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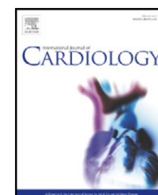

Acute exercise induce endothelial nitric oxide synthase phosphorylation via Akt and AMP-activated protein kinase in aorta of rats: Role of reactive oxygen species

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ABSTRACT

Background: Acute exercise increases reactive oxygen species (ROS) levels, including hydrogen peroxide (H_2O_2). H_2O_2 promotes endothelial nitric oxide synthase (eNOS) activation and phosphorylation in endothelial cells. With this in mind, the present study was designed to evaluate *ex vivo* eNOS phosphorylation in rat aortas incubated with H_2O_2 and to test this hypothesis *in vivo* in the aortas of rats submitted to acute exercise.
Methods: For *ex vivo* studies, six groups of aortic tissue were formed: control, H_2O_2, N-acetylcysteine (NAC), LY294002, compound C, and LY294002 plus compound C. While incubation with H_2O_2 increased Akt, AMPK and eNOS phosphorylation, pre-incubation with NAC strongly reduced the phosphorylation of these enzymes. For *in vivo* studies, male Wistar rats were divided into four groups: control, cont + NAC, exercise, and exer + NAC. After a 3 h swimming session, animals were decapitated and aortas were excised for biochemical and immunoblotting analysis.
Results: Acute exercise increased superoxide levels and dichlorofluorescein (DCF) concentrations, and this increase was related to phosphorylation of Akt, AMPK and eNOS. On the other hand, use of NAC reduced superoxide levels and DCF concentration. Reduced superoxide levels and DCF in the exer + NAC group were associated with decreased Akt, AMPK and eNOS phosphorylation. These results appear to be connected with vascular function because VASP phosphorylation increased in acute exercise and decreased in exer + NAC.
Conclusion: Our results indicate that ROS induced by acute exercise play the important role of activating eNOS, a process apparently mediated by Akt and AMPK.

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1. Introduction

Endothelial dysfunction, defined by decreased endothelium-dependent vasodilatation, is associated with a high number of cardiovascular events. In vascular disease, endothelial dysfunction has been linked to decreased nitric oxide (NO) bioavailability [1]. Endothelial dysfunction may be therapeutically reversible, even with exercise alone, as increased endothelium-dependent vasodilatation is associated with exercise [2]. Nevertheless, limited information is available on the mechanisms behind this association. A previous study has suggested the importance of endogenous NO production as a benefit of exercise [3], pointing to its key role in maintaining cardiovascular homeostasis.

NO is derived from L-arginine in a reaction catalysed by endothelial NO synthase (eNOS). Acute exercise increases the activation of eNOS [4], but it remains to be established how exercise mediates this induction. In addition, studies have demonstrated that H_2O_2 promotes eNOS phosphorylation in endothelial cells [5,6]. Considering exercise as a therapeutic approach, these findings are relevant because physical activity is also associated with increased oxidative stress [7].

The generation of reactive oxygen species (ROS) is a normal process in aerobic organisms. Physical exercise – characterised by a remarkable increase in oxygen consumption with concomitant production of ROS – presents a challenge to antioxidant systems. However, while chronic exercise has been widely associated with reduced ROS [8–10], it has been argued that acute exercise can induce an acute state of oxidative stress [7,11]. Clearly, exercise causes physical stress to the body, and numerous studies have shown that oxidative stress biomarkers are increased following acute exercise [12–14]. This is interesting because reactive oxygen species produced during repeated exercise bouts serve as stimulating stressors able to trigger hormetic responses [15,16]. In endothelial cell function, basal intracellular levels of H_2O_2, typically within the range of 25–75 μM, are normal [17,18]. However, under pathological conditions, excessive ROS production has detrimental consequences to the vascular wall [19].

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As mentioned above, eNOS is activated by H_2O_2. In addition, eNOS has been found to be phosphorylated at several consensus motifs by a number of kinases, including PKB/Akt and AMP-activated protein kinase (AMPK) [4,20,21]. Studies reported that H_2O_2 promoted eNOS phosphorylation in endothelial cells. On the other hand, acute and exhaustive exercise increases levels of oxidants. In addition, exercise induces eNOS activation, but the underlying mechanisms of this process remain unclear. The aim of this study was therefore to evaluate eNOS phosphorylation *ex vivo* in isolated aortas incubated in the presence of H_2O_2 and to test this hypothesis *in vivo* in aortas of rats submitted to acute exercise.

2. Methods

2.1. Animals

Two-month-old male Wistar rats weighing 250–300 g were obtained from our breeding colony (UNESC) and housed in a temperature-controlled room with a reverse light–dark cycle. Tap water and standard rodent laboratory diet were supplied *ad libitum*. The animals were looked after in accordance with the guiding principles in the care and use of animals, and protocols were approved by the Ethics Committee (protocol number—91/2009) of the Universidade do Extremo Sul Catarinense (UNESC). For *ex vivo* study, rats were killed, thoracic aorta were divided in six groups (*pool* of two aortas per group/well) and pre-incubated immediately in i) no H_2O_2 (control), ii) with H_2O_2 (H_2O_2), iii) N-acetylcysteine, iv) LY294002, v) compound C or vi) LY294002 plus compound C for 30 min. After pre-incubation, all groups (except the control group) were incubated with 200 μM of H_2O_2 for 30 min. At the end, aortas were immediately homogenised for immunoblotting. This experiment was repeated on five different days (n = 5).

For the *in vivo* tests, the animals were randomly assigned to four groups: control (cont) (n = 20), control plus NAC (cont + NAC) (n = 20), acute exercise (exercise) (n = 20), and acute exercise plus NAC (exer + NAC) (n = 20). These animals were used for the analyses of superoxide levels and DCF concentration (n = 5 for each parameters – ten rats). The last experiment was used for immunoblotting (ten rats—pool of two aortas, i.e., n = 5). Rats were killed by decapitation and aorta tissue was removed, homogenised in specific buffer and stored at −70 °C for western blot and biochemical analysis.

2.2. NAC supplementation and acute exercise protocol

N-acetylcysteine was supplemented by gavage at 25 mg per kg/bw for five days. The rats began the adaptation period on the 3rd day of supplementation. The animals were habituated in a swimming pool for 10 min on the 3rd and 4th days of supplementation to mitigate stress caused by the new environment. On the 5th day of supplementation, the rats swam for 3 h without overload or a rest period. The water temperature was maintained at ~32 °C. The untrained animals were put in an empty swimming pool for the same length of time as the acute exercise group. Immediately after the exercise protocol, the rats were killed and thoracic aorta was surgically excised. Excess blood and all adjacent tissue were removed to reveal the smooth muscle tissue and the endothelium.

2.3. Biochemical assays

2.3.1. Superoxide anion assay

Superoxide anion was measured according to a method previously described [22] and expressed as nmol/min/mg protein. Superoxide was calculated based on adrenaline oxidation rate determined by absorbance at 780 nm.

2.3.2. Dichlorofluorescein assay

2′,7′-dichlorofluorescein diacetate (DCFH-DA) was used to estimate intracellular ROS concentration. DCF was prepared from DCF-DA as previously described [23]. Fluorescence was monitored on a SpectraMax spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA) with excitation at 488 nm and emission at 525 nm. Autofluorescence was corrected for with the inclusion of parallel blanks (DCFH alone in buffer). DCFH-DA was purchased from Molecular Probes, Inc. (Eugene, OR, USA).

2.3.3. Protein determination

The amounts of protein for biochemical analysis were assayed by Lowry's method [24].

2.4. Aorta incubation experiment

To study the influence of H_2O_2 on Akt, AMPK, eNOS and VASP phosphorylation, aorta from rats were pre-incubated in RPMI-1640 medium (Sigma Chemical Co, St. Louis, MO, U.S.A.) without (control and H_2O_2 groups) or with N-acetylcysteine (20 mM), LY294002 (20 μM) or compound C (20 μM) for 30 min at 37 °C under 5% O_2 and 95% CO_2. After the pre-incubation period, 200 μM of H_2O_2 was added to the RPMI medium (except for the control group) and incubated for 30 min [21]. After the final incubation period, the aorta was immediately homogenised in extraction buffer and immunoblotting was performed.

2.5. Protein analysis by immunoblotting

Immediately after the acute exercise protocol or the end of the incubation period, aortas were homogenized in extraction buffer (1% Triton-X 100, 100 mM Tris, pH 7.4, containing 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF and 0.1 mg of aprotinin/ml) at 4 °C (Polytron MR 2100, Kinematica, Switzerland). The extracts were centrifuged at 9,000 g and 4 °C (5804R, Eppendorf AG, Hamburg, Germany) for 40 min to remove insoluble material. The supernatants were used for protein quantification, according to the Bradford method. Proteins were denaturated by boiling in Laemmli sample buffer containing 100 mM DTT, run on SDS-PAGE, transferred to nitrocellulose membranes. Membranes were blocked, probed and blotted with primary antibodies. Antibodies used for immunoblotting were anti-Akt, anti-AMPK, anti-VASP, anti-phospho-Akt^{ser473} and anti-phospho-VASP^{ser239} (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-eNOS, anti-phospho-AMPK^{Thr172} and anti-phospho-eNOS^{ser1177} antibodies were from Cell Signaling Technology (Beverly, MA, USA). Chemiluminescent detection was performed with horseradish peroxidase-conjugate secondary antibodies (Thermo Scientific, Rockford, IL, USA). Autoradiographs of membranes were taken for visualization of protein bands. The results of blots are presented as direct comparisons of area of apparent bands in autoradiographs and quantified by densitometry using the Scion Image software.

2.6. Statistical analysis

Biochemical data were expressed as means and mean standard errors (SEM), and analysed statistically by one-way variance analysis (ANOVA), followed by the Bonferroni *post-hoc* test. The results of blot analysis were expressed as means of area of bands ± SEM. Differences between the groups were evaluated using one-way analysis of variance (ANOVA) followed by the Bonferroni *post-hoc* test. A probability of less than 0.05 was considered significant. The software used for analysis of the data was the Statistical Package for the Social Sciences (SPSS) version 16.0 for Windows.

3. Results

3.1. Ex vivo effects of H_2O_2 on Akt, AMPK, eNOS and VASP phosphorylation in freshly isolated aorta

Aorta tissue was first incubated in RPMI medium in the presence or absence of H_2O_2 (200 μM) with or without NAC or inhibitors specific to Akt and AMPK phosphorylation. In the presence of H_2O_2, Akt phosphorylation increased compared to the control group, while NAC pre-treatment reduced Akt phosphorylation (Fig. 1A). To establish a direct link between Akt phosphorylation and eNOS activation upon exposure of the aorta to H_2O_2, we used specific inhibitors of Akt phosphorylation. Akt phosphorylation was observed to decrease in aortas pre-incubated with both LY294002 and LY294002 plus compound C when compared with the H_2O_2 group (Fig. 1A). The levels of Akt phosphorylation remained unchanged in aortas pre-incubated with compound C (Fig. 1A).

Incubation of aortas with H_2O_2 (H_2O_2 group) also led to increased AMPK phosphorylation compared to the control group. Pre-treatment of aortas with NAC reduced AMPK phosphorylation when compared with the H_2O_2 group (Fig. 1B). In aortas pre-incubated with compound C and LY294002 plus compound C, the AMPK phosphorylation decreased compared to the H_2O_2 group (Fig. 1B). The pre-incubation of aortas with LY294002 did not alter AMPK phosphorylation levels (Fig. 1B).

Similar results were observed for eNOS phosphorylation (Fig. 1C). Hydrogen peroxide led to an increase in eNOS phosphorylation when compared with the control group. In the presence of NAC, the phosphorylation of eNOS decreased compared to the H_2O_2 group (Fig. 1C). When aortas were pre-incubated with LY294002, compound C and LY294002 plus compound C, a marked reduction in eNOS phosphorylation compared with the H_2O_2 group was observed (Fig. 1C).

To determine if these results were connected with vascular function, we measured VASP (vasodilator-stimulated phosphoprotein) phosphorylation (Fig. 1D). In the presence of H_2O_2, VASP phosphorylation increased compared with the control group, while NAC

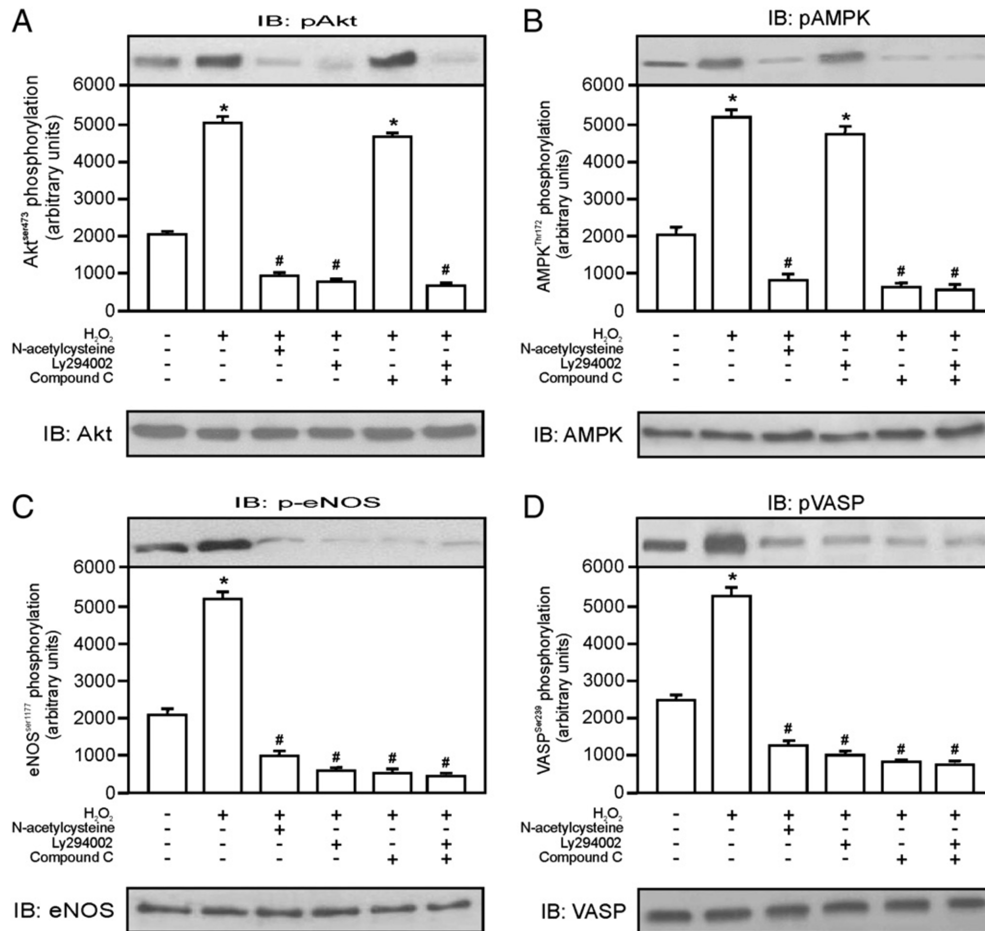

Fig. 1. *Effects ex vivo of H_2O_2 on Akt, AMPK, eNOS and VASP phosphorylation in freshly isolated aorta.* The aortic tissue was pre-incubated for 30 min without (control and H_2O_2 groups) and with: N-acetylcysteine, LY294002, Compound C or LY294002 plus Compound C. After this period, the aorta of all groups (except control group) were incubated with 200 μM H_2O_2 for 30 min. Immediately after incubation, aortas were homogenized and immunoblot performed to measure the levels of the phosphorylation (top panel) of Akt (A), AMPK (B), eNOS (C) and VASP (D). The lower panel shows bands representative of Akt, AMPK, eNOS and VASP protein levels. The results are presented as arbitrary units. Bars are presented as means of area of bands ± SEM of five different incubation experiments per group, $^*p<0.05$, H_2O_2 group versus control and $^\#p<0.05$, NAC and inhibitors treatments versus H_2O_2 group.

pre-treatment reduced these values (Fig. 1D). Furthermore, the phosphorylation of VASP diminished when aortas were pre-incubated with LY294002 and compound C. The use of LY294002 plus compound C also reduced VASP phosphorylation when compared with the H_2O_2 group, but a non-significant effect was observed for the combination compared to inhibitors alone (Fig. 1D).

3.2. Superoxide levels and DCF concentration in aorta of rats

Acute exercise increased superoxide levels compared with the control (non-exercised) group (Fig. 2A). On the other hand, NAC (exer + NAC group) reduced superoxide levels compared to the exercise group (Fig. 2A). Additionally, acute exercise increased DCF concentrations compared with the control group (Fig. 2B). However, the DCF concentration decreased in exercised rats supplemented with NAC compared with rats that only exercised (Fig. 2B).

3.3. In vivo effects of acute exercise on Akt, AMPK, eNOS and VASP phosphorylation in aorta of rats

Exercise increased Akt phosphorylation when compared with control and cont + NAC groups. On the other hand, the levels of phosphorylation of Akt decreased when NAC was supplemented (exer + NAC) (Fig. 3A). Acute exercise increased AMPK phosphorylation compared with control and cont + NAC groups. In addition, AMPK phosphorylation reduced in aorta of rats exercised and previously supplemented with NAC (exer + NAC group) (Fig. 3B). Exercise increased eNOS phosphorylation compared with control and cont + NAC groups, but then decreased in rats exercised and supplemented with NAC (exer + NAC) (Fig. 3C). To evaluate the importance of vascular function in these results, we also analysed VASP phosphorylation (Fig. 3D). As seen, VASP phosphorylation behaved similarly to eNOS phosphorylation.

4. Discussion

The beneficial effects of exercise to endothelial function can be mediated in a number of ways, including oxidant/antioxidant balance. However, these benefits depend on the type of exercise performed [25]. For example, acute exercise activates ROS production [7,11–14] and eNOS phosphorylation [4]. In this sense, our goal was to study *ex vivo* redox modulation of eNOS in aorta and determine if acute exercise caused eNOS phosphorylation *in vivo* through a similar mechanism. Based on the presented data, increased vascular oxidative stress and subsequent activation of Akt and AMPK is suggested to be the mechanism involved in eNOS phosphorylation.

A well characterised pathway that modulates eNOS function through phosphorylation is the phosphatidylinositol 3-kinase (PI3K)/ Akt cascade [26–28]. The Akt/eNOS phosphorylation axis was thus explored in aorta exposed to H_2O_2. A large increase in H_2O_2-stimulated

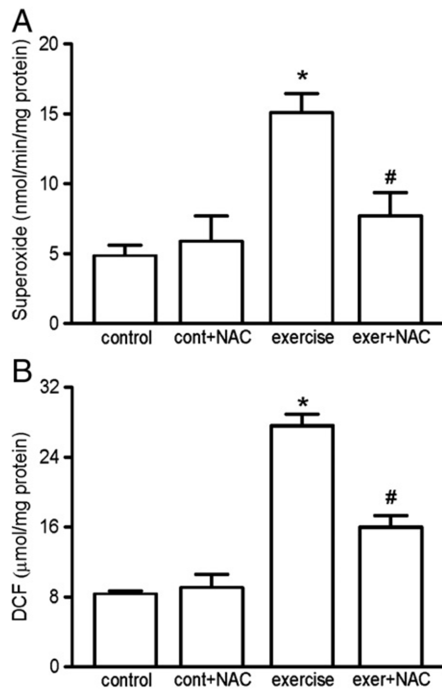

Fig. 2. *Superoxide levels and DCF concentration in aorta of rats.* Superoxide levels (A) and diclorofluorescein concentration (B). Superoxide levels are expressed as nmol/min/mg of protein (A) and DCF is expressed as μmol/mg of protein (B). Bars represent means ± SEM of five rats per group. *$p<0.05$, exercise versus control group; #$p<0.05$, exer + NAC versus exercise group.

phosphorylation of Akt was subsequently observed. To better evaluate the participation of Akt in eNOS phosphorylation, a specific inhibitor of Akt (i.e., LY294002) was also employed. Pre-incubation with LY294002 drastically reduced Akt phosphorylation and subsequently eNOS phosphorylation. Interestingly, the use of NAC reduced Akt and eNOS phosphorylation by a magnitude equal to LY294002. These results are in concordance to literature [5,6]. An elegant study by Hu and colleagues investigated the effects of H_2O_2 on cultured endothelial cells and revealed that Akt largely activated eNOS, though this effect was reversed when cells were pre-treated with the PI3K inhibitor wortmaninn [20]. These authors also observed that AMPK was significantly activated by H_2O_2.

AMPK is a heterotrimeric serine/threonine kinase that can be activated by a rise in the AMP/ATP ratio [29]. Active AMPK may phosphorylate metabolic enzymes that switch on ATP-generating catabolic pathways and switch off anabolic pathways. A pioneering study on the role of AMPK in vasculature revealed that mammalian AMPK provides an interesting means of integrating metabolic stress signals within endothelial cells and myocytes to control eNOS, thereby potentially modulating the local circulatory system to improve nutrient supply and suppress mechanical activity of the muscle [30]. In the present study, the incubation of aortas with H_2O_2 led to an increase in AMPK phosphorylation. Pre-treatment of aortas with N-acetylcysteine (NAC) markedly reduced this AMPK phosphorylation. The involvement of AMPK was also investigated utilising the specific inhibitor compound C. In the presence of compound C, H_2O_2 failed to increase eNOS phosphorylation, demonstrating the importance of AMPK in this process. The use of NAC again reduced AMPK and eNOS phosphorylation to a magnitude equal to that observed

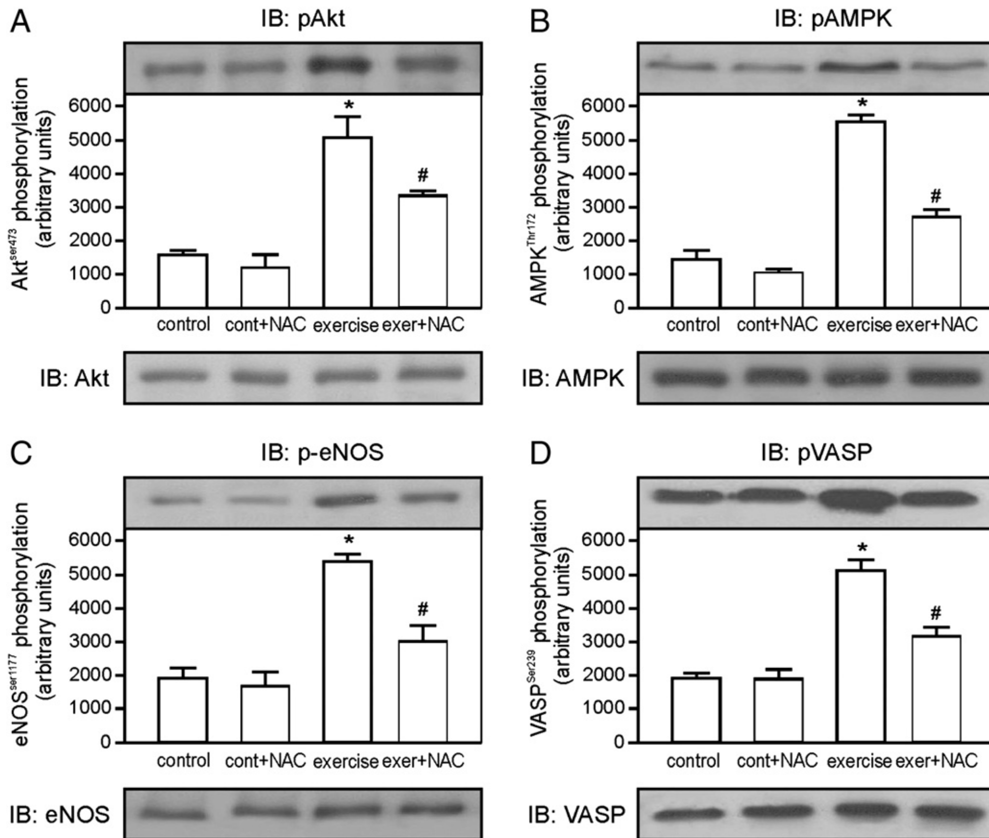

Fig. 3. *Effect in vivo of acute exercise on Akt, AMPK, eNOS and VASP phosphorylation in aorta of rats.* The levels of the phosphorylation (panel upper) the Akt (A), AMPK (B), eNOS (C) and VASP (D) were measured by immunoblot in aorta of rats killed immediately after acute exercise. The lower panel shows bands representative of Akt, AMPK and eNOS protein levels. The results are presented as arbitrary units. Bars are presented as means of area of bands ± SEM of five rats per group, *$p<0.05$, exercise versus control group; #$p<0.05$, exer + NAC versus exercise group.

with compound C. These results demonstrate the involvement of ROS in eNOS phosphorylation and suggest that Akt and AMPK are members of an underlying mechanism. Jin and colleagues investigated the signalling mechanisms involved in AMPK activation in endothelial cells stimulated with H_2O_2. The authors observed important involvement of this enzyme in eNOS phosphorylation [21].

Endothelium-dependent relaxation is not exclusively regulated by nitric oxide. To analyse the significance of the results on vascular function (vasorelaxation), we measured VASP phosphorylation. Analysis of VASP phosphorylation at serine 239 is a useful biomonitor of cGK (cGMP-dependent protein kinase) activity and thus NO effects in cultured endothelial and smooth muscle cells [31]. VASP is then a biochemical marker capable of monitoring NO bioavailability in vascular tissues [32]. The results demonstrate increased phosphorylation of VASP in the presence of H_2O_2. When aortas were pre-incubated with NAC or Akt and AMPK inhibitors, however, these values were drastically reduced.

The literature clearly indicates that endurance exercise reduces ROS production due to an increase in endogenous antioxidant production [8,9]. On the other hand, acute exercise seems to increase ROS levels [7,12–14]. In this sense, we evaluated if acute exercise increases ROS in the aorta of rats, which, at least partially, could favour eNOS phosphorylation. Acute exercise was subsequently observed to markedly increase superoxide levels and DCF concentrations. 2′,7′-dichlorofluorescein diacetate (DCFH-DA) was used as a fluorescent probe for indirect detection of ROS. After deacetylation of DCFH-DA, it can be oxidised by ROS to the fluorescent compound DCF. Studies have shown that the oxidation of DCFH is quantitatively proportional to the concentration of H_2O_2 generated [33]. The higher superoxide levels and DCF concentrations associated with no oxidative damage in lipid and protein (data not show) demonstrated that the exercise protocol used was effective at modulating redox balance. Thus, once it was established that the exercise protocol affected the redox state, we speculated whether the results obtained following acute exercise would be similar to the data from isolated aortic tissue directly subjected to hydrogen peroxide (i.e., similarly increased levels of phosphorylation of Akt, AMPK and eNOS). In fact, acute exercise was observed to markedly increase Akt, AMPK and eNOS phosphorylation. On the other hand, the results in the acute exercise plus NAC group suggest that partial elimination of ROS by N-acetylcysteine supplementation reduced the phosphorylation levels of Akt, AMPK, and eNOS. In addition, the *in vivo* results, like the *ex vivo* results, correlated with vascular function because exercise increased VASP phosphorylation, and NAC supplementation reduced it.

Activation of eNOS is not exclusively mediated by Akt and AMPK. However, mice deficient in AMPK [34] and Akt [35] showed reduced eNOS phosphorylation. Furthermore, ROS is not the only trigger for eNOS phosphorylation; viscosity and blood velocity at the endothelial wall (endothelial shear stress) have also been suggested for inducing eNOS phosphorylation [36]. Methodologically it is difficult to rule out the involvement of shear stress in this process. eNOS can be phosphorylated at sites other than serine 1177. However, a preliminary investigation into the relationship of acute exercise, Akt, AMPK and eNOS observed that Akt and AMPK primarily phosphorylated eNOS at serine 1177 in arteries from treadmill-running (50 min) rats [4]. The authors thus argued that defects in vascular kinase signalling to eNOS that precipitate decreases in NO bioavailability might be restored by exercise. In pathophysiological states, such as hypertension or obesity, whether ROS-induced acute exercise increase Akt and AMPK phosphorylation, activating eNOS and increasing NO bioavailability, is to be investigated.

The present study does not try to show how H_2O_2 increases the phosphorylation of Akt and AMPK, which is more appropriate for future studies. However, it has been suggested that ROS preferentially oxidise phosphatases, which could explain the increase in Akt and AMPK phosphorylation [37,38]. For example, serine protein phosphatase PP2A, which has been implicated in the negative regulation of Akt and AMPK, has a redox-sensitive cysteine residue that is potentially susceptible to inhibition by H_2O_2 [37]. The phosphatase and tensin homologue PTEN, which dephosphorylates the 3-phosphate of inositol phospholipids generated by PI3K, is inactivated by exposure to oxidants through the formation of a disulphide linkage between two essential cysteine residues in its active site [39].

Increased endothelium-dependent vasodilatation is associated with exercise. Nevertheless, limited information is available on the mechanisms behind this relationship. The endogenous production of NO is involved in the beneficial effects of exercise, but it is not yet known how the induction of vascular eNOS by exercise is mediated. On the other hand, it has been shown that H_2O_2 promotes eNOS phosphorylation in endothelial cells. This is relevant concerning exercise, which is associated with an increase in oxidative stress within vasculature. Taken together, our results indicate that ROS play an important role in eNOS phosphorylation, a process apparently mediated by Akt and AMPK. This mechanism is likely the route through which exercise activates eNOS. The reported results are relevant to the understanding of mechanisms that may explain the effects of exercise on cardiovascular health.

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