Unexpected Effect of Proton Pump Inhibitors
Elevation of the Cardiovascular Risk Factor Asymmetric Dimethylarginine

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Background—Proton pump inhibitors (PPIs) are gastric acid–suppressing agents widely prescribed for the treatment of gastroesophageal reflux disease. Recently, several studies in patients with acute coronary syndrome have raised the concern that use of PPIs in these patients may increase their risk of major adverse cardiovascular events. The mechanism of this possible adverse effect is not known. Whether the general population might also be at risk has not been addressed.

Methods and Results—Plasma asymmetrical dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide synthase. Elevated plasma ADMA is associated with increased risk for cardiovascular disease, likely because of its attenuation of the vasoprotective effects of endothelial nitric oxide synthase. We find that PPIs elevate plasma ADMA levels and reduce nitric oxide levels and endothelium-dependent vasodilation in a murine model and ex vivo human tissues. PPIs increase ADMA because they bind to and inhibit dimethylarginine dimethylaminohydrolase, the enzyme that degrades ADMA.

Conclusions—We present a plausible biological mechanism to explain the association of PPIs with increased major adverse cardiovascular events in patients with unstable coronary syndromes. Of concern, this adverse mechanism is also likely to extend to the general population using PPIs. This finding compels additional clinical investigations and pharmacovigilance directed toward understanding the cardiovascular risk associated with the use of the PPIs in the general population. (Circulation. 2013;128:845-853.)

Key Words: dimethylarginine dimethylaminohydrolase ■ endothelium ■ N,N dimethylarginine ■ nitric oxide ■ proton pump inhibitors

Proton pump inhibitors (PPIs) are effective antagonists of gastric acid secretion used to treat a number of gastroesophageal disorders, including dyspepsia, gastroesophageal reflux disease, Zollinger-Ellison syndrome, Barrett esophagus, and Helicobacter pylori infection of the upper gastrointestinal tract.1-3 Biologically, PPIs are administered as uncharged prodrugs and require activation by parietal cells of the stomach to form positively charged active (sulfenamide and sulfenic acid) drugs. In this form, the PPIs irreversibly bind to the gastric proton pump and inhibit acid secretion.4,5 The high oral bioavailability of PPIs and their efficacy in sustained suppression of gastric acid secretion have favored their use over other acid-suppressing drugs such as histamine receptor (H2 receptor) antagonists. According to the US Food and Drug Administration, ≈21 million people in the United States used ≥1 prescription PPIs in 2009.6 Most PPIs are now available over the counter, increasing their general use in the absence of medical supervision. In 2009, sales of PPIs grew to over $13 billion globally.7

PPIs are usually well tolerated when used intermittently in healthy subjects8,9 but may be associated with hypersecretion of gastric acid after their withdrawal.9 When used long term, they may be associated with bone fracture and low levels of blood magnesium.6,10 More worrisome are recent reports that PPIs may reduce the benefit of antiplatelet agents in patients with acute coronary syndromes (ACS).11-14 Initial concern focused on the reduced benefit of clopidogrel in ACS patients taking PPIs.11-14 This effect was attributed to the inhibition by PPIs of the hepatic enzyme (CYP2C19), which is required for activation of clopidogrel.12,15

However, in ACS patients, the PPIs also diminish the benefit of ticagrelor,16 a drug that does not require hepatic activation. Furthermore, recent studies indicate that every member of the PPIs increases cardiovascular risk in ACS patients, despite the fact that some of these PPIs do not significantly inhibit CYP2C19.12,14,17-19 Accordingly, the mechanism by which the PPIs may increase the risk of major adverse cardiovascular events (MACEs) in ACS patients is unknown. Furthermore, it is not known if the risk might extend to the larger population of ambulatory patients and consumers using PPIs.

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Here, we report our finding that PPIs inhibit the activity of dimethylarginine dimethylaminohydrolase (DDAH), an enzyme necessary for cardiovascular health. DDAH metabolizes asymmetrical dimethylarginine (ADMA), an endogenous and competitive inhibitor of nitric oxide (NO) synthase (NOS). By inhibiting endothelial NOS, ADMA would be anticipated to increase the risk of vascular inflammation and thrombosis, which may explain the increased risk of MACEs in patients taking PPIs. Indeed, elevated plasma ADMA is a risk factor for cardiovascular morbidity and mortality in patients with cardiovascular disease, as well as in healthy individuals.20–26 Here, we provide molecular, cellular, ex vivo, and in vivo data demonstrating direct inhibition of DDAH activity by PPIs. These data compel additional clinical investigations and pharmacovigilance directed toward understanding the cardiovascular risk associated with use of the PPIs in the general population.

Methods

Molecular and Biochemical Studies

High-Throughput Screening for DDAH Inhibitors

Recombinant human DDAH1 was generated and purified as previously described.27 A DDAH activity assay for high-throughput chemical screening was used to screen a library of 130,000 small molecules in the Stanford High Throughput Bioscience Center.27 Primary hits (inhibitors of DDAH activity) were validated using orthogonal biochemical assays (both colorimetric and fluorimetric activity assays) as we described previously.27 For the binding study described below, recombinant human DDAH1 purification was modified to include HEPES buffer elution (containing 10 mmol/L HEPES in PBS) to avoid competing amine groups during protein coupling.

Enzyme–Drug Binding Studies

To evaluate enzyme–drug binding and to study the nature of the interaction, we used surface plasmon resonance. First, recombinant human DDAH1 protein was amine coupled to a CM5 sensor chip. A vehicle (dimethyl sulfoxide) or PPI (omeprazole), dissolved in stock dimethyl sulfoxide, was diluted in phosphate buffer (100 mmol/L Na2HPO4; pH 6.5). The affinity of the PPI to recombinant human DDAH1 was monitored in real time by sensorgrams that reflect the binding of the compound with the coupled protein. The binding study was performed at 4 different biochemically relevant concentrations (12.5–100 μmol/L) or a known DDAH inhibitor (L-257)29 for 3 hours before switching to fully supplemented DMEM (supplemented with 10% FBS, 4 mmol/L HEPES, and penicillin/streptomycin; pH 7.6) at 37°C/5% CO2 until 24 hours. The cells were then washed and lysed, and the total cell lysate was recovered for estimation of protein concentration with the Coomassie Plus protein assay27 demonstrating direct inhibition of DDAH activity by PPIs. These data compel additional clinical investigations and pharmacovigilance directed toward understanding the cardiovascular risk associated with use of the PPIs in the general population.

The Effect of PPIs on Human Endothelial Cells

Human microvascular endothelial cells31 were plated on cell culture flasks in serum-free DMEM and treated with vehicle or PPIs (omeprazole or lansoprazole at 20-μmol/L final compound concentration) or a known DDAH inhibitor (L-257)29 for 3 hours before switching to fully supplemented DMEM (supplemented with 10% FBS, 4 mmol/L HEPES, and penicillin/streptomycin; pH 7.6) at 37°C/5% CO2 until 24 hours. The cells were then washed and lysed, and the total cell lysate was recovered for estimation of protein concentration with the Coomassie Plus protein assay27 for ADMA measurement. Similarly, the intracellular NO level was measured from cell lysates of primary microvascular endothelial cells with the Griess assay. In addition, the lysate (20 μg each) was also used to study the protein expression of DDAH1 (Abcam, Cambridge, MA), DDAH2 (Abcam), endothelial NOS (eNOS; BD Biosciences, San Jose, CA), and phosphorylated eNOS (Cell Signaling, Danvers, MA).27

In some studies, nitrogen oxides were measured in the conditioned medium from human saphenous vein segments harvested during coronary artery bypass graft surgery.26 The vessel segment was rinsed in Ringer lactated solution and then dissected into 0.5-cm sections before the pieces were transferred into each well of a 12-well plate containing endothelial cell media. The vessels were either kept under basal conditions or stimulated with the calcium ionophore A23187 (0.5 μmol/L) for 24 hours in the presence of omeprazole (3–100 μmol/L), L-257 (100 μmol/L), or vehicle. Release of NO into the conditioned media was measured with the Griess assay.

The Effect of PPI on Vascular Reactivity Ex Vivo

The effect of omeprazole on vascular reactivity was studied by isolating mouse thoracic aorta for functional studies as described.27 In brief, aorta was isolated from 10-week-old C57BL/6J mice, and the rings were mounted in myograph at a resting tension. Vessels that manifested reproducible relaxation in response to acetylcholine were either maintained in basal conditions or stimulated with the calcium ionophore A23187 (10−4 mol/L) or sodium nitroprusside (10−5−10−4 mol/L). Response curves to phenylephrine (force) or acetylcholine or sodium nitroprusside (percent relaxation) were plotted.

The Effect of a PPI In Vivo

The effect of a typical PPI, lansoprazole, on serum ADMA levels was investigated in male C57BL/6J mice (20 weeks old; Jackson Laboratories; Bar Harbor, ME). Animals were randomized to receive lansoprazole (30 mg·kg−1·d−1 lansoprazole; n=8) or vehicle (0.5% carboxymethylcellulose; n=8) by subcutaneous injection daily for 5 weeks.26 Whole blood was collected by tail clamping at baseline and weekly for ADMA measurement. At the time of death, a blood sample was collected by cardiac puncture. Serum was separated by centrifugation of the whole blood at 6000 rpm for 20 minutes at 4°C for measurement of ADMA as described above.

The respective animal studies described above were approved by the Imperial College London and Stanford University Administrative Panel on Laboratory Animal Care.

Statistical Methods

The number of animals needed in each study group was calculated using power and sample size calculation (PS version 3.0.14, Vanderbilt University). The in vivo experiment was designed to detect a difference in the experimental and control means (δ) of 0.27 with an estimated standard deviation (σ) of 0.18 at a significance level (α) of 0.05 with 80% power (β). Unless stated otherwise, all other statistical tests described in the study were performed with GraphPad Prism version 5 (GraphPad, La Jolla, CA). Data analysis was performed with 1-way ANOVA followed by Bonferroni post hoc correction. Unpaired Student t test was used to compare 2 groups. Statistical significance was noted at a value of P<0.05.
Results

High-Throughput Screen Identifies PPIs as DDAH Inhibitors

We screened ∼130,000 small molecules in the Stanford High Throughput Bioscience Center to search for modulators of DDAH activity. The enzymatic activity of DDAH was monitored with colorimetric and fluorometric assays as described. This screen identified ∼200 small molecules that inhibited DDAH by >30%. We were surprised to find among our hits 4 members of the PPI class (omeprazole, pantoprazole, lansoprazole, and tenatoprazole). Subsequently, these positive hits and additional members of the class (esomeprazole and rabeprazole) were validated with freshly prepared compounds and orthogonal assays as follows.

PPIs Directly Inhibit Human DDAH1 Activity

Using a microplate assay, we monitored the enzymatic activity of DDAH biochemically. In this assay, ADMA degradation by DDAH was examined by detecting the product (l-citrulline). In brief, recombinant human DDAH1 was mixed with ADMA in a 384-well format, and l-citrulline formation was quantified after the enzyme-substrate mix was incubated with the PPIs and color-developing reagent was added. The inhibitory activity of each of the PPIs was confirmed by use of a full-dose range of the agents. From these data, we calculated the IC\text{50} of each agent, as shown in the Table. These studies validated that the direct inhibition of DDAH by the PPIs (Figure 1) was a class effect (Figure 2A). These results were further confirmed with an orthogonal fluorometric assay (Figure 2B).

PPIs Bind to Purified Human DDAH1 Reversibly

The surface plasmon resonance study showed that omeprazole, but not vehicle, generated sensorgram signals, indicating a direct interaction between the PPI and DDAH (Figure 3). As expected, the vehicle control, serially diluted dimethyl sulfoxide, did not show binding. Moreover, in the enzymatic studies, we found rapid and almost complete reversibility of omeprazole inhibition of DDAH enzymatic activity when serially diluted (Figure I in the online-only Data Supplement). These data are consistent with a recent report that high levels of omeprazole reduced cellular NO release while increasing superoxide anion generation. The PPI enhanced an increase in cellular ADMA would be expected to reduce the activity of NOS. Indeed, omeprazole dose-dependently reduced the levels of nitrogen oxides in cultured endothelial cells (Figure 4B). In addition, we found that the expression of total eNOS and active eNOS (phospho-eNOS) in human microvascular endothelial cells was downregulated by omeprazole (Figure 5). Similarly, we found that omeprazole (and L-257) significantly inhibited nitric oxide release from isolated human saphenous veins in the presence or absence of an activator of NOS (Figure 6A and 6B). These data are consistent with a recent report that high levels of omeprazole reduced serum NO levels in an animal model of colorectal cancer.

The PPI Omeprazole Impairs Vascular Function

Omeprazole impaired vascular reactivity in a manner that was consistent with a reduction in NOS activity. The PPI enhanced

PPIs Increase Intracellular ADMA Concentration

We next studied the effect of PPIs (esomeprazole and lansoprazole) on intracellular ADMA in human endothelial cells. This study demonstrated that the PPIs increased intracellular ADMA (by ∼30%) compared with vehicle control. L-257 also increased ADMA as expected (Figure 4A). Notably, this effect of the PPIs was in the absence of any changes in DDAH expression. In brief, human microvascular endothelial cells were exposed to different concentrations of the PPI omeprazole (3–100 μmol/L) for 24 hours, and the protein expression of DDAH1 and DDAH2 was examined by Western blot as described. In this study, omeprazole did not regulate the expression of DDAH1 or DDAH2 (Figure II in the online-only Data Supplement). These data suggest that PPIs are able to increase intracellular levels of ADMA in endothelial cells, most likely by inhibiting DDAH activity (but not expression).

PPIs Reduce Intracellular NO Level

An increase in cellular ADMA would be expected to reduce the activity of NOS. Indeed, omeprazole dose-dependently reduced the levels of nitrogen oxides in cultured endothelial cells (Figure 4B). In addition, we found that the expression of total eNOS and active eNOS (phospho-eNOS) in human microvascular endothelial cells was downregulated by omeprazole (Figure 5). Similarly, we found that omeprazole (and L-257) significantly inhibited nitric oxide release from isolated human saphenous veins in the presence or absence of an activator of NOS (Figure 6A and 6B). These data are consistent with a recent report that high levels of omeprazole reduced serum NO levels in an animal model of colorectal cancer.

The asymmetrical dimethylarginine (ADMA) pathway. ADMA is derived from proteins (largely nuclear) containing methylated arginine residues. ADMA is largely (80%) metabolized by dimethylarginine dimethylaminohydrolase (DDAH). ADMA is a competitive inhibitor of nitric oxide (NO) synthase (NOS). Endothelial NOS (eNOS) is highly regulated and produces small amounts of NO locally to affect vascular homeostasis. Increased levels of ADMA (such as through possible inhibition by the proton pump inhibitors [PPIs]) could impair eNOS activity, reducing NO generation while increasing superoxide anion generation. The vasoprotective action of eNOS is lost, increasing the risk for adverse vascular events. In this setting, inflammatory cells are attracted into the vessel wall and express inducible NOS (iNOS), which generates superoxide anion and nitric oxide, which combine to form the cytotoxic free radical peroxynitrite anion. L-NMMA indicates N\textsuperscript{\textbeta}-monomethyl-l-arginine; and PRMTs, protein arginine methyltransferases.

Table. Determination of the IC\text{50} of PPIs

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC\text{50} μmol/L</th>
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<tbody>
<tr>
<td>Lansoprazole</td>
<td>51±3</td>
</tr>
<tr>
<td>Esomeprazole</td>
<td>52±5</td>
</tr>
<tr>
<td>Rabeprazole</td>
<td>53±5</td>
</tr>
<tr>
<td>Omeprazole</td>
<td>58±5</td>
</tr>
<tr>
<td>Tenatoprazole</td>
<td>61±1.5</td>
</tr>
<tr>
<td>Pantoprazole</td>
<td>63±5</td>
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The IC\text{50} of each of the PPIs was determined by use of an 8-point concentration range (0.78–100 μmol/L). Data are mean±SEM from triplicate values per group. IC\text{50} indicates half-maximal concentration; PPI, proton pump inhibitor.
the contraction to phenylephrine and blunted the relaxation to acetylcholine but did not affect the (endothelium-independent) vasorelaxation to sodium nitroprusside (Figure 7). These findings are consistent with a reduction in eNOS activity resulting from an accumulation of ADMA.33

**Lansoprazole Increases Circulating Levels of ADMA In Vivo**

We also studied the effect of lansoprazole on serum ADMA levels in mice. As early as 1 week after lansoprazole administration, we observed an increase in serum ADMA. This increase of ≈20% in the lansoprazole-treated group compared with the vehicle control was sustained throughout the 5-week study period (Figure 8). It is known that rodent DDAH shares >90% homology with the human isoforms37; therefore, the increase in circulating ADMA is likely mediated by an inhibition of DDAH activity in vivo.

**Discussion**

We find that PPIs as a class directly bind to and inhibit the activity of DDAH, the enzyme that degrades ADMA. This effect of the PPIs explains our subsequent observation that PPIs increase ADMA concentration in cultured human endothelial cells in association with a reduction in NO synthesis. Similarly, the PPI omeprazole reduced NO generated by human saphenous vein segments ex vivo. In addition, PPIs impaired endothelium-dependent vasodilation in isolated murine vessels. Furthermore, lansoprazole34 administered...
by subcutaneous injection increased serum ADMA levels in mice by ≈20%.

ADMA is an emerging risk factor for cardiovascular disease.20–26 Accordingly, an increase in plasma ADMA induced by PPIs may potentially explain the association of PPIs with cardiovascular events in patients with unstable coronary syndromes (ACS). Of perhaps greater concern, an elevation of plasma ADMA of this magnitude, if the data are translated to humans, might increase the hazard ratio for MACEs and mortality in adults not recognized to have cardiovascular disease. Our study in human endothelial cells showed an increase in ADMA levels by ≈30%, an elevation that is reported to significantly increase MACEs in humans. However, the dose we used (20 μmol/L) is 5- to 10-fold higher than the plasma concentration obtained with a typical oral dose of PPI. For example, a single oral dose of 30 mg lansoprazole would produce a maximum plasma concentration of 2 to 6 μmol/L, within ∼3 hours.38 Nevertheless, repeated dosing of PPIs to attain consistent suppression of gastric acidity19 could impair normal vascular endothelial function.

ADMA as a Risk Factor for Cardiovascular Disease

Indeed, previous studies have reported that a modest increase in plasma ADMA is associated with an increased risk for MACEs and mortality in patients with cardiovascular disease and in healthy ambulatory individuals.20,23,25,26 In patients with coronary or peripheral arterial disease, individuals in the upper tertile of plasma ADMA are more likely to incur MACEs and to have reduced longevity. In the AtheroGene study, ADMA (but not C-reactive peptide) was predictive of MACEs.39 Plasma ADMA is also a risk factor for the general population, as indicated by longitudinal community-based studies. In the Population Study of Women in Gothenburg, the top quintile of ADMA (≥0.71 μmol/L) was associated with a relative risk of 1.75 after adjustment for traditional cardiovascular risk factors, renal function, and homocysteine.40 In this study, an increase in plasma ADMA of 0.15 μmol/L (≈20% to 30%) increased MACEs by 30% over 24 years. Similar results were observed in the Framingham Offspring Study.23 Thus, the ADMA elevation that we observed in normal mice treated with PPI is of a magnitude that would significantly increase cardiovascular risk in a human. The PPI-induced elevation in a patient may even be larger if there is a loss of DDAH reserve resulting from the vascular oxidative stress of metabolic perturbations, as we have previously described.41 We have initiated a clinical study to determine the effect of PPIs on ADMA levels and endothelial function in healthy subjects and those with cardiovascular disease to directly address these questions.

Mechanisms of ADMA Elevation

ADMA is derived from the hydrolysis of proteins containing methylated arginine residues (Figure 1). Subsequently, ≈80% of ADMA is degraded by DDAH, and the remainder is excreted in the urine. Individuals with renal insufficiency have elevated plasma ADMA levels, and the magnitude of this elevation is correlated with low estimated glomerular filtration rate and is a predictor for MACEs and mortality in individuals with chronic kidney disease.41–44 However, it appears that the most common cause of plasma ADMA elevation is an impairment of DDAH activity. Because it contains a sulfhydryl moiety in its catalytic pocket,45–49 DDAH is highly sensitive to oxidative stress.50 Metabolic perturbations such as hyperlipidemia, hyperglycemia, and hyperhomocysteinemia increase endothelial oxidative stress and impair endothelial DDAH activity, impairing its degradation...
of ADMA.49-54 Endothelial and systemic levels of ADMA increase and contribute to impairment in eNOS. The impairment of eNOS is associated with an increase in oxidative stress and a dysregulation of vasomotor tone.55 Furthermore, given the anti-inflammatory and antiplatelet effects of endothelium-derived NO,6,7 the impairment of eNOS would be anticipated to increase the risk of MACEs, as suggested by studies of coronary and brachial artery vasoreactivity.18 To be sure, inducible NOS activation in the vessel wall may play a role in the pathophysiology of atherosclerosis, and ADMA would inhibit the pathological activity of this enzyme. Nevertheless, the aggregate effect of an increase in plasma ADMA appears to increase cardiovascular risk.

In addition to acquired impairment of DDAH activity, there is emerging evidence for genetic deficiency of the pathway. In the Kuopio Ischemic Heart Disease Risk Factor Study (KIHDS), Finnish men with a functional polymorphism of the DDAH1 gene had elevated plasma ADMA, a 50-fold increased risk for coronary heart disease, and a 5-fold increase in the prevalence of hypertension compared with noncarriers.59 Furthermore, retrospective analyses of clinical samples from stroke and coronary heart disease patients identified functional genetic polymorphism in the DDAH1 promoter, resulting in an \( \approx 40\% \) reduction in the transcriptional activity of DDAH in endothelial cells and subsequent elevation in plasma ADMA (by \( \approx 30\% \) to \( 40\% \)) compared with control subjects. This loss of function has been associated with significantly increased risk of stroke and coronary artery disease (by \( \approx 40\% \) each) even after adjustment for traditional risk factors.60 In addition, DDAH polymorphisms have been correlated to ADMA levels in diabetic patients61 and to susceptibility to preeclampsia.62 Although encouraging, these small studies require confirmation in genome-wide association studies.

**Proposed Mechanism for DDAH Inhibition by PPIs**

The action of PPIs is dependent on a covalent and irreversible inhibition of the proton (H+/K+ ATPases) pump of parietal cells in the stomach. The PPIs become positively charged (sulfenic acid derivatives) on interaction with gastric acid in the stomach and covalently and irreversibly bind to active-site cysteines. Interestingly, the biochemical enzymatic assays and cell culture studies in the present study were conducted at nearly physiological pH (6.0–7.6). The DDAH inhibitory activity at this nonacidic pH indicates that the PPIs do not necessarily need to be converted to an activated form, as seen during the inhibition of gastric pumps, to interfere with the DDAH pathway.

**DDAH possess a highly conserved catalytic triad containing a critical cysteine (Cys) residue (Cys273 in DDAH1 and Cys276 in DDAH2).**37 The reactive cysteine residue is crucial in the metabolism of ADMA, forming a transient covalent bond with the carbon in the guanidino residue of the substrate.35 Site-directed mutagenesis study revealed that substitution of the catalytic cysteine by alanine abolishes the catalytic activity of the enzyme.48 Because our Biacore and inhibitor-dilution study show rapid and nearly complete reversibility of the PPIs and subsequent recovery of DDAH enzymatic activity, it seems likely that the interaction of DDAH with a PPI is noncovalent. However, increasing substrate concentration of the reaction appears to influence the inhibitory activity of PPIs, suggesting that although reversible, their mode of inhibition might still be through interaction with the active site of DDAH but likely apart from Cys273. To understand the precise mechanism of interaction, we are resolving the structure of DDAH cocrystallized with a PPI.

**Role of DDAH Inhibition in the Potential Adverse Cardiovascular Effects of PPIs**

Our proposed biological mechanism for the association between PPIs and MACEs is more consistent with the available human data than previously proposed drug-drug interactions. Although several of the PPIs may inhibit the hepatic enzyme CYP2C19, which activates clopidogrel, other antiplatelet agents not dependent on such activation (eg, ticagrelor) also manifest diminished efficacy when combined with a PPI, even after adjustment for confounding effects.15 Furthermore, it is unlikely that the PPI-induced change in gastric pH is impairing absorption or action of antiplatelet agents because a similar reduction in intragastric pH is achieved with the H2 receptor antagonist without increased cardiovascular risk.14,17

Thus, a PPI-induced impairment of DDAH activity, with subsequent dysregulation of vascular NOS, may be a more likely explanation for the association with MACEs and mortality. The present report may heighten the concerns of the US Food and Drug Administration about the possible association of PPIs with MACEs.63 Consistent with this hypothesis, it is worth noting that drugs that reduce circulating levels of ADMA such as angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, and insulin sensitizers24,64-67 are associated with a reduction in cardiovascular risk.

Finally, given the experimental findings, we recently used a novel data-mining approach to examine the risk of MI in patients with gastroesophageal reflux disease treated with either a PPI or an H2 receptor antagonist independently of
Conclusions
We provide biochemical, cellular, ex vivo, and in vivo data revealing that commonly prescribed PPIs directly interact with and significantly inhibit human DDAH activity, thereby increasing endothelial and serum ADMA levels. The increase in ADMA levels would be anticipated to impair vascular NOS activity, to increase oxidative stress, to reduce vasodilator function, and to impair vasoprotective mechanisms. Such disruption of vascular homeostasis may explain the increased MACEs and mortality associated with the prolonged use of PPIs in large clinical trials of patients with ACS. Of concern is the effect that a long-term elevation of ADMA levels may have on the general population using PPIs. Taken together, our preclinical and epidemiological observations raise serious concerns that should be actively and urgently explored to delineate the potential cardiovascular risk associated with use of the PPIs in the general population. However, it is important to recognize that the present study cannot establish a cause-and-effect relationship between PPI use and elevation of cardiovascular risk in ACS patients or the general population. This is rather a hypothesis-generating observation that warrants further prospective investigation.

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Disclosures
Drs Cooke and Ghebremariam are inventors on patents owned by Stanford University that protect the use of agents that modulate the NOS/DDAH pathway therapeutically. The other authors report no conflicts.
References

In several clinical trials, the use of proton pump inhibitors appeared to increase the risk of major adverse cardiovascular events in patients with acute coronary syndromes. These observations are controversial, and the mechanism of the adverse effects is obscure. This article provides evidence that proton pump inhibitors interfere with the clearance of asymmetrical dimethylarginine (ADMA), the endogenous antagonist of nitric oxide synthase. By increasing ADMA, proton pump inhibitors reduce the synthesis of nitric oxide in the mouse and in human vessels ex vivo. This effect of the proton pump inhibitors is concerning because endothelium-derived nitric oxide is critical for vascular health. Endothelium-derived NO is strongly and additively associated with serum ADMA concentrations in individuals with type 2 diabetes. Atherosclerosis. 2008;200:336–344.

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Figure S1

1hr at RT pre-incubation prior to 100-fold dilution with substrate containing buffer

100X DDAH
1X; 10X or 100X IC_{50} Inhibitor

1X DDAH
0.01X; 0.1X or 1X IC_{50} Inhibitor

% DDAH Activity

[Omeprazole]

DDAH (0.3 μM)

% DDAH Activity

[L-257]

DDAH (0.3 μM)

*
Supplemental Figure Legend:

**Figure-S1:** Dilution assay demonstrating reversible inhibition of DDAH activity by the PPI omeprazole (IC$_{50}$ ~ 60 µM). The graph shows recovery of DDAH activity upon dilution of the inhibitor. Data are Mean ± SEM of quadruplicates at each concentration (*p<0.05).

**Figure-S2:** PPIs do not regulate endothelial DDAH expression. The influence of PPIs on the expression of endothelial DDAH1 and DDAH2 proteins was studied by Western blot using lysate (20 µg per lane) from endothelial cells exposed to the PPI omeprazole or vehicle. The expression was normalized to β-actin (ACTB).