Original Article

Reduced Expression of Heme Oxygenase-1 in Patients with Coronary Atherosclerosis

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Heme oxigenase-1 (HO-1) is known to be an inducible cytoprotective enzyme that copes with oxidative stress. However, changes in HO-1 expression and their association with human diseases have not been studied. To test the hypothesis that the capacity to upregulate HO-1 in response to oxidative stress is an intrinsic marker for susceptibility to coronary atherosclerosis, we assessed stimulation-induced change in HO-1 expression in blood cells in 110 patients who underwent coronary angiography, comparing the results with the extent of coronary atherosclerosis and $(GT)_n$ repeat polymorphism in the HO-1 gene promoter region, which is believed to affect the gene expression level. The extent of coronary atherosclerosis was assessed by coronary score. Mononuclear cells were incubated with 10 μmol/l hemin or vehicle for 4 h to maximally stimulate HO-1 expression, then the HO-1 expression level was determined by real-time polymerase chain reaction (PCR). The difference between the HO-1 mRNA levels of hemin- and vehicle-treated cells (*Δ*HO-1 mRNA) was taken as an index of the capacity to upregulate HO-1 mRNA. The coefficient of variance of *Δ*HO-1 mRNA was 7.2%. Consistent with previous studies, *Δ*HO-1 mRNA was significantly lower in patients carrying a long $(GT)_n$ repeat. *Δ*HO-1 mRNA negatively and significantly correlated with the coronary score (r^2=0.50, p<0.01). In conclusion, the capacity to upregulate HO-1 expression may be determined, at least in part, by genetics, and reduced ability to induce HO-1 may be involved in the mechanism of coronary atherosclerosis. (*Hypertens Res* 2007; 30: 341–348)

***Key Words*: atherosclerosis, hemoxygenase-1 gene expression, oxidative stress**

Introduction

Heme oxygenase (HO) is the rate-limiting enzyme catalyzing the cleavage of heme to free iron, biliverdin and carbon monoxide. Free iron is subsequently sequestered to ferritin, and biliverdin is rapidly converted to bilirubin (*1*, *2*). Two major isoforms of the enzyme have been identified in mammals (*3*): HO-1 and HO-2. HO-2 is constitutively expressed in all tissues in order to cope with the continuous release of heme in cell metabolism. On the other hand, HO-1 is an inducible form of the enzyme and the activity of HO-1 (V_{max}) is approximately one order of magnitude greater than that of HO-2 (*3*). Upregulation of the HO-1 protein in mammals is mostly dependent on transcriptional activation of the HO-1 gene (*4*) and occurs in response to stimuli such as hypoxia and oxidative stresses (*5*, *6*). HO-1 is considered to be a cytoprotective factor in two respects. First, free heme, a substrate to be diminished by HO-1, is highly reactive and cytotoxic. Second, carbon monoxide and bilirubin, the two end products, are

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a mediator inhibiting the inflammatory process (*7*) and a scavenger for reactive oxygen (*8*), respectively. HO-1 gene–deficient mice display reduced stress defense (*9*), a pro-inflammatory tendency (*9*), and susceptibility to atherosclerotic lesion formation (*10*). Consistent with these findings, Yachie *et al*. (*11*) reported a case of human HO-1 deficiency associated with endothelial cell injury, vulnerability to stress, anemia, and growth retardation. Conversely, experimental HO-1 gene delivery has been shown to result in amelioration of atherosclerosis (*12*), injury-induced vascular neointima formation (*13*), ischemic heart disease (*14*), renal ischemia/reperfusion injury (*15*), hypoxia-induced lung injury (*16*) and liver ischemia/reperfusion injury (*17*), as well as improvement of vascular function (*18*).

Although accumulating evidences indicate that HO-1 plays a beneficial role in animals, it is not clear whether HO-1 has a similar biological role in humans. For example, it has been reported that hypoxia down-regulates rather than up-regulates HO-1 expression in human cells *in vitro* (*19*). In addition, it has been shown (*20*) that there is a $(GT)_n$ repeat length polymorphism in the promoter region of the HO-1 gene, raising a hypothesis that the capacity of HO-1 expression is, at least in part, determined genetically by the polymorphism. Accordingly, it has been reported that the short $(GT)_n$ repeats are associated with lower plasma levels of inflammation markers (*21*) and reduced risk of restenosis after percutaneous interventions on femoral (*22*) and coronary arteries (*23*), aortic aneurysm (*24*), and cerebrovascular events (*25*). These studies were designed based on the assumption that short $(GT)_n$ repeats may be associated with high HO-1 expression. However, the relationship between the $(GT)_n$ repeats length and HO-1 expression level has not been directly studied. Obviously, the measurement of HO-1 mRNA expression in human blood cells, including monocytes, is one method to assess individual ability to upregulate HO-1. Monocytes are one of the major cell types involved in the pathogenesis of atherosclerosis (*26*) and are also known to express HO-1 abundantly (*27*). However, there has been no study on the expression of HO-1 in relation either to $(GT)_n$ repeat polymorphism or human diseases.

In the present study, we tested the hypothesis that humans are heterogeneous in their ability to upregulate HO-1 and that a reduced ability to upregulate HO-1 in response to oxidative stress may be involved in the mechanism of atherosclerosis. We assessed $(GT)_n$ repeat polymorphism as a possible determinant of activation of the HO-1 gene in combination with the actual HO-1 mRNA expression level in peripheral blood mononuclear cells (PBMCs) of patients with coronary artery disease.

Methods

Study Population

The subjects of the present study were 110 patients with suspected coronary atherosclerosis who underwent elective coronary angiography (CAG) at Hiroshima University Hospital. We did not include patients with unstable angina and/or acute coronary syndromes.

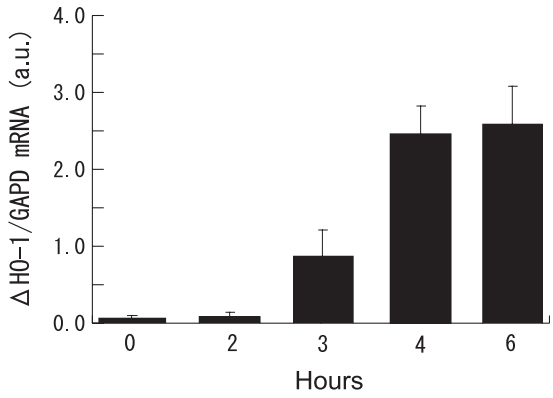

Fig. 1. *Time course of ΔHO-1 mRNA expression in peripheral blood mononuclear cells during incubation in hemin-supplemented medium (hemin: 10 μmol/l). Results are the means±SD;* n = *10. a.u., arbitrary units; GAPD, glyceraldehyde-3-phosphate dehydrogenase.*

The subjects were screened for the presence of hypertension (systolic and/or diastolic blood pressure ≥140/90 mmHg), diabetes (fasting plasma glucose ≥126 mg/dl and/or HbA1c ≥6.5%), and hypercholesterolemia (plasma low-density lipoprotein [LDL] cholesterol level ≥160 mg/dl by Friedewald's formula). Subjects were also considered to have hypertension, diabetes, or hypercholesterolemia when they had previously been diagnosed with such diseases and/or were receiving appropriate therapy.

All participants were Japanese and gave written informed consent after understanding the rationale and potential risks of the study. This study was approved by the Ethics Committee of Hiroshima University.

Coronary Score

Lesions were graded as proposed by the American Heart Association (*28*) by two independent experts who assigned a coronary score to each angiogram. Briefly, a score of 1 was given for 25%, 2 for 50%, 4 for 75%, 8 for 90%, and 16 for 99.9% stenosis, and a score of 32 was given in cases of total obstruction. Then each score was multiplied by a factor that was assigned based on the localization of the lesion and the results were summed up.

Blood Sampling and Isolation of PBMCs

Blood was collected on the day of CAG. The subjects were instructed not to take any medication for at least 10 h before blood collection. The blood samples were placed into tubes with EDTA as an anti-coagulant and kept at 4°C. Plasma was

Table 1. Effects of Commonly Used Drugs

Drug	ΔHO-1/GAPD mRNA (a.u.)
Vehicle (DMSO, 0.1%)	2.84±0.45
Carvedilol (100 nmol/l)	3.01±0.64
Losartan (100 nmol/l)	2.90±0.25
Pravastatin (10 nmol/l)	3.00±0.25
Benidipine (10 nmol/l)	3.11±0.30
Sodium nitroprusside (10 nmol/l)	2.80±0.95

Values are mean±SD, $n=10$. All changes against vehicle are not significant. a.u., arbitrary unit; DMSO, dimethylsulfoxyde; GAPD, glyceraldehyde-3-phosphate dehydrogenase.

Table 2. Characteristics of the Patients ($n=110$)

Age and BMI	
Age (years)	65.6±12.2
BMI (kg/m^2)	23.3±3.26
Risk factors (*n* (%))	
Male sex	75 (68)
Hypertention	60 (55)
Hypercholesterolemia	46 (42)
Diabetes	35 (32)
Current smoker	37 (34)
Family history of CAD	27 (25)
CAG (*n* (%))	
Normal coronary	42 (38)
One-vessel disease	30 (27)
Two-vessel disease	22 (20)
Three-vessel disease	16 (15)

Data are mean±SD or number of subjects (%). BMI, body mass index; CAD, coronary artery disease; CAG, coronary angiography.

separated by centrifugation and stored at −80°C for ELISA to determine levels of 8-isoprostane (Cayman Chemical, Ann Arbor, USA), TNF-α and IL-10 (R&D Systems, Minneapolis, USA) as systemic markers of oxidative stress, inflammation and HO-1 associated cytokine (*7*), respectively.

PBMCs were isolated from the buffy coat by centrifugation through a Ficol gradient (MUTO Pure Chemicals, Tokyo, Japan). Then the cells were washed twice with the incubation medium and placed into polystyrene dishes (1.5 cm in diameter) containing 1 ml RPMI-1419 medium supplemented with 10% FBS and 100 mg/ml of penicillin-streptomycin mixture (Gibco, Carlsbad, USA).

Treatment of PBMCs with Hemin

To study the effect of oxidative stress on HO-1 expression, we divided the isolated PBMCs into two portions and treated the cells with 10 μmol/l hemin (Sigma-Aldrich, Chicago, USA) or a vehicle (dimethylsulfoxyde [DMSO]) for 4 h at 37°C. Hemin is known to be immediately reduced to heme within cells. We used hemin because 1) heme is a pro-oxidant that is known to induce HO-1 (*29*); 2) it has been implicated in the pathogenesis of atherosclerosis (*30*); and 3) it is thought to be an endogenous mediator of stress signals that lead to upregulation of HO-1 mRNA (*31*, *32*). The incubation time to stimulate HO-1 expression was determined on the basis of results of preliminary experiments described below (Fig. 1). After the incubation, total RNA was isolated from PBMCs to analyze HO-1 mRNA expression by the guanidine-thyocyanate method using TriZol Reagent according to a manufacturer's protocol (Invitrogen, Carlsbad, USA). Genomic DNA was simultaneously isolated from the same sample to analyze $(GT)_n$ repeat length polymorphism.

Assessment of $(GT)_n$ Repeat Length Polymorphism in the HO-1 Gene Promoter

The $(GT)_n$ repeat length polymorphism in the HO-1 gene promoter region was assessed by polymerase chain reaction (PCR) as previously described by Kimpara *et al*. (*33*). To facilitate amplification of the GT-rich template, we used LA Taq DNA polymerase for GT-rich templates (Takara Bio, Otsu, Japan). The precise size of the PCR product, *i.e.*, the $(GT)_n$ repeat number, was estimated using a microelectrophoresis system (Bioanalyzer 2100; Agilent Technologies, Palo Alto, USA). This system is sensitive to differences in molecular sizes of more than two nucleotide bases; the size of the PCR product was verified by direct sequence analysis (ABI Prism 310; Applied Biosystems, Foster, USA).

As in the previous studies (*22*, *23*, *34*), the $(GT)_n$ repeat length ranged from 16 to 41 and showed a bimodal distribution, with the median length of the short repeat being around 23 pairs and that of the long repeat being around 30 pairs. Thus, as in the previous studies, each of the two alleles of the gene was classified as a long (L) or short (S) genotype based on whether the repeat length was more or less than 26 pairs, respectively.

Assessment of HO-1 mRNA Expression

First-strand cDNAs were synthesized from approximately 1 μg of total RNA isolated from PBMCs with the use of MLV reverse transcriptase and random hexamer primers according to the manufacturer's instructions (RT-PCR Core Kit; Takara Bio). HO-1 mRNA expression was quantified by real-time PCR (Light Cycler Instrument; Roche Diagnostics, Basel, Switzerland) utilizing glyceraldehyde-3-phosphate dehydrogenase (GAPD) as an internal standard. Primers were synthesized according to the published sequences (*35*). Standard curves were prepared for the target and reference genes by amplification of diluted plasmid DNA containing the target sequences (pGem+ vector; Promega Corp., Madison, USA). The relative amounts of the target gene and the reference gene were calculated by the crossing point method in the manual

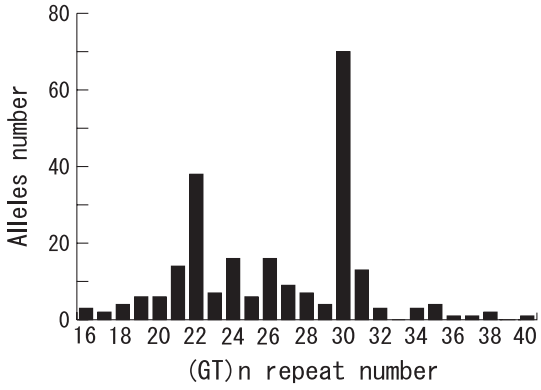

Fig. 2. *Bimodal distribution of* $(GT)_n$ *repeat alleles in 110 patients (*n = *220 alleles) with peaks at around 22 and 30 repeats.*

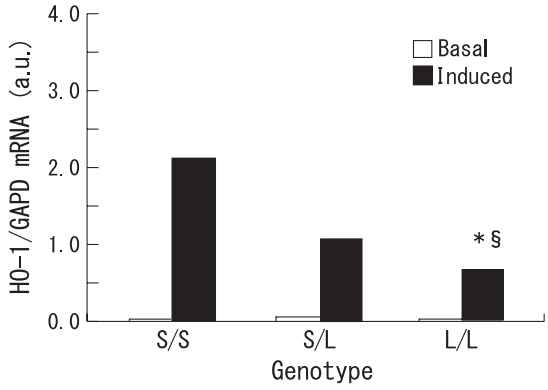

Fig. 3. *Basal and hemin-stimulated HO-1 mRNA expression levels in three* $(GT)_n$ *repeat length polymorphism genotypes. Results are presented as the means.* *p < *0.05* vs. *the S/S genotype group;* §p < *0.05* vs. *the S allele carriers group (combined S/S and S/L genotype groups), Mann-Whitney* U *test. a.u., arbitrary units; GAPD, glyceraldehyde-3-phosphate dehydrogenase.*

mode. Results are expressed as the target/reference ratio. Several of the PCR products were sequenced on an automated DNA sequencer (ABI Prism 310). The inter-assay reproducibility of the stimulated HO-1 mRNA level measurements was acceptable: the coefficient of variance (CV) was 7.2%.

The difference between the HO-1 mRNA levels of hemin- and vehicle-treated cells was considered to reflect the capacity of cells to upregulate HO-1 mRNA, and it was expressed as *Δ*HO-1 mRNA.

Preliminary Experiments to Determine the Optimal Incubation Time of PBMCs

To determine the optimal incubation time for stimulating HO-1 expression, we incubated PBMCs with 10 μmol/l hemin for 2, 3, 4 and 6 h at 37°C (Fig. 1). The results showed that 4 h was the minimum-required incubation time to fully stimulate HO-1 expression. In addition, since most subjects were receiving medications, we investigated the possible influence of major medications on HO-1 expression *in vitro*. While PBMCs were being incubated with hemin, one of the following agents or a vehicle was added to the medium: an angiotensin receptor blocker (losartan; Banyu Seiyaku, Tokyo, Japan) at 100 nmol/l, a β-blocker (carvedilol; Daiichi Seiyaku, Tokyo, Japan) at 100 nmol/l, a hydroxymethylglutaryl-CoA (HMG-CoA) reductase inhibitor (pravastatin; Sigma-Aldrich, Chicago, USA) at 10 nmol/l, a calcium channel blocker (benidipine; Kyowa Hakko, Tokyo, Japan) at 10 nmol/l or a vasodilator (sodium nitroprusside; RBI, Natick, USA) at 10 nmol/l. As shown in Table 1, the stimulated HO-1 expression level was not significantly altered by any of the tested agents.

We also measured the HO-1 mRNA expression level in the monocyte fraction that was purified by centrifugation of the PBMCs using an OptiPrep gradient (Axis-Shield, Oslo, Norway). The cell-count-adjusted HO-1/GAPD mRNA ratios after hemin treatment in cells prepared by these two methods were almost identical, suggesting that HO-1 expression in mononuclear cells represents that in monocytes.

Statistical Analysis

Differences between groups were assessed by the Mann-Whitney test. When analyzing the correlation between *Δ*HO-1 mRNA expression and coronary score or other known risk factors, simple linear regression and multiple regression analysis were applied. Analysis was performed using StatView v.5.0 software (SAS Institute, Cary, USA). Data are expressed as the means±SD when appropriate. Values of $p<0.05$ were considered statistically significant.

Results

The background of the subject population and an outline of the angiographic data are shown in the Table 2. Serum levels of HbA1c in subjects with diabetes *vs*. those without it were 7.46±1.5% *vs*. 5.27±0.5% ($p<0.05$), respectively. Total cholesterol levels in subjects with hypercholesterolemia *vs*. those without it were 217.8±51 *vs*. 179.6±25 mg/dl (n.s.). Systolic/diastolic blood pressures in subjects with hypertension *vs*. those without it were 142.8±22/76.9±15 mmHg *vs*. 124.2±17/70.9±14 mmHg (n.s.).

$(GT)_n$ Repeat Length Polymorphism and HO-1 mRNA Expression

In the studied cohort, the $(GT)_n$ repeat number ranged from 16 to 40 and the distribution of the $(GT)_n$ repeat numbers was bimodal, with peaks around 23 and 30 (Fig. 2). These findings were all consistent with previous observations in both Cauca-

Table 3. Multivariate Predictors of CAD

Parameters	Correlation coefficient	*p* value*
Age	1.054	0.35
Male gender	2.341	0.21
BMI	1.120	0.18
Hypertension	0.730	0.27
Hyperlipidemia	2.031	0.16
Diabetes	3.714	0.02
ΔHO-1 mRNA	0.631	0.01

CAD, coronary artery disease; BMI, body mass index; ΔHO-1 mRNA, difference between HO-1 mRNA levels of hemin- and vehicle-treated cells. **p* value is based on multiple regression analysis using coronary score parameter as a dependent variable.

sian and Japanese populations (*22*, *23*, *34*). We divided the subjects into three genotype groups (S/S, S/L, and L/L) as described above, then compared the basal and stimulated levels of HO-1 mRNA among them. Although the basal HO-1 mRNA levels did not differ among the three groups, the stimulated HO-1 mRNA level (and therefore ΔHO-1 mRNA) was significantly reduced in L/L genotype carriers compared with the values in S/S genotype carriers and in the combined group of S/S and S/L genotype carriers (Fig. 3), suggesting that the L genotype, in the absence of the S allele, is associated with a lowered capacity for HO-1 gene upregulation in response to stress. This result is consistent with the previous *in vitro* observation that increasing the length of $(GT)_n$ sequences in the gene promoter compromised the expression of the HO-1 gene (*36*).

HO-1 mRNA Expression and Coronary Atherosclerosis

Having established that HO-1 mRNA expression is, at least in part, determined by genetics, we investigated the mRNA expression levels in comparison with the coronary score. There was no correlation between basal HO-1 mRNA expression levels and coronary scores. However, the ΔHO-1 mRNA level ($r=0.50$, $p<0.05$, Fig. 4a) as well as the stimulated HO-1 mRNA level ($r=0.53$, $p<0.05$) showed a strong negative correlation with coronary scores. These results indicate that the ability to induce HO-1 is impaired in subjects with greater coronary scores. Because the coronary score does not always correlate with the severity of coronary lesions or the susceptibility to coronary artery disease, we compared the ΔHO-1 mRNA between patients with clinically established coronary artery disease (one-, two- and three-vessel disease) and subjects without significant coronary lesions (Fig. 4b). ΔHO-1 mRNA was reduced with increasing number of involved coronary arteries, confirming that the reduced ability to upregulate HO-1 is associated with progression of coronary atherosclerosis.

To determine whether reduction of ΔHO-1 mRNA is an independent risk factor for coronary atherosclerosis, we evaluated the impact of ΔHO-1 mRNA on the coronary score among known coronary risk factors using multiple regression analysis (*37*) (Table 3). Among the variables of age, gender, body mass index, ΔHO-1 mRNA, presence of hypertension, diabetes and current smoking, only the presence of diabetes and ΔHO-1 mRNA were independent risk factors affecting the coronary score.

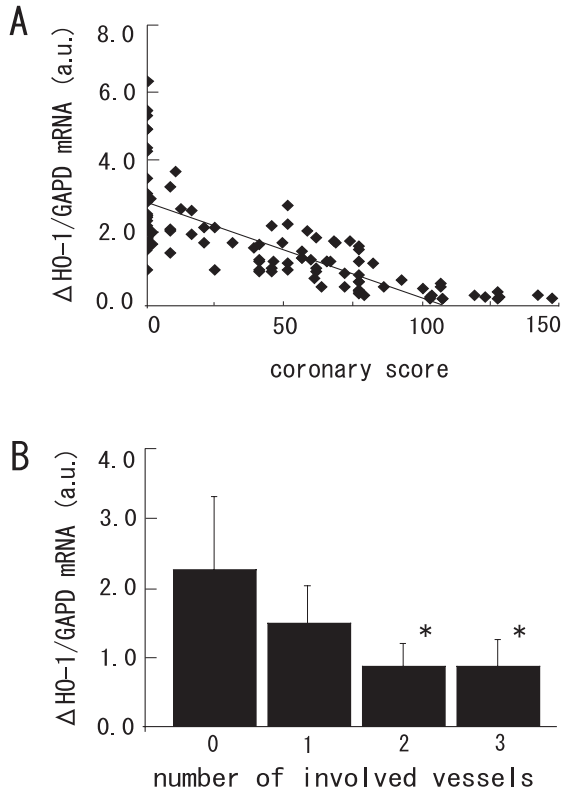

Fig. 4. *A: Correlation between the capacity of cells to upregulate HO-1 in response to hemin (ΔHO-1 mRNA) and coronary scores.* $n=110$, $r^2=0.50$, $p<0.01$. *B: ΔHO-1 mRNA levels in patients with coronary artery disease (1–3 vessel diseases) and control subjects with intact coronary arteries (0 vessel disease). Values are the means±SD.* *$p<0.05$ vs. *0 vessel disease group. a.u., arbitrary units; GAPD, glyceraldehyde-3-phosphate dehydrogenase.*

Relationship of HO-1 Genotype and mRNA Expression with Systemic Oxidative Stress and Inflammation

Finally, we investigated the relationship between the HO-1 expression level and systemic oxidative stress or inflammation. No significant correlation was found between basal HO-1 mRNA levels and serum levels of 8-isoprostane, TNF-α or IL-10, or between the stimulated HO-1 mRNA level (or ΔHO-1 mRNA) and these factors. Similarly, there were no

significant differences in the circulating levels of 8-isoprostane, TNF-α and IL-10 among S/S, S/L and L/L genotype carriers.

Discussion

Oxidative stress is determined by the balance between the production and elimination of oxidants. Thus, the ability to eliminate oxidants is an important determinant of the susceptibility to many diseases associated with oxidative stress. However, little attention has been paid to individual variability of anti-oxidant defense. Upregulation of HO-1 is one of the major defense mechanisms against oxidative stress. In the present study, we tested the hypothesis that individual capacity to upregulate HO-1 is genetically determined and that reduced HO-1 expression may contribute to the development of coronary atherosclerosis. Consistent with the notion that transcriptional activity of the HO-1 gene decreases with increasing numbers of $(GT)_n$ repeats (*36*), patients with the L/L genotype in the present study showed a lower level of ΔHO-1 mRNA than patients carrying other genotypes, indicating that the capacity for HO-1 upregulation is, at least in part, genetically regulated. Consistent with this notion, the ΔHO-1 mRNA results were reproducible (the CV was 7.2%). Furthermore, we demonstrated that ΔHO-1 mRNA was directly correlated with the coronary score, suggesting that reduced HO-1 response may be involved in the mechanism of atherosclerosis. Therefore it is tempting to speculate that the maximum capacity to upregulate HO-1 may be an intermediate phenotype reflecting the ability to handle oxidative stress and susceptibility to the progression of coronary artery disease.

In atherosclerotic lesions, oxidative stress in the form of reactive oxygen species derived from various sources, including mitochondria, NADPH oxidase, and xantine oxidase, stimulates signals provoking inflammatory reactions and other pro-atherosclerotic processes (*38*). HO-1, expressed in the endothelium (*39*, *40*) and monocytes/macrophages (*41*), is upregulated upon exposure to reactive oxygen species, or oxidized LDL (*42*), and it antagonizes the pro-atherosclerotic processes through the actions of its catalytic end-products, carbon monoxide and bilirubin. It is of note that heme, either endogenous in hemorrhagic atherosclerotic lesions or derived from the denatured hemoglobin within them, significantly contributes to the lesion formation and potently induces HO-1 (*30*). In the endothelium, upregulated HO-1 inhibits cytokine-induced expression of adhesion molecules, thereby silencing endothelial activation and platelet aggregation (*43*). In monocytes/macrophages, augmented HO-1 induction results in attenuation of monocyte chemoattractant protein-1 expression (*44*) and a reduction in the chemotaxis elicited either by oxidized LDL (*45*) or angiotensin II (*46*). These observations support the notion that upregulation of HO-1 in monocytes significantly contributes to inhibition of atherosclerosis. Although we studied mononuclear cells instead of monocytes, the HO-1 mRNA expression level in the mononuclear cell fraction was almost identical to that in the further purified monocytes (see Methods). Similarly, vascular cells were not directly studied. However, given the significant association between HO-1 expression level and genetics, it is conceivable that other cell types, including endothelial cells, share similar phenotypes related to HO-1 expression.

We used hemin to stimulate HO-1 expression since heme, a reduced product of hemin, represents a potent endogenous inducer of the HO-1 gene (*31*) and is also implicated in atherosclerosis (*30*). The mechanism whereby heme stimulates HO-1 expression involves its interaction with transcription factor Bach1. HO-1 gene expression is normally suppressed by Bach1 (*29*), which has high-affinity binding sites for heme (*31*). Upon association with heme, Bach1 loses its DNA-binding activity and is exported out of nuclei, which results in unlimited upregulation of HO-1 transcription depending on the extent of stimuli (*29*). This inactivation of Bach1 is a common process in the signaling pathways of HO-1 upregulation, including that derived from oxidized LDL (*47*). Thus, it is reasonable to study the role of HO-1 in the mechanisms of atherosclerosis based on the response of HO-1 mRNA to hemin.

We investigated whether HO-1 expression affects systemic levels of inflammatory cytokines or oxidative stress markers. The level of IL-10 was evaluated since this cytokine has been reported to be controlled by HO-1 (*7*, *48*). However, none of these parameters were significantly related to either the basal or the stimulated level of HO-1. Overproduction of reactive oxygen species (ROS) as a possible cause of atherosclerosis and the protection against such stress by HO-1 occur locally in the vasculature and are mainly intracellular events (*49*). Systemic measurements of the markers of inflammation (*50*) and oxidative stress were not sufficiently sensitive to determine the relationship with HO-1 expression.

The simulated HO-1 expression level and ΔHO-1 mRNA, albeit reproducible in our assay, may be influenced by numerous factors other than genetics. However, multiple logistic regression analysis revealed that both ΔHO-1 mRNA and the stimulated HO-1 level were independent risk factors for an increase in the coronary score. Importantly, we assessed maximal stimulation of HO-1 expression *in vitro* (Fig. 1) to evaluate the intrinsic capacity to upregulate HO-1. HO-1 upregulation is not a parameter influenced by *in vivo* fluctuations of humoral and hemodynamic factors. However, we cannot exclude the possibility that the reduction of the stimulated HO-1 expression level is merely a consequence of atherosclerosis. It remains to be determined whether HO-1 expression can be a marker for susceptibility to atherosclerosis, and further studies with a prospective design will be needed for this purpose.

In summary, we have demonstrated that ΔHO-1 mRNA in mononuclear cells is reproducible within an individual but variable among individuals. ΔHO-1 mRNA is consistent with the level estimated from the gene polymorphism. Furthermore, ΔHO-1 mRNA was reduced with increasing severity of

coronary atherosclerosis, a condition coincident with overt oxidative stress. These results suggest that ΔHO-1 mRNA may be useful as an intermediate phenotype that could help to assess a patient's ability to antagonize oxidative stress. Few attempts have been made to evaluate oxidative status based on the ability to eliminate oxidants. In that sense, we here propose a novel concept and method to indirectly evaluate the oxidative status in clinical patients.

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