial lysosomes.
- Subsequently protein aggregates accumulate with increased oxidative stress.
- Endothelial dysfunctions with telomere shortening and accelerated senescence are observed

These studies reveal that chronic exposure of human endothelial cells to PPIs disturbs proteostasis and accelerates senescence. Accelerated vascular aging may be a mechanistic link for the increased association of cardiac, neurological and renal morbidity in PPI users. Although short term use of PPIs is relatively safe and very effective for GERD, the long-term use of PPIs without medical supervision should be re-examined. Clinical studies to assess the long-term effects of PPIs on vascular health are indicated.

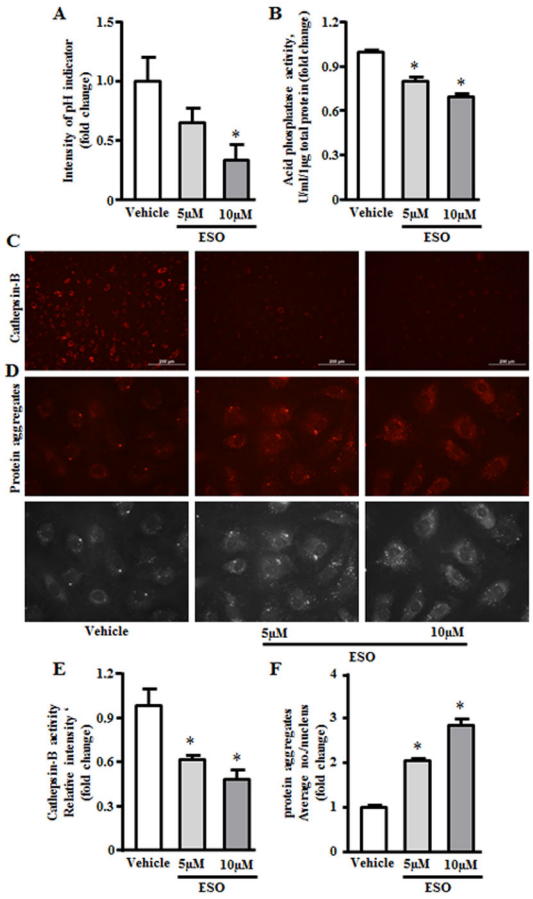


**Figure 1. Esomeprazole impairs proteostasis**

**(A)** Intensity of pHrodo™ Green AM fluorescence, which is inversely proportional to lysosomal pH (n=4). (**B**) Acid phosphatase assay (n=4). (**C&E**) Intracellular cathepsin-B activity assessed by Magic Red® fluorescence dye (n=4). (**D&F**) Intracellular protein aggregates assessed by PROTEOSTAT® assay (fluorescent staining in upper panel and corresponding phase-contrast image on lower panel) and quantification (n=4). *p< 0.05 vs vehicle (DMSO).

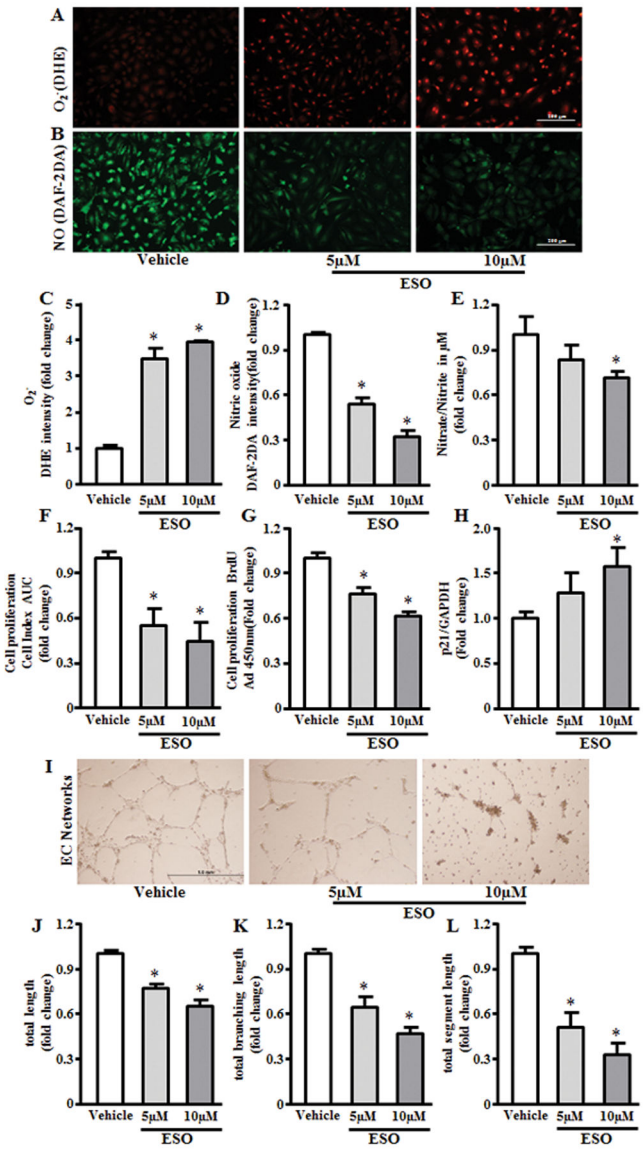


**Figure 2. Esomeprazole impairs endothelial function**

**(A&C)** Superoxide anion generation assessed by dihydroethidium staining (n=4). (**B&D**) Nitric oxide generation assessed by di-amino fluorescein 2-diacetate (DAF-2DA) staining (n=4). (**E**) Total nitrate/nitrite levels assessed by Greiss reaction (n=6). (**F**) Measurement of cell proliferation using real time cell analyzer which generates cell index (CI) values represented as area under curve (n=5). (**G**) Cell proliferation assessed by BrdU assay (n=8). (**H**) p21 mRNA expression using RT-PCR (n=4). (**I–L**) Angiogenic capacity of ECs reflected by network formation in growth factor depleted matrigel. *p< 0.05 vs vehicle (DMSO).

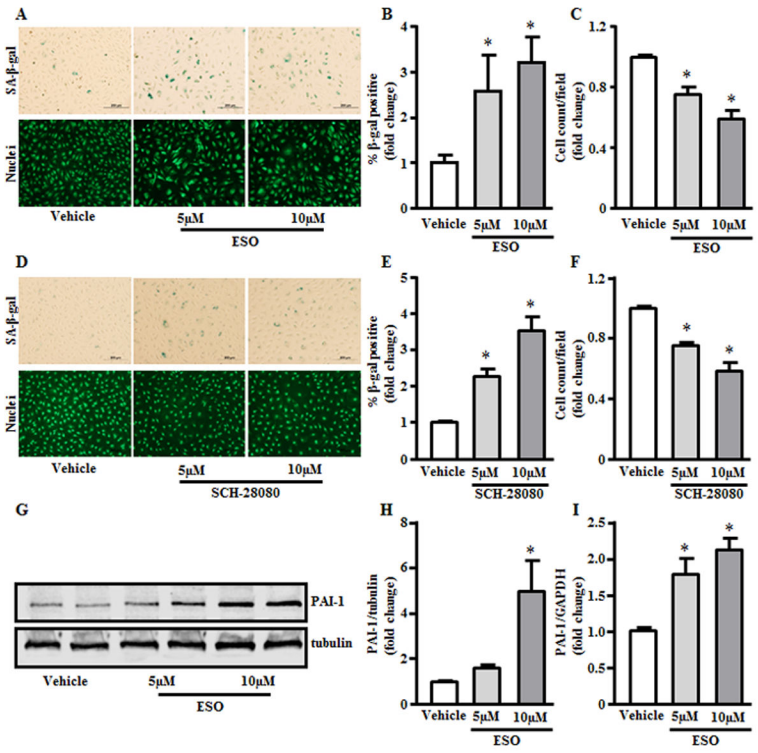


**Figure 3. PPIs accelerate endothelial senescence**

**(A&D**) Senescent cell number detected by staining for senescence associated-β-galactosidase (SA-β-gal; upper panel) and for SYTO-13 to detect cell nuclei for total cell count (lower panel). (**B,C,E** and **F**) Respective quantification for % positive SA-β-gal cells and average cell count per field (n=6). (**G** and **H**) PAI-1 protein expression by Western blot analysis (n=3). (**I**) PAI-1 mRNA expression quantified by RT-PCR (n=6). *p< 0.05 vs vehicle (DMSO).

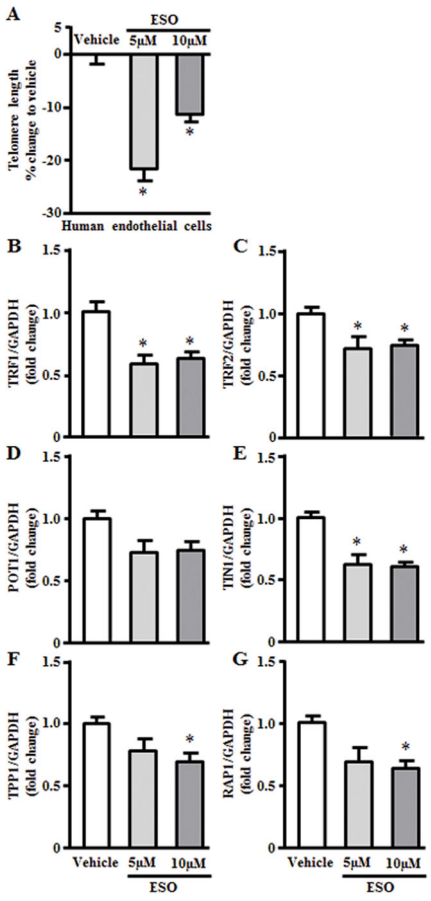


**Figure 4. PPIs reduce telomere length and expression of shelterin complex subunits**

Relative telomere length assessed by mmqPCR (**A)** in human MVEC (n=6) (**B–G**) Expression of shelterin complex genes assessed by RT-PCR (n=6). *p< 0.05 vs vehicle (DMSO)