Modulation by Peroxynitrite of Akt- and AMP-activated kinase-dependent Serine\textsuperscript{1179} Phosphorylation of Endothelial Nitric Oxide Synthase

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Running Title: AMP kinase and eNOS phosphorylation

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Summary

Peroxynitrite (ONOO\textsuperscript{−}), a nitric oxide-derived oxidant, uncouples endothelial nitric oxide synthase (eNOS) and increases enzymatic production of superoxide anions (O\textsubscript{2}\textsuperscript{−}, Zou, et al, J. Clin. Invest. 109:817-826, 2002). Here we studied how ONOO\textsuperscript{−} influences eNOS activity. In cultured bovine aortic endothelial cells (BAEC), ONOO\textsuperscript{−} increased basal and agonist-stimulated serine\textsuperscript{1179} (Ser\textsuperscript{1179}) phosphorylation of eNOS, whereas it decreased nitric oxide production and bioactivity. However, ONOO\textsuperscript{−} strongly inhibited the phosphorylation and activity of Akt, which is known to phosphorylate eNOS-Ser\textsuperscript{1179}. Moreover, expression of an Akt dominant negative mutant did not prevent ONOO\textsuperscript{−}-enhanced eNOS-Ser\textsuperscript{1179} phosphorylation. In contrast to Akt, ONOO\textsuperscript{−} significantly activated 5′-AMP-activated kinase (AMPK), as evidenced by its increased Thr\textsuperscript{172} phosphorylation, as well as increased Ser\textsuperscript{92} phosphorylation of acetyl CoA carboxylase, a downstream target of AMPK. Associated with the increased release of O\textsubscript{2}\textsuperscript{−}, ONOO\textsuperscript{−} significantly increased the co-immunoprecipitation of eNOS with AMPK. Further, overexpression of the AMPK-constitutive active adenovirus significantly enhanced ONOO\textsuperscript{−}-upregulated eNOS-Ser\textsuperscript{1179}-P as well as the release of O\textsubscript{2}\textsuperscript{−}. In contrast, overexpression of a dominant-negative AMPK mutant attenuated the ONOO\textsuperscript{−}-enhanced eNOS-Ser\textsuperscript{1179} phosphorylation and O\textsubscript{2}\textsuperscript{−} release. We conclude that ONOO\textsuperscript{−} inhibits Akt and increases AMPK-dependent Ser\textsuperscript{1179} phosphorylation of eNOS resulting in enhanced O\textsubscript{2}\textsuperscript{−} release.
Introduction:

Mammalian AMP-activated protein kinase (AMPK) belongs to a family of protein kinases that has been highly conserved in animals, plants and yeast, and which plays a key role in the regulation of energy homeostasis (1,2). AMPK is a heterotrimeric enzyme, consisting of a catalytic subunit (α) and two regulatory subunits (β and γ) (3, 4). AMPK is activated by cellular stresses, such as shock, hypoxia and ischemia, that deplete ATP and which in turn elevate the ratio of AMP to ATP (2, 5). In addition to allosteric activation by AMP, AMPK is phosphorylated and activated by an upstream kinase, termed AMPK kinase (AMPKK) (6). Activation of AMPK results from phosphorylation of Thr^{172} in the activation loop of the catalytic α-subunit, although other phosphorylation sites have been reported (7). Whether or not AMPK is regulated by mechanisms other than the AMP/ATP ratio remains elusive.

Once activated, AMPK phosphorylates multiple downstream substrates aimed at conserving existing ATP levels. AMPK reduces further ATP expenditure by inhibiting key enzymes in biosynthetic pathways such as acetyl-CoA carboxylase (ACC), which is important in fatty acid synthesis, and 3-hydroxy-3-methyl-CoA reductase in cholesterol synthesis (2, 8-10). Despite the recent observation that AMPK phosphorylates endothelial nitric oxide synthase (eNOS) on Ser^{1179} (based on the bovine eNOS sequence and equivalent to human eNOS-Ser^{1177}) and activates rat cardiac eNOS in vitro (11, 12), the mechanism and functional implications of AMPK-mediated eNOS phosphorylation remains unknown.

Peroxynitrite (ONOO⁻), a highly reactive oxidant formed by the diffusion-controlled reaction of superoxide anion (O_2⁻) and nitric oxide (NO), is formed during
sepsis, inflammation, diabetes, ischemia-reperfusion, and atherosclerosis, and contributes to these pathophysiological processes (13-17). In a recent study we showed that ONOO− oxidizes the zinc-thiolate cluster of eNOS, inhibiting its NO synthetic activity, but increasing the NADPH oxidase activity and $O_2^−$ production by the enzyme (17). In the present study, we further examined how ONOO− regulates eNOS activity. We show that in bovine aortic endothelial cells (BAEC), ONOO− enhances $O_2^−$ release partially by increasing eNOS-Ser1179 phosphorylation while inhibiting NO release from the uncoupled enzyme. ONOO− inhibits Akt/PKB activity, but in contrast, activates AMPK, as shown by increased Thr172 phosphorylation of AMPK and Ser92 phosphorylation of acetyl CoA carboxylase (ACC), a downstream target of AMPK. In addition, ONOO− significantly increases association of eNOS and AMPK. Furthermore, by expressing an AMPK dominant-negative mutant, the enhanced activity of AMPK was demonstrated to be required for the increased release of $O_2^−$ by the eNOS.

**Methods and Materials**

**Materials.**

Bovine aortic endothelial cells (BAEC) and cell culture media were obtained from Clonetics Inc. (Walkersville, MD). Confluent BAEC cells were maintained in 2% FCS and growth factors before use. $^3$H-L-arginine was purchased from New England (Boston, MA). Dowex AG50W-X8 columns were obtained from Bio-Rad (Hercules, CA). Cyclic GMP ELISA kit, bovine recombinant NOS, and sepiapeterin were obtained from Cayman Chemical (Ann Arbor, MI). Poly- and mono-clonal antibodies against eNOS were obtained from BD Transduction Laboratory (San Diego, CA). Antibodies against anti-
phospho-acetyl CoA carboxylase (ACC) (Ser\textsuperscript{79}) were from Upstate Biotechnology (Lake Placid, NY). Akt assay kit and antibodies against, Akt, phospho-Akt (Thr\textsuperscript{308} or Ser\textsuperscript{473}), phosphor-eNOS (Ser\textsuperscript{1177}), phospho-GSK-3\(\alpha/\beta\) (Ser\textsuperscript{21/9}), phospho-AMPK (Thr\textsuperscript{172}), AMPK were obtained from Cell Signaling Inc. (Beverly, MA). H89, KT5823, ODQ, tetrahydrobiopeterin (BH\textsubscript{4}), and NADPH were purchased from Calbiochemical (La Jolla, CA). L-arginine, HEPES, FAD, FMN, EDTA, L-arginine, 2-mercaptoethanol, 5-HT, calcium ionophore A23187, cytochrome c, catalase, zaprinast were obtained from Sigma (St Louis, MO). Other chemicals and organic solvents of highest grade were obtained from Fisher Scientific (Morris Plains, NJ).

Methods

Adenoviral Transfection

BAEC cells were transfected with adenovirus expressing a dominant-negative mutant Akt (Akt-DN) (18), constitutive AMPK (19), or dominant-negative AMPK (mutation of LY45 to Arg, 20). BAEC cells were infected in medium with 2% FCS overnight. The cells were then washed and incubated in fresh EGM medium with 2% FCS for an additional 24 h prior to experimentation. Using these conditions, transfection efficiency was typically >80% as determined by GFP expression.

Peroxynitrite Synthesis

ONO\textsuperscript{-} was synthesized using a quenched-flow reaction as previously described (17). The concentrations of ONO\textsuperscript{-} were determined spectrally in 0.1 M NaOH \((\varepsilon_{302}=1670 \text{ M}^{-1}\text{S}^{-1})\). ONO\textsuperscript{-} was diluted in 0.1 M NaOH before use in order to avoid a sharp shift of pH.
Treatment of bovine aortic endothelial cells with ONOO⁻

Confluent BAEC cell were treated with ONOO⁻, as described previously (17). Briefly, to confluent cells in 6-well plates was added 950 µL of 100 mmol/L HEPES buffer, pH 7.4. 50 µL of concentrated ONOO⁻ in 0.1 mol/L NaOH was evenly, but quickly added into 6-well plates in rapidly rotating orbital shakers at room temperature. There was no pH shift during treatment with ONOO⁻. The same volumes of 0.1 mol/L NaOH or decomposed ONOO⁻ (ONOO⁻ was first decomposed in 1 mol/L Tris buffer, pH 7.4, and kept for 5 min or overnight) were used as controls.

Detection of Ser¹¹⁷⁹ phosphorylation of eNOS in BAEC cells

eNOS is a homodimeric enzyme. Under native conditions, eNOS dimers, which are sensitive to temperature but resistant to SDS, run as dimers (∼270 kD) in low-temperature SDS-PAGE (17). When denatured by boiling, eNOS dimers become dissociated and run as monomers with a molecular weigh of ∼135 kD in room temperature SDS-PAGE. Unless otherwise indicated, in order to distinguish the effects of ONOO⁻ on SDS resistant eNOS dimers, unboiled samples were examined by low-temperature SDS-PAGE.

The low temperature SDS-PAGE was performed according to Ref 17. Briefly, after being washed twice with ice-cold PBS buffer, ONOO⁻-treated BAEC cells were lysed and sonicated twice. Protein lysates were mixed with 3-fold loading buffer and loaded on 6% gels without boiling. Proteins were separated either with low-temperature SDS-PAGE under reducing (with β-mercaptoethanol). Proteins were blotted onto nitrocellulose membranes and incubated with a polyclonal antibody against phospho-Ser¹¹⁷⁹ of eNOS (eNOS-Ser¹¹⁷⁹-P, 1:1000, 4°C overnight). Ser¹¹⁷⁹ phosphorylation of
eNOS was visualized by using the appropriate horseradish peroxidase-linked secondary antibodies and ECL reagents. In order to test the specificity of antibody to eNOS-Ser$^{1179}$-P for native proteins bovine recombinant eNOS purified from SF9 cells were treated with ONOO$^-$ and no staining was found with ONOO$^-$ treated or non-treated recombinant eNOS, indicating that ONOO$^-$ did not increase non-specific binding of eNOS with the antibody.

For room temperature SDS-PAGE, cell extracts were mixed with β-mercaptoethanol-containing Laemmi buffer (3 x) and boiled for 10 min. Proteins were separated in room temperature and Western blotted onto nitrocellulose. eNOS-Ser$^{1179}$-P was detected as described above.

**Assays of L-arginine uptake and eNOS activity**

The nitric oxide synthase activity was assayed as described previously (18). Briefly, 5 min after being treated with ONOO$, BAEC cells in 6-well plates were washed twice with 2 ml PBS buffer pH 7.5 and 1 ml PBS buffer with 0.1 mmol/L CaCl$_2$ was added. The cells requiring L-NAME were pre-incubated with L-NAME (500 µmol/L) for 60 min. The eNOS activity was assayed by incubating BAEC cells with 10 µM L-arginine plus 5 µCi of $^3$H L-arginine in the presence of 1 µmol/L calcium ionophore A23187. After 15 min the medium was removed and the cells were washed with 1 ml of PBS. The combined medium and PBS were countered for the radioactivity to determine the cellular $^3$H-uptake. After that, the cells were lysed with 250 µl of 100% ethanol for 3 min and were added with 2 ml ice-cold stop buffer (20 mM sodium acetate, pH 5.5, 1 mM L-citrulline, 2 mM EDTA, 2 mM EGTA). The lysate in stop buffer was then subjected to anion exchange chromatography using 2 ml of Dowex AG50W-X8 columns.
(0.4 g/ml, Bio-Rad) pre-equilibrated with stop buffer. L-citrulline was eluted three times with 1 ml of stop buffer and the eluent was collected for the determination of $^3$H-L-citrulline by liquid scintillation counting. Data is reported as the extent of the conversion of $^3$H L-arginine to $^3$H-L-citrulline that is sensitive to pre-treatment of the BAEC for 30 min with L-NAME and expressed as % inhibition.

**Cyclic GMP assay**

Confluent BAEC cells were treated with ONOO- (50 μmmol/L). After being washed twice with 3 ml of PBS buffer, cells were stimulated with agonists for 15 min. Cells were scraped with cell scrapers on ice and quickly frozen –80°C before assay. The cellular cGMP contents were assayed by using ELISA kits obtained from Cayman Chemicals (Ann arbor, MI), as described previously (16).

**Akt activity assay**

Akt activity was assayed by using an Akt kinase assay kit obtained from Cell Signaling Inc. (Beverly, MA), as described by the supplier. Briefly, an antibody to Akt was used to selectively immunoprecipitate Akt from cell lysates. The resulting immunoprecipitates were then incubated with a GSK fusion protein in the presence of ATP and kinase buffer. This allows immunoprecipitated Akt to phosphorylate GSK-3. Phosphorylation of GSK was measured by Western blotting using a phospho-GSK-3α/β (Ser21/9) and used as index of Akt activity.

**Detection of O$_2^-$ release in cultured endothelial cells**

The release of O$_2^-$ release in cultured BAEC cells was assayed by SOD-inhibitable cytochrome c reduction by measuring the absorbance at 550 nm ($\varepsilon_{550}=21$ mM$^{-1}$ s$^{-1}$) as described previously (16, 17).
**Immunoprecipitation and Western blotting of eNOS and AMPK**

Co-immunoprecipitation studies for determining the interaction of AMPK with eNOS were performed as described previously (16, 17). Briefly, confluent BAEC cells in 6-well dishes were pretreated with ONOO⁻ as described above. The cells were washed three times with cold PBS buffer (pH 7.4) immediately after being treated with ONOO⁻ and 100 µl of lysis buffer was added. Cell lysates (1 mg/ml) were incubated with the antibodies against eNOS (15 µg/ml) or AMPK (15 µg/ml) overnight. Cell proteins were loaded with 3-fold sample buffer and boiled for 5 min. The proteins were loaded on 6% SDS-PAGE, transferred onto nitrocellulose membranes, and blotted with primary antibody overnight at 4°C. The proteins were visualized by using the appropriate horseradish peroxidase-linked secondary antibodies and ECL reagents.
Results and Discussion

Increase by ONOO$^{-}$ of eNOS-Ser$^{1179}$ phosphorylation

Ser$^{1179}$ phosphorylation of eNOS has been widely considered as an important mechanism for increasing NO production under conditions such as fluid shear stresses (21, 22, 23), growth factors such as VEGF (24, 25), insulin-like growth factor-1 (IGF-1) (24), estrogen (26, 27) or oxidants such as hydrogen peroxide (H$_2$O$_2$) (18). Mutation of Ser$^{1179}$ to alanine attenuates agonist-induced NO release (21) because phosphorylation of Ser$^{1179}$ in the reductase domain of eNOS enhances the rate of electron flux from the reductase to the oxygenase domain of the enzyme, thus increasing the rate of NO synthesis (28).

All three NOS isoforms are dimeric enzymes comprised of two identical monomers, which are bridged by a zinc tetrathiolate (ZnS$_4$) cluster. Under native conditions, eNOS dimers, which are sensitive to temperature but resistant to SDS, run as dimeric enzyme under reducing conditions in low-temperature SDS-PAGE (17). When denatured, eNOS dimers become dissociated and run as monomers with a molecular weigh of $\sim$135 kD in normal temperature SDS-PAGE.

ONOO$^{-}$ significantly increased O$_2^{-}$ release in BAEC (17) but the mechanism remained unknown. We first addressed whether or not eNOS Ser$^{1179}$ phosphorylation contributed to ONOO$^{-}$-induced O$_2^{-}$ release. BAECs were treated with ONOO$^{-}$ and samples were boiled in presence of $\beta$-mercaptoethanol for 10 min and eNOS was separated by room temperature SDS-PAGE. As shown in Figure 1a, ONOO$^{-}$, but not decomposed ONOO$^{-}$, significantly up-regulated eNOS-Ser$^{1179}$–P in BAEC.
Our previous studies (17) demonstrated that ONOO\textsuperscript{−} oxidizes the ZnS\textsubscript{4} cluster, resulting in zinc release and formation of disulfide bonds between the monomeric units. The zinc-depleted eNOS dimers were dissociated under reducing conditions as observed with low-temperature SDS-PAGE. In BAEC under the conditions of this study both eNOS dimer, corresponding to a 260 kDa protein, and eNOS monomer (135 kDa) were observed, indicating that the ZnS\textsubscript{4} cluster of the enzyme is partially oxidized (Figure 1b). We first addressed whether or not the integrated status of eNOS protein (i.e. dimer vs monomer) affected the phosphorylation of Ser\textsuperscript{1179}. As shown in figure 1b, treatment of BAEC with ONOO\textsuperscript{−} caused a dose-dependent decrease in eNOS dimers and increase in eNOS monomers. On the other hand, ONOO\textsuperscript{−} caused a dose-dependent increase in eNOS-Ser\textsuperscript{1179}-P (Figure 1b). Despite the fact that fewer eNOS dimers were detected by low temperature SDS-PAGE after treatment with ONOO\textsuperscript{−}, the amount of phosphorylated eNOS dimer was significantly increased. ONOO\textsuperscript{−} decomposed in 1 M Tris, pH 7.5 for 10 min before addition to the cells did not affect the Ser\textsuperscript{1179} phosphorylation of eNOS (not shown). Thus, ONOO\textsuperscript{−} has at least two effects on eNOS; it oxidizes the ZnS\textsubscript{4} cluster and increases the phosphorylation of Ser\textsuperscript{1179}. As shown In Figure 1b, eNOS-Ser\textsuperscript{1179}-P was mainly detected in eNOS dimers in contrast to weak staining in eNOS monomers. The reason why eNOS monomers did not stain with the antibody against Ser\textsuperscript{1179}-P is unknown. It might be due to a lowered affinity of eNOS monomers with the antibody against phosphorylated Ser\textsuperscript{1179}, compared with eNOS dimmers. Similarly, we have previously found (17) that eNOS monomers have a lowered affinity with the antibody against eNOS.
As shown in figure 1c, the increased Ser\textsuperscript{1179} phosphorylation of eNOS was detected in cells harvested immediately after being treated with ONOO\textsuperscript{-}, but phosphorylation persisted for up to 3 h after treatment with ONOO\textsuperscript{-}.

**Inhibition of NO production and bioactivity by ONOO\textsuperscript{-}**

The catalytic mechanisms of NOS involve flavin-mediated electron transport from C-terminal-bound NADPH to the N-terminal heme center where oxygen is reduced and incorporated into the guanidino group of L-arginine giving rise to NO and L-citrulline. Therefore the formation of L-citrulline can be used as an index of NO release.

Because increased eNOS-Ser\textsuperscript{1179} phosphorylation is thought to increase NO production, we next determined the effect of ONOO\textsuperscript{-} on NO production and bioactivity in BAEC by monitoring the release of L-citrulline and cyclic GMP. As shown in figure 2a, ONOO\textsuperscript{-} significantly inhibited the production of NO. ONOO\textsuperscript{-} did not affect \textsuperscript{3}H-arginine uptake (data not shown), and supplementation with exogenous L-arginine (up to 1 mmol/L for 3 h) did not restore ONOO\textsuperscript{-}-induced inhibition of NO release (data not shown) indicating that the effect of ONOO\textsuperscript{-} is not due to decreased uptake or availability of L-arginine.

The availability of tetrahydrobiopterin (BH\textsubscript{4}) is essential for NO synthetic activity of eNOS although its role in NOS catalysis is not understood. There is evidence that ONOO\textsuperscript{-} depletes BH\textsubscript{4} and thereby promotes eNOS uncoupling. To investigate whether oxidation of BH\textsubscript{4} was involved, sepiapterin (100 \(\mu\)mol/L), which is converted to BH\textsubscript{4} by the salvage pathway, or BH\textsubscript{4} (100 \(\mu\)mol/L) was immediately added into the cells after ONOO\textsuperscript{-} treatment and incubated for an additional 3 h. However, neither sepiapterin nor BH\textsubscript{4} restored the NO synthetic activity (L-citrulline formation) of eNOS in ONOO\textsuperscript{-}.
treated cells (data not shown) suggesting that depletion of BH$_4$ by ONOO$^-$ was unlikely involved in ONOO$^-$-mediated eNOS dysfunction. Furthermore, BH$_4$ (100 µmol/L), which was added into cells immediately before ONOO$^-$ addition, did not block ONOO-induced inhibition on L-citrulline formation (data not shown). Since our previous results (17) have demonstrated that supplementation with BH$_4$ (100 µmol/L) did not prevent loss of recombinant eNOS dimers after ONOO$^-$ exposure (17), these results indicated that zinc-thiolate cluster of eNOS is a preferentially target for ONOO$^-$.

We have previously demonstrated that treatment of BAEC with L-NAME prevents the increased O$_2^-$ release (17) indicating that it is derived from eNOS. We next investigated whether or not increased Ser$^{1179}$ phosphorylation of eNOS increased release of O$_2^-$.

As shown in figure 2b, ONOO$^-$ significantly increased O$_2^-$ release in cells exposed to ONOO$^-$, suggesting that after Ser$^{1179}$ phosphorylation the accelerated electron transfer from the reductase to oxygenase domains of eNOS leads to increased O$_2^-$ release.

NO exerts its biological effects in part via activation of guanylyl cyclase (GC) to produce cyclic GMP (29). Therefore, NO bioactivity was assessed in BAEC treated with ONOO$^-$ by measuring cyclic GMP levels after stimulation with agonists including calcium ionophore, A23187 (1 µmol/L), angiotensin-II (Ang-II, 1 µmol/L) and 5-hydroxytryptamine (5-HT, 1 µmol/L), which normally increase NO production in a calcium-dependent manner. Similar to cells treated with ONOO$^-$ under basal conditions, eNOS-Ser$^{1179}$-P was increased in the BAEC stimulated with A23187, angiotensin-II, or 5-HT. However, ONOO$^-$ significantly attenuated the agonist-induced cyclic GMP formation (Fig. 2c) indicating that ONOO$^-$ decreased agonist-induced NO bioactivity in BAEC cells.
Inhibition of Akt activity by ONOO⁻

Various enzymes, such as Akt/protein kinase B (PKB) (18-24), protein kinase A (PKA, 23, 30), and AMPK (11, 12) have been reported to phosphorylate Ser^{1179} of eNOS both *in vitro* and *in vivo*. Akt/PKB (18, 21-27) and protein kinase A (23, 30) phosphorylate eNOS-ser^{1179}, resulting in increased NO release in cells stimulated with growth factors (21-27) or hydrogen peroxide (18).

We therefore addressed whether or not ONOO⁻ activated Akt and thereby phosphorylated eNOS-Ser^{1179}. Surprisingly, low concentrations of ONOO⁻ significantly inhibited Akt activity as indicated by decreased Akt-dependent GSK-3 phosphorylation (Fig. 3a). Furthermore, overexpression of an Akt dominant negative mutant, which prevents Akt activation (18, 25), did not attenuate ONOO⁻-stimulated eNOS-Ser^{1179}-P (Fig. 3b). In addition, ONOO⁻ decreased the phosphorylation of Akt-Ser^{473} (Fig. 3c), which is involved in the regulation of Akt activity. Taken together, these results indicate that eNOS-Ser^{1179}-P caused by ONOO⁻ was accompanied by decreased Akt activity, and therefore not dependent on Akt.

Role of guanylyl cyclase and protein kinase A and G in ONOO⁻-induced eNOS phosphorylation

Although it is several-fold less potent than NO in doing so, ONOO⁻ does activate guanylyl cyclase, and consequently protein kinase G (31). We therefore addressed whether or not ONOO⁻ increased eNOS-Ser^{1179}-P by activating protein kinase G. As shown in figure 3d, ODQ (1 μmol/L), an inhibitor for cyclic GMP production, did not decrease the ONOO⁻ induced eNOS- Ser^{1179}-P. Zaprinast (50 μmol/L), which inhibits phosphodiesterase, preventing the degradation of cyclic GMP, also did not influence the
effect of ONOO− (data not shown). Furthermore, inhibition of protein kinase G by KT5823 (1 μmol/L) did not affect ONOO−-induced of eNOS-Ser^{1179}-P indicating that Ser^{1179} phosphorylation of eNOS by ONOO− is independent of protein kinase G.

Protein kinase A is reported to phosphorylate eNOS-Ser^{1179} in response to increased shear stress (22, 23). Preincubation with H89 (1 μmol/ L), a potent inhibitor of protein kinase A, did not attenuate ONOO− induced eNOS-Ser^{1179} phosphorylation, suggesting that activation of protein kinase A is unlikely to be responsible.

Activation of AMPK by ONOO−

Chen et al. have reported that AMPK phosphorylates eNOS-Ser^{1179} in vitro and in ischemic cardiac myocytes (11). In order to investigate whether or not ONOO− causes phosphorylation of eNOS-Ser^{1179} by activating AMPK, BAEC cells were treated with different concentrations of ONOO− and cell proteins were stained with a specific antibody against phosphorylated Thr^{172} of AMPK that is reported to be essential for the AMPK activity (7). As shown in figure 4a, ONOO− dose-dependently increased the phosphorylation of AMPK-Thr^{172}. Similar to phosphorylation of eNOS-Ser^{1179}, ONOO− increased phosphorylation of AMPK-Thr^{172} immediately after treatment, lasting at least 30 min (Fig. 4b). Moreover, ONOO− also increased the prolonged phosphorylation of Ser^{79} of ACC (Fig. 4b), a downstream target that is known to be phosphorylated by AMPK (2, 9, 10). This suggests that ONOO− treatment activates AMPK in BAEC.

AMPK-dependent eNOS phosphorylation following ONOO−.

Further evidence for the role of AMPK-dependent eNOS-Ser^{1179} phosphorylation was obtained in experiments in which constitutively active AMPK (AMPK-CA) and dominant-negative AMPK (AMPK-DN) mutants were expressed with adenoviral vectors.
As shown in figure 5a, overexpression of AMPK-CA, which alone slightly increased e-NOS-Ser\textsuperscript{1179} phosphorylation (data not shown), did not influence the effect of ONOO\textsuperscript{-} on eNOS-Ser\textsuperscript{1179}-P. In contrast, overexpression of the AMPK-DN attenuated ONOO\textsuperscript{-}-upregulated eNOS-Ser\textsuperscript{1179}-P. These results indicate that ONOO\textsuperscript{-}-induced eNOS-Ser\textsuperscript{1179}-P requires AMPK activity.

**ONOO\textsuperscript{-}-upregulated eNOS-Ser\textsuperscript{1179} phosphorylation contributes to increased superoxide anion production**

We next addressed whether or not phosphorylation of eNOS-Ser\textsuperscript{1179} induced by ONOO\textsuperscript{-} contributes to increased release of O\textsubscript{2}\textsuperscript{-}. Overexpression of the AMPK-CA significantly enhanced ONOO\textsuperscript{-}-upregulated eNOS-Ser\textsuperscript{1179}-P as well as the release of O\textsubscript{2}\textsuperscript{-}. In contrast, overexpression of AMPK-DN, which attenuated ONOO\textsuperscript{-}-induced eNOS-Ser\textsuperscript{1179}-P, only partially blocked ONOO\textsuperscript{-}-induced O\textsubscript{2}\textsuperscript{-} release indicating that the AMPK-dependent eNOS-Ser\textsuperscript{1179}-P accounts for only part of the increased release of O\textsubscript{2}\textsuperscript{-} from eNOS caused by ONOO\textsuperscript{-}. Because we showed that ONOO\textsuperscript{-} also increased O\textsubscript{2}\textsuperscript{-} from recombinant eNOS in which no phosphorylation occurs, it is likely that the increased production of O\textsubscript{2}\textsuperscript{-} from eNOS is accounted for both by oxidation of the Zn-S\textsubscript{4} group as well as eNOS-Ser\textsuperscript{1179}-P.

**Increased association of eNOS with AMPK caused by ONOO\textsuperscript{-}**

Previous studies (11) have demonstrated that AMPK co-immunoprecipitates with eNOS. As shown in figure 5c, ONOO\textsuperscript{-} increased the association of AMPK and eNOS. Similar results were obtained either with immunoprecipitation of eNOS and staining for AMPK, or with immunoprecipitation of AMPK and staining for eNOS.
Ser\textsuperscript{1179} phosphorylation of eNOS has been widely considered to be an important mechanism for increased NO production under the influence of fluid shear stress (21-24), growth factors (24-27), or H\textsubscript{2}O\textsubscript{2} (18). Although mutation of Ser\textsuperscript{1179} to alanine attenuates agonist-induced eNOS activity (21), our results suggest that Ser\textsuperscript{1179} phosphorylation can only be regarded as an indicator of the activation status of eNOS regardless of whether it is making NO or O\textsubscript{2}\textsuperscript{−}. Whether or not Ser\textsuperscript{1179} phosphorylation leads to increased NO release is likely dependent on the redox status of the ZnS\textsubscript{4} cluster of eNOS, as well as possibly its substrate, L-arginine, or the cofactor, BH\textsubscript{4}. It is likely that the increased O\textsubscript{2}\textsuperscript{−} release observed from eNOS exposed to ONOO\textsuperscript{−} originates from the increased amounts of eNOS with oxidized ZnS\textsubscript{4} centers. Ser\textsuperscript{1179} phosphorylation of eNOS likely accounts in part for the increase in catalytic activity of eNOS in cells exposed to ONOO\textsuperscript{−}. The phosphorylation of Ser\textsuperscript{1179} in eNOS with intact ZnS\textsubscript{4} clusters might produce some NO, but it is likely that at least some NO is rapidly converted to ONOO\textsuperscript{−} upon reacting with O\textsubscript{2}\textsuperscript{−}. ONOO\textsuperscript{−} produced in this manner would not only likely further the oxidation of ZnS\textsubscript{4} clusters, increasing the uncoupling of the enzyme and O\textsubscript{2}\textsuperscript{−} production, but also oxidize other endothelial proteins, as we previously demonstrated for prostacyclin synthase (16, 17). Because our previous studies showed that \textit{in vivo}, eNOS is partially oxidized in tissues of diabetic mice, it is highly likely that ONOO\textsuperscript{−} regulates eNOS activity and its product formation \textit{in vivo}. In addition, other regulators of eNOS function may be dependent on the redox status of the ZnS\textsubscript{4} cluster. For example, the mechanisms described here are likely important to understand the recent observation that inhibition of HSP90 with geldanamycin significantly increases O\textsubscript{2}\textsuperscript{−} production while increasing Ser\textsuperscript{1179} phosphorylation of eNOS (32).
In summary, the main finding of the present study is that ONOO⁻ inhibits Akt-dependent and increases AMPK-dependent phosphorylation of eNOS-Ser^{1179}. ONOO⁻ activates AMPK and increases the association of AMPK with eNOS whereas it inhibits Akt-activity. AMPK-dependent phosphorylation of eNOS-Ser^{1179} likely contributes to increased $O_2^-$ and ONOO⁻ production by eNOS. We conclude that phosphorylation of eNOS-Ser^{1179} reflects only the activated state of eNOS, rather than whether it can generate NO or $O_2^-$, which rather depends on the integrity of the ZnS₄ cluster of the protein.
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Footnotes:

ACC, acetyl CoA carboxylase; Akt-DN: a dominant-negative mutant Akt; AMPK, 5’-AMP activated-kinase; AMPK-CA, constitutively active AMPK; AMPK-DN, dominant-negative AMPK mutants; Ang-II, angiotensin-II; BH₄, tetrahydrobiopterin; eNOS, endothelial nitric oxide synthase; 5-HT, 5-hydroxytryptamine; β-ME, β-mercaptoethanol; NO, nitric oxide; O₂⁻, superoxide anion; ONOO⁻, peroxynitrite; eNOS-Ser¹¹⁷⁹-P, serine1179 phosphorylated nitric oxide synthase;
Legends to Figures

Figure 1. **ONOO− upregulates eNOS-Ser\textsuperscript{1179} phosphorylation.** Cultured BAEC cells were treated with ONOO− (0-50 µmol/L), decomposed ONOO− (ONOO− were added in 1M Tris, pH 7.4 for 10 min before being added into samples), or the NaOH vehicle (100 mmol/L) as described in Methods. Ten min after treatment cells were harvested and lysed. Proteins in boiled samples were separated under reducing or non-reducing conditions by SDS-PAGE at room temperature (Fig. 1a) or in low-temperature SDS-PAGE with unboiled samples (Fig. 1b, 1c, and 1d). (a). Representative blot of eNOS-Ser\textsuperscript{1179}-P in room temperature SDS-PAGE with boiled samples. BAEC cells were boiled for 10 min in presence of β-mercaptoethanol. Proteins were separated at room temperature by SDS-PAGE and Western blotted. eNOS-Ser\textsuperscript{1179}-P was detected as described in Methods. (b). Representative blots of reducing gels of eNOS dimer and monomers and Ser\textsuperscript{1179} phosphorylation of eNOS obtained from cells immediately after exposure to ONOO−. eNOS dimers and monomers were separated by low temperature SDS-PAGE (6 %) under reducing gels (+β-mercaptoethanol, +β-ME). The blot represents those from 10 independent experiments. (c). Time-dependent phosphorylation of eNOS-Ser\textsuperscript{1179}-P by ONOO− in cultured BAEC cells. Increased phosphorylation was observed immediately after and for up to 180 min following exposure to ONOO− (50 µmol/L). The blot represents three individual experiments; D: decomposed ONOO−, Di-eNOS: eNOS dimer; eNOS: eNOS monomer.

Figure 2. **ONOO− increases eNOS-Ser\textsuperscript{1179} phosphorylation but decreases NO bioactivity in BAEC cells.** (a). ONOO− (50 µmol/L) inhibited the rate of eNOS-
dependent L-citrulline formation \((n=12, *P<0.01)\) and increased \(O_2^-\) release in ONOO\(^{-}\)-treated BAEC cells \((n=12, *P<0.01)\). L-citrulline and \(O_2^-\) release were assayed as described in Materials and Methods. (b). ONOO\(^{-}\) (50 µmol/L) increased eNOS-Ser\(^{1179}\)-P following treatment of BAEC with ONOO\(^{-}\) and A23187, angiotensin (Ang-II), and 5-HT \((n=4)\). eNOS dimers and monomers were separated by low temperature SDS-PAGE (6 %) under reducing (+β-ME) conditions. eNOS phosphorylation was observed both with and without ONOO\(^{-}\) treatment following the agonists, but was greater following ONOO\(^{-}\) treatment; (c). ONOO\(^{-}\) treatment decreased cyclic GMP content following agonist stimulation of BAEC. Cyclic GMP was assayed as described in Materials and Methods. ONOO\(^{-}\) significantly decreased agonist-induced cyclic GMP production \((n=9, *p<0.01)\).

Figure 3. \textbf{ONOO}^- inhibits Akt activity and Akt phosphorylation in BAEC cells (a). ONOO\(^{-}\) dose-dependently inhibited Akt activity in BAEC cells as indicated by phosphorylation of Ser\(^{21/9}\) of GSK-3α/β. Akt activity was assayed by using an \textit{in vitro} Akt kinase kit as described in Methods. The blot represents those obtained in 5 independent experiments. (b). Overexpression of Akt-DN does not inhibit ONOO\(^{-}\)-upregulated eNOS-Ser\(^{1179}\)-P. ONOO\(^{-}\) (50 µmol/L) increased the detection of eNOS-Ser\(^{1179}\)-P, which was not blocked by overexpressing adenoviral Akt-DN. The blot represents those from 6 independent experiments. (c). ONOO\(^{-}\) decreased Akt phosphorylation in BAEC cells. The blot represents those of six independent experiments. (d). Inhibition of protein kinase A by H89 (1 µmol/L) or protein kinase G by (KT5823, 1 µmol/L) or guanylate cyclase with ODQ (1 µmol/L) did not affect ONOO\(^{-}\) increased eNOS Ser\(^{1179}\)-P. The blot represents those from 5 independent experiments.
Figure 4. ONOO\textsuperscript{−} increases phosphorylated AMPK and acetyl-CoA carboxylase (ACC-Ser\textsuperscript{79}) in BAEC. (a). ONOO\textsuperscript{−} (1-50 µmol/L) increased detection of AMPK-Thr\textsuperscript{172} phosphorylation. The blot represents those from 10 independent experiments. ONOO\textsuperscript{−} increases the AMPK-Thr\textsuperscript{172} phosphorylation in BAEC. The intensity (area x density) of the individual bands of phosphorylated AMPK in Western blots was quantitated by densitometry (Model GS-700, Imaging Densitometer, Bio-Rad). The results were calculated as percentage change compared to the corresponding band in control cells (n=5, *p<0.05). (b). ONOO\textsuperscript{−} time-dependently increases phosphorylated AMPK and phosphorylated ACC (Ser\textsuperscript{79}). The blot represents those from 6 independent experiments.

Figure 5. ONOO\textsuperscript{−} upregulates AMPK-dependent eNOS-Ser\textsuperscript{1179} phosphorylation and is associated with increased O\textsubscript{2}\textsuperscript{−} and ONOO\textsuperscript{−} (a). Overexpression of a dominant-negative AMPK mutant (AMPK-DN) inhibits ONOO\textsuperscript{−}-upregulated eNOS-Ser\textsuperscript{1179}-P. Neither control virus (cnt virus) nor AMPK-CA attenuated ONOO-induced eNOS-Ser\textsuperscript{1179}-P. eNOS dimers and monomers were separated by low temperature SDS-PAGE (6 %) under reducing gels (+β-ME). The blot represents those from five independent experiments. (b). Overexpression of AMPK-DN attenuates ONOO\textsuperscript{−}-upregulated O\textsubscript{2}\textsuperscript{−} release in BAEC. The cells, following exposure to ONOO\textsuperscript{−}, were rinsed twice with 2 mL PBS buffer, pH 7.4, and exposed to calcium ionophore A23187 (10 µmol/L) for 2 hours. The O\textsubscript{2}\textsuperscript{−} release was measured by SOD-inhibitable cytochrome c reduction as described in Methods (n=6, #P<0.05 vs control, *P<0.05 vs ONOO\textsuperscript{−}); (c). ONOO\textsuperscript{−} increases the association of eNOS and AMPK. Increased staining was observed for AMPK in
immunoprecipitates obtained with anti-eNOS antibody as well as for eNOS in
immunoprecipitates obtained with the AMPK antibody. The blots represent those from 6
independent experiments.
Figure 1

a. eNOS-Ser^{1179}-P

b. ONOO^- (µmol/L)

| Control | D | ONOO^- |
|---------|---|--------|
| ONOO^-  |   |        |
| 0       | 100 | 50     |
| 10      |     |        |
| 1       |     |        |

c. eNOS-Ser^{1179}-P

| ONOO^- (50 µmol/L) |
|---------------------|
| eNOS-Ser^{1179}-P   |
| 1                   |
| 5                   |
| 10                  |
| 30                  |
| 60                  |
| 120                 |
| 180                 |

Time (min)
Figure 2

(a) Cytosolic cGMP (pmol/mg protein) levels in control and treated cells.

(b) Superoxide anion (nmol/min/well) produced by decomposed ONOO-.

(c) Western blot analysis showing eNOS-Ser1179-P levels in control and ONOO- treated cells.

Figure 2

A23187  Ang-II  5-HT

control  ONOO-  control  ONOO-  control  ONOO-

cGMP (pmol/mg protein)

Ang-II  5-HT  A23187

* indicates significance compared to control.
Figure 3

(a) Western blot showing p-GSK-3β (Ser^{21/9}) levels in different concentrations of ONOO⁻ (µmol/L).

(b) Western blot showing eNOS-Ser^{1179}-P levels in control, ONOO⁻, ONOO⁻/Akt-DN conditions.

(c) Western blot showing p-Akt (Ser^{473}) levels in control, ONOO⁻, H89, KT5823, ODQ conditions.

(d) Western blot showing e-NOS-Ser^{1179}-P levels in control, ONOO⁻, H89, KT5823, ODQ conditions.
**Figure 4**

**a.**

| ONOO⁻ (µmol/L) | 0 | 1 | 10 | 50 | 100 |
|----------------|---|---|----|----|-----|
| AMPK p-AMPK (Thr¹⁷²) |   |   |    |    |     |

**b.**

| ONOO⁻ (50 µmol/L) | none | 1 | 5 | 10 | 30 | 60 | 120 | 180 |
|-------------------|-------|---|---|----|----|----|-----|-----|
| p-AMPK (Thr¹⁷²)   |       |   |   |    |    |    |     |     |
| p-ACC (Ser⁷⁹)     |       |   |   |    |    |    |     |     |

Time (min)
Figure 5

Panel a:

**ONOO- (50 µmol/L)**

- eNOS-Ser^{1179}\text{-P}

Panel b:

| Condition          | Superoxide Anion (nmol/min/well) |
|--------------------|----------------------------------|
| control            | 0                                |
| none               | 3                                |
| AMPK-DN            | 6                                |
| AMPK-CA            | 9                                |
| GFP virus          | 9                                |

Panel c:

- **IP:** eNOS
  - WB: AMPK
- **IP:** AMPK
  - WB: eNOS
Modulation by peroxynitrite of AKt- and AMP- activated kinase-dependent serine phosphorylation of endothelial nitric oxide synthase
Ming-Hui Zou, Xiu-Yun Hou, Chao-Mei Shi, Daisuke Nagata, Kenneth Walsh and Richard A. Cohen

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