Activation of 5'-AMP-activated Kinase Is Mediated through c-Src and Phosphoinositide 3-Kinase Activity during Hypoxia-Reoxygenation of Bovine Aortic Endothelial Cells

ROLE OF PEROXYNITRITE

This article has been withdrawn by the authors. Analysis performed by the Journal determined the following. The c-Src immunoblot in Fig. 1C was reused for AMPK in Fig. 3A. A portion of the c-Src immunoblot in Fig. 2D was reused in Fig. 2E for P70S6 kinase. The PDK1-P and AMPK-P immunoblots in Fig. 2E are the same. The second lane of the c-Src immunoblot on the left in Fig. 4D was reused in the first lane of the AMPK immunoblot on the right in the same figure panel. The authors state that the presentation of identical blots for PDK-1P and AMPK-P in Fig. 2E was an error. Because the original immunoblots cannot be recovered 16 years after publication, the authors cannot determine which immunoblot was used in error. However, the authors state in the legend to Fig. 2 that similar experiments performed at that time support the conclusions. For the other immunoblots in question, the withdrawing authors have carefully examined the immunoblots in question and disagree with the Journal. The authors offered to repeat the experiments in Fig. 2; however, the Journal declined the offer. Furthermore, the authors state that the results of this paper have been confirmed by the results of complementary experiments presented in the article, and that the principal observations of this article were further confirmed in publications from other laboratories (Quintero, M. et al. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 5379-5384; Emerling, B. M. et al. (2009) Free Radic. Biol. Med. 46, 1386-1391; Mackenzie, R. M. et al. (2013) Clin. Sci. (Lond.) 124, 403-411). The authors stand by the conclusions of the paper.

DN, dominant-negative; c-Src, c-Src kinase; eNOS, endothelial nitric oxide synthase; GFP, green fluorescent protein; H/R, hypoxia/reoxygenation; i-NAM, L-nitroarginine methyl ester; O₂⁻, superoxide anion; ONOO⁻, peroxynitrite; PDK1, phosphoinositide-dependent kinase 1; PI 3-kinase, phosphoinositide 3-kinase; SOD, superoxide dismutase.

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EXPERIMENTAL PROCEDURES

Materials

Bovine aortic endothelial cells (BAEC) and cell culture media were obtained from Clonetics Inc. (Walkersville, MD). The adenoviral constructs for SO21, SO22, and catalase were obtained from the University of Iowa Viral Vector Core facility. ATP, ADP, and AMP were obtained from Sigma. Peroxynitrite was obtained from Calbiochem (San Diego, CA). Antibodies against phosphoacetyl-CoA carboxylase (ACC) (Ser79), and the SAMS peptides were from Upstate Biotechnology (Lake Placid, NY). Polyclonal antibodies against c-Src, phosphorylated c-Src ( Tyr416), phosphorylated PD1K (Ser411), phosphorylated p70S6 kinase (Thr38/Ser424), p70S6 kinase, phospho-AMPK (Thr172), and AMPK were obtained from Cell Signaling Inc. (Beverly, MA). [14C]ATP (10 mCi/ml) was obtained from PerkinElmer Life Sciences. A monoclonal antibody toward c-Src, protein A-G agarose, and antibodies against AMPK subunits (α1, α2, β1 and 2, and y2 and 3) were obtained from Santa Cruz Biotechnology or Upstate Biotechnology. Other chemicals and organic solvents of highest grade were obtained from Fisher Scientific (Morris Plains, NJ).

Methods

Peroxynitrite Synthesis—ONOO raised either obtained from Calbiochem (San Diego, CA) or synthesized using a quenched-flow reaction as previously described (19). Briefly, an aqueous solution of 0.6 m sodium nitrite mixed rapidly with an equal volume of 0.7 m H2O2 containing 0.6 m HCl and immediately quenched with the same volume of 1.5 m NaOH. Residual H2O2 was removed by treatment with granulate manganese dioxido. All solutions were kept on ice. The concentrations of ONOO were determined spectrophotometrically in 0.1 m NaOH (εmax = 1670 m-1 cm-1).

Treatment of BAEC Cells with Peroxynitrite—To confute BAEC lines, 6-well plates were added 950 μL of 100 mmol/liter HEPES buffer, pH 7.4, after being rinsed twice with phosphate-buffered saline buffer, gassed only with 95% O2,5% CO2 for equivalent periods. After treatment of BAEC with ONOO- in 0.1 mol/liter NaOH solution, 50 μL of concentrated ONOO- in 0.1 mol/liter NaOH was quickly added to the plates while being rapidly shaken at room temperature. There was no p- with ONOO-. The same volumes of ONOO- used as controls (ONOO- was synthesized with NaOH, Tris buffer, pH 7.4, and 1% Triton X-100). After treatment of BAEC with ONOO- or vehicle, BAEC (100-cm2 dishes) were immediately washed with 2 ml of ice-cold phosphate-buffered saline buffer, gassed 5 times with 1% phosphoric acid. After the final wash, the filters were quickly dried, cut, and subjected to H/R as described above. After treatment, cells were immediately washed with 2 ml of ice-cold phosphate-buffered saline buffer and scraped with a rubber spatula in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTa, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride). The cell lysates were then sonicated twice for 10 s in an Ultrasonic dismembrator with output 10% (Model 500, Fisher Scientific) and then centrifuged at 14,000 × g for 20 min at 4 °C. The pellets were discarded and supernatants were assayed for protein concentration. Duplicate tubes with 200 μg protein from each sample were prepared and were mixed with 500 μl of IP buffer (lysis buffer plus 1% NaCl and 1% dithiothreitol). AMPK was then immunoprecipitated by adding 10 μg of polyclonal antibody against AMPK (Cell Signaling) and 25 μL of protein A-G agarose (Santa Cruz Biotechnology) and incubated at 4 °C. After centrifugation (14,000 × g, 1 min), the beads were washed with IP buffer and then twice with 10× reaction buffer (400 mM HEPES, pH 7.4, 800 mM NaCl, 50 mM MgCl2, 1 mM dithiothreitol). The AMPK activity was assayed by adding 50 μl of reaction mixtures, consisting of 5 μl of reaction buffer, 10 μl of SAMS peptide (1 mg/ml), 10 μl of ATP working stock consisting of 0.1 μl of 100 mM ATP, 1 μl of [14C]ATP, and 8.9 μl of H2O2, 25 μl H2O, or 25 μl of 400 μM AMP and incubated at 37 °C for 10 min. The beads were quickly washed and 25 μl of supernatant was spotted onto PS1 Whatman filter papers. The filters were then washed 4–5 times with 1% Triton X-100. After the final wash, the filters were quickly dried and counted in a liquid scintillation counter. The difference in cpm between the control and treated samples is calculated as the AMPK activity.

Assay of AMP Kinase Activity—Confuent BAEC were exposed to ONOO- or subjected to H/R as described above. After treatment, cells were immediately washed with 2 ml of ice-cold phosphate-buffered saline buffer and scraped with a rubber spatula in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTa, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride). The cell lysates were then sonicated twice for 10 s in an Ultrasonic dismembrator with output 10% (Model 500, Fisher Scientific) and then centrifuged at 14,000 × g for 20 min at 4 °C. The pellets were discarded and supernatants were assayed for protein concentration. Duplicate tubes with 200 μg protein from each sample were prepared and were mixed with 500 μl of IP buffer (lysis buffer plus 1% NaCl and 1% dithiothreitol). AMPK was then immunoprecipitated by adding 10 μg of polyclonal antibody against AMPK (Cell Signaling) and 25 μL of protein A-G agarose (Santa Cruz Biotechnology) and incubated at 4 °C. After centrifugation (14,000 × g, 1 min), the beads were washed with IP buffer and then twice with 10× reaction buffer (400 mM HEPES, pH 7.4, 800 mM NaCl, 50 mM MgCl2, 1 mM dithiothreitol). The AMPK activity was assayed by adding 50 μl of reaction mixtures, consisting of 5 μl of reaction buffer, 10 μl of SAMS peptide (1 mg/ml), 10 μl of ATP working stock consisting of 0.1 μl of 100 mM ATP, 1 μl of [14C]ATP, and 8.9 μl of H2O2, 25 μl H2O, or 25 μl of 400 μM AMP and incubated at 37 °C for 10 min. The beads were quickly washed and 25 μl of supernatant was spotted onto PS1 Whatman filter papers. The filters were then washed 4–5 times with 1% Triton X-100. After the final wash, the filters were quickly dried and counted in a liquid scintillation counter. The difference in cpm between the control and treated samples is calculated as the AMPK activity.

Dependent of Cellular ATP: Measurement of Cellular ATP, ADP, and AMP—After treatment with ONOO- or vehicle, BAEC (10-cm2 dishes) were immediately covered with 6 ml of ice-cold 1% trichloroacetic acid and kept on ice for 5 min. The cells were then scraped and centrifuged at 4 °C (5 min, 14,000 rpm). After centrifugation, the supernatants were neutralized by ether extraction, freeze-dried (SpeedVac) and then stored at −80 °C until they were re-dissolved in 0.5 ml water for assay. The contents of ATP, ADP, and AMP were assayed by bioluminescent methods as described previously (31).

Hypoxia Reoxygenation of BAEC—BAEC were cultured in 6-well plates. The cells were first transfected with adenoviral vectors for 2 days if required. The cells were placed in a water bath (37 °C, total volume of 1 liter) filled with 1 presaturated Krebs-Ringer’s buffer, gassed with 95% O2, 5% CO2. After 30 min incubation, the oxygen tension was reduced abruptly from 95% O2, 5% CO2 to 95% N2, 5% CO2 and was maintained for the indicated time. After this phase of hypoxia, 95% O2/5% CO2 was resumed (reoxygenation) for 30 min. After that the cells were washed with phosphate-buffered saline buffer twice and collected for Western blot and immunoprecipitation assays. Control BAEC were gassed only with 95% O2, 5% CO2 for equivalent periods.

Immunoprecipitation and Western Blots—Immunoprecipitation and Western blots were performed as described previously (21–22). To determine the interaction of c-Src and AMPK, c-Src was immunoprecipitated and stained for AMPK, or vice versa.

Quantification of Western Blot—The intensity (area × density) of the individual bands on Western blots was quantitated by densitometry (Model GS-700, Imaging Densitometer, Bio-Rad). The background was subtracted from the calculated area. The results were calculated as percentage change compared with the corresponding control band.
Fig. 1. ONOO\textsuperscript{–}-activated AMPK is c-Src-mediated, but independent of AMP/ATP ratios in BAEC. BAEC were treated for 1 min with the NaOH vehicle as control (C), decomposed ONOO\textsuperscript{−} (D, ONOO\textsuperscript{−} was added to 1 m Tris, pH 7.4 for 10 min before being added into samples) or ONOO\textsuperscript{−} (0–100 µmol/liter), as described under “Experimental Procedures.” a, exposure of BAEC to ONOO\textsuperscript{−} did not affect the contents of ATP, ADP, or AMP (n = 5, p > 0.05). b, effects of ONOO\textsuperscript{−} on the ADP/ATP ratios in BAEC. c, ONOO\textsuperscript{−} dose-dependently increased c-Src phosphorylation (Thr\textsuperscript{416}) (n = 4, #, p < 0.05). d, inhibition of c-Src with PP2 (10 µmol/liter) or overexpression of c-Src-DN attenuates ONOO\textsuperscript{−}-induced AMPK and ACC phosphorylation (Control versus GFP, n = 4, #, p < 0.05; GFP versus c-Src or PP2, n = 4, *, p < 0.05). e, ONOO\textsuperscript{−}-up-regulated AMPK-dependent phosphorylation of SAMS peptide is inhibited by either c-Src-DN overexpression and PP2 (Control versus GFP with ONOO\textsuperscript{−}, n = 5, #, p < 0.01; GFP plus ONOO\textsuperscript{−} versus ONOO\textsuperscript{−} plus c-Src-DN or PP2, n = 5, *, p < 0.01). f, ONOO\textsuperscript{−}-enhanced phosphorylation of PDK1 and p70S6 kinase is inhibited by either c-Src-DN overexpression or PP2 inhibited.

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rylation of SAMS peptide using [\textsuperscript{32}P]ATP assays (Fig. 1e). As shown in Fig. 1e, both PP2 and c-Src-DN strongly inhibited ONOO\textsuperscript{−} up-regulated phosphorylation of SAMS peptides.

In search of intermediates involved in the activation of AMPK by c-Src, we next addressed whether or not activation of c-Src by ONOO\textsuperscript{−} leads to PI 3-kinase activation. Phosphoinositide-dependent kinase-1 (PDK1), which is activated by PI 3-kinase lipid products serves as a link between PI 3-kinase and its downstream targets, such as p70S6 kinase (33, 34). As shown in Fig. 1f, ONOO\textsuperscript{−} increased the phosphorylation of
Fig. 2. ONOO\textsuperscript{−}-induced AMPK activation is PI 3-kinase-dependent.\textit{a}, growth factors, VEGF (50 ng/ml) and insulin (0.1 microunit/ml), did not cause any appreciable increase in phosphorylation of either AMPK or ACC in BAEC (Control versus ONOO\textsuperscript{−}, \(n = 6\), \(*\), \(p < 0.01\); VEGF or insulin versus ONOO\textsuperscript{−} plus VEGF or insulin, \(n = 3\), \(*\), \(p < 0.01\)).\textit{b}, either wortmannin (W, 100 nmol/liter) or LY294002 (L, 50 \(\mu\)mol/liter) inhibited ONOO\textsuperscript{−}-enhanced phosphorylation of PDK1, p70S6 kinase, AMPK, and ACC (Control versus ONOO\textsuperscript{−}, \(n = 6\), \(*\), \(p < 0.01\); ONOO\textsuperscript{−} versus ONOO\textsuperscript{−} plus wortmannin or LY294002, \(n = 6\), \(*\), \(p < 0.01\)).\textit{c}, either wortmannin or LY294002 abolished ONOO\textsuperscript{−}-enhanced phosphorylation of SAMS peptide (Control versus ONOO\textsuperscript{−}, \(n = 5\), \(*\), \(p < 0.01\); ONOO\textsuperscript{−} versus ONOO\textsuperscript{−} plus wortmannin or LY294002, \(n = 5\), \(*\), \(p < 0.01\)).\textit{d}, PP2, but neither wortmannin (100 nmol/liter) nor LY294002 (50 \(\mu\)mol/liter) inhibited phosphorylation of c-Src.\textit{e}, overexpression of a kinase-inactive mutant PDK1 blocked ONOO\textsuperscript{−}-up-regulated phosphorylation of p70S6 kinase, PDK1, AMPK, and ACC.\textit{f}, inhibition of PDK-1-KS blocked ONOO\textsuperscript{−}-up-regulated phosphorylation of AMPK, ACC, and P70S6 kinase. (Control versus ONOO\textsuperscript{−} or GFP plus ONOO\textsuperscript{−} \(n = 6\), \(*\), \(p < 0.01\); PDK1 plus ONOO\textsuperscript{−} versus ONOO\textsuperscript{−} or ONOO\textsuperscript{−} plus GFP, \(n = 6\), \(*\), \(p < 0.01\)).
AMPK in BAEC (22), the attenuation of PI 3-kinase prevented the ONOO⁻ activation. This extent as in the absence of the growth factor (Fig. 2a). Either wortmannin (100 nmol/liter) or LY294002 (50 μmol/liter) added for 10 min did not affect AMPK or ACC phosphorylation. ONOO⁻ phosphorylation (data not shown), both inhibitors prevented ONOO⁻ overexpression of the c-Src-DN mutant blunted the effect of PDK1 and p70S6 kinase. Inhibition of c-Src by PP2 or by overexpression of SOD to scavenge O₂⁻, or treating the cells with l-NAME, attenuated H/R-enhanced c-Src, AMPK, and ACC phosphorylation, as well as AMPK activity as assayed by SAMS peptide phosphorylation. Infection of cells with adenovirus encoding GFP alone had no effect on c-Src, AMPK, and ACC phosphorylation, as well as AMPK activation by ONOO⁻ linked activation of PI 3-kinase.

ONOO⁻ activates AMPK via its Downstream Kinase, PDK1—As shown in Fig. 2, e and f, overexpression of a PDK1-KD mutant prevented ONOO⁻-enhanced phosphorylation of both PDK1 and p70S6 kinase. We also found that the PDK-1-KD decreased ONOO⁻-enhanced phosphorylation of AMPK and ACC (Fig. 2, e and f) indicating that ONOO⁻ activates AMPK via PI 3-kinase and PDK1. Importantly, the PDK-1-KD had no effect on AMPK or ACC phosphorylation under basal conditions in cells not exposed to ONOO⁻ (data not shown). Thus, these data indicate that ONOO⁻ activates a signaling pathway involving c-Src, PI 3-kinase, and PDK1 that leads to activation of AMPK and ACC.

Coordinated Activation of c-Src, AMPK, and ACC by Hypoxia-Reoxygenation—Because AMPK is activated by cellular stresses such as ischemia (12–13) and because ischemia is associated with the generation of ONOO⁻ in endothelial cells (23–25), we determined if H/R activated a similar signaling sequence as does ONOO⁻. As shown in Fig. 3a, short (5 min) or long (15 min) increased phosphorylation of both c-Src, AMPK, and ACC following the initial period of stress. The increase in phosphorylated c-Src, AMPK, and ACC at 30 min (Fig. 3a, b, and c) was inhibited either by overexpression of the c-Src-DN mutant, also prevented H/R-enhanced phosphorylation of c-Src, AMPK, and ACC at 30 min (Fig. 3a, b, and c). These data indicate that phosphorylation of c-Src, ACC, and ACC are temporally coordinated during H/R, and that activation of AMPK and ACC after 30 min of hypoxia is less at a time when ATP depletion would be expected to be greatest.

ONOO⁻ Mediates H/R Activation of c-Src and AMPK—Because our studies indicate that ONOO⁻ can activate c-Src and AMPK, we next determined if endogenous generation of ONOO⁻ was involved in c-Src and AMPK activation caused by H/R. Production by BAEC of ONOO⁻ was inhibited either by overexpressing SOD to scavenge O₂⁻, or treating the cells with l-NAME (1 mmol/liter) to prevent formation of NO. As shown in Fig. 4, a and b, overexpression of SOD1 or SOD2, or treatment of the cells with l-NAME, attenuated H/R-enhanced c-Src, AMPK, and ACC phosphorylation, as well as AMPK activity as assayed by SAMS peptide phosphorylation. Infection of cells with adenovirus encoding GFP alone had no effect on H/R-induced phosphorylation, excluding a nonspecific effect of viral infection. Because we previously showed that neither NO nor O₂⁻ alone activated AMPK in BAEC (22), the attenuation of c-Src and AMPK phosphorylation by either l-NAME or overexpression of SOD1 and SOD2 is likely explained by the formation of ONOO⁻ during H/R, which then activates both c-Src and AMPK kinase in the coordinated fashion observed.

c-Src-mediated and PI 3-Kinase-dependent AMPK Activation in Hypoxia-Reoxygenated BAEC—We next determined whether or not c-Src and PI 3-kinase are involved in AMPK activation in H/R-treated cells due to the generation of ONOO⁻. Overexpression of the c-Src-DN mutant significantly blunted the H/R-enhanced AMPK phosphorylation (Fig. 4c). Furthermore, the c-Src-DN also blocked the increased phosphorylation of ACC during H/R (Fig. 4c). In parallel, overexpression of the PDK-1-KD mutant, also prevented H/R-enhanced phosphorylation of both AMPK and ACC (Fig. 4c). While it was
not feasible to treat BAEC exposed to H/R with wortmannin or LY294002 because of the large volume of buffer bathing the cells, these studies suggest that like chemically synthesized ONOO⁻/H₂O₂, H/R activates AMPK and ACC via a c-Src and PI 3-kinase-dependent mechanism. Our previous studies showed that ONOO⁻/H₂O₂ increased phosphorylation of another target of AMPK, eNOS-Ser1179 (22). In other studies, we also found that H/R increases eNOS-Ser 1179 phosphorylation, and that this is prevented by the c-Src-DN and PDK-1-KD mutants (data not shown).

We previously reported that ONOO⁻ increases the association of AMPK with its downstream target, eNOS. To better understand how ONOO⁻ and H/R activate AMPK, we determined if c-Src was also physically associated with AMPK after ONOO⁻, or after H/R treatment of BAEC. As shown in Fig. 4d, addition of ONOO⁻ to BAEC increased the association of c-Src
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**DISCUSSION**

H/R was one of the first identified physiological activators of AMPK (1–6, 12–15). It is generally believed that H/R activates AMPK by increasing the AMP/ATP ratio by inhibiting mitochondrial ATP generation (1–6). Although H/R activation of AMPK has been demonstrated in various tissues, and a mechanism other than AMP/ATP has been suggested, none has yet been identified. The present study has for the first time demonstrated that H/R via the generation of oxidants, likely ONOO−, activates AMPK via a c-Src-mediated, PI 3-kinase-dependent pathway. Although an increased AMP/ATP ratio obviously contributes to hypoxia induced AMPK activation in many tissues, ONOO− generation in BAEC during H/R provides a novel pathway to regulate AMPK independently of altered cellular AMP/ATP ratio.

The evidence that activation of AMPK in the early phase of H/R is likely to be explained by the increased formation of ONOO− rather than, or in addition to, altered AMP/ATP ratio is severalfold. First, phosphorylated AMPK and ACC were detected as early as the first 5–15 min of hypoxia. In addition, after 30 min of hypoxia there was a decreased level of phosphorylated AMPK and ACC compared with those at 5 and 15 min. Because prolonged periods of hypoxia would be expected to further increase the AMP/ATP ratio, these data suggest a mechanism other than AMP/ATP ratio that can temporally activate AMPK.

Reoxygenation of hypoxic BAEC, which is associated with the generation of ONOO− in endothelial cells (23–26), also activated AMPK and ACC for 5 to 30 min, suggesting that the AMP/ATP ratio is not the sole factor that determines AMPK activity.

Second, inhibition of ONOO− formation by overexpression of SOD (to scavenge O2−) or NOS inhibition with l-NAME (to prevent the formation of NO) attenuated H/R-enhanced phosphorylation of both AMPK and ACC. Because NO or O2− alone have no effect on AMPK activation (22), and addition of authentic ONOO− did not affect the AMP/ATP ratio, the attenuation of AMPK and ACC phosphorylation by l-NAME and SOD in cells exposed to H/R suggests the involvement of ONOO− and can not be explained by the AMP/ATP ratio.

Third, in parallel with increased AMPK phosphorylation, H/R also increases ACC phosphorylation. Inhibition of c-Src activity, with or without overexpression of a c-Src mutant also blocked both AMPK and ACC phosphorylation, indicating that of AMPK, and ACC, phosphorylation is dependent upon c-Src. These data suggest that H/R, via c-Src activates AMPK and downstream signaling that initiates AMPK activation. There is also no evidence to suggest that AMPK or PDK-1 is activated by altered AMP/ATP ratio.

Finally, the postulate that ONOO− generated during H/R can activate AMPK independently of AMP/ATP ratio is substantiated by the fact that synthetic ONOO− was shown to do so without a measurable change in AMP/ATP. Thus, our results suggest a signaling pathway initiated by ONOO− capable of activating AMPK independently of changes in AMP/ATP.

This novel activation scheme (Fig. 5) for ONOO− generated during H/R was anticipated from studies of the effect of synthetic ONOO−. Relatively low concentrations of ONOO− dose-dependently increased the phosphorylation of c-Src and AMPK. Most importantly, inhibition of c-Src activity with a selective c-Src inhibitor, PP2, or overexpression of a c-Src mutant attenuated ONOO−-stimulated AMPK and ACC phosphorylation. ONOO− increased phosphorylation of PDK1 and P70S6 kinase, two downstream targets of PI 3-kinase, confirming that ONOO− activates PI 3-kinase. Furthermore, either wortmannin or LY294002 abolished ONOO−-induced AMPK phosphorylation as well as AMPK activity, as evidenced both by phosphorylation of the SAMS peptide and phosphorylation of ACC. Moreover, overexpression of the inactive PDK1 mutant likewise blocked ONOO−-activated AMPK phosphorylation and activity, strongly indicating that as a consequence of ONOO−, AMPK is a downstream target of PI 3-kinase and PDK1.

Precisely how PI 3-kinase regulates AMPK remains a subject for further study. Because inhibition of PI 3-kinase either with wortmannin or LY294002, or by overexpressing PDK-1-K5, did not affect basal AMPK phosphorylation in quiescent cells, nor did activation of PI 3-kinase by growth factors such as insulin and VEGF increase phosphorylation of AMPK in the absence of ONOO−, it is apparent that activation of PI 3-kinase alone is...
not sufficient to activate AMPK under normal conditions. However, the fact that the PDK1-KD or pharmacological inhibitors of PI 3-kinase prevented ONOO⁻-induced activation of AMPK, strongly suggests that PI 3-kinase plays a key role in cells challenged with the reactive nitrogen species.

Another novel finding of the present study is that oxidants such as ONOO⁻ might be important in regulating metabolic enzymes such as ACC. Phosphorylation and inactivation of ACC (35–36) and/or phosphorylation and activation of malonyl-CoA decarboxylase (37) by ONOO⁻-induced activation of AMPK will lead to a decrease in the concentration of malonyl-CoA (25–37). Because malonyl-CoA is a potent allosteric inhibitor for carnitine palmitoyltransferase I (CPT 1) (1–23) or physiological exercise (38–40) may contribute to accelerated fatty acid oxidation, leading to the development of metabolic disorders such as diabetes mellitus (21, 22).

It has been shown that exaggerated formation of ONOO⁻ may contribute to metabolic disorders such as diabetes mellitus (21, 22), and that oxidants, which have been considered as toxic and damaging to cells in high concentrations, may be important in regulating metabolic enzymes such as ACC. Phosphorylation and inactivation of ACC might be important in regulating metabolic disorders such as diabetes mellitus (21, 22).

In conclusion, we have demonstrated that H/R activates AMPK via c-Src, PI 3-kinase, and PDK1 might therefore be implicated in regulating cellular energy status particularly for cells recovering from hypoxia-reoxygenation. Another novel finding of the present study is that oxidants such as ONOO⁻ might be important in regulating metabolic enzymes such as ACC. Phosphorylation and inactivation of ACC might be important in regulating metabolic disorders such as diabetes mellitus (21, 22).

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