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Oxidative stress induces phosphorylation of neuronal NOS in cardiomyocytes through AMP-activated protein kinase (AMPK)

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ABSTRACT

Neuronal nitric oxide synthase (nNOS) plays a critical role in regulating cardiomyocyte function. nNOS was reported to decrease superoxide production in the myocardium by inhibiting the function of xanthine oxidoreductase. However, the effect of oxidative stress on nNOS in cardiomyocytes has not been determined. We report here that brief exposure of HL-1 cardiomyocytes to hydrogen peroxide (H_2O_2) induces phosphorylation of nNOS at serine 1412. This increase in phosphorylation was concomitant with increased nitric oxide (NO) production. Prolonged exposure to the oxidant, however, resulted in decreased expression of the protein. H_2O_2 treatment for short periods also stimulated phosphorylation of AKT and AMPK. H_2O_2-induced phosphorylation of nNOS was reduced when AMPK activity was inhibited by compound C, suggesting that AMPK is a mediator of oxidative stress-induced phosphorylation of nNOS. However, inhibition of AKT activity by the pan AKT inhibitor, AKTi, had no effect on nNOS phosphorylation caused by H_2O_2. These data demonstrate the novel regulation of nNOS phosphorylation and expression by oxidative stress.

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1. Introduction

Increased production of reactive oxygen species (ROS) has been shown to exert detrimental effects on various cell types. Indeed, oxidative stress has been implicated in many disease processes, including diabetic cardiomyopathy [1], atherosclerosis [2], and neurodegenerative diseases [3]. However, several recent studies have strongly advocated for critical signaling roles of *low levels* of ROS [4]. Among the various reactive oxygen species, superoxide and hydrogen peroxide (H_2O_2) have received the most attention as critical messengers, and the greater stability of the latter suggests this ROS species as a primary candidate for initiating changes in cellular signaling events [5].

Nitric oxide (NO) produced by the nitric oxide synthases (NOSs) is a critical signaling molecule in regulating cardiomyocyte function, modulating function of proteins involved in excitation contraction coupling including L-type Ca^{2+} channel (LTCC), troponin I and phospholamban. Furthermore, NO mediated-S-nitrosation modulates function of key myocardial proteins, including ryanodine receptor Ca^{2+} release channel (RyR), sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) and L-type Ca^{2+} channel (LTCC) [6]. Little is known, however, about the interplay between ROS and NO in regulating cellular signaling in cardiomyocytes.

Cardiomyocytes express both neuronal NOS (nNOS) [7] and endothelial NOS (eNOS) [8]. Differential biological functions of these NOSs are achieved by targeting of these enzymes to distinct subcellular compartments. eNOS is predominantly localized to the caveolae, whereas nNOS is localized to the sarcoplasmic reticulum and the plasma membrane. Similar to eNOS, nNOS is phosphorylated under various conditions by several different kinases, which modulates its function. Phosphorylation at serine 847 by Cam Kinase II was shown to decrease enzymatic activity [9,10], whereas phosphorylation at serine 1412 by AKT was reported to increase its activity in cortical neurons [11]. Purified nNOS S1412D, a phosphomimetic mutant, exhibited a higher rate of electron transfer and heme reduction, but the net NO production was diminished, likely due to faster heme-NO inhibitory complex formation [12].

Several reports indicate a role for nNOS-derived NO in basal and β adrenergic receptor (β3-AR)- regulated myocardial contraction. Apart from regulating myocardial contraction, nNOS inhibits superoxide production by xanthine oxidoreductase (XOR) [13], mediates β3-AR agonist-induced cardioprotection through suppression of ROS [14], and mediates the anti-hypertrophic and

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antioxidant response of β3-AR in cardiomyocytes [15]. These observations suggest that myocardial nNOS maintains a balance between NO and ROS. Although several studies have reported a role for nNOS in inhibiting ROS in cardiomyocytes, the effect of oxidant stress on nNOS expression and phosphorylation is not known. At least two splice variants of nNOS are present in heart tissue, nNOSα and nNOSμ [16]. The cardiomyocyte cell line used in the present study likely expresses both of these variants; however we will not distinguish between them, referring to them collectively as nNOS.

To explore the effect of oxidant stress on nNOS in cardiomyocytes, we treated HL-1 cardiomyocytes with H_2O_2 and examined nNOS protein levels and phosphorylation status. We report herein that transient exposure of HL-1 cardiomyocytes to this oxidant results in nNOS phosphorylation mediated by AMP activated protein kinase (AMPK). Subsequent prolonged exposure to the oxidant caused decreased expression of the nNOS protein itself.

2. Materials and methods

2.1. Materials

Claycomb medium, serum for HL-1 cells, fibronectin, gelatin and norepinephrine were from Sigma (St Louis, MO). Penicillin, streptomycin and trypsin were from Life Technologies (Grand Island, NY). AKTi and compound C were obtained from Merck Millipore (Billerica, MA), AKTi, phospho-AKT (Serine 473), phospho-AMPK (Threonine 172) and phospho-ACC (Serine 79) antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Phospho-S1417 nNOS antibody (recognizes mouse nNOSα at S1412 and mouse nNOSμ at S1446; for simplicity, we will refer to these residues as S1412) was from Abcam (Cambridge, MA) and nNOS antibody was from BD Biosciences (San Jose, CA). Horse radish peroxidase-linked mouse and rabbit secondary antibodies were from Santa Cruz Biotechnology Inc. (Dallas, TX) and Thermo Fisher Scientific (Waltham, MA).

2.2. Cell culture

HL-1 cardiomyocytes were obtained from the laboratory of Dr. William Claycomb (Louisiana State University Health Science Center, New Orleans, LA, USA) and were grown in Claycomb media with 10% FBS, pencillin, streptomycin, L-glutamine and norepinephrine as previously described (W.C. Claycomb, PNAS, 1998). Cells were plated overnight in full growth media on gelatin- and fibronectin-coated plates and were then treated with 200 μM of H_2O_2. Cells were treated with inhibitors (compound C or AKTi) 1 h prior to H_2O_2 treatment.

2.3. Western blotting

Whole cell lysates were collected from HL-1 cells after the treatment period in 2x Laemmeli buffer containing protease and phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA). Equal amounts of proteins were subjected to SDS-polyacrylamide gel electrophoresis (4–15% Tris Glycine gels; Biorad, Hercules, CA), and proteins were transferred onto PVDF membranes (Merck Millipore, Billerica, MA). Membranes were blocked in 5% BSA in TBST (Tris-buffered saline with 0.1% tween 20) and were then incubated with primary antibodies overnight, followed by washing with TBST and incubation with horse radish peroxidase-conjugated secondary antibody for an hour at room temperature. Blots were developed using the Immobilon western chemiluminiscent substrate (Merck Millipore, Billerica, MA) and images were analyzed using Image Studio from LI-COR (Lincoln, NE).

2.4. NO detection

HL-1 cells were incubated with 5 μM of DAF 2-DA for 15 min prior to the addition of 200 μM of H_2O_2. After the treatment, cells were washed with PBS to remove excess DAF 2-DA. Images were then obtained using an Olympus IX70 fluorescence microscope.

2.5. Calf intestinal phosphatase assay

Cell lysate was collected in Laemmeli buffer without phosphatase inhibitor after treatment with hydrogen peroxide and was then incubated with calf intestinal phosphatase for 30 min at 37 °C. Phosphatase was then inactivated by incubating the lysate at 65 °C for 10 min.

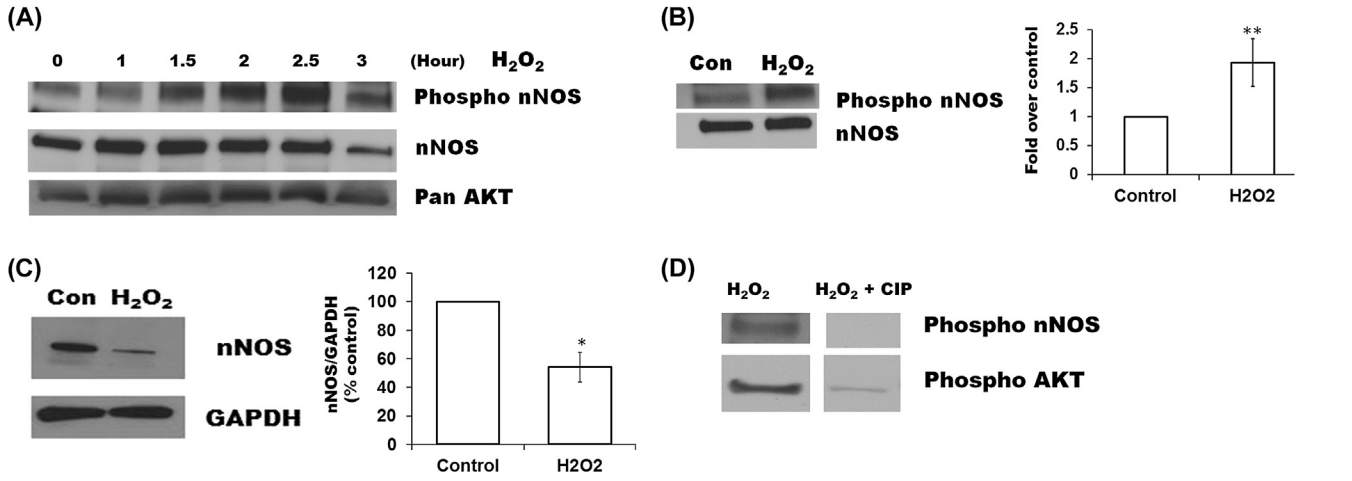

Fig. 1. Effect of H_2O_2 on nNOS phosphorylation in cardiomyocytes. (A) HL-1 cells were treated with 200 μM of H_2O_2 for 0, 1, 1.5, 2, 2.5 and 3 h. Cell lysates collected after treatment were subjected to immunoblotting using antibodies against phospho-nNOS, total nNOS and pan AKT. (B) Representative blot showing nNOS phosphorylation after 1.5 h of treatment with 200 μM of H_2O_2. Bar graph shows ratio of phospho-nNOS to total nNOS from 3 independent experiments, Control versus H_2O_2,**, $P < 0.01$. (C) Whole cell lysate after a 24h treatment with 200 μM of H_2O_2 was subjected to immunoblotting using nNOS and GAPDH antibodies. Images were quantified using Image Studio software from LI-COR and show the ratio of total nNOS to GAPDH from $n = 3$ experiments, Control versus H_2O_2,*, $P < 0.05$. (D) Whole cell lysate after H_2O_2 treatment was incubated with and without calf intestinal phosphatase and was then subjected to immunoblotting using phospho-nNOS and phospho-AKT (Serine 473) antibody.

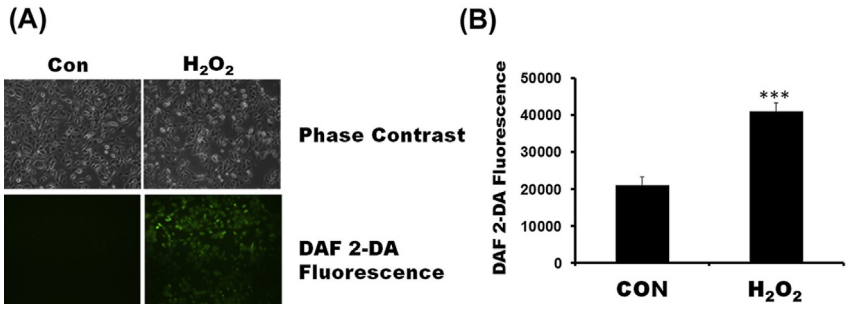

Fig. 2. Effect of H_2O_2 on NO bioavailability in HL-1 cells. HL-1 cells were incubated with 5 μM DAF 2-DA for 15 min before treatment with 200 μM H_2O_2 for 1.5 h. (A) Phase contrast (Upper panel) and fluorescence images (Lower panel) of untreated and treated cells were obtained using an IX70 Olympus fluorescence microscope after washing excess DAF 2-DA with PBS (B) DAF 2-DA fluorescence intensity was read in control and treated cells in a TECAN microplate reader (TECAN Group Ltd. CH-8708, Mannedorf), excitation- 495 nm, and emission-550 nm. Bar graph shows DAF 2-DA fluorescence intensity in control and H_2O_2-treated cells from n = 3 experiments, Control versus H_2O_2, ***, $P < 0.001$.

3. Results and discussion

3.1. The phosphorylation of neuronal NOS (nNOS) is induced by hydrogen peroxide treatment in the cardiomyocyte cell line, HL-1

To explore the effect of oxidative stress on nNOS phosphorylation, HL-1 cardiomyocytes were treated with 200 μM of H_2O_2 for various periods of time. We chose this model as this immortalized cardiac cell line maintains contractile activity and exhibits differentiated cardiac morphological, electrophysiological and biochemical properties [17]. We used the lowest possible dose of H_2O_2 (200 μM) that elicited cellular signaling changes. Increased nNOS phosphorylation was observed after a 1.5-h treatment with the oxidant (Fig. 1A and B). This increase in phosphorylation was constant through 2.5 h of treatment with the oxidant, but started to diminish by 3 h. nNOS protein expression also decreased after 3 h of treatment with the oxidant (Fig. 1A). Longer periods of treatment (24 h) with the oxidant resulted in a significant decrease in the expression of nNOS (Fig. 1C).

In contrast to our finding, Sartoretto et al. [18], reported that brief exposure to H_2O_2 (10 μM) induced activation of eNOS but not nNOS in adult mouse cardiac myocytes, which could be due to the differences between the cell line and the primary cells and/or the dose of oxidant and the duration of exposure to oxidant stress. In addition, these authors did not probe for nNOS phosphorylation, and their conclusion was based on the fact that the H_2O_2-induced increase in NO production was abrogated in cardiomyocytes from eNOS null mice but not in nNOS null mice.

It is interesting to note that, although a brief exposure of HL-1 cells to oxidant stress resulted in phosphorylation of nNOS with no change in the level of total nNOS, longer exposure (>3 h) to H_2O_2 resulted in decreased expression of the protein itself (Fig. 1C). It is possible that, during initial exposure to oxidant stress, cells upregulate the activity of nNOS to protect themselves from oxidative stress. In this regard, it is noteworthy that nNOS knockout mice showed higher levels of superoxide in their myocardium than wild-type mice [13]. However, persistent oxidant stress likely causes the decrease in expression of the protein, perhaps due to activation of calpains or other proteases, which likely contribute to cardiomyocyte apoptosis.

To ensure that the band detected by the phospho-specific antibody is indeed due to phosphorylation, cell lysates were treated with calf intestinal phosphatase (CIP) to remove any phosphorylation. As shown in Fig. 1D, the band corresponding to nNOS phosphorylation disappeared with the phosphatase treatment, confirming that the nNOS phospho-specific antibody was indeed detecting only the phosphorylated protein. As expected, the band corresponding to phospho-AKT was also greatly diminished with phosphatase treatment, confirming that the phosphatase was active and was affecting AKT phosphorylation as well.

3.2. nNOS phosphorylation by H_2O_2 is concomitant with increased NO production

Since the phosphorylation of nNOS by insulin at this particular residue (S1412) was shown to increase NO production in muscle cells [19], we measured NO bioavailability in HL-1 cells using the NO-specific cell permeable fluorescent probe DAF 2-DA.

HL-1 cells treated with H_2O_2 for 1.5 h showed a marked increase in DAF 2-DA fluorescence over the cells treated with vehicle (Fig. 2A). Quantification of the fluorescence intensities in the control and H_2O_2-treated cells indicated that NO levels in the cell were approximately doubled by oxidant treatment (Fig. 2B). The increase in NO bioavailability caused by H_2O_2 could have been a result of increased activity of nNOS alone or both eNOS and nNOS, as eNOS was also shown to be phosphorylated and activated by H_2O_2 [18]. It remains to be seen whether increased NO produced by these two distinct NOS variants under conditions of oxidative stress perform unique cellular function by being sequestered in distinct subcellular compartments or synergize each other's function.

3.3. Phosphorylation of nNOS is mediated through AMPK

To determine which kinase might be responsible for phosphorylation of nNOS under oxidant stress, we examined the phosphorylation of AKT and AMPK during different periods of treatment with H_2O_2. Both AKT and AMPK were activated in response to H_2O_2 treatment as seen by increased phosphorylation

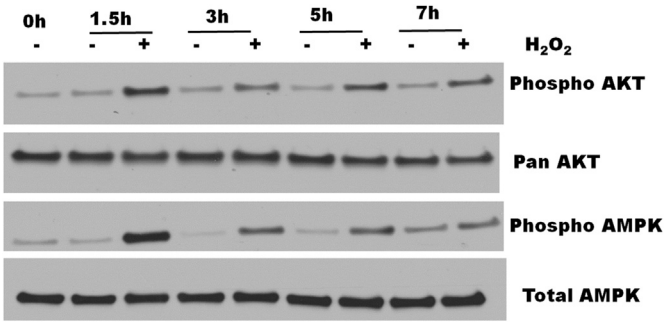

Fig. 3. Effect of H_2O_2 on AKT and AMPK phosphorylation. HL-1 cells were incubated with 200 μM of H_2O_2 for 0, 1.5, 3, 5 or 7 h. Whole cell lysates after treatment were subjected to immunoblotting using antibodies for phospho-AKT (serine 473), pan AKT, phospho-AMPK (threonine 172) and total AMPK.

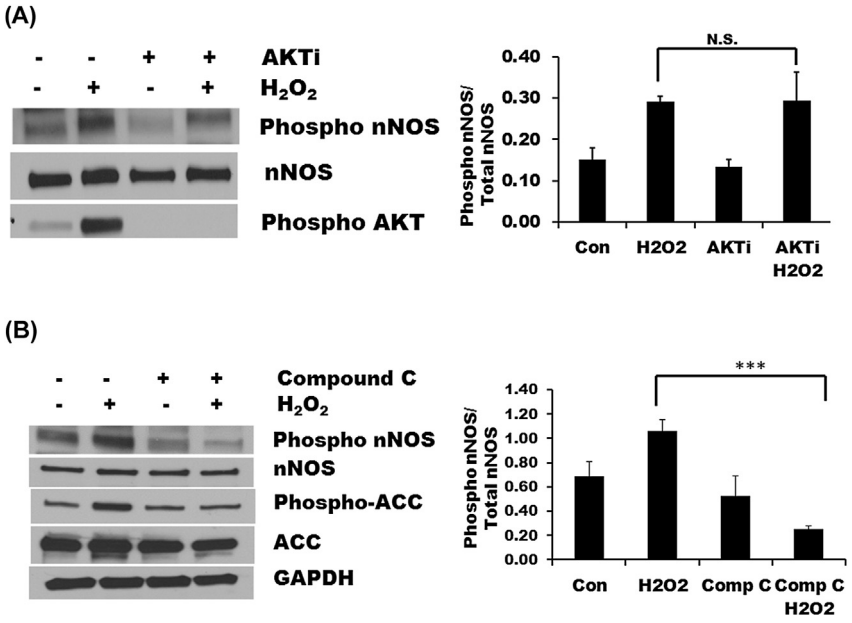

Fig. 4. Effect of AKT or AMPK inhibitor on H_2O_2-induced phosphorylation of nNOS in HL-1 cells. (A) Cells were pretreated with 20 μM of AKTi before addition of 200 μM of H_2O_2. Whole cell lysate after 1.5 h of treatment was probed with phospho-nNOS, nNOS and phospho-AKT antibodies. Right Panel shows quantification of phospho-nNOS to total nNOS from 3 independent experiments, H_2O_2 versus AKTi + H_2O_2 not significant (N.S.) (B) Cells were treated with compound C (40 μM) before H_2O_2 treatment. Cell lysates after treatment were probed with antibodies for phospho-nNOS, nNOS, phospho-ACC (Serine 79), ACC and GAPDH. Right Panel shows quantification of band intensities of phospho-nNOS to total nNOS from n = 3 experiments. H_2O_2 versus compound C + H_2O_2, ***, $P < 0.001$.

of these kinases by 1.5 h of treatment with the oxidant (Fig. 3). It is interesting to note that this timing coincides with that of H_2O_2-mediated nNOS phosphorylation (Fig. 1B).

The involvement of AKT in inducing nNOS phosphorylation by H_2O_2 was explored using AKTi, a specific inhibitor of all AKT isoforms. This inhibitor blocked AKT phosphorylation and activation; however, it failed to inhibit phosphorylation of nNOS, indicating that AKT is not the kinase responsible for nNOS phosphorylation promoted by H_2O_2 (Fig. 4A). Since both AMPK and AKT were shown to phosphorylate eNOS in cardiomyocytes [18], the involvement of AMPK in nNOS phosphorylation caused by H_2O_2 in HL-1 cells was also explored. Cells were treated with AMPK inhibitor, compound C, before treatment with H_2O_2. To test the efficacy of compound C in inhibiting the AMPK pathway, we probed for the phosphorylation (Serine 79) of acetyl-CoA carboxylase (ACC), a direct target of the AMPK pathway. Compound C prevented H_2O_2-induced phosphorylation of both nNOS and ACC (Fig. 4B), suggesting that AMPK is involved in regulating nNOS phosphorylation during H_2O_2 treatment.

Whether nNOS is a direct substrate for AMPK or AMPK causes the phosphorylation of nNOS through other signaling intermediates is unknown at this time. eNOS phosphorylation observed in the adult primary cardiomyocytes was shown to be caused by both AKT and AMPK, whereas the nNOS phosphorylation observed in the current study appears to require AMPK, but not AKT. It is interesting to note that in cardiac myocytes and in vascular endothelial cells, AKT activation appeared to be regulated by the AMPK pathway, as inhibition of AMPK activity either by compound C [18] or by small interfering RNA mediated knockdown of AMPK α1 [20] decreased agonist-induced AKT phosphorylation. Although we observed similar activation of AMPK and AKT within 1.5 h of treatment with the oxidant, AMPK appeared to be the kinase responsible for nNOS phosphorylation caused by the oxidant stress, but not AKT. Furthermore, it is quite possible, even probable, that phosphorylation of eNOS and nNOS may be regulated uniquely by different kinases.

It will be interesting to determine how phosphorylation at this residue, under conditions of increased oxidative stress, results in alteration of nNOS subcellular localization and its interaction with other proteins, which could produce further diverse cellular changes. Future studies will characterize upstream kinases/proteases that are involved in the regulation of nNOS phosphorylation and expression.

Conflict of interest

The authors declare no conflict of interest.

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