Brief UltraRapid Communication

Proton Pump Inhibitors Accelerate Endothelial Senescence

Gautham Yepuri, Roman Sukhovershin, Timo Z. Nazari-Shafti, Michael Petrascheck, Yohannes T. Ghebre, John P. Cooke

Rationale: **Proton pump inhibitors (PPIs) are popular drugs for gastroesophageal reflux, which are 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.**

Conclusions: **Our data may provide a unifying mechanism for the association of PPI use with increased risk of cardiovascular, renal, and neurological morbidity and mortality. (*Circ Res*. 2016;118:e36-e42. DOI: 10.1161/CIRCRESAHA.116.308807.)**

Key Words: aging ■ cardiovascular diseases ■ proteostasis deficiencies ■ proton pump inhibitors

Proton pump inhibitors (PPIs), such as esomeprazole (Nexium), are widely used drugs for the treatment of gastroesophageal reflux disease. In the United States, these drugs are sold over 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 ≤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 study, we provide evidence that chronic exposure to proton pump inhibition accelerates senescence in human endothelial cells (ECs), a unifying mechanism that may explain the association of adverse cardiovascular, renal, and neurological effects with the use of PPIs.

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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 Data Supplement.

Results

The PPI esomeprazole impairs human lysosomal function and proteostasis.

We cultured human microvascular ECs continuously for 3 passages (passages 4–6) in media containing a clinically relevant concentration of the PPI esomeprazole (5 and 10 μmol/L) or vehicle (DMSO). Using a pH-sensitive fluorescent dye

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From the Department of Cardiovascular Sciences, Center for Cardiovascular Regeneration, Houston Methodist Research Institute, TX (G.Y., R.S., T.Z.N-.S., J.P.C.); Department of Chemical Physiology, Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA (M.P.); and Department of Radiation Oncology, Baylor College of Medicine, One Baylor Plaza, Houston, TX (Y.T.G.).

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Correspondence to John P. Cooke, MD, PhD, Department of Cardiovascular Sciences, Center for Cardiovascular Regeneration, Houston Methodist Research Institute, 6670 Bertner Ave, Mail Stop: R10-South, Houston, TX 77030. E-mail jpcooke@houstonmethodist.org

Nonstandard Abbreviations and Acronyms	
ECs	endothelial cells
EndoMT	endothelial to mesenchymal transition
NO	nitric oxide
PPIs	proton pump inhibitors

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 esomeprazole, 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 ≈4.80.[14,15] Indeed, the activity of lysosomal cathepsin-B and acid phosphatase was reduced in ECs treated chronically with esomeprazole (Figure 1B, 1C, and 1E). 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 esomeprazole-treated ECs (Figure 1D and 1F). These studies indicate that PPIs impair endothelial lysosomal acidification, enzyme activity, and proteostasis.

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 with EC treated with vehicle, those treated chronically with esomeprazole produced more superoxide anion as measured by dihydroethidium and generated less NO as measured by diamino fluorescein 2-diacetate staining. This impairment in EC function was confirmed by a decrease in total nitrate levels as detected by Griess colorimetric assay (Figure 2A–2E) in the esomeprazole-treated group. We also observed a decrease in the expression of *DDAH1/2* (dimethylarginine dimethylaminohydrolase, isoforms 1 or 2), *eNOS* (endothelial nitric oxide synthase), and *iNOS* (inducible nitric oxide synthase) (Online Figure IIIA–IIID); 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 esomeprazole dose-dependently impaired cell proliferation as measured by 5-bromo-2′-deoxyuridine 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 esomeprazole as low as 1 μmol/L significantly reduced EC proliferation as measured by real-time cell analyzer (Online Figure IV). Consistent with these observations, we observed that chronic esomeprazole treatment increased the expression of cell cycle inhibitor *p21* gene (Figure 2H). Finally, we noted that esomeprazole impaired the angiogenic capacity of ECs as measured by network formation on growth factor–depleted matrigel (Figure 2I–2L). These results indicate that esomeprazole impairs multiple endothelial functions.

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 effect of chronic treatment with esomeprazole or with SCH-28080 (another H^+K^+ ATPase inhibitor with a potency similar to omeprazole, IC_{50} of 2.5 and 4.0 μmol/L, respectively). We found that senescence-associated β-galactosidase (SA-β-gal)–positive cells were increased by comparison to vehicle (Figure 3A, 3B, 3D, and 3E) as early as P6 in both esomeprazole- and SCH-28080–treated groups. Also, we observed a decrease in total cell count per microscopic field (Figure 3E and 3F) 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 on treatment with ranitidine (Online Figure VA–VC; ranitidine is a H_2 histamine receptor antagonist, which is used as an alternative treatment for gastroesophageal reflux disease). We further investigated the expression of 331 genes from 5 different molecular pathways (cellular senescence, EC biology, angiogenesis, transforming growth factor-β–bone morphogenic protein, and epithelial to mesenchymal transition signaling pathways) involved in esomeprazole-induced endothelial dysfunction using polymerase chain reaction array. We observed that 52 genes were upregulated (>2-fold increase) and 49 genes were downregulated (>0.5-fold of control value). In general, the changes in gene expression were consistent with those observed in endothelial senescence, for example, increased expression of genes involved in endothelial-to-mesenchymal transition (EndoMT), inflammation, and increased oxidative stress (Online Tables 1 and 2). We selected several of these genes for validation. Plasminogen activator inhibitor is a well-known marker for endothelial dysfunctions, for example, increased thrombogenicity, immune activation, oxidative stress, and senescence.[24] We found that plasminogen activator inhibitor message and protein expression were upregulated in esomeprazole-treated cells (Figure 3G–3I). We also found that genes associated with EndoMT, including *TWIST1*, *COL1A1*, and *SMAD3* (Online Figure VIA–VIC), were upregulated, together with a decline in the expression of von Willebrand factor (Online Figure VID), a marker for vascular endothelium. In additional studies, after treating ECs with esomeprazole (5 or 10 μmol/L) or vehicle for 3 passages, we discontinued treatment and maintained the ECs in an endothelial growth medium at the same passage for ≈3 months. At the 3-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 esomeprazole, with most high-power fields, showing some cell loss or EndoMT (Online Figure VII). To conclude, chronic exposure to a PPI induces endothelial dysfunction consistent with EndoMT and senescence.

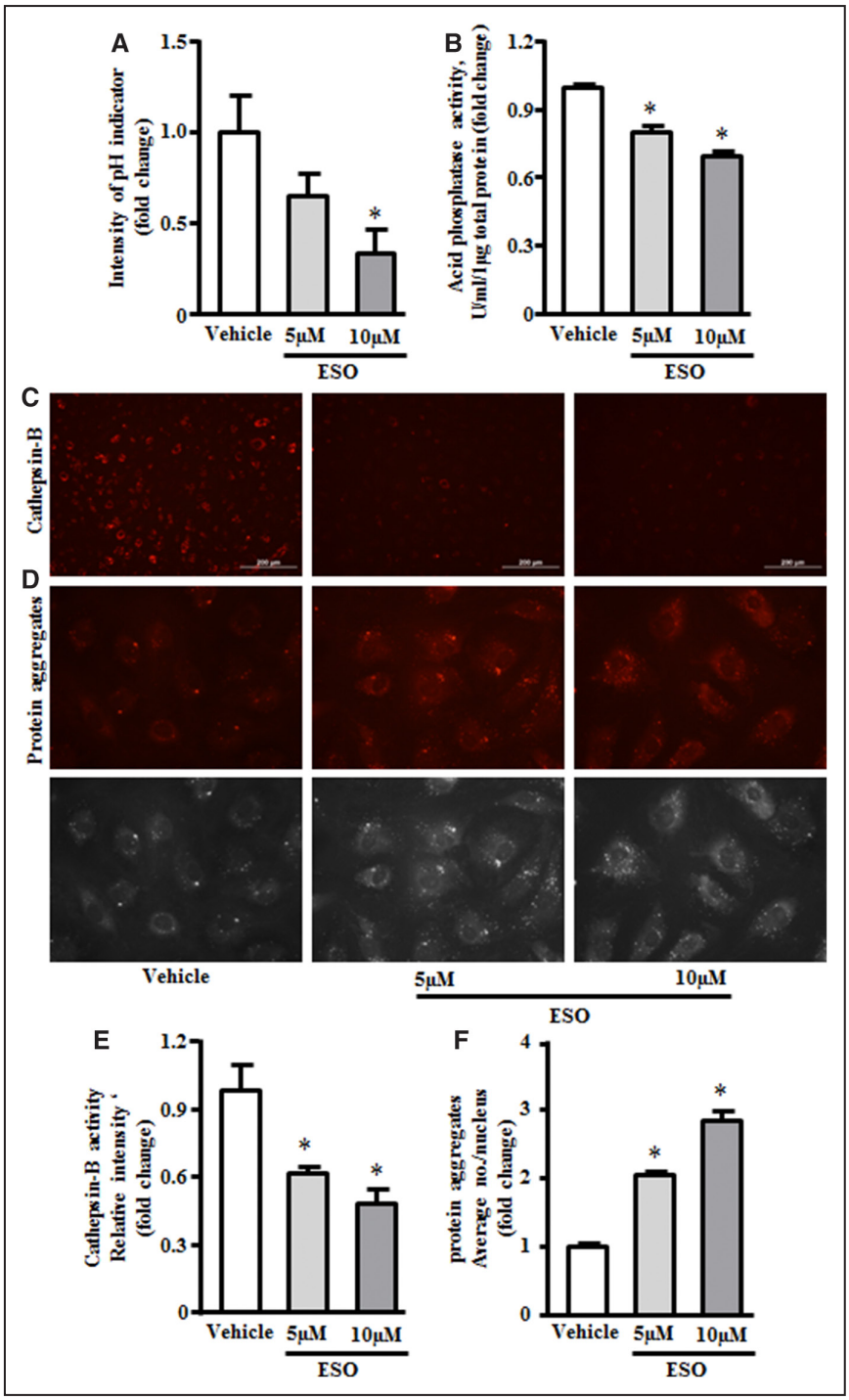

Figure 1. Esomeprazole impairs proteostasis. A, Intensity of pHrodo Green AM fluorescence, which is inversely proportional to lysosomal pH (n=4). **B**, Acid phosphatase assay (n=4). **C** and **E**, Intracellular cathepsin-B activity assessed by Magic Red fluorescence dye (n=4). **D** and **F**, Intracellular 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). ESO indicates esomeprazole.

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, ECs in either group did not manifest telomerase expression (data not shown) or activity (Online Figure VIII). Using monochrome multiplex quantitative polymerase chain reaction as we have previously described,[27] we observed a significant decrease in telomere length in esomeprazole-treated group compared with 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 6 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 6 genes of the shelterin complex (Figure 4C–4H), 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

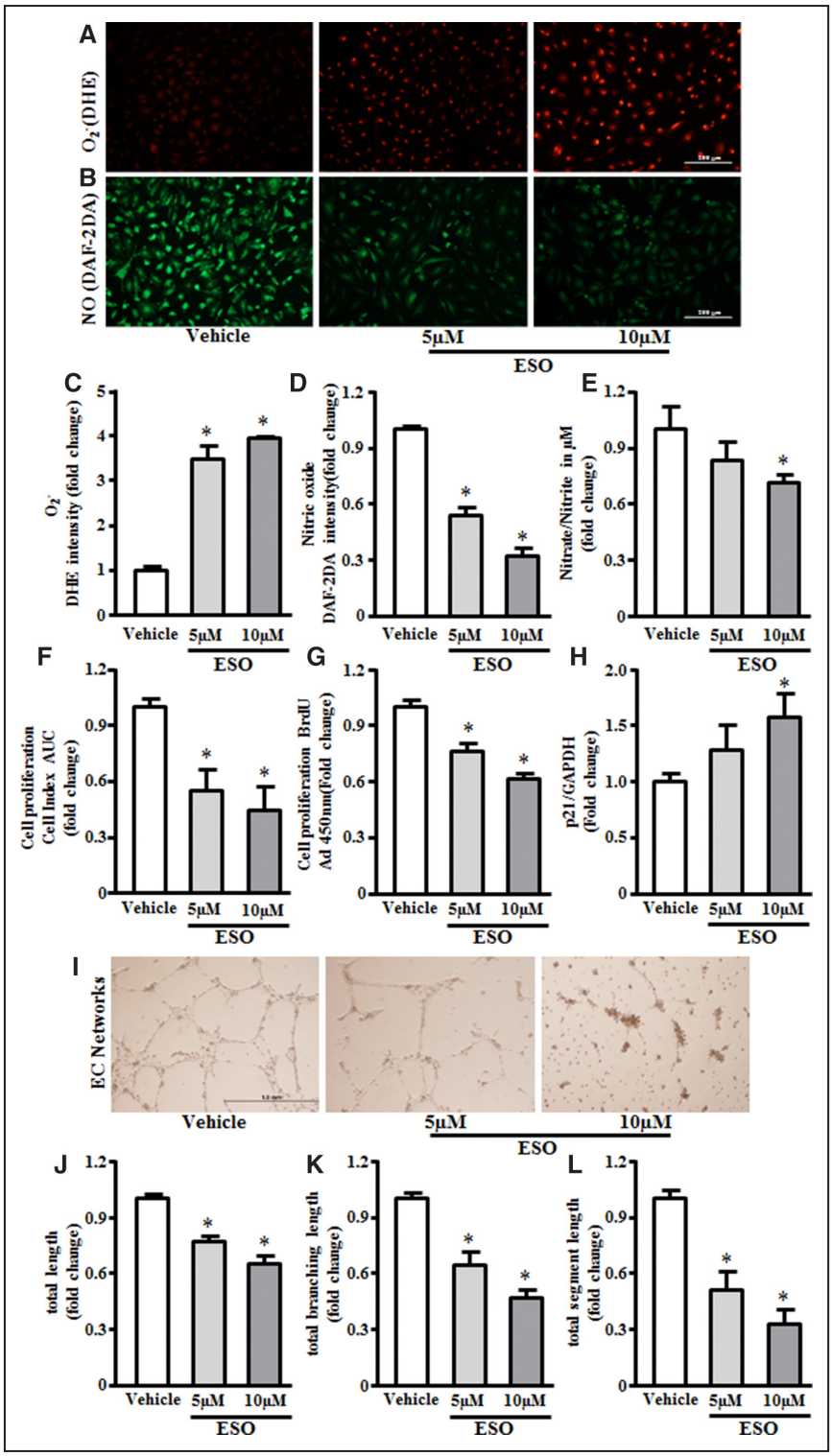

Figure 2. Esomeprazole impairs endothelial function. A and **C**, Superoxide anion generation assessed by dihydroethidium staining (n=4). **B** and **D**, Nitric oxide generation assessed by diamino fluorescein 2-diacetate (DAF-2DA) staining (n=4). **E**, Total nitrate/nitrite levels assessed by Greiss reaction (n=6). **F**, Measurement of cell proliferation using real-time cell analyzer, which generates cell index values represented as area under curve (AUC; n=5). **G**, Cell proliferation assessed by 5-bromo-2′-deoxyuridine (BrdU) assay (n=8). **H**, p21 mRNA expression using reverse transcription polymerase chain reaction (n=4). **I–L**, Angiogenic capacity of endothelial cells reflected by network formation in growth factor depleted matrigel. **P*<0.05 vs vehicle (DMSO). DHE indicates dihydroethidium; ESO, esomeprazole; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; and NO, nitric oxide.

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 EndoMT. Our results in primary human ECs 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.

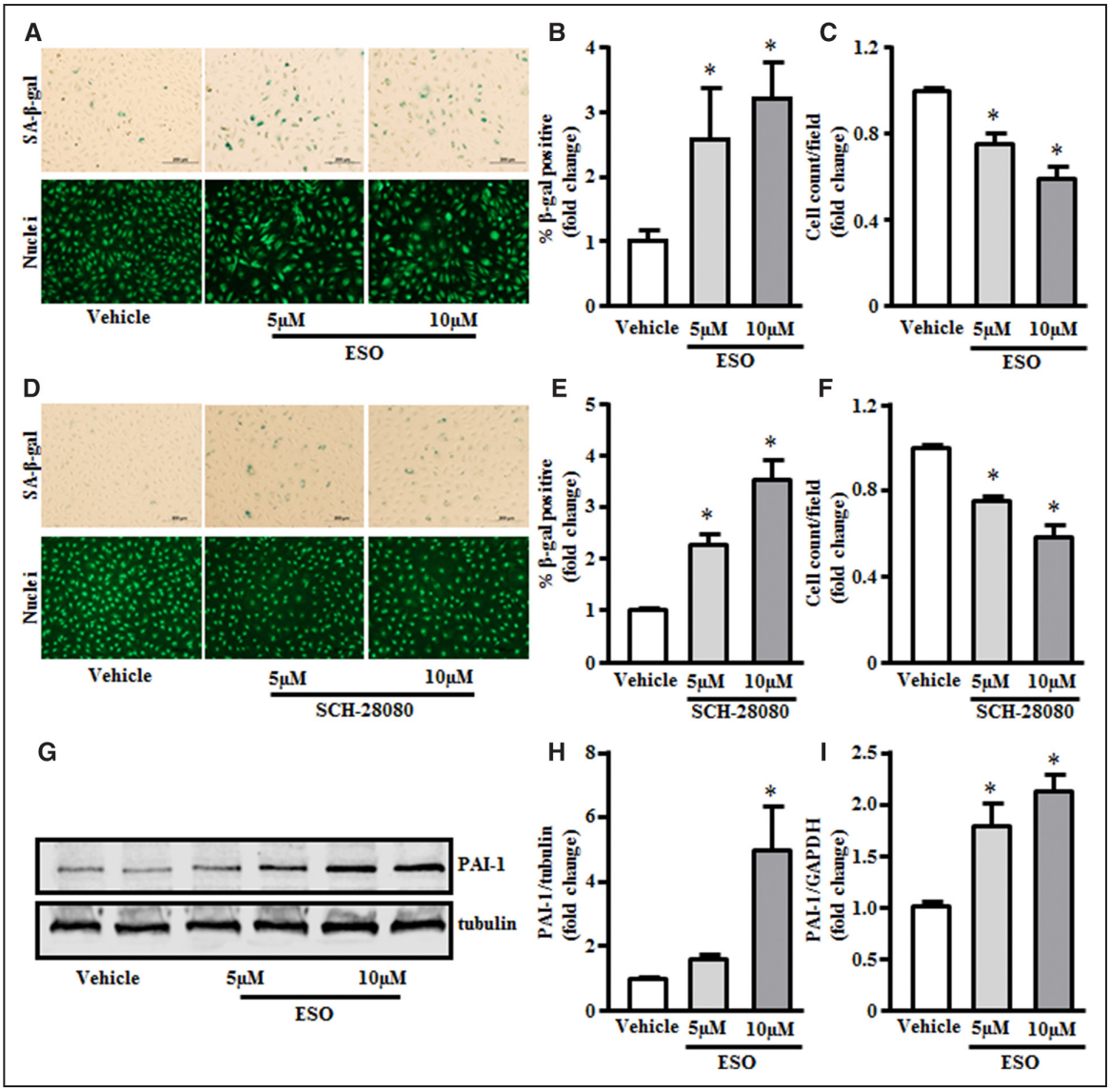

Figure 3. Proton pump inhibitors (PPIs) accelerate endothelial senescence. A and **D**, Senescent cell number detected by staining for senescence-associated β-galactosidase (SA-β-gal; **top**) and for SYTO-13 to detect cell nuclei for total cell count (bottom). **B**, **C**, **E** and **F**, Respective 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**, Plasminogen activator inhibitor (PAI-1) mRNA expression quantified by reverse transcription polymerase chain reaction (n=6). **P*<0.05 vs vehicle (DMSO). ESO indicates esomeprazole; and GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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 40-mg dose.[30,31] The metabolism of esomeprazole is dependent on the isoenzyme CYP2C19, which exhibits polymorphism. About 3% of whites and 23% of Asians are poor metabolizers and may experience a 3-fold increase in plasma concentration of esomeprazole.[30,32]

In addition, we find that chronic PPI exposure upregulates genes that are involved in 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 on 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 gastroesophageal reflux disease, which acts by a different mechanism than the PPIs, does not have an adverse effect on endothelial aging.

A limitation of this study is that 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 think that 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 ECs 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.

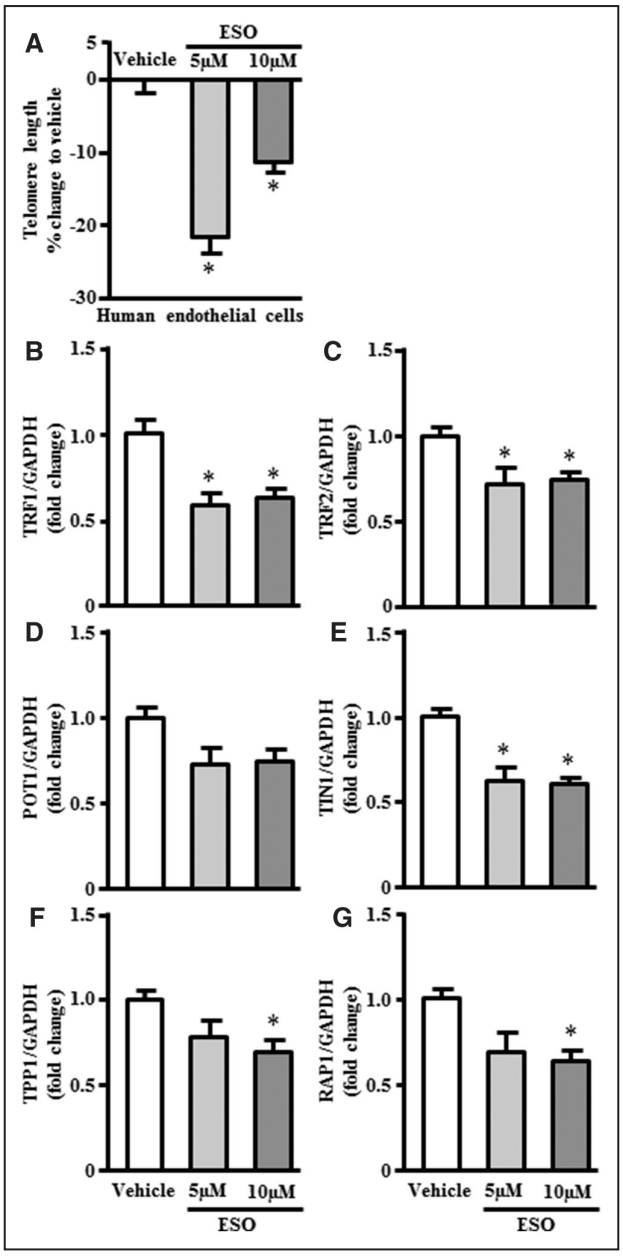

Figure 4. Proton pump inhibitors reduce telomere length and expression of shelterin complex subunits. A, Relative telomere length assessed by monochrome multiplex quantitative polymerase chain reaction (PCR) in human microvascular endothelial cells (n=6). **B**–**G**, Expression of shelterin complex genes assessed by reverse transcription PCR (n=6). *P<0.05 vs vehicle (DMSO). ESO indicates esomeprazole; and GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

To conclude, we found that chronic exposure of human ECs to the PPIs, esomeprazole or SCH-28080, accelerates endothelial aging. This adverse effect seems to be because of 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 revisit the specificity and the safety of these agents.

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Disclosures

None.

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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 the 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 effective for gastroesophageal reflux disease, 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.