Mechanism of 4-HNE Mediated Inhibition of hDDAH-1: Implications in No Regulation†

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ABSTRACT: Nitric oxide synthase is inhibited by NG-methylated derivatives of arginine whose cellular levels are controlled by dimethylarginine dimethylamino-hydrolase (DDAH). DDAH-1 is a Zn(II)-containing enzyme that through hydrolysis of methylated l-arginines regulates the activity of NOS. Herein, we report the kinetic properties of hDDAH-1 and its redox-dependent regulation. Kinetic studies using recombinant enzyme demonstrated K_m values of 68.7 and 53.6 μM and V_{max} values of 356 and 154 nmols/mg/min for ADMA and L-NMMA, respectively. This enzymatic activity was selective for free ADMA and L-NMMA and was incapable of hydrolyzing peptide incorporated methylarginines. Subsequent studies performed to determine the effects of reactive oxygen and reactive nitrogen species on DDAH activity demonstrated that low level oxidant exposure had little effect on enzyme activity and that concentrations approaching $\geq$100 μM were needed to confer significant inhibition of DDAH activity. However, exposure of DDAH to the lipid oxidation product, 4-HNE, dose-dependently inhibited DDAH activity with 15% inhibition observed at 10 μM, 50% inhibition at 50 μM, and complete inhibition at 500 μM. Mass spectrometry analysis demonstrated that the mechanism of inhibition resulted from the formation of Michael adducts on His 173, which lies within the active site catalytic triad of hDDAH-1. These studies were performed with pathophysiologicaly relevant concentrations of this lipid peroxidation product and suggest that DDAH activity can be impaired under conditions of increased oxidative stress. Because DDAH is the primary enzyme involved in methylarginine metabolism, the loss of activity of this enzyme would result in impaired NOS activity and reduced NO bioavailability.

Nitric oxide (NO[1]) is an important regulator of vascular homeostasis. Endothelium-derived NO is responsible for vasodilatation and has anti-proliferative and anti-atherogenic effects on the vascular wall (*1*−*3*). Dysregulation of NO biosynthesis has been noted in multiple disease states including hypercholesterolemia (*4*) and atherosclerosis (*5*). A growing volume of literature implicates the endogenous nitric oxide synthase (NOS) inhibitors asymmetric dimethyl arginine (ADMA) and N^G-monomethyl arginine (L-NMMA) in the impaired NO production associated with these diseases (*6*, *7*). These methylarginines are produced by the proteolysis of methylated arginine residues on proteins. Six isoforms of the protein−arginine methyl transferase's (PRMTs) family of enzymes have been identified and are responsible for the methylation of arginine residues on proteins. Free ADMA and L-NMMA, formed during routine proteolysis are subsequently hydrolyzed by dimethylarginine dimethylamino hydrolase (DDAH) (*8*). DDAH metabolizes ADMA to L-citrulline and dimethylamine, and L-NMMA to L-citrulline and monomethylamine. There are two isoforms of DDAH with distinct tissue localization patterns (*9*). DDAH-1 has been implicated as a critical regulator of methyarginine levels and NO production in the vasculature (*10*). The activity of DDAH has been shown to be altered in a variety of disorders involving endothelial dysfunction. In chronic heart failure, DDAH-1 activity decreases, but protein levels remain constant, suggesting that post-translational modifications are responsible for the modulation of DDAH activity (*11*). Unfortunately, little is known with regard to the enzyme kinetics of hDDAH-1. To date, enzymatic studies of DDAH have utilized either recombinant bacterial DDAH-1 or DDAH-1 partially purified from porcine brain homogenates. Results using these preparations have determined that DDAH-1 hydrolyses ADMA at a faster rate than L-NMMA with reported K_m values of 0.18 and

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[1] Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; eNOS, endothelial NOS; L-NMMA, N^G-methyl-L-arginine; ADMA, asymmetric dimethylarginine; PRMT, protein−arginine methyl transferases; DDAH, dimethylarginine dimethyl-aminohydrolase; BAEC, bovine aortic endothelial cells; 4-HNE, 4-hydroxy-nonenal; DEANONOate, 2-(*N*,*N*-dimethylamino)-diazenolate-2-oxide; DTT, dithiothreitol; EDTA, ethylenediamine-tetraacetic acid; $ONOO^-$, peroxynitrite.

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0.36 mM, respectively, and is responsible for >90% of ADMA metabolism (*12*−*14*). DDAH-1 contains a Zn(II) binding site with endogenously bound Zn(II) inhibiting the catalytic activity of DDAH (*15*). In addition to the zinc regulatory domain, it has been demonstrated that NO inhibits DDAH activity through S-nitrosylation of Cys249 located within the active site of DDAH (*16*).

Evidence suggests that DDAH-1 activity is inhibited in a variety of cardiovascular diseases and that this inhibition contributes to impaired NO bioavailability (*17*−*19*). Therefore, it is of critical importance to elucidate the pathways and determine the effector molecules involved in DDAH-1 dysregulation. In atherosclerosis, levels of lipid hydroperoxides such as 4-hydroxy-2-nonenal (4-HNE) are elevated. A biologically active major aldehyde of membrane lipid peroxidation, 4-HNE, is formed during inflammation, and oxidative stress and can reach tissue concentrations of 10 μM to 5 mM (*20*). Plasma levels of 4-HNE, normally 5−10 μM, can increase by as much 50-fold under disease conditions (*21*). 4-HNE is a highly reactive aldehyde and has been shown to inhibit the function of multiple proteins mainly through the formation of Michael or Schiff base adducts (*22*). A potent electrophile, 4-HNE preferentially reacts with cysteine and histidine residues on proteins to form Michael adducts (*23*). Therefore, in the present study we have carried out a comprehensive evaluation hDDAH-1 enzyme kinetics and determined the effects of the lipid peroxidation product, 4-HNE, on DDAH activity as we have recently observed that 4-HNE inhibits endothelial cell NO production (*24*).

EXPERIMENTAL PROCEDURES

DDAH Expression and Purification. A bacterial expression system for human DDAH-1, pDEST17-hDDAH1 in BL21star *E. coli*, was generated using Gateway technology (Invitrogen). This expression system generates human DDAH-1 fused in frame to an N-terminal His6-tag. After growth and induction in 2 L of terrific broth and induction by IPTG, the bacterial pellet was resuspended in 160 mL of lysis buffer consisting of MAC buffer A (20 mM phosphate, 500 mM NaCl, and 30 mM imidazole at pH 7.4), with the addition of EDTA-free complete protease inhibitor (Roche), 1 mM DTT, 1 mM $MgCl_2$, 10 ug DNase, and 10 ug lysozyme. The cells were homogenized with two passes through an Emulsiflex C3 (Avestin) at 12−15 kpsi, and the insoluble material was removed by centrifugation at 4 °C (1 h at 48,000*g*). The supernatant was loaded onto a 1 mL HisTrap column (GE Bioscience) and equilibrated in MAC buffer A. The column was washed with 30 column volumes of 95% MAC A and 5% MAC buffer B (20 mM phosphate, 500 mM NaCl, and 250 mM imidazole at pH 7.4) and eluted with 100% MAC buffer B. The fractions containing hDDAH1 were desalted and exchanged into 25 mM bis-tris propane at pH 7.4 (IEX buffer A), using a HiTrap desalting column (GE Bioscience). The desalted fractions were loaded onto a 1 mL HiTrap Q sepharose column (GE Bioscience) and eluted with a linear gradient from 0 to 12% IEX buffer B (IEX buffer A plus 1 M NaCl) over 30 column volumes. All chromatography was performed at 4 °C using an AKTA purifier 10 (GE Biosciences). The fractions containing hDDAH 1 were pooled and concentrated using ultrafiltration. The concentrated fractions were brought up to 10% v/v glycerol and 50 mM sodium phosphate at pH 7.4, fast frozen, and stored at −80 °C.

DDAH Activity Assay. A modified version of the method designed by Knipp and Vašák was designed (*25*). For kinetic measurements of enzyme activity, purified hDDAH (1.0 μg) was incubated for 5 min at 37 °C in 50 mM imidazole buffer (pH 7.5) in the presence of 1.0−1000 μM substrate with a total reaction volume of 100 μL. Following incubation, 200 μL of freshly prepared COLDER was added to each sample, and the plate was incubated for 15 min at 95 °C in an oven. The plate was allowed to cool to room temperature and cittruline formation was measured at 540 nm. For the determination of DDAH hydrolytic activity on peptide incorporated ADMA, 10 μg of hDDAH was incubated in the presence of peptides (1 mM) containing 5 random amino acid sequences with ADMA at the 3 position (xxADMAxx). Incubation of the reaction mixture (100 μL) was carried out at 37 °C for 2 h. At the termination of the incubation period, DDAH was removed using a 3,000 MW centricon filter, and the peptide eluent was subjected to acid hydrolysis. Acid hydrolysis was carried out by adding an equal volume of concentrated HCl to the eluent followed by incubation at 105 °C for 18 h. Following acid hydrolysis, samples were neutralized and citrulline formation measured as described above. The standard curve for citrulline was linear with an $R^2 = 0.99$.

DDAH Radioisotope Activity Assay. DDAH activity was measured from the conversion of L-[^{14}C]NMMA to L-[^{14}C]-citrulline. Purified hDDAH-1 was incubated with 4-HNE in 50 μL of PBS for 2 h at 37 °C in a water bath. A final volume of 100 μL was reached by adding the reaction buffer (50 mM Imidazole, 20 μM L-[^{14}C]NMMA, 200 μM L-NMMA at pH 7.4) to each sample. The samples were then incubated in a water bath at 37 °C for 1 h. Following the incubation, the reaction was stopped with 1 mL of ice-cold stop buffer using 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2 ethanesulfonic acid (HEPES) with 2 mM EDTA at pH 5.5. Separation of L-[^{14}C]citrulline from L-[^{14}C]NMMA was performed using the cation-exchange resin Dowex AG50WX-8 (0.5 mL, Na^+ form, Pharmacia). The L-[^{14}C]citrulline in the eluent was then determined using a liquid scintillation counter.

Proteomic Analysis of hDDAH-1. In Gel Digestion Manual. Gels were digested with sequencing grade trypsin from Promega (Madison WI) or sequencing grade chymotrypsin from Roche T(Indianapolis, IN)T using the Montage In-Gel Digestion Kit from Millipore (Bedford, MA) following the manufacturer's recommended protocols. Briefly, bands were trimmed as close as possible to minimize background polyacrylamide material. Gel pieces were then washed in 50% methanol/5% acetic acid for 1 h. The wash step is repeated once before gel pieces are dehydrated in acetonitrile. The gel bands were rehydrated and incubated with dithiothreitol (DTT) solution (5 mg/mL in 100 mM ammonium bicarbonate) for 30 min prior to the addition of 15 mg/mL iodoacetamide in 100 mM ammonium bicarbonate solution. Iodoacetamide was incubated with the gel bands in the dark for 30 min before removed. The gel bands were washed again with cycles of acetonitrile and ammonium bicarbonate (100 mM) in 5 min increments. After the gels were dried in a speed vac, the protease was driven into the gel pieces by rehydrating them in 50 μL of sequencing grade modified trypsin or chymotrypsin at 20 μg/mL in 50 mM ammonium

bicarbonate for 10 min. Then, 20 μL of 50 mM ammonium bicarbonate was added to the gel bands, and the mixture was incubated at room temperature for overnight. The peptides were extracted from the polyacrylamide with 50% acetonitrile and 5% formic acid several times and pooled together. The extracted pools were concentrated in a speed vac to ~25 uL.

Mass Spectrometry LTQ. Capillary-liquid chromatography-nanospray tandem mass spectrometry (NanoLC/MS/MS) was performed on a Thermo Finnigan LTQ mass spectrometer equipped with a nanospray source operated in positive ion mode. The LC system was a UltiMate Plus system from LC-Packings A Dionex Co. (Sunnyvale, CA) with a Famos autosampler and Switchos column switcher. Solvent A was water containing 50 mM acetic acid, and solvent B was acetonitrile. Five microliters of each sample was first injected on to the trapping column (LC-Packings A Dionex Co, Sunnyvale, CA) and washed with 50 mM acetic acid. The injector port was switched to inject, and the peptides were eluted off of the trap onto the column. A 5 cm 75 μm ID ProteoPep II C18 column (New Objective, Inc. Woburn, MA) packed directly in the nanospray tip was used for chromatographic separations. Peptides were eluted directly off the column into the LTQ system using a gradient of 2−80% B over 50 min, with a flow rate of 300 nL/min. The total run time was 60 min. The MS/MS was acquired according to standard conditions established in the lab. Briefly, a nanospray source operated with a spray voltage of 3 KV and a capillary temperature of 200 °C was used. The scan sequence of the mass spectrometer was based on the TopTen method; briefly, the analysis was programmed for a full scan recorded between 350 and 2000 Da, and a MS/MS scan to generate product ion spectra to determine amino acid sequence in consecutive instrument scans of the 10 most abundant peak in the spectrum. The CID fragmentation energy is set to 35%. Dynamic exclusion is enabled with a repeat count of 30 s, exclusion duration of 350 s, and a low mass width of 0.5 and high mass width of 1.50 Da.

RESULTS

hDDAH-1 Protein Purification. An *E. coli* bacterial expression system was used to produce the hDDAH-1 enzyme. Following the lysis of bacterial cells, FPLC was used to purify hDDAH-1 from a crude protein homogenate. The homogenate was passed through a nickel metal affinity column to bind His-tagged hDDAH-1 followed by a desalting column and then anion exchange chromatography. Purity was determined using SDS−PAGE and Coomassie Brilliant Blue staining. Results demonstrated a 37 kd band consistent with hDDAH-1 with greater than 95% purity (Figure 1A). The yield was approximately 1 mg/L. We found that the purified protein was prone to precipitation after a freeze/thaw cycle, but with the addition of glycerol and the phosphate buffer prior to freezing, there was no precipitation.

Enzyme Kinetics of hDDAH-1. The activity of hDDAH-1 was analyzed by colorimetric detection of citrulline formation from ADMA and NMMA as described previously (*25*). DDAH contains a zinc binding domain, and catalytic activity is inhibited by endogenously bound Zn(II); thus, 50 mM imidazole buffer was used for activity assays. Our purified enzyme exhibited the known properties of DDAH, hydrolyzing the NOS inhibitors ADMA and NMMA to citrulline and showing no activity toward SDMA. In addition, it has been hypothesized that protein methylation may serve as a signaling mechanism and that DDAH may regulate this process through protein demethylation. Therefore, experiments were performed to assess the ability of DDAH to hydrolyze peptide-incorporated methylarginines using ran-

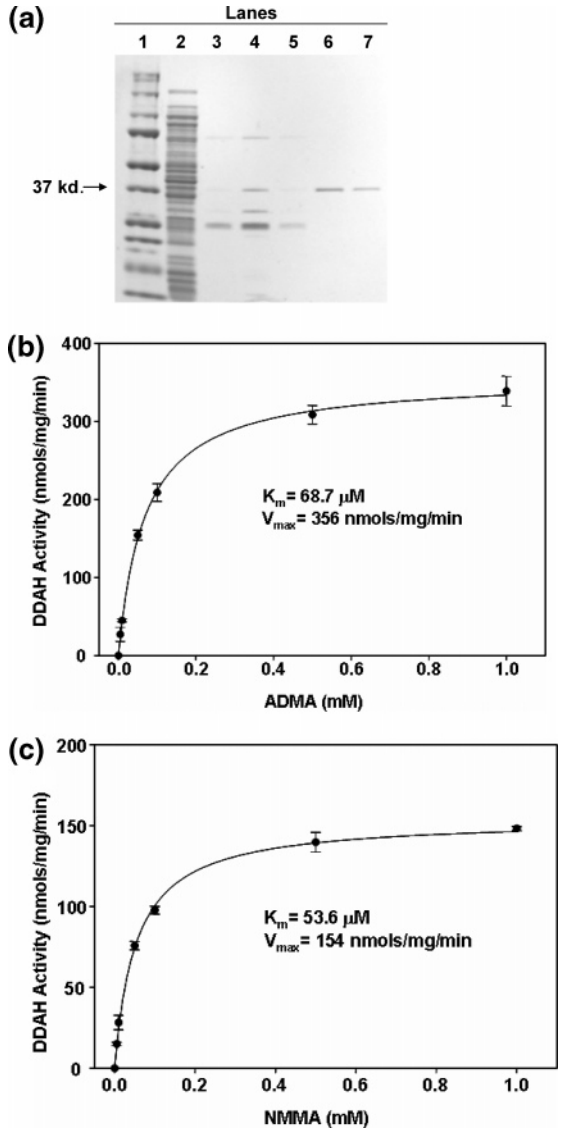

FIGURE 1: hDDAH-1 purification and enzyme kinetics. (A) DDAH purification. SDS−PAGE (4−20%) gel electrophoresis and Coomassie stained eluents following column chromatographic separation of *E. coli* hDDAH-1 expression system. Lane 1, MW ladder; lane 2, crude protein homogenate; lanes 3−5, eluents following passage through His-Trap column and desalting column; lanes 6−7, eluents following passage through ion-exchange column. The eluents from lanes 6 and 7 were used for enzymatic studies. (B) hDDAH-1 enzyme kinetics. Purified hDDAH-1 (10 μg/mL) was incubated in the presence of varying concentrations of ADMA (0.1−1000 μM). hDDAH-1 activity was measured by the conversion of ADMA to citrulline. The K_m and V_{max} were fitted using the Michaelis−Menton equation. For ADMA, the K_m was found to be 68.7 μM, and the V_{maz} was found to be 356 nmols/mg/min. (C) Purified hDDAH-1 (10 μg/mL) was incubated in the presence of varying concentrations of L-NMMA (0.1−1000 μM). The K_m and V_{max} were fitted using the Michaelis−Menton equation. For L-NMMA, the K_m was found to be 53.6 μM, and the V_{maz} was found to be 154 nmols/mg/min. hDDAH-1 activity was measured by the conversion of ADMA to citrulline. $n = 6$.

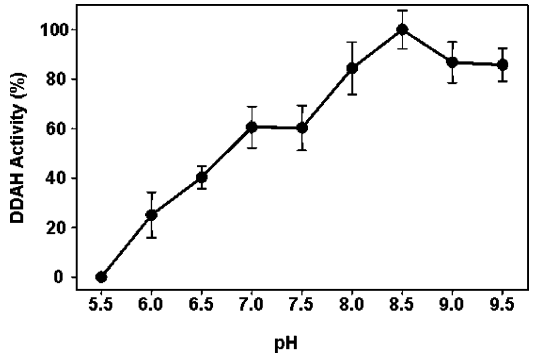

FIGURE 2: Effects of pH on hDDAH-1 enzyme activity. Purified hDDAH-1 was incubated in the presence of 500 μM ADMA at varying pH (5.5−9.5). Bis-Tris (25 mM) and 25 mM Bis-Tris propane were used as the buffering system. hDDAH-1 activity was measured by the conversion of ADMA to citrulline. The results represent % activity compared to pH 8.5. $n = 3$.

dom five amino acid polypeptides with ADMA at position 3. Results demonstrated that DDAH activity is selective for the free methylarginines ADMA and L-NMMA and cannot hyrdrolyze peptide-incorporated methylarginines. Measurements of DDAH enzyme kinetics were performed using a microplate colorimetric activity assay, and the data points were fitted using the Michaelis−Menton equation. The results demonstrated a K_m of 68.7 μM and a V_{max} of 356 nmols/mg/min when ADMA was used as the substrate (Figure 1B). Similar experiments were repeated with L-NMMA as the substrate, and the results demonstrated a K_m value of 53.6 and V_{max} equal to 154 nmol/mg/min (Figure 1C). Our results differed from previous studies of DDAH kinetics in which the enzyme was purified from either *Pseudomonas* or porcine brain homogenates. These studies observed K_m values of 0.18 for ADMA and 0.36 mM for NMMA (*38*). Thus, we are among the first to determine the catalytic activity of human DDAH-1, and these values differ from the values previously reported for DDAH-1 from other species.

pH Dependence of hDDAH-1. In order to determine the effects of pH on hDDAH-1, enzyme activity measurements were performed in the presence of 25 mM bis-tris-propane; 25 mM bis-tris buffer with pH ranging from 5.5 to 9.5. The enzyme was found to be active from pH 6 to 9.5 with maximal activity observed at pH 8.5. (Figure 2). Acidic conditions were found to greatly decrease the catalytic activity of hDDAH-1 with almost complete inhibition of enzyme activity at pH 5.5. Maximal activity was measured at pH 8.5 demonstrating that under alkaline conditions activity of the enzyme is increased. This is in contrast to previous studies in which maximal activity was measured at acidic pH values. These findings have important relevance in disease states in which the cellular environment becomes acidic, such as myocardial ischemia, in which cellular pH has been demonstrated to drop to pH 5.5. These results would suggest that under these conditions, DDAH activity would be largely inhibited and may contribute to the endothelial dysfunction and impaired NO synthesis observed in reperfusion injury.

Effect of Oxidants on hDDAH-1 Activity. It has been suggested that the activity of DDAH-1 may be influenced by the redox environment and that exposure of the enzyme to reactive oxygen and nitrogen species may result in oxidant-induced post-translational modifications of critical cysteine residues resulting in loss of enzyme activity. As a result of decreased DDAH activity, methylarginine levels would be expected to rise and result in decreased NOS derived NO. Therefore, we carried out a series of studies aimed at determining the dose-dependent effects of NO, $ONOO^-$, •OH, and H_2O_2 on DDAH activity. NO studies were carried out using the NO donor compound *DEANONOate*. For $ONOO^-$ and H_2O_2 studies, the authentic oxidant was used, and in the case of H_2O_2, experiments were performed in the presence of 100 μM DTPA to prevent Fenton reactions. The •OH radical-generating system consisted of H_2O_2 (1−1000 μM) in the presence of Fe-NTA (20 μM), which has been shown to effectively redox cycle the Fe and result in efficient •OH generation. Exposure of hDDAH-1 to pathophysiological levels of reactive oxygen was found to have little to no effect on the activity of the enzyme (Figure 3A). A modest inhibition of 15% was observed following exposure to authentic $ONOO^-$ at concentrations ranging from 10 to 100 μM. Higher levels of inhibition were observed at supraphysiological concentrations (1 mM) of H_2O_2 (43%), ONOO− (25%), and •OH (47%) (Figure 3A). As previously demonstrated, exposure of hDDAH-1 to NO resulted in significant inhibition at concentrations at or above 100 μM (*16*). Overall, hDDAH-1 was found to be largely resistant to inhibition by physiological/pathologically relevant levels of reactive oxygen and reactive nitrogen species. However, because DDAH activity has been shown to be inhibited in a variety of

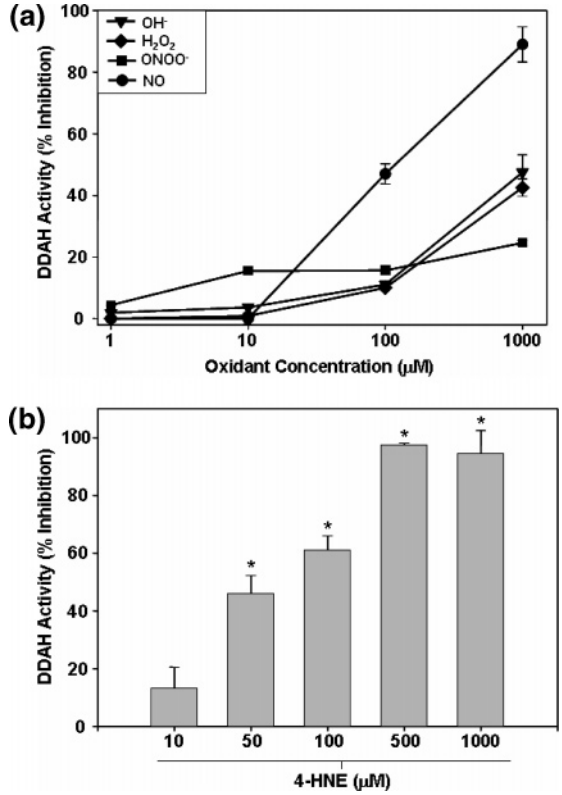

FIGURE 3: Effects of oxidants and lipid hydroperoxides on hDDAH-1 activity. (A) Purified hDDAH-1 was exposed to varying concentrations (1−1000 μM) of OH^-, H_2O_2, $ONOO^-$, and NO. hDDAH-1 activity was measured by the conversion of ADMA to citrulline. (B) Increasing concentrations of 4-HNE (10−1000 μM) were found to dose-dependently inhibit the activity of hDDAH-1. The results are presented as % inhibition compared to vehicle-treated hDDAH-1. * indicates significance at $p < 0.05$. $n = 3$.

cardiovascular diseases associated with oxidant stress, we carried out further studies aimed at studying the effects of oxidatively modified lipids on enzyme activity.

Effect of 4-HNE on hDDAH-1 Activity. Among the proposed mechanisms for the impaired NOS activity observed in endothelial dysfunction associated with coronary artery disease are elevated levels of oxidatively modified lipids (*26*, *27*). Polyunsaturated fats in cholesterol esters, phospholipids, and triglycerides are subjected to free radical-initiated oxidation. These polyunsaturated fatty acid peroxides can yield a variety of highly reactive smaller molecules such as the aldehyde 4-hydroxy-2-nonenal (4-HNE) upon further oxidative degradation. Therefore, studies were carried out in order to determine the dose-dependent effects of 4-HNE on hDDAH-1 activity. The results demonstrated that exposure of hDDAH-1 to 4-HNE (10 μM−1 mM) at 37 °C caused a dose-dependent inhibition of enzyme catalytic activity with 50% inhibition observed at 50 μM and near complete inhibition at 500 μM (Figure 3B). These results demonstrate that at pathologically relevant levels, 4-HNE significantly inhibits DDAH activity. This observation represents a new mechanism for DDAH regulation and would be expected to significantly inhibit NOS-derived NO production.

Proteomic Analysis of hDDAH-1. Sequence information from the MS/MS data was processed by converting the raw dta files into a merged file (.mgf) using MGF creator (merge.pl, a Perl script). The resulting mgf files were searched using Mascot Daemon by Matrix Science (Boston, MA). Data processing was performed following the guidelines in *Molec. Cell. Proteomics* (published online). Assigned peaks have a minimum of 10 counts (S/N of 3). The mass accuracy of the precursor ions were set to 2.0 Da, given that the data was acquired on an ion trap mass analyzer, and the fragment mass accuracy was set to 0.5 Da. Considered modifications (variable) were methionine oxidation and carbamidomethyl cysteine. Protein identifications were checked manually, and proteins with a Mascot score of 40 or higher with a minimum of two unique peptides from one protein having a $-b$ or $-y$ ion sequence tag of five residues or better were accepted. HNE modification was searched using PEAKS (Bioinformatics Solutions, Waterloo, ON Canada) programs. A peptide modified with HNE will result in a mass increase of 156.1150 Da on cysteine and histidine residues, if compared with the unmodified peptides. Therefore, the program was set to search peptides with a mass shift of 156.1150 Da as well as the unmodified peptides. Cysteine carbamido-methylation and methionine oxidation were also considered as variable modification. The mass accuracy of the precursor ions were set to 2.1 Da, and the fragment mass accuracy was set to 0.5 Da. On the basis of the predicted *m*/*z* ratios, the results demonstrated that exposure of hDDAH-1 to 50 μM 4-HNE for 1 h resulted in Michael adduct formation at histidine residues 15 and 173 (Figure 4A and B). Interestingly, His 173 lies within the active site catalytic triad of DDAH-1, and mutation of this amino acid has been demonstrated to result in the near complete loss of enzyme catalytic activity (*28*, *29*).

DISCUSSION

Endothelium-derived nitric oxide (NO) has been demonstrated to function as a critical effector molecule in the maintenance of vascular function (*30*−*32*). In the vasculature, NO is derived from the oxidation of L-arginine, catalyzed by the constitutively expressed enzyme, endothelial nitiric oxide synthase (eNOS) (*33*, *34*). Altered NO biosynthesis has been implicated in the pathogenesis of a variety of cardiovascular diseases including atherosclerosis, heart failure, renal failure, diabetes, preeclampsia, and pulmonary hypertension; it is possible that accumulation of the endogenous NOS inhibitors, asymmetric dimethylarginine (ADMA) and NG-monomethy arginine (NMMA), may be involved in the reduced NO generation observed in these conditions (*8*, *17*, *19*). ADMA and L-NMMA are endogenous inhibitors of NOS, exerting their inhibitory effects through competitive inhibition of L-arginine binding and thus preventing L-arginine from being oxidized by NOS to form NO. Metabolism of these endogenous methylarginines is carried out by dimethylarginine dimethylamino hydrolase (DDAH), an enzyme which hydrolyzes the conversion of ADMA to L-citrulline and dimethylamine. Dysregulation of this enzyme has been implicated in a variety of disorders associated with endothelial dysfunction (*17*, *19*). To date, only a few studies have been published regarding DDAH enzymology and regulation; these studies have been carried out using enzyme purified from either *Pseudomonas* or from porcine brain homogenates (*16*, *28*, *29*, *35*, *36*). Despite the potential importance of this enzyme in NOS regulation, there is a paucity of information regarding the kinetics and cellular regulation of the human isoform. In this regard, we have recently expressed and purified hDDAH-1 and have measured the precise kinetic parameters of this enzyme. Results from these studies demonstrated K_m values of 68.7 and 53.6 μM and V_{max} values of 356 and 154 nmols/mg/min for ADMA and L-NMMA, respectively. The maximal enzymatic activity correlates well with previously published reports in which values of 350−400 nmols/mg/min were reported (*37*). However, the K_m values obtained differ from those previously published using DDAH purified from other species, in which values of 180−360 μM were measured (*38*). This has important physiological consequences if one considers that under normal conditions, total intracellular levels of methylarginies in the endothelium are reported to be between 10 and 20 μM (*18*). This would suggest that under physiological conditions, intracellular methylarginine levels are close to the K_m of DDAH, and as a result, loss of enzyme activity would likely result in significant accumulation of endogenous methylarginines. This would be expected to have significant impact on NOS-derived NO, as we have previously demonstrated that normal intracellular levels of methylarginines are present at levels sufficient to basally inhibit NOS by 10−15% (*18*). Thus, inhibition of DDAH and subsequent methylarginine accumulation would be expected to result in significant NOS inhibition and may represent a novel mechanism for NOS regulation and may play a role in the pathophsyiology of endothelial dysfunction.

In addition to measuring the precise kinetic parameters of hDDAH-1, we also evaluated the effects of pH on enzyme activity. Studies were performed using BIS-TRIS-propane/bis-tris buffer with the pH ranging from 5.5 to 9.5. Results demonstrated that hDDAH-1 was maximally active at pH 8.5 and that the activity decreased with pH, and almost complete inhibition was observed at pH values less than 6. This is in contrast to previous studies in which maximal

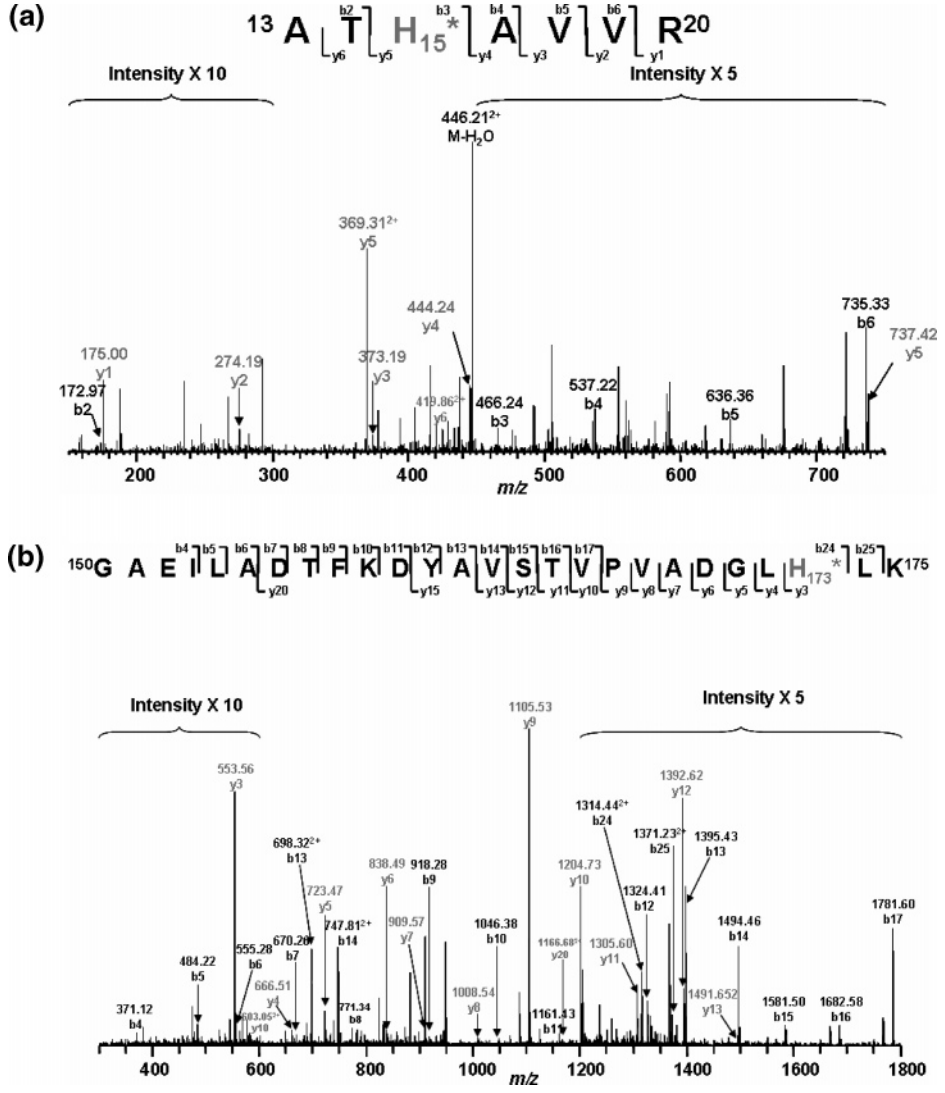

FIGURE 4: MS/MS spectra of a tryptic peptide generating the sequence b/y-ion series from the in-gel digest of the hDDAH-1 reacted with 4-HNE. (a) The peptide observed at m/z 455.2^{+2} corresponds to the aa sequence 13−20 with H15 modified by HNE with the y1−y 6 ions labled and the b2−b6 ions labeled. (b) The peptide observed at m/z 969.9^{+3} corresponds to the aa sequence 150−175 with H173 modified by HNE with the y3−y13, y15, and y 20 ions labeled along with the corresponding b4−b17 and b24−b25 ions labeled.

activity was measured at slightly acidic pH values (*39*). These finding have important relevance in disease states in which the cellular environment becomes acidic, such as myocardial ischemia, in which cellular pH has been demonstrated to drop to pH 5.5 (*40*). These results would suggest that under these conditions, DDAH activity would be largely inhibited and may contribute to the endothelial dysfunction and impaired NO synthesis observed in reperfusion injury.

Recently, Jiang et.al have demonstrated that exposure of endothelial cells to the anti-oxidant Probucol decreases ADMA levels and enhances DDAH activity (*41*). These results suggest that DDAH activity may be modulated by oxidative stress. In this regard, the regulation of cellular homeostasis through post-translational modification of proteins is one of the major responses to oxidative and nitrosative stress. Lysine, arginine, proline, and threonine side chains can be oxidatively converted to reactive aldehydes or ketone groups (carbonylation) causing inactivation, cross-linking, or protein breakdown. Proteins containing cysteine thiol groups are particularly susceptible to oxidation by free radicals, electrophiles, and NO donors. Oxidation of these critical thiol groups can increase or decrease the activity of proteins and represents not only a major mechanism of normal cell signaling but also a mechanism by which disease can interfere with protein function. Therefore, we sought to determine the effects of various reactive oxygen and reactive nitrogen species on DDAH activity. Following exposure of purified hDDAH-1 to varying concentrations (1 μM−1 mM) of NO, $ONOO^-$, •OH, and H_2O_2, DDAH activity was measured. Exposure of hDDAH-1 to pathophysiological levels of reactive oxygen and reactive nitrogen species was found to have only modest effects on enzyme activity. A modest inhibition of 10−20% was observed following exposure to 100 μM $ONOO^-$, •OH, and H_2O_2. At concentrations of 1 mM, exposure to the reactive oxygen species •OH and H_2O_2 resulted in a ∼50% inhibition of DDAH activity; however, these levels do not represent physiologically or pathologically relevant concentrations. Consistent with previous findings, exposure of zinc-free hDDAH-1 to the NO donor DEANONOate resulted in significant inhibition (*16*, *35*). Overall, hDDAH-1 was found to be largely resistant to inhibition by physiological/pathologically relevant levels of reactive oxygen and reactive nitrogen species. However, because DDAH activity has been shown to be inhibited in a

variety of cardiovascular diseases associated with oxidant stress (*8*, *17*, *19*), we carried out further studies aimed at studying the effects of oxidatively modified lipids on enzyme activity.

Among the proposed mechanisms for the impaired NOS activity observed in endothelial dysfunction associated with coronary artery disease are elevated levels of oxidatively modified lipids (*26*, *27*). Polyunsaturated fats in cholesterol esters, phospholipids, and triglycerides are subjected to free radical initiated oxidation. These polyunsaturated fatty acid peroxides can yield a variety of highly reactive smaller molecules such as the aldehyde 4-hydroxy-2-nonenal (4-HNE) upon further oxidative degradation (*42*). 4-HNE is a major biologically active aldehyde formed during the lipid peroxidation of w6 polyunsaturated fatty acids, which has been shown to accumulate in membranes at concentrations from 10 μm to 5 mM (*43*). In this regard, it has been demonstrated that exposure of endothelial cells to 4-HNE increases ADMA levels and inhibits DDAH-1 activity (*24*, *44*). It is hypothesized that this inhibition is mediated through electrophilic addition of 4-HNE to the active site cysteine of DDAH-1. Therefore, we carried out a series of studies in order to determine the dose-dependent and mechanistic effects of 4-HNE on hDDAH-1 activity. Results demonstrated that exposure to 4-HNE resulted in a dose-dependent inhibition of hDDAH-1 activity with 15% inhibition observed at 10 μM and near complete inhibition at 500 μM 4-HNE. Mass spectrometry analysis was then performed in order to determine the mechanisms through which 4-HNE elicits its effects. Previous studies have shown that exposure of proteins to 4-HNE can result in the formation of Michael addition adducts on cysteine and histidine residues (*45*−*47*). Results from our studies definitively demonstrated that exposure of hDDAH-1 to 50 μM 4-HNE for 1 h resulted in Michael adduct formation at histidine residues 15 and 173. This has important functional consequences as His 173 lies within the active site of DDAH-1, forming part of the catalytic triad (Asp127; His173; Cys274), and mutation of this amino acid has been demonstrated to result in near complete loss of enzyme catalytic activity (*28*, *29*). Although the reaction of 4HNE with cysteine is generally thought to be much faster than the reaction with histidine, we did not observe any 4HNE-Cys adducts.

Overall, this study demonstrates that the activity of hDDAH-1 is regulated by its redox environment. Interestingly, the observed oxidant effects are not due to direct radical−DDAH interactions but instead are a consequence of the formation of lipid oxidation products, which occurs secondary to increased free radical production. The levels of 4-HNE used in this study represent concentrations of 4-HNE observed under conditions of inflammation and oxidative stress and suggest that DDAH may play a critical role in mediating the endothelial dysfunction observed in pathological conditions involving oxidative stress through its effects on NO production.

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