0026-895X/10/7702-280–287$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2010 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 77:280–287, 2010

Vol. 77, No. 2
61077/3552831
Printed in U.S.A.

An Inhibitor of Protein Arginine Methyltransferases, 7,7′-Carbonylbis(azanediyl)bis(4-hydroxynaphthalene-2-sulfonic acid (AMI-1), Is a Potent Scavenger of NADPH-Oxidase–Derived Superoxide

Feng Chen and David J. R. Fulton

Vascular Biology Center and Department of Pharmacology, Medical College of Georgia, Augusta, Georgia (D.F., F.C.); and Department of Forensic Sciences, Xi'an Jiaotong University School of Medicine, Xi'an shaanxi, People's Republic of China (F.C.)

Received September 15, 2009; accepted November 9, 2009

ABSTRACT

The methylation of proteins is an important post-translational mechanism that has been established to influence the activity of nuclear and nucleic acid binding proteins. Much less is known about the importance of protein methylation in the regulation of cytosolic proteins. Increased methylation of proteins is observed in cardiovascular disease and occurs in conjunction with elevated production of reactive oxygen species. However, the nature of the relationship between reactive oxygen species and protein methylation is poorly understood. Therefore, the goal of the current study was to determine whether protein methylation influences the catalytic activity of the NADPH oxidases (Nox), which are a family of enzymes responsible for the generation of superoxide. We found that the selective inhibitor of protein arginine methyltransferases 7,7′-carbonylbis(azanediyl)bis(4-hydroxynaphthalene-2-sulfonic acid (AMI-1) was a potent antagonist of Nox-derived superoxide production. However, structurally and mechanistically dissimilar inhibitors of protein methylation and coexpression of protein arginine methyltransferase 1 did not influence Nox activity. Rather, the effect of AMI-1 was rapidly reversible and could be demonstrated in an assay using chemically synthesized superoxide. We conclude that protein methylation does not regulate the activity of NADPH-oxidases and that AMI-1 is a potent antioxidant with a greater potency than 4,5-dihydroxy-1,3-benzenedisulfonic acid (Tiron) and 4-hydroxy-2,2,6,6-tetramethylpiperydine-1-oxyl (Tempol).

The post-translational modification of proteins is a potent mechanism that provides robust and diverse changes in function by influencing protein folding, inter- and intramolecular binding, stability, and subcellular localization. Protein methylation is becoming increasingly recognized as a functionally important post-translational modification and has been shown to share many characteristics with phosphorylation. Methylation occurs on a variety of amino acids, most commonly the nitrogen groups of lysine and in particular arginine residues (Bedford and Clarke, 2009). The formation of methylated arginine residues is catalyzed by a family of enzymes collectively known as protein arginine methyltransferases (PRMTs), of which there are at least nine members. Considerably less is known about the process of demethylation of arginine residues, and because of its slow turnover, arginine methylation was generally thought to be an irreversible modification (Byvoet, 1972). Enzymes that are capable of arginine demethylation have been identified (Chang et al., 2007), which opposes the simplistic view of methylation as a static post-translational modification and suggests that, for some proteins at least, methylation can dynamically affect function.

The methylation of nuclear proteins, including the abundant histones and heterogeneous nuclear ribonucleoproteins, are perhaps the best characterized examples of arginine methylation (Littau et al., 1965; Liu and Dreyfuss, 1995) and are important for cellular proliferation, pluripotency, and differentiation (Wu et al., 2009). Cytosolic proteins can also be modified by methylation, and several PRMT isoforms can

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.109.061077.

ABBREVIATIONS: PRMT, protein arginine methyltransferase; Nox, NADPH-oxidase; AMI-1, 7,7′-carbonylbis(azanediyl)bis(4-hydroxynaphthalene-2-sulfonic acid; MTA, 5′-deoxy-5′-(methylthio)adenosine; Sinefungin, 6,9-diamino-1-(6-amino-9*H*-purin-9-yl)-1,5,6,7,8,9-hexadeoxy-D-glycero-α-L-talo-decafuranuronic acid; eNOS, endothelial nitric-oxide synthase; iNOS, inducible nitric-oxide synthase; L-012, 8-amino-5-chloro-7-phenylpyrido(3,4-*d*)pyridazine-1,4(2*H*,3*H*)dione; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PMS, phenazine methosulfate.

be localized to the cytosol (Pahlich et al., 2006; Bedford and Clarke, 2009). However, compared with nuclear proteins, there are significant gaps in our knowledge about the importance of protein methylation in the regulation of cytosolic protein function.

Cardiovascular disease can result in the accumulation of methylated arginine residues, and elevated levels of free asymmetric dimethylarginine in the plasma predict a negative cardiovascular outcome (Miyazaki et al., 1999; Zoccali et al., 2001). One explanation for this is that asymmetric dimethylarginine is a catalytic inhibitor of endothelial nitric-oxide synthase and increases superoxide production (Druhan et al., 2008). Indeed protein methylation is associated with increased levels of reactive oxygen species (Sydow and Münzel, 2003), but the effects of protein methylation on the enzymes that produce superoxide are not known.

Therefore, the goal of the current study was to identify whether inhibitors of methylation or selective inhibitors of arginine methylation can influence the activity of the NADPH oxidase family of enzymes. To achieve this, we have chosen to use a selective inhibitor of arginine methyltransferases (AMI-1), a nonselective inhibitor of protein methyl-

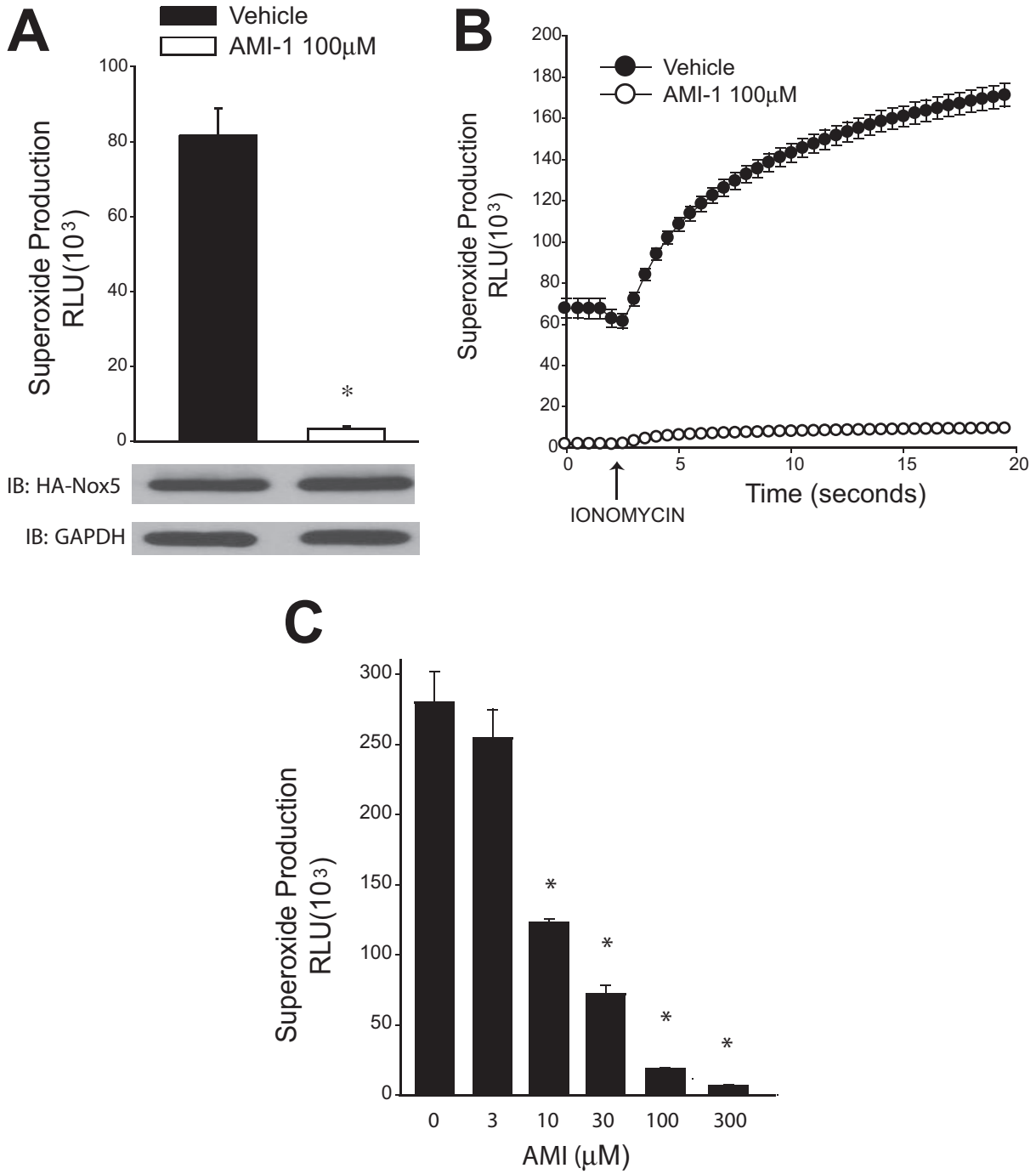

Fig. 1. AMI-1 is a potent inhibitor of Nox5-derived superoxide. Superoxide release was monitored from COS-7 cells transfected with Nox5 in the presence and absence of AMI-1 at the indicated concentrations under unstimulated or basal conditions (A), after stimulation with ionomycin (B), and dose-dependent inhibition (C). Results are presented as mean ± S.E.M. (n = 4–8); *, $P < 0.05$ versus vehicle.

transferases (MTA) (Williams-Ashman et al., 1982), and also an inhibitor of total cellular methylation (sinefungin) (Cheng et al., 2004).

Materials and Methods

Cell Culture, Transfection, and Treatment. COS-7 cells were grown in Dulbecco's modified Eagle's medium containing penicillin (100 U/ml), streptomycin (100 mg/ml), and 10% (v/v) fetal bovine serum and transfected with Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Cells were exposed to different concentrations of AMI-1 sodium salt hydrate (Sigma, St. Louis, MO), MTA (5′-deoxy-5′-(methylthio)adenosine; Sigma), sinefungin (6,9-diamino-1-(6-amino-9*H*-purin-9-yl)-1,5,6,7,8,9-hexadeoxy-D-glycero-α-L-talo-decafuranuronic acid; Thermo Fisher Scientific, Waltham, MA), Tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt monohydrate; Thermo Fisher Scientific), and Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl; Thermo Fisher Scientific) for 1 to 24 h.

DNA Constructs. Nox5 (GenBank accession number AF325189), Nox1, eNOS, and iNOS have been described previously (Jagnandan et al., 2005, 2007; Zhang et al., 2006, 2008). The protein arginine methylation transferase 1 (PRMT1; GenBank accession number NM_198318.2) was generated by polymerase chain reaction from a human cDNA library using the following primers (containing a hemagglutinin tag on forward): forward, 5′-CACCATGTACCCATACGATGTTCCAGATTACGCCGCGGCAGCCGAGGCCGCGAACT-3′; reverse, 5′-CACAGTCACGATGTGCCCTC-3′. DNA sequences were confirmed by automated sequencing.

Measurement of Reactive Oxygen Species. COS-7 cells were transfected with cDNAs encoding Nox5/Nox1(with NoxO1/NoxA1) or control plasmids (RFP or LacZ); 48 h later, cells were replated into white tissue culture-treated 96-well plates (Thermo Fisher Scien-

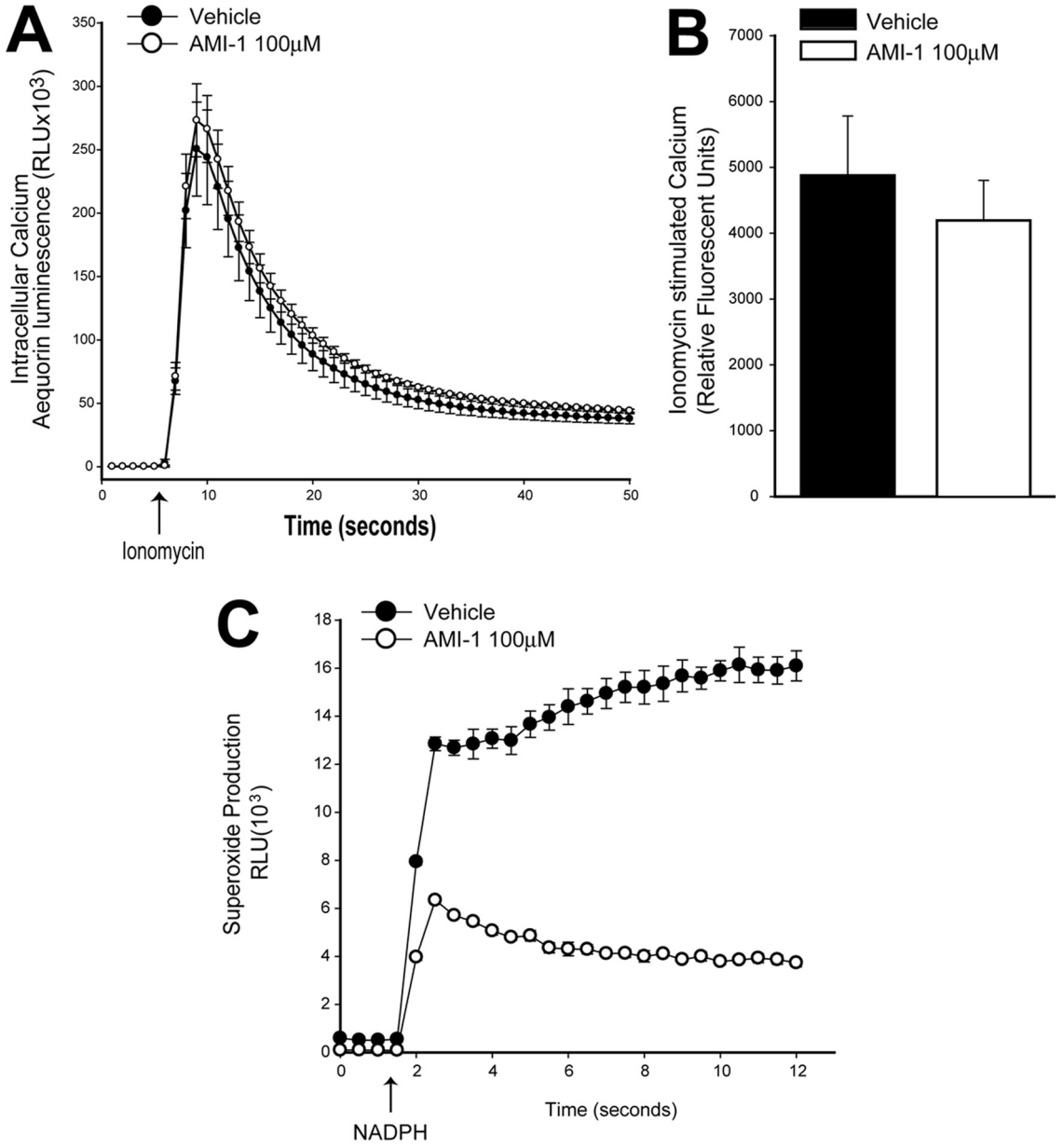

Fig. 2. AMI-1 exerts a direct effect of Nox5 and does not modify the level of intracellular calcium. Calcium was monitored inside COS-7 cells transfected with aequorin (A) or in untransfected cells using the calcium-indicator dye (B) in the presence and absence of AMI-1 (100 μM). C, the activity of Nox5 in cell-free extracts was determined in the presence and absence of AMI-1 (100 μM). Arrow indicates injection of NADPH. Results are presented as mean ± S.E.M. ($n = 8$). *, $P < 0.05$ versus vehicle.

tific) at a density of ~5 × 10^4 cells/well. The cells were incubated at 37°C in phenol-free Dulbecco's modified Eagle's medium (Sigma) containing L-012 (400 μM; Wako) for approximately 10 min, and luminescence was quantified over time using a LUMIstar Galaxy luminometer (BMG Labtech, Durham, NC). The relative light units quantified from the luminescence of L-012 are reflective of changes in the superoxide produced by Nox enzymes (Jagnandan et al., 2007).

Measurement of Hydrogen Peroxide. COS-7 cells were transfected with Nox cDNAs or control plasmids (LacZ) and hydrogen peroxide was measured using the Amplex Red assay with excitation of 530 to 560 nm and emission detection at ~590 nm. Cells were incubated at 37°C with 50 μM Amplex Red, 0.125 U/ml horseradish peroxidase in phenol-free Dulbecco's modified Eagle's medium (Sigma) for 10 min. Relative light units were calculated after subtraction of control groups (LacZ/catalase).

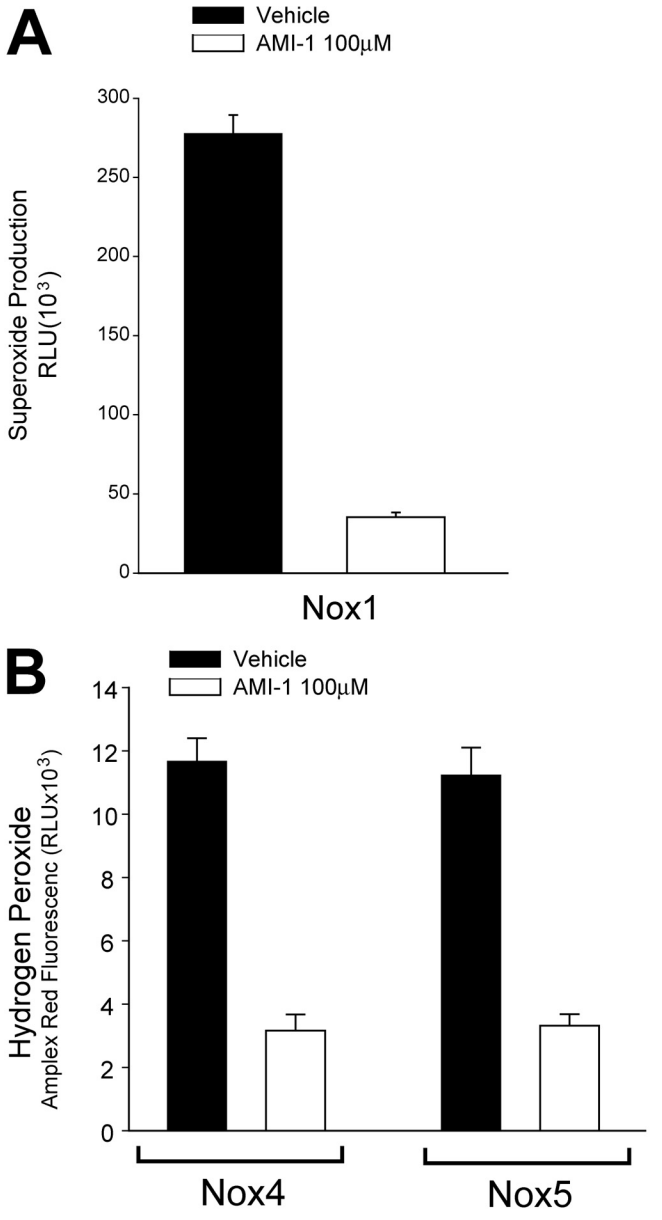

Fig. 3. Superoxide produced by other Nox is inhibited by AMI-1. A, COS-7 cells were cotransfected with Nox1 together with NoxO1/NoxA1 and superoxide measured in the presence and absence of AMI-1 (100 μM). B, H_2O_2 was monitored from cells expressing Nox4 or Nox5 in the presence and absence of AMI-1 (100 μM). Results are presented as mean ± S.E.M. (n = 8). *, $P < 0.05$ versus vehicle.

NO Release. Media (100 μl) containing nitrite and nitrate (primarily NO_2^-) was ethanol-precipitated to remove proteins and refluxed in sodium iodide/glacial acetic acid to convert NO_2^- to NO. NO was measured via specific chemiluminescence after reaction with ozone (Sievers NO analyzer; GE Analytical Instruments, Boulder, CO). Net NO_2^- from cells transfected with eNOS or iNOS was calculated after subtracting NO_2^- levels from cells lacking NOS activity (LacZ).

Western Blot Analysis. Cells were washed twice with phosphate-buffered saline, lysed on ice in 50 mM Tris-HCl, pH 6.8, 2% SDS, 30% glycerol, 6% β-mercaptoethanol, and 0.02% bromphenol blue. Lysates were clarified at 13,000 rpm for 10 min at 4°C and size-fractionated by 10% SDS-polyacrylamide gel electrophoresis and immunoblotted with antibodies to hemagglutinin (Roche Applied Science, Indianapolis, IN). All reagents were acquired from Sigma-Aldrich.

Intracellular Ca^{2+} Measurements. Intracellular calcium was measured using aequorin, as described previously (Church and Fulton, 2006). In brief, cells were transduced with an aequorin adenovirus and 24 h later reconstituted with coelenterazine (5 μM) for 1 h in calcium-free Hanks' solution. The cells were then exposed to 1 μM ionomycin, and luminescence was recorded using a luminescence plate reader (LumiSTAR Galaxy). Alternatively, calcium was measured by Fluo-4 Direct (Invitrogen), and fluorescence was measured with a LumiSTAR Galaxy plate reader with excitation at 494 nm and emission at 516 nm. Calcium levels were calculated after addition of ionomycin (1 μM) and subtraction of basal fluorescence.

Cell-Free Nox Activity Assays. COS-7 cells expressing Nox5 were lysed in a MOPS (30 mM, pH 7.2)-based buffer containing KCl (100 mM), Triton X-100 (0.3%), and protease inhibitors (Sigma). Adherent cells were rocked gently, and the lysis buffer was aspirated and then washed with phosphate-buffered saline (4°C). Remaining fractions were resuspended in the MOPS buffer with 0.3 mM EGTA to remove residual calcium and sonicated at low power and centrifuged at 14,000 rpm (4°C). The supernatant was then aspirated, and the pellet was resuspended in MOPS buffer with mild sonication. The cell-free extract was aliquoted into buffers containing L-012 (400 μM), 1 mM $MgCl_2$, 100 μM FAD (Sigma), and 1 mM $CaCl_2$. After a brief period of equilibration with or without agonists, reduced NADPH (Sigma) was injected to a final concentration of 200 μM, and the production of reactive oxygen species was monitored over time as described previously (Jagnandan et al., 2007).

Antioxidant Assay. The generation of superoxide from NADH (Sigma) and PMS (Sigma) has been described previously (Ewing and Janero, 1995). In brief, PMS (3.3 μM) was prepared in 50 mM phosphate buffer containing 400 μM L-012 (Wako Pure Chemicals, Osaka, Japan), pH 7.4, in the presence and absence of AMI-1 (100 μM). NADH was injected at a final concentration of 78 μM, and luminescence was quantified over time.

Statistical Analysis. Superoxide release data are expressed as means ± S.E.M. Comparisons were made using two-tailed Student's t test or analysis of variance, with a post hoc test where appropriate. Differences were considered significant at $p < 0.05$.

Results

AMI-1 Inhibits Superoxide Generated by NADPH Oxidase 5. To determine whether protein methylation can influence the activity of NADPH oxidases and thus superoxide production, we incubated COS-7 cells expressing Nox5 with vehicle (H_2O) or the selective PRMT inhibitor AMI-1 and measured superoxide output with the use of L-012 enhanced chemiluminescence (Imada et al., 1999). At 100 μM, AMI-1 virtually eliminated measurable superoxide production from unstimulated cells (Fig. 1A) and cells stimulated with ionomycin (Fig. 1B). The ability of AMI-1 to suppress the superoxide signal was not related to a change in the expression of Nox5 as shown by Western blot (Fig. 1A, bot-

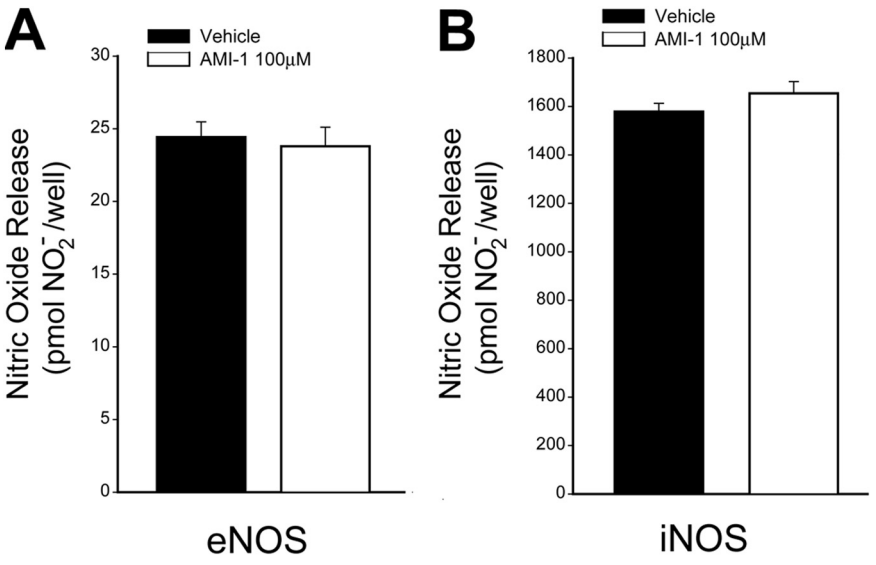

Fig. 4. NO production is unaffected by AMI-1. COS-7 cells were transfected with eNOS (A) or iNOS (B), and NO release was measured by NO-specific chemiluminescence in the presence and absence of AMI-1 (100 μM). Results are presented as mean ± S.E.M. (n = 4).

tom). The inhibition of Nox5-derived superoxide was inhibited by AMI-1 in a dose-dependent manner with an IC_{50} of approximately 8.3 μM (Fig. 1C). The ability of AMI-1 to suppress superoxide levels could be due to a number of factors, including the direct inhibition of enzyme activity and indirect inhibition through the alteration of second messengers, such as calcium, or through an ability to react directly with superoxide.

AMI-1 Directly Inhibits Nox5 and Does Not Modify Intracellular Calcium Levels. Nox5 is a calcium-dependent enzyme that generates superoxide in response to an elevation in intracellular calcium. Therefore, we next investigated whether AMI-1 exerts its inhibitory actions by inhibiting the mobilization of intracellular calcium. Two separate approaches were employed to monitor intracellular calcium: the calcium-dependent bioluminescent protein aequorin and the calcium indicator dye Fluo4-acetoxymethyl ester. As shown in Fig. 2A, AMI-1 did not modify the levels of basal or ionomycin-stimulated calcium in COS-7 cells. Becuase AMI-1 might also influence the activity of the protein aequorin, we also measured intracellular calcium using Fluo4-acetoxymethyl ester. As shown in Fig. 2B, AMI-1 did not reduce the ability of ionomycin to elevate calcium levels in COS-7 cells. Because calcium levels are unaffected by AMI-1, we next tested whether AMI-1 can directly influence Nox5 activity. This was achieved using a cell-free activity assay using partially purified Nox5 and the addition of cofactors FAD, calcium, and NADPH. As shown in Fig. 2C, AMI-1 directly inhibited superoxide production from Nox5, which demonstrates that cytosolic factors such as the level of intracellular calcium or the presence of other proteins are not required for its inhibitory actions. However, we cannot yet exclude an action of AMI-1 that is specific for the Nox5 isoform or the inhibition of all NADPH-flavoprotein–dependent processes.

AMI-1 Inhibits Other Nox Isoforms, but Not Nitric-Oxide Synthases. Therefore, we next tested whether AMI-1 could influence the activity of other NADPH oxidase isoforms. AMI-1 was effective at inhibiting superoxide production from cells expressing Nox1 and its activating subunits NoxO1 and NoxA1 (Fig. 3A) and also inhibited hydrogen peroxide produced from Nox4 and Nox5 (Fig. 3B). To determine whether AMI-1 inhibits other NADPH-dependent enzymes, we examined whether it can influence the activity of the nitric-oxide synthases eNOS and iNOS. AMI-1 had no effect on the ability of either eNOS or iNOS to generate nitric oxide (Fig. 4), demonstrating that it has a selective action on either the activity of the Nox family of enzymes as a group or the superoxide that they generate.

Mechanistically Dissimilar Inhibitors of Protein Methylation and Coexpression of PRMT1 Do Not Influence the Level of Nox5-Derived Superoxide. To test whether the inhibitory actions of AMI-1 are shared by structurally and mechanistically dissimilar inhibitors of arginine methylation, we next evaluated the effects of sinefungin (Fig. 5A) and MTA (Fig. 5B). In contrast to AMI-1, both MTA and sinefungin failed to inhibit the level of superoxide produced by Nox5. Likewise, overexpression of the arginine methyltransferase PRMT1 with Nox5 did not modify superoxide levels (Fig. 5C) or alter Nox5 expression (Fig. 5C, bottom). The combination of increased PRMT1 expression together with inhibition of methylation with sinefungin (Fig. 5D) or MTA (data not shown) was also ineffective, and these results argue against the importance of direct protein methylation in the control of Nox5 activity.

The Ability of AMI-1 to Inhibit Nox5-Derived Superoxide Is Rapidly Reversible. Because protein methylation is regarded as a lasting or more permanent type of post-translational modification, we next evaluated whether the effects of AMI-1 were rapidly reversible and thus unrelated to inhibition of protein methylation. In COS-7 cells expressing Nox5, the addition of AMI-1 instantaneously inhibited superoxide production (data not shown). However, the short-term removal of AMI-1 after a change of media completely abrogated this ability (Fig. 6A). This reversible action of AMI-1 was also apparent in cell-free activity assays (Fig. 6B), and these data suggest that inhibition of protein methylation and subsequent corruption of enzyme activity is not the primary mechanism of inhibition.

AMI-1 Is a Potent Antioxidant. Because AMI-1 was not modifying Nox5 activity directly, we next determined whether it could be acting as a scavenger of reactive oxygen species. To achieve this, we examined the effect of AMI-1 on a nonenzymatic, superoxide-generating assay and found that AMI-1 was a very effective scavenger of superoxide (Fig. 7A).

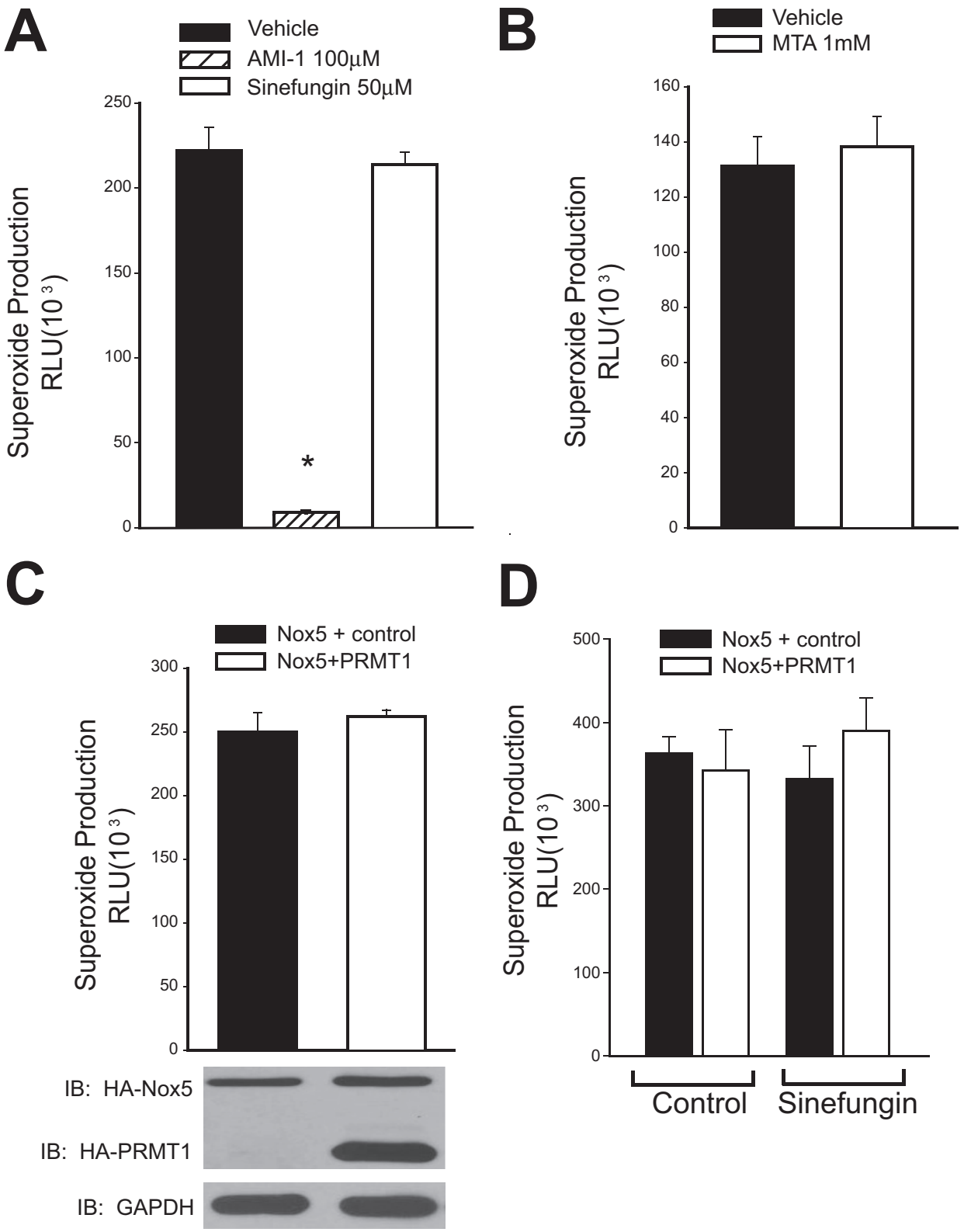

Fig. 5. Structurally and mechanistically distinct inhibitors of protein methylation and coexpression of PRMT1 do not affect Nox5-derived superoxide. Superoxide release from COS-7 cells transfected with Nox5 in the presence and absence of AMI-1 (100 μM) and sinefungin (50 μM) (A); MTA (1 mM) (B); cotransfected with PRMT1 (C); and cotransfected with PRMT1 in the presence and absence of sinefungin (50 μM) (D). Results are presented as mean ± S.E.M. (n = 8).

We next compared the potency of the antioxidant actions of AMI-1 versus established scavengers of superoxide, Tiron (Devlin et al., 1981) and Tempol (Krishna et al., 1996). We found that AMI-1 was a considerably more effective inhibitor of superoxide production than Tiron or Tempol (Fig. 7B). Collectively, these results suggest that AMI-1 is a potent

scavenger of superoxide and that arginine methylation plays little if any role in the regulation of Nox activity.

Discussion

Methylation is one of the most abundant post-translation modifications of proteins, and most of our knowledge of protein methylation is based on the abundant methylation of nuclear proteins such as histones, transcription factors, and coregulators (Lee and Stallcup, 2009). However, evidence for the regulation of cytosolic proteins by methylation is still emerging, with important roles demonstrated in signal transduction, RNA processing, metabolism, and oxidative stress (Philips et al., 1993; Liu and Dreyfuss, 1995; Abramovich et al., 1997; Gary and Clarke, 1998; An et al., 2009). Increased levels of reactive oxygen species stimulate protein methylation (Sydow and Münzel, 2003); interestingly, some of the most abundant methylated proteins in the cytosol are those involved in the defense against oxidative metabolites (An et al., 2009). Therefore, the goal of the current study was to determine whether the activity of the Nox enzymes, which are responsible for the majority of reactive oxygen species production in cells (Griendling et al., 2000), is influenced by methylation. We found that the selective inhibitor of protein arginine methylation AMI-1 potently reduced the levels of superoxide produced by Nox5 and other Nox isoforms.

However, this ability of AMI-1 to reduce superoxide levels was not due to a direct effect on Nox activity, in that it was both rapid and rapidly reversible, and methylation has been reported to be a more permanent modification of proteins (Byvoet, 1972). To rule out potential secondary effects of AMI-1, we also examined whether it modified the levels of

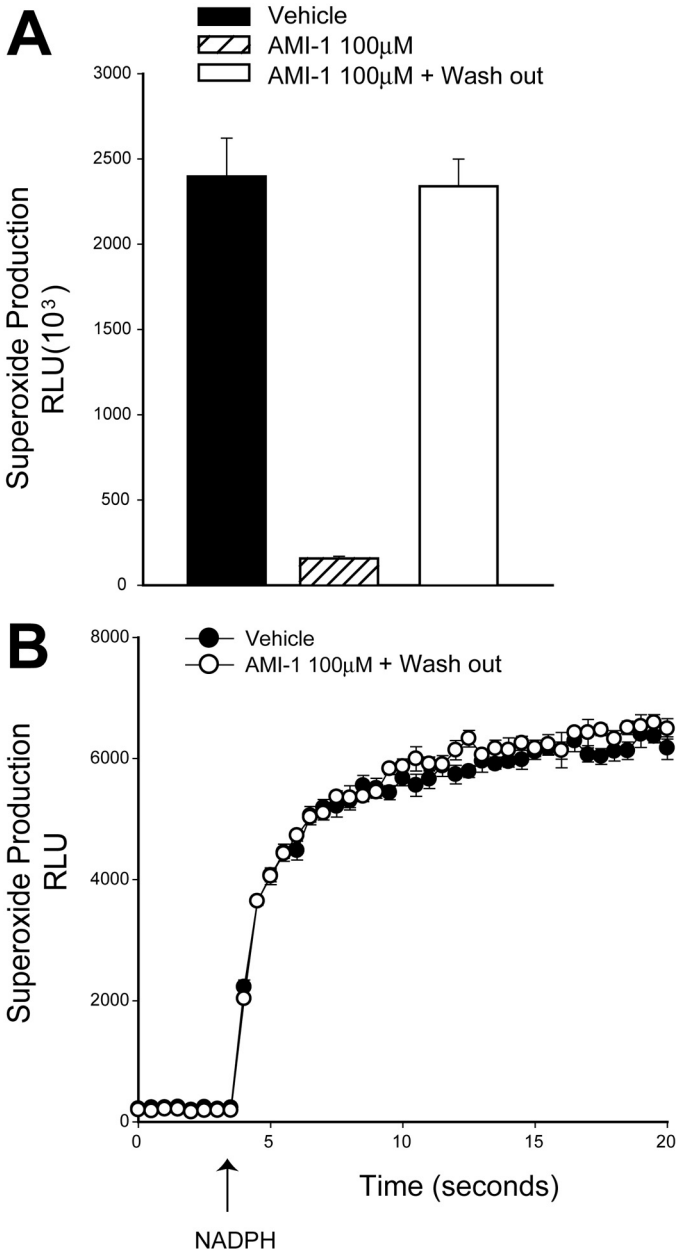

Fig. 6. The inhibitory effect of AMI-1 is rapidly reversible. A, superoxide release from Nox5-transfected COS-7 cells in the presence of AMI-1 (100 μM) and after washout. B, superoxide release from a cell-free activity assay in which cells were exposed to AMI-1 (100 μM), washed, and then disrupted by detergents. Results are presented as mean ± S.E.M. (n = 8).

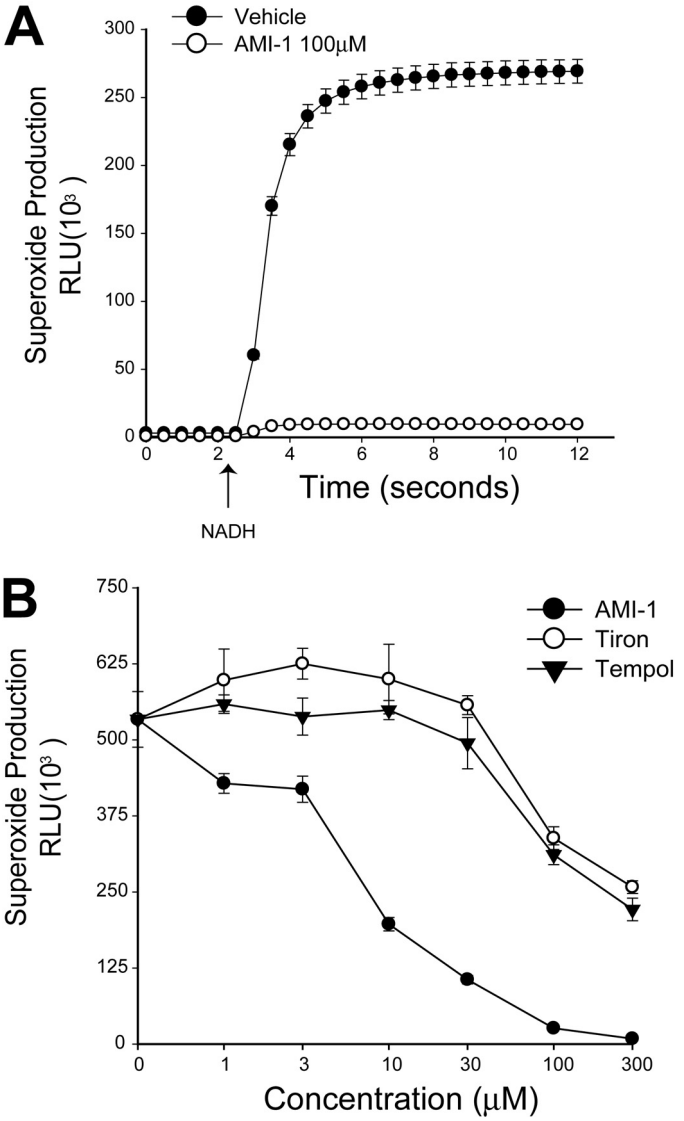

Fig. 7. AMI-1 has potent antioxidant activities. A, superoxide generation from the addition of NADH (arrow) to PMS as measured by L-012 chemiluminescence in the presence and absence of AMI-1 (100 μM). B, comparison of the antioxidant potencies of AMI-1 versus Tempol and Tiron in COS-7 cells expressing Nox5 (n = 4–8).

intracellular calcium or the activity of other NADPH-dependent enzymes, eNOS and iNOS, and found that it was without effect. Mechanistically and structurally distinct inhibitors of protein methylation sinefungin and MTA failed to acutely modify the activity of the Nox enzymes, further suggesting that AMI-1 was not directly modifying the enzymatic activity of Nox5, and this contention was supported by studies with coexpression of the protein arginine methyltransferase PRMT1 and Nox5. PRMT1 has a broad range of substrates and is responsible for the methylation of >85% of proteins and resides in both the cytosol and nucleus (Bedford and Clarke, 2009). However, increased expression of PRMT1 did not influence Nox activity. Therefore we next examined whether AMI-1 could inhibit superoxide from a nonenzymatic source and found that rather than directly inhibiting the catalytic activity of Nox enzymes, AMI-1 was acting as an antioxidant. Indeed, AMI-1 was considerably more efficacious at inhibiting superoxide than the established inhibitors of superoxide, Tiron and Tempol. Thus the ability of AMI-1 to scavenge superoxide directly provides a robust mechanism to account for the reduced levels of superoxide seen from cells expressing the different NADPH oxidase isoforms.

A potential caveat to our findings is that protein methylation may have a slow turnover (Byvoet, 1972) and that the short-term inhibition of methyltransferases may not provide the level of discrimination needed to observe an effect on activity. However, when cells were exposed to MTA for longer times (>24 h), there was no further effect on superoxide produced by Nox5, suggesting that the time of incubation was not a limiting factor.

The concentration (IC_{50}) of AMI-1 required to inhibit the major arginine methyltransferase (PRMT1) was similar to that required to scavenge superoxide (~8 μM) (Cheng et al., 2004). Although increased reactive oxygen species have been shown to correlate with increased methylation and increased PRMT activity (Yamagata et al., 2008), it is unlikely that reactive oxygen species scavenging underlies the selective ability of AMI-1 to inhibit arginine versus lysine methyltransferases. Sinefungin and MTA have both been shown to be effective inhibitors of both arginine and lysine methylation (Maher, 1993; Cheng et al., 2004) but do not exhibit antioxidant properties. Furthermore, only PRMT3 and PRMT5, and not PRMT1 or PRMT4, are sensitive to the reducing agent *N*-ethylmaleimide (Frankel and Clarke, 2000), indicating that the modification of cysteine sulfhydryl groups is not a requisite step in the ability of AMI-1 to inhibit these enzymes. A further caveat of these studies is that they have relied on the use of L-012 to detect superoxide. It is therefore possible but quite unlikely that AMI-1 could be interacting specifically with L-012 to reduce its ability to measure superoxide. The exact nature of the antioxidant ability of AMI-1, specifically whether it induces the dismutation of superoxide into hydrogen peroxide or acts in another manner, remains to be determined.

In summary, although increased production of reactive oxygen species promotes methylation of proteins and accumulation of methylated arginine residues, we found no evidence to support a role for arginine methylation in the activity of Nox enzymes. Furthermore, we found that a selective inhibitor of protein arginine methyltransferases is a potent scavenger of reactive oxygen species. This has important implications when studying the effects of this or related compounds in the context of cardiovascular disease or cancer, where oxidative stress may be increased.

References

Abramovich C, Yakobson B, Chebath J, and Revel M (1997) A protein-arginine methyltransferase binds to the intracytoplasmic domain of the IFNAR1 chain in the type I interferon receptor. *EMBO J* **16:**260–266.

An S, Yun M, Park YG, and Park GH (2009) Proteomic identification of cytosolic proteins that undergo arginine methylation during rat liver regeneration. *Electrophoresis* **30:**2412–2421.

Bedford MT and Clarke SG (2009) Protein arginine methylation in mammals: who, what, and why. *Mol Cell* **33:**1–13.

Byvoet P (1972) In vivo turnover and distribution of radio-N-methyl in arginine-rich histones from rat tissues. *Arch Biochem Biophys* **152:**887–888.

Chang B, Chen Y, Zhao Y, and Bruick RK (2007) JMJD6 is a histone arginine demethylase. *Science* **318:**444–447.

Cheng D, Yadav N, King RW, Swanson MS, Weinstein EJ, and Bedford MT (2004) Small molecule regulators of protein arginine methyltransferases. *J Biol Chem* **279:**23892–23899.

Church JE and Fulton D (2006) Differences in eNOS activity because of subcellular localization are dictated by phosphorylation state rather than the local calcium environment. *J Biol Chem* **281:**1477–1488.

Devlin RG, Lin CS, Perper RJ, and Dougherty H (1981) Evaluation of free radical scavengers in studies of lymphocyte-mediated cytolysis. *Immunopharmacology* **3:**147–159.

Druhan LJ, Forbes SP, Pope AJ, Chen CA, Zweier JL, and Cardounel AJ (2008) Regulation of eNOS-derived superoxide by endogenous methylarginines. *Biochemistry* **47:**7256–7263.

Ewing JF and Janero DR (1995) Microplate superoxide dismutase assay employing a nonenzymatic superoxide generator. *Anal Biochem* **232:**243–248.

Frankel A and Clarke S (2000) PRMT3 is a distinct member of the protein arginine *N*-methyltransferase family. Conferral of substrate specificity by a zinc-finger domain. *J Biol Chem* **275:**32974–32982.

Gary JD and Clarke S (1998) RNA and protein interactions modulated by protein arginine methylation. *Prog Nucleic Acid Res Mol Biol* **61:**65–131.

Griendling KK, Sorescu D, and Ushio-Fukai M (2000) NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res* **86:**494–501.

Imada I, Sato EF, Miyamoto M, Ichimori Y, Minamiyama Y, Konaka R, and Inoue M (1999) Analysis of reactive oxygen species generated by neutrophils using a chemiluminescence probe L-012. *Anal Biochem* **271:**53–58.

Jagnandan D, Church JE, Banfi B, Stuehr DJ, Marrero MB, and Fulton DJ (2007) Novel mechanism of activation of NADPH oxidase 5. calcium sensitization via phosphorylation. *J Biol Chem* **282:**6494–6507.

Jagnandan D, Sessa WC, and Fulton D (2005) Intracellular location regulates calcium-calmodulin-dependent activation of organelle-restricted eNOS. *Am J Physiol Cell Physiol* **289:**C1024–1033.

Krishna MC, Russo A, Mitchell JB, Goldstein S, Dafni H, and Samuni A (1996) Do nitroxide antioxidants act as scavengers of $O_2^{\cdot}$ or as SOD mimics? *J Biol Chem* **271:**26026–26031.

Lee YH and Stallcup MR (2009) Minireview: protein arginine methylation of nonhistone proteins in transcriptional regulation. *Mol Endocrinol* **23:**425–433.

Littau VC, Burdick CJ, Allfrey VG, and Mirsky SA (1965) The role of histones in the maintenance of chromatin structure. *Proc Natl Acad Sci U S A* **54:**1204–1212.

Liu Q and Dreyfuss G (1995) In vivo and in vitro arginine methylation of RNA-binding proteins. *Mol Cell Biol* **15:**2800–2808.

Maher PA (1993) Inhibition of the tyrosine kinase activity of the fibroblast growth factor receptor by the methyltransferase inhibitor 5′-methylthioadenosine. *J Biol Chem* **268:**4244–4249.

Miyazaki H, Matsuoka H, Cooke JP, Usui M, Ueda S, Okuda S, and Imaizumi T (1999) Endogenous nitric oxide synthase inhibitor: a novel marker of atherosclerosis. *Circulation* **99:**1141–1146.

Pahlich S, Zakaryan RP, and Gehring H (2006) Protein arginine methylation: Cellular functions and methods of analysis. *Biochim Biophys Acta* **1764:**1890–1903.

Philips MR, Pillinger MH, Staud R, Volker C, Rosenfeld MG, Weissmann G, and Stock JB (1993) Carboxyl methylation of Ras-related proteins during signal transduction in neutrophils. *Science* **259:**977–980.

Sydow K and Münzel T (2003) ADMA and oxidative stress. *Atheroscler Suppl* **4:**41–51.

Williams-Ashman HG, Seidenfeld J, and Galletti P (1982) Trends in the biochemical pharmacology of 5′-deoxy-5′-methylthioadenosine. *Biochem Pharmacol* **31:**277–288.

Wu Q, Bruce AW, Jedrusik A, Ellis PD, Andrews RM, Langford CF, Glover DM, and Zernicka-Goetz M (2009) CARM1 is required in embryonic stem cells to maintain pluripotency and resist differentiation. *Stem Cells* **27:**2637–2645.

Yamagata K, Daitoku H, Takahashi Y, Namiki K, Hisatake K, Kako K, Mukai H, Kasuya Y, and Fukamizu A (2008) Arginine methylation of FOXO transcription factors inhibits their phosphorylation by Akt. *Mol Cell* **32:**221–231.

Zhang Q, Church JE, Jagnandan D, Catravas JD, Sessa WC, and Fulton D (2006) Functional relevance of Golgi- and plasma membrane-localized endothelial NO synthase in reconstituted endothelial cells. *Arterioscler Thromb Vasc Biol* **26:**1015–1021.

Zhang Q, Malik P, Pandey D, Gupta S, Jagnandan D, Belin de Chantemele E, Banfi B, Marrero MB, Rudic RD, Stepp DW, et al. (2008) Paradoxical activation of endothelial nitric oxide synthase by NADPH oxidase. *Arterioscler Thromb Vasc Biol* **28:**1627–1633.

Zoccali C, Bode-Böger S, Mallamaci F, Benedetto F, Tripepi G, Malatino L, Cataliotti A, Bellanuova I, Fermo I, Frölich J, et al. (2001) Plasma concentration of asymmetrical dimethylarginine and mortality in patients with end-stage renal disease: a prospective study. *Lancet* **358:**2113–2117.

Address correspondence to: Dr. David J. R. Fulton, Vascular Biology Center, Medical College of Georgia, 1459 Laney Walker Blvd, Augusta, GA 30912. E-mail: dfulton@mcg.edu