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EFFECTS OF ASPIRIN& SIMVASTATIN AND ASPIRIN, SIMVASTATIN & LIPOIC ACID ON HEME OXYGENASE-1 IN HEALTHY HUMAN SUBJECTS

Adil E. Bharucha, M.B.B.S., M.D.¹, Kyoung Moo Choi¹, Jessica Saw², Simon J. Gibbons¹, Gianrico Farrugia, M.D.¹, David Carlson⁴, and Alan R Zinsmeister, Ph.D.³

¹Enteric Neurosciences Program, Division of Gastroenterology and Hepatology, Mayo Clinic and Mayo Foundation, Rochester, Minnesota, USA

²Mayo Medical School, Mayo Clinic and Mayo Foundation, Rochester, Minnesota, USA

³Division of Biomedical Statistics and Informatics, Mayo Clinic and Mayo Foundation, Rochester, Minnesota, USA

⁴GeroNova Research, Inc. Richmond, Ca

SUMMARY

Background—Heme-oxygenase 1 (HO-1) degrades heme and protects against oxidative stress. In vitro and animal models suggest that HO-1 is beneficial in several diseases (e.g., post-operative ileus, gastroparesis, acute pancreatitis, and colitis). However the only drugs (i.e., heme and heme arginate) which pharmacologically up-regulate HO-1 in humans are expensive and can only be administered intravenously. Our aims were to compare the effects of placebo, aspirin and simvastatin alone, and with α -lipoic acid, on HO-1 protein concentration and activity in humans.

Methods—This randomized, double-blind, placebo-controlled study compared the effects of 3 oral regimens administered for 7 days, ie, placebo; aspirin (325 mg twice daily) and simvastatin (40 mg twice daily); aspirin, simvastatin, and the sodium salt of R- α -lipoic acid (NaRLA, 600 mg three times daily) on markers of HO-1 activation (i.e., plasma HO-1 protein concentration and venous monocyte HO-1 protein activity) in 18 healthy subjects (14 females). Markers of HO-1 activation were evaluated at baseline, days 2 and 7.

Key Results—Baseline HO-1 protein concentrations and activity were similar amongst the three groups. Compared to placebo, aspirin and simvastatin combined, or together with NaRLA did not

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Address for correspondence and reprint requests: Adil E. Bharucha, M.D., Clinical Enteric Neuroscience Translational and Epidemiological Research Program (C.E.N.T.E.R.), Mayo Clinic, 200 First St. S.W., Rochester, MN 55905, Telephone: 507-538-5854, Fax: 507-538-5820, bharucha.adil@mayo.edu.

DISCLOSURES

NaRLA and placebos were donated by courtesy of GeroNova Research, Inc. DC has a financial relationship with (is employed by) GeroNova Research, Inc, which might have an interest in the submitted work.

AEB performed the research

AEB, GF, and DC designed the research study

KMC, SJG, and GF analyzed the blood samples for HO-1 protein concentration and activity.

ARZ conducted the statistical analysis.

JS, AEB, and DC wrote the paper.

All authors approved the final version of this manuscript.

affect HO-1 protein concentration or activity at 2 or 7 days. HO-1 protein concentrations and activity were correlated on day 7 ($r = 0.75$, $p = 0.0004$) but not at baseline and on day 2.

Conclusions—At therapeutic doses, aspirin, simvastatin, and α -lipoic acid do not increase plasma HO-1 protein concentration or venous monocyte HO-1 activity in healthy humans.

Keywords

aspirin; simvastatin; alpha lipoic acid; hemeoxygenase; humans; HO-1

Heme oxygenase-1 (HO-1), is the inducible isoform of the rate-limiting enzyme that catabolizes heme, mitigates oxidative stress, injury, and inflammation (1). These beneficial effects are exerted via several mechanisms, including removal of free heme, which is cytotoxic, and converting it into equimolar amounts of free iron (Fe^{2+}), biliverdin and carbon monoxide (CO) (2). Both biliverdin and its by product bilirubin have anti-inflammatory and antioxidant properties as does CO, which also exerts vasorelaxant effects. HO-1 is an inducible heat-shock protein that can be up-regulated by a variety of cellular stressors (1).

HO-1 has beneficial effects in vitro and animal models (2) of several diseases, eg, heme-induced acute renal injury due to rhabdomyolysis and cisplatin nephrotoxicity (3), atherosclerosis (4), various gastrointestinal disorders (e.g., post-operative ileus, radiation-induced intestinal injury, gastroparesis, acute pancreatitis, and colitis (5), vascular proliferation (6), and multiple sclerosis (7). In non-obese diabetic mice, M2 macrophages that express HO-1 prevented diabetes-induced delayed gastric emptying (8). Hemin induced HO-1, reduced reactive oxygen species, rapidly restored Kit, which is a marker of interstitial cells of Cajal, and neuronal nitric oxide synthase expression, and completely normalized gastric emptying in that model (9). Thus, induction of HO-1 might be a therapeutic option for patients with diabetic gastroparesis.

However, there are only 2 reports describing pharmacological up-regulation of HO-1 in humans, i.e., in healthy subjects, by hemin (8) and by heme arginate (10), which are expensive and can only be administered intravenously. There is an urgent need to identify compounds that can be administered orally, are safe, inexpensive, and can increase HO-1 activity in humans. After eliminating compounds (e.g., probucol, rapamycin, and heavy metals), which have toxic effects limiting use in humans (11), we selected 3 compounds i.e., aspirin, simvastatin, and α -lipoic acid, which have been shown to induce HO-1 in vitro, for further assessment in humans.

Aspirin induces HO-1 by prostaglandin-independent actions, which involve more than one signalling pathway, and are partly mediated by its metabolite 15-epi-lipoxin A4, as well as endothelial NO synthase and NO; this effect is not shared by other non steroidal anti-inflammatory drugs (NSAIDs), such as indomethacin (12). Up-regulation of HO-1 by statins in vitro is tissue specific, dose dependent (13), and may explain the anti-inflammatory effects of simvastatin in humans (14–18). R-(+)- α lipoic acid (R-LA) is a naturally occurring antioxidant found in foods (eg, organ meats, broccoli, and spinach) and is widely available as a dietary supplement. The racemic form, RS (+/-)-LA is unnatural and has been

used as an experimental drug treatment for many maladies, including diabetic neuropathy (19, 20). Both RS-LA and RLA activate the transcription factor (Nrf2), which is a potent inducer of HO-1 expression; RS-LA induced HO-1 expression in human monocytes, in vitro (21, 22).

Since aspirin, simvastatin and LA individually up-regulate HO-1 in various models, the hypothesis of this study was that combinations of aspirin and simvastatin or aspirin, simvastatin and RLA can safely and possibly synergistically increase HO-1 expression to levels consistent with a therapeutic response in healthy human subjects.

MATERIALS AND METHODS

Subjects

Eighteen healthy subjects, aged 23 to 63 years (mean age 40 ± 3 years; 14 female, mean body mass index 24.5 ± 0.78 kg/m²), were recruited by public advertisement and provided written consent to participate in this study which was approved by the Mayo Clinic Institutional Review Board. A gastroenterologist interviewed and examined all subjects to ensure they were healthy lifetime non-smokers and did not have any of the following: clinical evidence of significant cardiovascular, gastrointestinal, hematological, neurological, psychiatric or other disease that might interfere with the objectives of the study and/or pose safety concerns, symptoms of a functional gastrointestinal disorder by questionnaire (23), prior gastrointestinal surgery other than appendectomy, cholecystectomy, hysterectomy, tubal ligation, or inguinal hernia repair, medication use (except for stable doses of birth control pill, L-thyroxine, or estrogen replacement therapy) prior hypersensitivity to simvastatin or significant gastrointestinal side effects with aspirin. In addition, subjects with abnormal serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and bilirubin at baseline were excluded.

Study Design

In this double-blind placebo-controlled pilot study, subjects were randomized to one of 3 regimens (i.e., placebo; aspirin plus simvastatin; aspirin plus simvastatin, plus sodium salt of RLA [NaRLA]) in 18 healthy subjects. After fasting overnight, subjects were randomized to receive one of the three regimens and were instructed not to consume alcohol, vitamin C (grapefruit and orange juice), multivitamins, or any medications except as listed above. All participants were instructed to take 3 medications (or corresponding placebo tablets) daily for 7 days. The following fasting blood tests were checked at baseline, 2, and 7 days after starting medications: (i) markers of HO-1 activation i.e., plasma HO-1 protein concentration, venous monocyte HO-1 protein activity, and serum bilirubin and (ii) serum aspartate (AST) and alanine aminotransferase (ALT) were assessed to identify hepatic and muscle toxicity due to simvastatin. On days 2 and 7, blood samples were drawn 4 hours after taking medications.

Medications

All medications or matching placebo were administered with meals for 7 days: simvastatin (40 mg twice daily, Lupin Pharmaceuticals), enteric coated aspirin (325 mg twice daily,

Major Pharmaceuticals), and NaRLA (600 mg capsules three times daily, GeroNova Research, Inc). RLA was administered as NaRLA because plasma concentrations of α lipoic acid are considerably higher when it is administered in this form than as RS-LA, suggesting the possibility of a stereoselective transport absorptive mechanism which is more effective when S-ALA is absent (24). Because ascorbic acid can induce HO-1 activity in vitro (25), all multivitamins were discontinued for 1 week before the study.

Measurement of HO-1 Protein Concentration and HO-1 Activity

Plasma HO-1 protein concentrations and activity were measured as previously described (8). Briefly, for measuring plasma HO-1 protein, blood was collected in EDTA-containing tubes and centrifuged (1000g, 20 min, room temperature (RT) and HO-1 concentrations were determined from 100 μ L of plasma using an HO-1 (human) enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences, Farmingdale, NY). This assay can detect HO-1 in the range of 0.78 to 25 ng/mL of un-diluted plasma.

HO-1 activity was assessed by measuring enzyme activity in microsomes extracted from the buffy coat of peripheral blood cells (26) (27). This assay is an established cell-free system that uses purified microsomes and defined substrate concentrations and can detect HO-1 activity rates as low as 100 pmol bilirubin production/mg protein/h (27).

Statistical Analysis

Because there were no previous reports of the primary responses, the sample size was based on practical considerations. All subjects were included in this intent to treat analysis. Associations between HO-1 protein concentration and separately HO-1 activity and treatment group were assessed by Kruskal-Wallis test. Associations between HO-1 protein concentration and HO-1 activity were evaluated by Spearman's correlation coefficient. Data are reported as mean $\pm$ SEM.

RESULTS

Demographics

The distribution of age, gender, and BMI was comparable among study groups (Table 1). All subjects completed study assessments and none reported any side effects,

Effects on HO-1 Protein Concentration and Activity

Baseline HO-1 protein concentration levels and activity were similar among the three groups (Figures 1 and 2). Compared to placebo, aspirin and simvastatin combined, or together with NaRLA did not affect HO-1 protein concentration or activity at 2 or 7 days. HO-1 protein concentrations and activity were correlated on day 7 ($r = 0.75$, $p = 0.0004$) but not at baseline ($r = 0.02$, $p = 0.92$) or on day 2 ($r = 0.09$, $p = 0.73$).

Effects on Safety Parameters

Laboratory tests did not reveal any significant changes in serum transaminases, ALT and AST during therapy (Table 1).

DISCUSSION

Despite substantial evidence in experimental and animal models suggesting that increasing HO-1 is beneficial for preventing and/or treating various conditions (1, 2), there are only 2 studies showing that HO-1 activity can be pharmacologically increased in humans. These 2 studies evaluated hemin and heme arginate, which are expensive and can only be administered intravenously (8, 10). Aspirin, simvastatin, and LA individually increased HO-1 activity in vitro (28–32). However, in contrast to hemin and heme arginate, this combination did not increase plasma markers of HO-1 protein concentration and activity in this study. The lack of an effect on plasma HO-1 markers does not exclude the possibility of local effects, for e.g., on gastric mucosa or in disease states. Indeed, aspirin and other NSAIDs have been shown to up-regulate HO-1 in cultures of guinea pig gastric mucosal cells (33).

A previous uncontrolled study reported an approximately two-fold increase in HO-1 activity after treatment with aspirin at doses ranging from 81 mg to 1300 mg daily for 12 weeks in 70 patients with metabolic syndrome. However, that study was uncontrolled and dose effects were not statistically significant (34). In the present study, which was limited to healthy subjects, HO-1 concentration and activity also increased by a small amount even in subjects treated with placebo, which highlights the need for controlled studies and also highlights the inducibility of HO-1. Venipuncture and a large number of compounds may induce low level expression of HO-1 (2) and may explain the small increase noted in the placebo group. The significant correlation between HO-1 protein concentration and activity on day 7 also supports the inference that the observed increase in HO-1 expression on day 7 was real. We do not have a robust explanation for the lack of a correlation between HO-1 protein concentration and activity on day 2. Perhaps protein concentrations in some samples were low; hence HO-1 activity was close to the sensitivity of the activity assay. HO-1 activity and protein were measured in the buffy coat and plasma respectively. HO-1 is not secreted but is found in plasma as are several other non-secreted proteins (35). Plasma concentrations of HO-1 are correlated with HO-1 induction and are an accepted method to measure HO-1 (35). Plasma bilirubin and venous carboxyhemoglobin concentrations were not measured because hemin increased HO-1 activity by approximately 15-fold but did not affect serum bilirubin or venous carboxyhemoglobin concentrations (8).

In this study, plasma HO-1 concentrations were within the range of 1.0–1.9 ng/mL (IQR), reported for normal healthy subjects (36), even after starting treatment. The observed effects of placebo on HO-1 protein and activity were much smaller than the effects of hemin, which increased HO-1 protein concentrations and activity by approximately 5-fold and 15-fold respectively (8). As a therapeutic target, the goal should be to induce HO-1 to a factor at least 5 fold above baseline, which is the magnitude associated with resistance to oxidative injury, disease or hemin treatment (8, 36, 37). The assays of HO-1 protein concentration and activity utilized in this study are sufficiently sensitive to detect small and larger changes making it highly unlikely that a larger change was missed. It seems unlikely that these compounds negated each other's effects.

The dosages used in this study were designed to achieve therapeutic plasma concentrations without compromising safety. However, these dosages were generally lower than those used in animal and cell-based studies of HO-1 (12, 32, 38, 39). In animal studies, a similar duration of treatment increased HO-1 protein concentrations, often within 24 hours. While subject to the caveat that there are differences in the bioavailability and metabolism of drugs between humans and animals, these observations suggest it is unlikely that the duration of therapy was insufficient. In large clinical trials, the racemic mixture, RS-LA reduced neuropathic deficits and pain in diabetic neuropathy (40). These trials administered the RS-LA intravenously (i.e., 600 mg i.v. daily) or orally (i.e., 600 mg up to tid) (19, 20). This study employed the sodium salt of the R-enantiomer of LA, which due to high water solubility and rapid absorption provides higher plasma concentrations than the free acid form of RS-LA when administered orally (24). Hence, this regimen of LA should have provided equivalent if not higher concentrations of R-LA than those that were effective in diabetic neuropathy.

In summary, compared to placebo, aspirin and simvastatin, and aspirin, simvastatin and, RLA did not induce HO-1 in healthy human subjects. These findings reinforce the importance of verifying in vitro data in humans and the need to identify other pharmacological modulators of HO-1. Further studies with other pharmacological agents and combinations known to increase HO-1 activity and expression in vitro and animal models, for example probucol (41), interleukin-10 (42), and bardoxolone (43) are necessary to identify other drugs with the potential to induce HO-1 activity in healthy humans.

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Key Messages

General statement - This is important because Heme oxygenase-1 (HO-1) mitigates oxidative stress, injury, and inflammation. HO-1 has beneficial effects in animal/in vitro models of acute renal injury, atherosclerosis, post-operative ileus, gastroparesis, acute pancreatitis, and colitis. The only known pharmacological activators of HO-1 in humans (heme derivatives) are expensive; orally-administered HO-1 modulators are necessary. Hence, safe, effective, and inexpensive methods for modulating HO-1 in humans are urgently required. While aspirin, simvastatin and α lipoic acid activate HO-1 in vitro models, the present study demonstrates that at therapeutic doses, aspirin, simvastatin and α lipoic acid do not upregulate HO-1 activity in healthy humans.

Figure 1.

Effect of aspirin, simvastatin, alpha lipoic acid and placebo on venous plasma HO-1 protein concentrations (mean $\pm$ SEM). There were no significant differences amongst the three groups.

Figure 2.

Effect of aspirin, simvastatin, alpha lipoic acid and placebo on venous plasma HO-1 activity plotted (mean $\pm$ SEM). There were no significant differences findings amongst the three groups.

Table 1

Demographics Features and Drug Effects on Efficacy and Safety Parameters

	Aspirin/Simvastatin/Placebo	Aspirin/Simvastatin/R-ALA	Placebo/Placebo/Placebo
N	6	7	5
Gender (No of females)	5	5	4
Age, yr	32.2 ± 4.5	39.1 ± 5.5	30.2 ± 6.0
BMI, kg/m ²	24.4 ± 1.1	23.8 ± 1.4	25.7 ± 1.6
Hemoglobin, gm/dl, Baseline	12.65 ± 0.19	13.16 ± 0.30	13.44 ± 0.70
Hemoglobin, gm/dl, Day 2	12.83 ± 0.22	13.23 ± 0.37	13.52 ± 0.68
Hemoglobin, gm/dl, Day 7	11.9 ± 0.31	12.86 ± 0.27	12.76 ± 0.42
Bilirubin (total/direct), mg/dl, Baseline	0.62 ± 0.12/0.15 ± 0.02	0.61 ± 0.07/.14 ± 0.02	0.50 ± 0.06/0.14 ± 0.02
Bilirubin (total/direct), mg/dl, Day 2	0.78 ± 0.17/0.2 ± 0.04	0.71 ± 0.15/0.16 ± 0.03	0.56 ± 0.11/0.13 ± 0.02
Bilirubin (total/direct), mg/dl, Day 7	0.57 ± 0.11/0.17 ± 0.03	0.57 ± 0.07/.16 ± 0.02	0.42 ± 0.06/0.1 ± 0
AST, IU/L, Baseline	24.5 ± 1.57	21.29 ± 1.58	24.2 ± 1.98
AST, IU/L, Day 7	23.33 ± 1.02	21.00 ± 1.75	22.6 ± 2.01
ALT, IU/L, baseline	23 ± 2.50	17.57 ± 2.75	18.4 ± 2.62
ALT, IU/L, Day 7	18.5 ± 2.25	19.14 ± 2.32	19 ± 2.39